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Situation of Colicin Receptors in Surface Layers of Bacterial Cells

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SUMMARY

The susceptibilities to a series of colicins of *Escherichia coli* B, two *Proteus mirabilis* strains and their respective stable L-forms were compared. Certain stable L-forms are known to be completely devoid of their cell walls and consequently are completely phage-resistant. Nevertheless, such L-forms show the same or even higher degrees of susceptibility to colicins than do their parent bacteria; they also display exactly the same specific patterns of susceptibilities as do the corresponding normal bacillary forms. Colicins may become adsorbed directly to the cytoplasmic membranes of L-forms. The attachment of a colicin molecule to an adsorption site localized in the bacterial cell wall therefore cannot be regarded as a compulsory initial step leading to the killing of the cell (and the specificity of the interaction between colicins and bacterial cells cannot be determined by cell-wall receptors). Adsorption of colicins to *P. mirabilis* (normal and L-forms) is not possible after killing them with formaldehyde. The time of rescue by trypsin of cells which have adsorbed colicin is decreased to zero with L-forms. Obviously, the 'lethal' adsorption of colicins is to receptors in the cytoplasmic membrane.

INTRODUCTION

Both bacteriophages and colicins have been thought to attach to a bacterial cell in similar or even identical ways; namely, by means of receptor sites localized in the outer plastic layer of the cell wall. It is possible to demonstrate unequivocally that the first step in the infection of a sensitive bacterium by a phage particle is always its irreversible adsorption to a specific receptor site in the cell wall. Consequently, a bacterium sensitive to a certain phage will become resistant through the loss of its phage receptors. This may be shown experimentally by comparing the susceptibilities of lysozyme-induced protoplasts of Gram-positive bacteria and of penicillin-induced stable L-forms of Gram-negative bacteria with the corresponding bacillary forms. (Pl. 1, fig. 1). Both protoplasts and certain stable L-forms completely lack cell walls (Pl. 1, fig. 3): consequently, they do not adsorb phages and hence are absolutely phage-resistant (Martin, 1964; McQuillen, 1960; Taubeneck, 1961, 1962, 1963). On the other hand, spheroplasts of Gram-negative bacteria, which show an incomplete biosynthesis of the murein layer, nevertheless retain the essential components of their cell walls (Pl. 1, fig. 2) and hence remain sensitive to bacteriophages (Böhme & Taubeneck, 1958; Hofschneider, 1960).

Whilst the localization and function of phage receptors in the surface layers of the

bacterial cell is relatively well established, this is by no means true for the receptors of colicins. Until now the theory of colicin-fixing receptors formulated by Fredericq (1949) has been generally accepted. According to this theory, the lethal unit of a colicin becomes fixed to the sensitive cell by means of a specific receptor in its cell wall. This adsorption, irreversible under the usual conditions, is supposed to represent the first essential step in the interaction of any colicin with a sensitive cell, irrespective of its specific biochemical effect (Nomura, 1964). Every type of colicin is bound to a different receptor. Those cells which do not possess receptors for a given colicin, or receptors which have mutated to an ineffective state, are resistant to the colicin in question. The capacity of the wall to bind a colicin would thus primarily determine the sensitivity of the cell to it. This theory therefore envisages colicin receptors as being completely analogous to phage receptors, and, according to Fredericq (1949), certain phages may even share common receptors with certain colicins. This latter finding, however, has recently been seriously questioned by some authors (Reeves, 1965; Šmarda & Schuhmann, 1967). It has also been found that specific antibodies against the bacteria of one sensitive strain prevented the action of colicin E 2 on this strain, but not on the bacteria of another sensitive strain, although the cell-wall receptor antigen should be the same in both cases (Mayr-Harting, 1964). Finally, the kinetics of colicin adsorption is different from that of phages (Fredericq, 1952*b*). Mainly for these reasons we hold that the present concept of colicin receptors is not a complete and definitive one (Šmarda, 1966*a*).

Our preliminary observations showed that some stable L-forms of *Proteus mirabilis*, although completely phage-resistant, displayed a remarkable sensitivity to certain colicins (U. Taubeneck, unpublished results). We therefore made further experiments to determine whether the adsorption of different types of colicins to bacterial cell walls is in fact a pre-requisite for their action. For these experiments we chose the stable L-form of the *P. mirabilis* strain D 52, a strain whose chemical composition has been most thoroughly analysed. Thus, Martin (1964) found no trace of diaminopimelic acid (also Weibull *et al.* 1967), muramic acid, neutral sugars or lipids; i.e. of any typical bacterial cell-wall components. It was concluded, therefore, that the cells of this particular stable L-form represent true protoplasts. This is in agreement with the finding (Taubeneck, 1961) that this stable L-form is not capable of adsorbing any phages which infect the parent rod form. In particular, phage BF23, which supposedly shares a common receptor with colicins of the E group, has no effect on the L-form.

In the work reported here, ultrathin sections of the D 52 stable L-form of *Proteus mirabilis* showed no organized material outside the cytoplasmic membrane in the electron microscope (Pl. 1, fig. 3), a result which has also been reported by Weibull (1965). These experiments also included observations on a stable L-form of *Escherichia coli* B which had recently been isolated and which had been shown to be resistant to all phages active on *E. coli* B rods (Taubeneck & Schuhmann, 1966). (Unfortunately we had no stable L-form of a colicin-resistant mutant strain at our disposal.)

METHODS

Bacterial strains. The colicinogenic strains used are listed in Table 1. The indicator strains were: *Escherichia coli* ϕ, *E. coli* B and a stable L-form of *E. coli* B, *E. coli* 17, *Proteus mirabilis* VI, *P. mirabilis* D 52 and stable L-forms of *P. mirabilis* VI and D 52.

Nutrient media and cultivation. Broth cultures (beef extract with 1 % peptone and 0·5 % NaCl, pH 7·8) were grown in 500 ml. flasks. 50 ml. broth were inoculated with 0·5 ml. of an overnight broth culture of the bacteria or with 5 ml. of a stable L-form culture respectively and incubated at 37° on a rotary shaker overnight. In contrast to the stable *P. mirabilis* L-forms, the stable L-form of *E. coli* B could be grown on solid media only. Broth suspensions were prepared by mechanical dispersion of surface growth from agar cultures. For double-layer plates, media with 1·5 % or 2 % agar were used as base layers, with 0·7 % or 0·9 % as top layers. The *P. mirabilis* L-forms were cultivated with the addition of 10 % (v/v) horse serum, the *E. coli* B L-forms with 10 % (v/v) serum and 3·5% sucrose. Plates inoculated with a suspension of this latter L-form had to be incubated for 6–7 days.

Table 1. *Colicinogenic strains of different enterobacteria used and the colicins produced by them*

Colicinogenic strain		Colicin produced	Colicinogenic strain		Colicin produced
Escherichia coli	CA 7	V, M	*Paracolobactrum coliforme*	CA 57	C
	CA 18	B		CA 62	E 1, I
	CA 23	D	*Citrobacter freundii*	CA 31	A
	CA 38	E 3, I	*Shigella sonnei*	P 9	E 2, I
	CA 42	E 2		P 9 (O)	E 2
	CA 46	G		P 9 (T)	E 2, I
	CA 53	I	*Shigella dispar*	P 14	E 1
	CA 58	H		P 15	S 4
	K 235	K, ?			
	18	Q, D, I			
	11	Q, E 1, I			
	CL 136	E 1			
	CL 137	E 2			

Colicins. As colicins we used filtrates (membrane filters 'Synthesia' HUFS—pore diameter 0·3–0·5 μ) of overnight broth cultures of colicinogenic strains. The strain specificity of the bactericidal effects was tested on 50 strains of *Escherichia coli* and 20 strains of *Proteus vulgaris* and *P. mirabilis*; the filtrate of P 9 (O) inhibited 31 strains and that of CA 46 inhibited 27. The complete abolition of the action by trypsin indicated that the antibacterial activity of the filtrates was exclusively due to colicins.

Most experiments were made with colicins E 2 (from *Shigella sonnei* P 9 (O)) and G (from *Escherichia coli* CA 46). As indicator for colicin E 2 we generally used *E. coli* ϕ, for colicin G a stable L-form of *P. mirabilis* D 52, these strains being most strongly affected by the respective colicins. The molecular weight of colicin E 2 is 60,000 (Helinski, quoted by Maeda & Nomura, 1966), the figures for colicin G being probably lower than 50,000 (Geuther, personal communication). Colicin E 2 interferes with DNA synthesis and induces the breakdown of DNA (Nomura, 1964) and of ribosomal RNA (Nose, Mizuno & Ozeki, 1966). Nothing is known about the mechanism of action of colicin G, but it is not accompanied by any provable change of the submicroscopic structure of the cytoplasmic membrane (Gumpert & Šmarda, 1968).

Demonstration of the action of colicins on agar. In the first place we tested for the presence of colicins by a drop method with agar double layers with indicator bacteria embedded in the soft top layer. For quantitative experiments a more sensitive method

was used, namely the formation of inhibition zones round chloroform-killed macrocolonies of colicinogenic bacteria (Fredericq, 1957). Despite its much higher sensitivity, the results obtained with this method need not necessarily agree with those obtained by estimation of the killing action of the same colicins in liquid medium.

Colicin titration. To estimate the titre approximately, we looked for the highest dilution causing the last, weak, but still visible action; as a titre of colicin in 'arbitrary units' (Jacob, Siminovitch & Wollman, 1952) the inverse value of this dilution was evaluated.

In all experiments where an accurate titration of colicins E 2 and G was necessary, the punch-hole method (van Horn, 1961) was used. Its principle lies in a comparison of the width of inhibition zones formed by diffusing colicin solutions of various standard concentrations, with those of the unknown samples. The absolute titre values of a standard solution were ascertained from the number of the surviving indicator bacteria (*E. coli* ϕ rods for colicin E 2 and *P. mirabilis* D 52 L-form for colicin G) in broth suspensions (Mayr-Harting, 1964). In both cases we could confirm that 1 arbitrary unit corresponded roughly to 5×10^7 lethal units (l.u.).

RESULTS

Action of colicins on normal forms and stable L-forms of Escherichia coli B, *Proteus mirabilis* D 52 *and P. mirabilis* VI

Individual colicins reveal different activity spectra, but primarily only strains of the genera *Escherichia* and *Shigella* are inhibited. Proteus species are supposed to be resistant to colicins with the exception of colicin H (Fredericq, 1948). In fact, 58% of the strains of *P. morganii* tested were sensitive to colicin H (Vieu, 1960). Preliminary experiments showed, however, that some strains of *P. mirabilis* were sensitive to several other colicins (Šmarda, Taubeneck & Schuhmann, 1966). Thus it became possible to compare the action of all colicinogenic strains used not only on the normal rod form and stable L-form of *E. coli* B, but also on normal rods and stable L-forms of *P. mirabilis* VI and D 52. All colicins were tested simultaneously on the classical indicator strain *E. coli* ϕ. In the sensitivity tests Fredericq's (1957) method was used primarily. Top layers were inoculated with 1×10^8 to 2×10^9 living normal forms, or 7×10^8 to $3{\cdot}5 \times 10^9$ *P. mirabilis* L-forms, the concentration of the *E. coli* B L-form being lower.

The results with 16 well-known colicinogenic strains out of those 21 tested are given in Table 2.

Summing the results, we find that all 21 colicins tested acted on *Escherichia coli* ϕ. *E. coli* B was not affected by the following colicins of group E, E 1 (of CL 136 and II), E 2 (of P 9 and CL 137), colicin M, and probably E 3. On the other hand, *Proteus mirabilis* VI and D 52 were affected by colicins B, D, E 1 (of P 14 and CA 62), E 2 (of P 9), G and H. All the stable L-forms used are qualitatively as sensitive (or resistant) to colicins as are the corresponding rod forms. But, with the exception of colicin B, the L-forms of *P. mirabilis* were more sensitive to colicins than the rods. With colicin B no difference was observed. The stable L-forms of *P. mirabilis* were even more sensitive to colicins E 1 (CA 62), G and H than the classical indicator, *E. coli* ϕ. The stable *E. coli* B L-form was, in several cases, also more sensitive than were *E. coli* B rods.

The time course of the action of colicins E 2 and G on the stable L-form of Proteus mirabilis D 52

The L-forms from an overnight broth culture of *Proteus mirabilis* D 52 were re-suspended in fresh broth at $1{\cdot}7 \times 10^{10}$ to $1{\cdot}96 \times 10^{10}$ colony-formers/ml. To ensure an excess of colicin, E 2 at $4{\cdot}26 \times 10^{11}$ l.u./ml. and colicin G at $2{\cdot}28 \times 10^{10}$ l.u./ml. were used. The suspensions of the stable L-form and solutions of the colicins were mixed 1 + 1 (v/v) and the mixtures incubated at 37°. At appropriate intervals, samples were

Table 2. *Action of 13 colicinogenic strains on Escherichia coli φ and B, and Proteus mirabilis D 52, rod form and stable L-form*

Diameter of the inhibition zone: 0 mm., −; 1–5 mm., +; 6–15 mm., ++; > 15 mm., +++.

	Indicator strains				
	E. coli			*P. mirabilis* D 52	
Colicinogenic strains (colicins)	φ	B	Stable L-form of B	Rod form	Stable L-form
CA 31 (A)	+	N.T.*	N.T.	−	−
CA 18 (B)	++	++	++	++	++
CA 57 (C)	++	N.T.	N.T.	−	−
CA 23 (D)	++	++	++	++	+++
P 14 (E 1)	++	+	++	++	+++
CL 136 (E 1)	++	−	−	−	−
CA 42 (E 2)	++	+†	+++†	−	−
P 9 (o) (E 2)	++	−	−	+++	+++
CL 137 (E 2)	++	−	−	−	−
CA 38 (E 3+I)	++	+	+++	−	−
CA 46 (G)	++	++	+++	+++	+++‡
CA 58 (H)	++	++	+++	++	+++‡
CA 53 (I)	++	++	++	−	−
K 235 (K+?)	++	+	++	−	−
P 15 (S 4)	+	N.T.	N.T.	−	−
CA 7 (V+M)	++	++	++	−	−

* N.T. — not tested.
† Inhibition zones formed only when macrocolonies not chloroform-killed.
‡ Inhibition zone—as opposed to that formed by the rod form—completely clear.

taken for counting the L-form colonies and, after rapid removal of the organisms by membrane ultrafiltration, for assay of free colicin by the punch-hole method. The results are given in Fig. 1 and 2. There was a marked difference in the effect of the two colicins.

Immediately after mixing with colicin E 2 the number of L-form colonies decreased by about tenfold. After 10 min. the surviving elements started to divide again very slowly, although about 63 % of free unadsorbed colicin remained in the mixture. A very similar course was shown by the titre of free colicin E 2. After an immediate decrease it increased again markedly during the first 10 min.

The action of colicin G was much stronger and more prolonged. This agent decreased the number of living L-forms by 4 orders of magnitude during 40 min. Also, adsorption was accomplished immediately after mixing. Afterwards the titration curve showed only an insignificant tendency to increase.

Adsorption and action of colicins E 2 and G on the rods of Escherichia coli ϕ and 17 and on the rods and stable L-forms of Proteus mirabilis D 52

The method was standardized according to Mayr-Harting (1964). Colicin suspension was mixed 1 + 1 (v/v) with a suspension of bacteria in broth, incubated for 60 min. at 37° and then for at least 90 min. at 4°. Then the counting of survivors and the assay of the remaining free colicin by the punch-hole method were done, typical results are presented in Table 3.

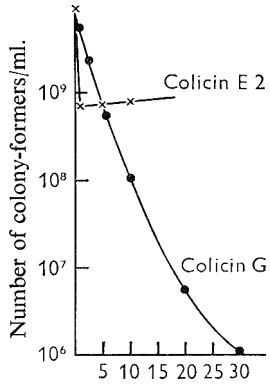

Fig. 1

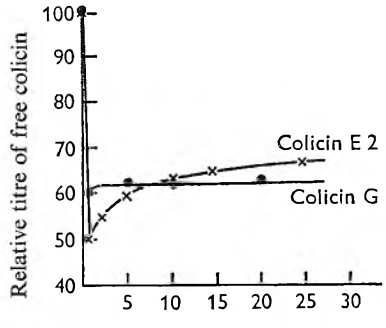

Fig. 2

Fig. 1. Survival of stable L-forms of *Proteus mirabilis* D 52 in suspensions containing colicins E 2 or G. For techniques, see the text.

Fig. 2. Relative titre of free colicins E 2 and G in suspensions of stable L-forms of *Proteus mirabilis* D 52. For techniques see the text.

Table 3. *Adsorption and action of colicins E 2 and G to living bacteria*

Colicin	Bacteria	Original no. of colony-formers/ml. of suspension with colicin	No. of colony-formers killed/ml.	Original colicin titre/ml. of suspension with bacteria (l.u.)	Decrease of colicin titre (%)
E 2	*Escherichia coli ϕ*	$3{\cdot}58 \times 10^9$	$3{\cdot}54 \times 10^9$	$1{\cdot}34 \times 10^{10}$	100
	E. coli 17	$6{\cdot}62 \times 10^9$	0	$1{\cdot}34 \times 10^{10}$	3
	Proteus mirabilis D 52 rod form	$5{\cdot}54 \times 10^9$	0	$1{\cdot}34 \times 10^{10}$	8
	P. mirabilis D 52 stable L-form	$4{\cdot}53 \times 10^9$	$1{\cdot}37 \times 10^9$	$1{\cdot}34 \times 10^{10}$	16
G	*E. coli ϕ*	$9{\cdot}35 \times 10^9$	0	$1{\cdot}14 \times 10^{10}$	13
	E. coli 17	$5{\cdot}17 \times 10^9$	0	$1{\cdot}14 \times 10^{10}$	4
	P. mirabilis D 52 rod form (in cultivation medium)	$6{\cdot}65 \times 10^9$	0	$1{\cdot}14 \times 10^{10}$	100
	P. mirabilis D 52 rod form (in fresh medium)	$8{\cdot}40 \times 10^9$	$1{\cdot}20 \times 10^9$	$1{\cdot}14 \times 10^{10}$	13
	P. mirabilis D 52 stable L-form	$5{\cdot}80 \times 10^9$	$5{\cdot}79 \times 10^9$	$1{\cdot}14 \times 10^{10}$	42

Colicin E 2 acted vigorously on *Eschericha coli ϕ*, and $3{\cdot}58 \times 10^9$ bacteria/ml. adsorb 100 % of this colicin, i.e. $1{\cdot}34 \times 10^{10}$ l.u./ml. The adsorption capacity was probably still not saturated by this amount. It was confirmed that *E. coli* 17 was resistant to

colicins E 2 and G and that it did not adsorb them significantly. The action of colicin E 2 on the rods of *Proteus mirabilis* D 52 and of colicin G on the rods of *E. coli* ϕ was so weak that, in the heavy suspensions used, no killing was detected. In this case, too, the decrease of the titres of these colicins was only about 10 %.

Colicin G did not affect *Proteus mirabilis* D 52 rods when applied in the original culture medium, but it vanished completely from the solution. After resuspending the bacteria in fresh broth, however, some bacteria were killed and only about 13 % of the colicin was adsorbed. This paradox may be explained by the finding of a specific extracellular inhibitor for colicin G which is produced and excreted in broth cultures of *P. mirabilis* D 52 (Šmarda, 1966*b*). The stable L-form of *P. mirabilis* D 52 was substantially more sensitive to both colicins than the rod form and its adsorption capacity was higher; the number of elements killed depended directly on the amount of colicin adsorbed. The lethal effect of colicin G on the L-form of *P. mirabilis* was extremely strong, although less than 1 l.u./organism was adsorbed. The data suggest that colicin G molecules can possibly be released from the inactivated L-forms and are then able to kill new ones.

Table 4. *Adsorption of colicins E 2 and G to dead bacteria*

Colicin	Bacteria	No. of organisms/ml. of suspension with colicin	No. of organisms surviving formaldehyde/ml.	Original colicin titre/ml. of suspension with bacteria (l.u.)	Decrease of colicin titre (%)
E 2	*Escherichia coli* ϕ	$1{\cdot}85\times10^{9}$	0	$4{\cdot}26\times10^{10}$	100
	Proteus mirabilis D 52 rod form	$1{\cdot}84\times10^{10}$	0	$4{\cdot}26\times10^{10}$	0
	P. mirabilis D 52 stable L-form	$3{\cdot}72\times10^{8}$	10^{2}	$4{\cdot}26\times10^{10}$	0
G	*E. coli* ϕ	$9{\cdot}35\times10^{9}$	10^{3}	$1{\cdot}14\times10^{10}$	13
	P. mirabilis D 52 rod form	$8{\cdot}40\times10^{9}$	0	$1{\cdot}14\times10^{10}$	0
	P. mirabilis D 52 stable L-form	$2{\cdot}60\times10^{9}$	0	$1{\cdot}14\times10^{10}$	4

Adsorption of colicins E 2 and G to rods of Escherichia coli ϕ and rods and stable L-forms of Proteus mirabilis D 52, previously killed with formaldehyde

If the adsorption of colicins to the stable L-form of *Proteus mirabilis* D 52 is really reversible, this would be in complete contrast to colicin adsorption by rods of *Escherichia coli* ϕ. To examine this difference, we made a series of adsorption experiments with killed organisms. The same bacterial suspensions (only the resistant strain, *E. coli* 17 was omitted) and stable L-form suspensions and the same colicin G solution were used as in the experiments with living bacteria and their L-forms. The titre of colicin E 2 was $4{\cdot}26\times10^{10}$ l.u./ml. According to Mayr-Harting (1964), 0·2 ml. of 3 % (w/v) formaldehyde was added to 3 ml. of each suspension, stirred, and incubated for 1 hr at 37°. Then the bacteria were spun down, once washed with broth, resuspended in 3 ml. fresh broth and used for adsorption under the same conditions as previously stated. The results are given in Table 4.

The comparison with the results of adsorption to living bacteria (Table 3) shows the difference between the reaction of the rods of *Escherichia coli* ϕ and of *Proteus mirabilis* D 52. While the degree of adsorption of colicins E 2 (as already stated by Mayr-Harting, 1964) and G to rods of *E. coli* ϕ was not changed by killing the bacteria

with formaldehyde, it was notably decreased in the case of normal forms and especially of stable L-forms of *P. mirabilis* D 52; here the capacity to bind colicin E 2 decreased from 16 % to zero, and from 42 % to 4 % in the case of colicin G.

Attempts to rescue stable L-forms of Proteus mirabilis D 52 by trypsin after the attachment of colicin E 2 and G

Trypsin (0·15–0·5 % (w/v) solution) was added to a mixture of *Proteus mirabilis* D 52 L-forms and colicins E 2 or G at different times. The activity of trypsin towards the colicins was determined separately before each experiment. Any errors arising from multiplication of organisms during the incubation with trypsin were excluded by

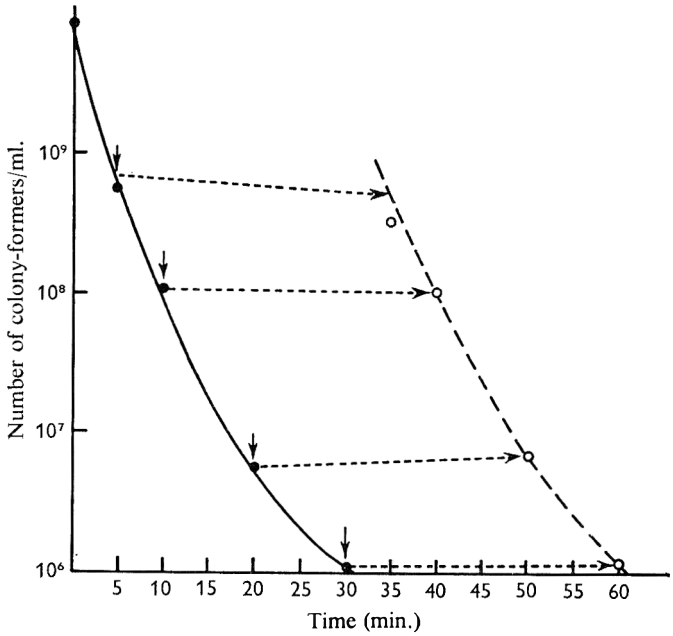

Fig. 3. Number of colony-formers in *Proteus mirabilis* D 52 L-form broth suspension with colicin G at 37°. At zero time, L-form suspension was mixed with colicin G. After 5, 10, 20 and 30 min. the survival count was made by plating (full circles); at the same times warmed trypsin solution was added to each sample (full arrows). The samples were incubated at 37° for a further 30 min. (dashed arrows) and the number of colony-formers again ascertained in each sample (empty circles) to check whether an increase of the number of survivors occurred after the action of trypsin.

control experiments. The results with colicin G are given in Fig. 3 which shows that there was no reactivation effect of trypsin in the case of the L-forms even after only short exposures to colicin G. Even when trypsin was added immediately after mixing the L-form with colicin E 2, no reactivation was observed. Thus, contrary to *Escherichia coli* rods, the stable L-forms of *Proteus mirabilis* were no rescued after the attachment of colicins E 2 or G.

DISCUSSION

Our results suggest that the bacterial cell wall plays quite different roles in the lethal actions of bacteriophages and colicins, respectively. Phage receptors in the walls of sensitive bacteria mediate a type-specific adsorption of phage particles. The attachment of the phage to the receptor site is necessary for successful infection of the bacterium. Bacteria without cell walls, i.e. protoplasts and stable L-forms of the protoplast type, are therefore not able to adsorb phages and consequently are phage-resistant (Taubeneck, 1961, 1963; Taubeneck & Schuhmann, 1966).

Receptor sites for colicins also mediate a strain-specific adsorption. The results of reactivation experiments (Nomura & Nakamura, 1962; Reynolds & Reeves, 1963; Šmarda, 1965) suggested that the lethal unit of colicin does not penetrate the cell, but remains attached to its surface. An accurate cytological interpretation of what is meant as 'bacterial surface' in this connexion has not yet been given. Nevertheless, this suggestion has been verified by experiments with radioactive colicins (Maeda & Nomura, 1966). It is thought (Nomura, 1964) that the primary change at a receptor site on the bacterial surface caused by a colicin is to induce a stimulus which is transferred by a specific (and until now hypothetical) mechanism, presumably through the cell wall and along the cytoplasmic membrane to the killing (biochemical) target. The biochemical changes induced in the organisms attacked by different colicins may differ according to the diverse stimuli induced by the respective substances. There is little doubt that the common seat of the relevant actions is the cytoplasmic membrane (Luria, 1964). The results presented here strongly suggest that adsorption of colicin to an adsorption site in the cell wall is not an essential prerequisite for its killing effect.

This evidence stems mainly from experiments in which the effects of colicin E 2 and G on normal forms of *Proteus mirabilis* D 52 and *Escherichia coli* B and on the stable L-forms of these strains were compared. The stable L-forms are resistant to all phages which attack their parent bacteria, and it has been shown by biochemical analysis that, at least in the case of the stable L-form of *P. mirabilis* D 52, no cell-wall components can be detected (Martin, 1964). Preliminary observations on the stable L-form of *E. coli* B have shown that besides its resistance to phages T 1–7, BF 23 and λ (Taubeneck & Schuhmann, 1966), this L-form contains only traces of diaminopimelic acid, which certainly come from intermediary metabolism and not from cell-wall murein.

In spite of the fact that they completely lack cell walls, stable L-forms of *Proteus mirabilis* D 52 and of *Escherichia coli* B are sensitive to the action of colicins B, D, some E 1 and E 2, G and H. Moreover, there is essentially no qualitative difference between the sensitivity of rods and L-forms. This suggests that colicins may adsorb directly to the cytoplasmic membrane which forms the true surface of our L-forms which was shown experimentally with the L-forms of *P. mirabilis*. Whether they adsorb to the site of action proper, or to any other site from which stimuli are transmitted to the killing or biochemical target, cannot yet be decided. In any case, the connexion of the receptor site in the cytoplasmic membrane and the killing target must be rapid and efficient, at least for colicin E 2.

The specificity of the susceptibilities of the L-forms to all the colicins examined is the same as that of the corresponding normal forms with complete cell walls. Thus, specific adsorption of a colicin to an adsorption site in the cell wall cannot be regarded as the start of interaction between the colicin and the bacterial metabolism,

at least not the necessary one, and there may be another explanation: the effective 'lethal' colicin receptors are not those localized in the cell wall, but those in the cytoplasmic membrane. Only those lethal units can exert their effect which come through the cell wall to the membrane. This is in complete agreement with the finding of Maeda & Nomura (1966) that nearly all the radioactivity of ^{14}C-labelled colicin E 2 after its adsorption on *Escherichia coli* W 3110, followed by their disruption and differential centrifugation, remained in the fraction containing cell walls and cell membranes.

The cross-resistance to certain colicins and phages has never been proved in 100 % of mutants examined. The cases where it actually exists could be explained, for example, by the assumption of a specific chemical structure in the cell wall necessary for both: binding a certain phage and enabling the passage of a certain colicin to the cytoplasmic membrane.

There is little doubt, however, that considerable amounts of colicin may be adsorbed to cell-wall structures. However, much of the adsorption to cell walls might represent a 'non-lethal binding to sites other than those which we have called effective (lethal) receptors' (quoted from Shannon & Hedges, 1967). The adsorption of a colicin to a cell wall does not necessarily require the viability of the organism; for example, formaldehyde-killed organisms of *Escherichia coli* adsorb colicins as well as do living ones, and adsorbed colicin is not released after killing the organism (Fredericq, 1952*a*; Maeda & Nomura, 1966). The adsorption capacity of formaldehyde-killed L-forms is extremely limited, and previously adsorbed colicin is set free again (at least to a certain extent) after the killing action of the colicin itself. Preliminary results suggest that it is also set free again after killing the L-forms with chloroform. The observation that normal Proteus rods also, after having been killed with formaldehyde, do not adsorb colicins cannot yet be explained. Perhaps *Proteus mirabilis* D 52 rods do not possess cell-wall adsorption sites for colicins E 2 and G, although corresponding cytoplasmic membrane receptors and biochemical targets are present. Further experiments are in progress to determine whether this is really due to the different mode of adsorption of colicins to rods and to stable L-forms or rather to a species difference between *E. coli* and *P. mirabilis*.

According to the view of Holland, Hill & Bellamy (1966), receptors for colicins of the E group are situated in the innermost layer of the cell wall, in close proximity to the cytoplasmic membrane. The colicins may, in fact, penetrate the wall of bacterial rods and act in the same way as on the L-forms, i.e. directly on the cytoplasmic membrane. The channels in the lipoprotein and lipopolysaccharide layer of the cell wall which have been shown recently (Bayer & Anderson, 1965) could be regarded as possible pathways of penetration. In this case, the stable L-forms should be expected to be attacked by the colicins more readily; this has been shown by our experiments.

It is known that colicins adsorbed to the cell wall may be digested by trypsin. When this digestion is performed within a certain time after the adsorption to sensitive organisms, the lethal action of the colicins may be prevented (Nomura & Nakamura 1962; Reynolds & Reeves, 1963; Šmarda, 1965). The time limit within which rescue of inactivated organisms is possible is considerably shortened after adsorption of colicins to spheroplasts (Obdržálek, Šmarda, Čech, & Adler in preparation) and it becomes zero or almost zero after their adsorption to stable L-forms of *Proteus mirabilis* D 52. This finding further emphasizes an immediate action of colicins on sensitive organisms if these lack their cell walls.

In view of the characteristics of colicin G (strain specificity of action, no direct effect on cytoplasmic membrane, etc.) it does not seem probable that it would be a phospholipase, like, for example, megacin A (Ozaki *et al.* 1966), although it is not directly excluded.

We conclude from our results that the bactericidal effect of colicins generally is not mediated through the fixation of a lethal unit of colicin to a cell-wall receptor (followed by the transmission of an as yet unknown stimulus to a killing/biochemical target presumably in the cytoplasmic membrane). This effect on the killing target is readily achieved by a direct and probably reversible contact of a colicin lethal unit with the cytoplasmic membrane, without any specific mechanism involving irreversible fixation to the cell wall as an essential step. In contrast to cell walls, cytoplasmic membranes of formaldehyde-fixed L-forms are not capable of adsorbing colicins. Thus, sensitivity of the organism to colicins is primarily determined by receptors in the cytoplasmic membrane and not in the wall, of the organism, as is the case with bacteriophages.

We are indebted to Dr Anna Mayr-Harting and Professors P. Fredericq and J. Sedlák and to the Czechoslovak Collection of Micro-organisms for some of the *Escherichia coli* strains. Dr Edith Schuhmann provided stable L-forms of *E. coli* B. The excellent technical assistance of Mrs Gerda Elske is gratefully acknowledged. Ultrathin cell sections for Pl. 1 fig. 1 and 3 were kindly prepared by Dr J. Gumpert, for Pl. 1, fig. 2 by Dr Marie Havelková.

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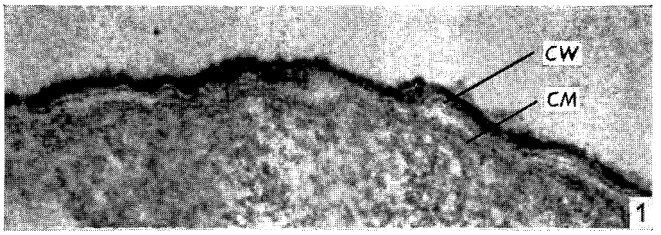
EXPLANATION OF PLATE

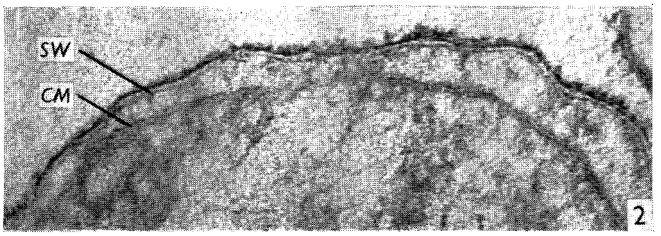
PLATE I

Fig. 1. Ultrathin section of a normal bacillary cell of *Proteus mirabilis* D 52. Kellenberger fixation. × 150,000. *CW*, Cell wall; *CM*, cytoplasmic membrane. Note the surface formed by a cell wall and a cytoplasmic membrane.

Fig. 2. Ultrathin section of a penicillin spheroplast of *Proteus mirabilis* D 52. Kellenberger fixation. × 150,000. *SW*, Spheroplast wall; *CM*, cytoplasmic membrane. Note the surface formed by a weakened wall and a cytoplasmic membrane.

Fig. 3. Ultrathin section of a stable L-form of *Proteus mirabilis* D 52. Kellenberger fixation. × 150,000, *CM*, Cytoplasmic membrane. Note the surface formed by a cytoplasmic membrane


CW
CM
1


SW
CM
2


CM
3

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Physiological and Biochemical Characteristics of Some Strains of Sulphate-reducing Bacteria

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SUMMARY

Fourteen previously unstudied strains of the genus *Desulfovibrio* and eight thermophilic strains of the genus *Desulfotomaculum* were allotted to species on the results of physiological and biochemical tests.

On DNA base composition the *Desulfovibrio* strains fell clearly into three groups, corresponding to *Dv. salexigens*, *Dv. desulfuricans* and *Dv. vulgaris*. NaCl-dependence appeared to be a constant and unique feature of *Dv. salexigens* strains, and 'sulphate-free' growth on pyruvate a constant characteristic of *Dv. desulfuricans*, although one strain of *Dv. vulgaris* also dismuted pyruvate. Fumarate dismutation showed no species correlation. 'Sulphate-free' growth on choline was often minimal and the test appeared to be of little value. A high degree of resistance to Hibitane was not a constant characteristic of *Dv. salexigens*.

The thermophilic strains showed great uniformity in DNA base composition, Hibitane resistance and 'sulphate-free' growth, and were all classified as *Desulfotomaculum nigrificans*.

INTRODUCTION

Saunders, Campbell & Postgate (1964) showed that 30 strains of non-sporulating dissimilatory sulphate-reducing bacteria could be divided into three groups defined by their deoxyribonucleic acid (DNA) base composition. With these and other data in mind, the classification of the dissimilatory sulphate-reducing bacteria was revised (Campbell & Postgate, 1965; Campbell, Kasprzycki & Postgate, 1966; Postgate & Campbell, 1966). The sporulating members were placed in a new genus, *Desulfotomaculum*, represented by three species: *Dt. nigrificans*, *Dt. ruminis* and *Dt. orientis* (Campbell & Postgate, 1965). The non-sporulating members were placed in the genus *Desulfovibrio*, represented by five species: *Dv. desulfuricans*, *Dv. vulgaris*, *Dv. salexigens*, *Dv. gigas* and *Dv. africanus* (Campbell *et al.* 1966; Postgate & Campbell, 1966).

The present paper reports the results of a study of the physiological and biochemical characteristics of 14 previously unstudied strains of the genus *Desulfovibrio* and eight thermophilic strains of the genus *Desulfotomaculum*.

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METHODS

Organisms. The following 22 strains of sulphate-reducing bacteria were obtained as ampoules of freeze-dried material from the National Collection of Industrial Bacteria, Torry Research Station, Aberdeen, Scotland (NCIB):

(1) Mesophilic strains (non-sporulating; growth temperature 30°). (*a*) Salt-water strains: NCIB 8309 (EL AGHEILA A), 8315 (NEW JERSEY SW-8), 8316 (NEW JERSEY SW-3), 8326 (CALIFORNIA 29:137:5), 8396 (EL AGHEILA 4), 8399 (VENEZUELA), 8400 (HOSSEGOR), 8402 (EL AGHEILA 2) and 9492 (ABERDOVEY). (*b*) Fresh-water strains: 8456 (DENMARK, a morphologically unusual strain showing predominantly long spirillar forms), 8457 (WOOLWICH) and 8458 (BYRON).

(2) Thermophilic strains (fresh-water strains; spore-bearers; growth temperature 55°): 8356 (HOLLAND CT), 8357 (DELFT 48 T), 8359 (DELFT 3 T), 8360 (DELFT 13 T), 8361 (DELFT 15 T), 8394 (DELFT 1 T), 8706 and 8788 (unnamed strains).

Cultivation. Cultures were grown anaerobically, from freeze-dried specimens, at 30° or 55° as appropriate, in the medium of Baars (1930) containing 1·0 g. Difco yeast extract/l. and 5 mM-cysteine hydrochloride. NaCl 25·0 g./l. was added for the salt-water organisms. Details of the preparation, sterilization and pH adjustment of this medium were as given by Saleh, Macpherson & Miller (1964). After revival, stock cultures of the mesophilic strains were maintained in the medium C of Butlin, Adams & Thomas (1949) as modified by Professor J. R. Postgate (see Baker, Papiska & Campbell, 1962), and subcultured weekly. The thermophilic strains gave better growth in Baars medium, in which stock cultures were therefore maintained. Stock and experimental cultures were grown in Pyrex test tubes or flasks plugged with cottonwool and incubated in McIntosh and Fildes anaerobic jars in an atmosphere (except when stated otherwise) of 99% (v/v) H_2 + 1% (v/v) CO_2.

Stock cultures, and all experimental cultures except those in the Hibitane test, were tested for aerobic and anaerobic contaminants (Postgate, 1953).

Pigment detection. For cytochrome c_3 demonstration, fully grown 200 ml. cultures in medium C were concentrated by centrifugation and examined with the Hartridge reversion spectroscope after the addition of sodium dithionite. A strong absorption band at about 554 mμ and a weaker one at 525 mμ indicated the presence of cytochrome c_3. Desulfoviridin was tested for by the method of Postgate (1959*a*).

Maximum growth temperature. In view of the significance of thermophily in the taxonomy of sulphate-reducing bacteria, the limiting growth temperature was determined for the strains stated by the NCIB to be mesophilic. Test-tube cultures in medium C were incubated in anaerobic jars immersed in a thermostat tank initially at 37° ± 0·2°; the maximum growth temperature was observed by making serial subcultures with incubation temperature increments of 0·5°.

Resistance to Hibitane. The bacteriostatic concentration of Hibitane (Imperial Chemical Industries Ltd., Wilmslow, Cheshire) towards each strain in Baars medium was determined by the method of Saleh (1964).

Adaptation to growth in salt-water or freshwater media. Serial subcultures of the mesophilic strains were made in medium C with increments or decrements of 5·0 g./l. in NaCl concentration. Three successive subcultures were made in the final medium before adaptation was considered to be proved. In later stages of the experiment,

growth often took more than a week to appear; results were therefore not recorded as negative until three weeks after inoculation.

Pyruvate dismutation test. Strains were examined for sulphate-free growth by using the pyruvate medium of Postgate (1963*a*) containing cysteine hydrochloride. The sodium pyruvate used was recrystallized from 80% (v/v) ethanol in water. The cultures were incubated under nitrogen. Persistence of growth through five subcultures was taken as evidence of ability to grow by pyruvate dismutation.

Choline utilization. Strains were examined for anaerobic growth (under N_2) on choline, with and without 26 mM-sodium sulphate, by using the medium of Baker *et al.* (1962) containing cysteine. The same criterion of substrate utilization was used as in the pyruvate test (smell of trimethylamine is an unsuitable criterion of growth in the case of large numbers of cultures incubated together, since the gas penetrates into cultures in which no growth has occurred).

Estimation of growth. In the pyruvate, choline, and NaCl adaptation tests, growth was often scanty and therefore difficult to measure turbidimetrically or by microscopic examination. Growth in the final subculture was thus visually graded $-$, $\pm$ (turbidity just discernible in daylight), or $+$.

Buoyant density measurements. DNA was isolated and purified as described by Saunders *et al.* (1964). The buoyant density of the DNA was determined by CsCl density gradient centrifugation (Saunders *et al.* 1964) with ^{15}N-labelled *Pseudomonas aeruginosa* DNA ($\rho = 1{\cdot}742$ g. cm.$^{-3}$) as a density reference standard. Buoyant densities and base compositions of the DNA were calculated according to the equations of Sueoka (1961).

RESULTS

All results except those of the pigment tests are summarized in Table 1.

Pigment tests. All the mesophilic strains contained cytochrome c_3 and desulfoviridin; the thermophilic strains contained neither pigment.

Inhibition by Hibitane. Saleh (1964) tested the ABERDOVEY strain against Hibitane and found the bacteriostatic concentration to be 10 μg./ml.; in our experiment with this strain it was 100 μg./ml. None of our strains ascribed to the species *Desulfovibrio salexigens* on DNA base composition grew in the presence of 50 μg. Hibitane/ml. Since the members of this species tested by Saleh resisted 1000 μg./ml., we checked our experimental technique by re-testing one of the strains of *Dv. salexigens* that Saleh had tested (CALIFORNIA 43:63; NCIB 8364). Our result confirmed his finding with this strain.

NaCl adaptation. Only one strain isolated from a freshwater habitat was unable to grow in 2·5% (w/v) NaCl. At the first attempt this strain (BYRON) did not survive in 2% NaCl; in a second attempt, starting again with a culture in NaCl-free medium, growth occurred in the presence of 2·0% NaCl but not 2·5%.

Pyruvate dismutation. This test was made at Urbana, Illinois, U.S.A., and at Teddington, Middlesex, England; somewhat different results were obtained. In the case of the 'doubtful' dismuter AUSTRALIA and the 'positive' dismuter EL AGHEILA A (Teddington results) the fifth subculture was examined microscopically: typical vibrios were seen. The cultures were uncontaminated.

Choline utilization. The whole choline utilization experiment was performed twice. In some cases, growth was obtained in one experiment but not the other; these are

Table 1. *Physiological and biochemical characteristics of 22 strains of sulphate-reducing bacteria*

The mesophilic strains are arranged in three groups according to their DNA base composition (% guanine+cytosine, G+C), and their classification derived from this (see Postgate & Campbell, 1966) is shown.

Name of strain	NCIB no.	Max. growth temperature	Hibitane: bacteriostatic concentration (μg./ml.)	NaCl adaptation	Pyruvate dismutation: Urbana expts	Pyruvate dismutation: Teddington expts	Choline dismutation	Growth on choline+sulphate	% G+C in DNA	Classification from DNA composition
Mesophilic										
NEW JERSEY SW-8	8315 (S)	42·5°	25	Down to 0·25 %	−	−	−	−	44·7	*Desulfovibrio salexigens*
NEW JERSEY SW-3	8316 (S)	42·5°	25	Down to 0·25 %	−	−	−	±	45·6	
AUSTRALIA	8329 (S)	39·0°	5	Down to 0·75 %	−	±	−	+	45·6	
EL AGHEILA 2	8402 (S)	39·0°	50	Down to 0·5 %	−	−	−	±	44·6	
CALIFORNIA 29:137:5	8326 (S)	43·5°	100	Down to 0	±	±	±	±	56·3	*Desulfovibrio desulfuricans*
CALIFORNIA 29:137:11	8339 (S)	44·5°	50	Down to 0	+	+	−	±	56·3	
EL AGHEILA 4	8396 (S)	44·0°	2·5	Down to 0	+	+	±	+	56·3	
HOSSEGOR	8400 (S)	43·5°	10	Down to 0	+	+	±	−	56·3	
BYRON	8458	39·0°	5	Up to 2·0 %	+	+	±	+	56·6	
ABERDOVEY	9492 (S)	42·5°	100	Down to 0	+	+	±	+	54·4	
EL AGHEILA A	8309 (S)	44·5°	2·5	Down to 0	−	+	−	+	60·2	*Desulfovibrio vulgaris*
VENEZUELA	8399 (S)	46·0°	100	Down to 0	−	−	−	−	60·8	
DENMARK	8456	44·5°	25	Up to 2·5 %	−	−	−	−	60·2	
WOOLWICH	8457	45·0°	25	Up to 2·5 %	−	−	±	±	60·2	
Thermophilic										
HOLLAND CT	8356	*	< 0·1	*	+	+	−	−	46·6†	*Desulfotomaculum nigrificans*‡
DELFT 48 T	8357	*	< 0·1	*	±	±	−	−	46·6	
DELFT 3 T	8359	*	< 0·1	*	+	+	−	−	45·6	
DELFT 13 T	8360	*	< 0·1	*	+	+	−	−	45·6	
DELFT 15 T	8361	*	< 0·1	*	+	±	−	−	45·6	
DELFT 1 T	8394	*	< 0·1	*	+	+	−	−	45·6	
Unnamed	8706	*	< 0·1	*	+	+	−	−	45·6	
Unnamed	8788	*	< 0·1	*	+	±	−	−	45·6	

(S) = strain isolated from a salt-water environment. − = no growth. ± = doubtful or scanty growth. + = growth.
* Test not made; † all % G+C values for the thermophils taken from Saunders & Campbell (1966); ‡ see text.

recorded as ± in Table 1, as also are strains which gave scanty growth. Some strains, recorded as negative, produced scanty growth and died out on or before the fifth subculture. Where positive growth occurred, it was much less than in sulphate-free pyruvate medium.

DNA-base composition. As judged by DNA base composition all 14 Desulfovibrio strains could be placed in the three groups of Saunders *et al.* (1964). Both marine and freshwater strains appear in group I (60% guanine+cytosine, G+C) and group II (54–56% G+C); the obligate salt-water strains all belonged to group III (45–46% G+C). The DNA base compositions of the *Desulfotomaculum nigrificans* strains were reported previously by Saunders & Campbell (1966).

DISCUSSION

The physiological and biochemical data show that the 14 strains of non-sporulating dissimilatory sulphate-reducing bacteria studied here belong to the genus *Desulfovibrio*; by using the taxonomic criteria outlined by Postgate & Campbell (1966), it was possible to place the strains into three species: *Dv. vulgaris*, *Dv. desulfuricans* and *Dv. salexigens*. The thermophilic sporulating sulphate reducers were identified as *Desulfotomaculum nigrificans* (Campbell & Postgate, 1965).

Saleh (1964) reported that the Hibitane resistance of Desulfovibrio strains showed a correlation with the DNA base composition. This apparent correlation did not hold for the strains examined in the present work. Moreover, it has been found that a given strain can show a tenfold variation in its Hibitane resistance at different times (or in the hands of different workers). Thus Hibitane resistance is a less useful taxonomic character for this group of organisms than was originally thought, though very high resistance still predominates in the *Dv. salexigens* group and relatively low resistance in the *Dv. vulgaris* and *Dv. desulfuricans* groups.

The readiness with which our marine and freshwater strains of *Desulfovibrio desulfuricans* and *Dv. vulgaris* could be adapted to grow either in the absence or presence of NaCl confirms the contention of Postgate & Campbell (1966) that salt relations are not of taxonomic significance in these two species. Our obligate salt-requiring strains were classified as *Dv. salexigens* (see Postgate & Campbell, 1966), though it is interesting that three of our four strains grew at lower NaCl concentrations than that stated by these authors to be required by *Dv. salexigens* (> 0·6%). It was not determined at this stage whether our strains required Na^+ or Cl^-; a more comprehensive study of the salt relations of this species is under way.

The problems of using carbon source utilization data for speciation in the genus *Desulfovibrio* were discussed by Postgate (1959*b*), Macpherson & Miller (1963), Postgate (1965), and Postgate & Campbell (1966). The ability to grow on choline or pyruvate in the absence of sulphate appears to separate *Dv. desulfuricans* from other species of the genus *Desulfovibrio*. However, the difficulty in obtaining repeatable results with choline, coupled with the scanty growth of many strains of *Dv. desulfuricans* on this substrate, makes the use of this character questionable. Miller & Wakerley (1966) reported that the ability to grow on sulphate-free fumarate media is possessed by strains which have now been shown to belong to four of the five species of the genus *Desulfovibrio*. Fumarate dismutation is therefore not a valid taxonomic character for this group of organisms. Thus, except for dismutation of pyruvate, the utilization of carbon substrates does not appear to be useful in characterizing these organisms.

Strain EL AGHEILA A presents a taxonomic problem similar to that of Postgate's (1963*b*) oxamate-utilizing strain MONTICELLO 2. In its ability to grow on pyruvate in the absence of sulphate, EL AGHEILA A resembles *Desulfovibrio desulfuricans*; its DNA base composition, however, places it with the *Dv. vulgaris* group. We have given greater weight to the DNA base composition and have classified EL AGHEILA A as *Dv. vulgaris* (it should be noted that EL AGHEILA A does not utilize oxamate when tested by the method of Postgate, 1963*b*).

Our results with *Desulfotomaculum nigrificans* strains in sulphate-free medium confirm the previous observations of Postgate (1963*a*) and Akagi (1964) with this species.

With this study of 22 previously unclassified strains, most of the strains of the genera *Desulfovibrio* and *Desulfotomaculum* at present available from recognized culture collections have now been examined. The findings are consistent with the working classification schemes proposed for these two genera by Campbell & Postgate (1965) and Postgate & Campbell (1966).

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The Composition of Normal and Elongated *Mycobacterium lepraemurium*

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SUMMARY

A process is described for preparing suspensions of *Mycobacterium lepraemurium* from livers of infected mice, free from traces of host tissue. The organisms doubled in length when incubated in 'elongation medium' for 12–14 days but did not multiply. Analyses of normal and elongated organisms were made for lipid, phosphorus, nucleic acids, hexosamines, neutral sugars and α-amino nitrogen. A doubling of dry weight occurred during elongation, and there was a net synthesis of each of the components measured. Lipid and neutral sugar more than doubled, phosphorus, nitrogen and total nucleic acid rather less than doubled and hexosamine and DNA increased by about one third.

INTRODUCTION

The development of elongated forms by various bacteria grown in adverse conditions is a familiar phenomenon. When Hart & Valentine (1960, 1963) found that *Mycobacterium lepraemurium*, an organism previously uncultivated *in vitro*, would elongate in a simple medium, it seemed possible that some small modification of the medium might allow multiplication of the organism. Ultraviolet and electron microscopy seemed to confirm the vital nature of the elongation process (Hart, 1965), and the increase in length was accompanied by a proportional increase in weight, as measured by electron scattering (Valentine, 1962). On the other hand, measurement of protein, nucleic acids and muramic acid in the bacteria suggested that none of these constituents was synthesized during elongation (Hart, 1965). This paper describes an attempt to resolve this paradox by a more detailed analysis of larger quantities of the organism.

METHODS

Suspension of organisms. Mycobacterium lepraemurium, strain DOUGLAS, was obtained aseptically from experimentally infected mice (Parkes albino strain). A crude suspension of bacteria in bovine serum albumin (0·1 % in 0·9 % NaCl) from the homogenized livers of two mice was digested at 37° with Pronase (40 μg./ml.) in 20 ml. 0·05 M-tris in Gey solution (pH 7·6). After 4 hr the bacteria were collected by centrifugation, suspended in tris HCl buffer (0·1 M, pH 7·2) and applied to sucrose density gradients. The gradients consisted of 5 ml. of 13·5 % (w/v) sucrose in 0·1 M-tris buffer (pH 7·2) layered on 5 ml. of 50 % (w/v) sucrose in the same buffer, and allowed to stand for 12–20 hr before use. The gradients were centrifuged at 1000*g* for 1 hr, the bands of bacteria removed through the side of the tubes (cellulose nitrate) into sterile syringes, and the bacteria washed and suspended in a measured small volume of 0·1 % albumin in 0·9 % NaCl. About 2×10^{12} bacteria were usually obtained

(about 20 mg.). Suspensions for analysis were washed several times in 0·1 M-tris HCl (pH 7·2) containing 0·05 % Tween 80, then in 0·05 % Tween 80 and finally suspended in water.

Elongation of organisms. Volumes containing 2×10^{11} organisms of purified *Mycobacterium lepraemurium*, determined by direct counts of dilutions of the suspensions (Hart & Rees, 1960), were inoculated into 200 ml. of 'elongation medium' at pH 6·4 (Hart & Valentine, 1963), containing benzylpenicillin 0·24 mg./ml. in Thompson bottles. The bottles were incubated undisturbed at 37° for 12–14 days. Bacterial contamination of the suspensions was excluded before and after elongation by examining smears stained by the Ziehl–Neelsen method, and by inoculating samples into Headley–Wright broth. For analysis the elongated bacteria were collected and washed in the same way as the normal ones. Suspensions were usually counted to check recoveries.

Analysis of bacterial constituents. To avoid transferring very small amounts of dry material, analyses were performed on measured volumes of suspensions with known dry weights and (in most batches) known bacterial populations. For dry weights bacteria were dried for 16 hr at 80° in weighed glass vials. Populations were determined by direct counting of stained suspensions of bacteria (Hart & Rees, 1960). Amounts of bacterial constituents were calculated as % of the dry weight of organisms.

Size of organisms. Lengths of bacteria were measured with the electron microscope.

Lipids. Dried material was repeatedly extracted at room temperature with chloroform + methanol (2 + 1 by vol., 4 × 1 ml., 24 hr each extraction). The residual material was dried at 80° and reweighed.

Phosphorus. Samples were ashed and phosphorus contents measured by the method of Chen, Toribara & Warner (1956). 'Non-lipid phosphorus' was measured in the insoluble residue after extraction with chloroform + methanol (see above), and nucleic acid phosphorus in ashed nucleic acid extracts (see below).

α-Amino nitrogen. Samples were hydrolysed in 4 N-HCl for 16 hr at 105°. α-Amino nitrogen in the hydrolysates was measured with ninhydrin (Rosen, 1957), with DL-alanine as standard.

Nucleic acids. Nucleic acids were extracted from bacterial samples with two or three portions of 5 % trichloroacetic acid at 90° for 10 min. The DNA in these extracts was measured with diphenylamine (Burton, 1956), with deoxyribose as a standard, and RNA with orcinol (Schneider, 1957) using ribose as a standard. Total nucleic acids were determined spectrophotometrically. Bacterial samples were extracted successively as follows, supernatant extracts being discarded in each case: 0·2 N-$HClO_4$ for 10 min. at 0°; 75 % (v/v) aqueous ethanol (buffered with 0·1 M-ammonium acetate) for 15 min. at 37°; buffered ethanol + ether (1 + 1, v/v) for 15 min. at 37°. After this preliminary treatment nucleic acids were extracted from bacterial samples with trichloroacetic acid (as above), the solutions were made 0·1 N in $HClO_4$ and the trichloroacetic acid was removed by ether extraction. Nucleic acids were determined from the extinction at 260 mμ of these solutions, with sodium ribonucleate from yeast as a standard. Contamination with protein was detected as extinction at 232 mμ (Munro & Fleck, 1966).

Hexosamines. Samples were hydrolysed in 4 N-HCl for 4 hr at 105°. Hexosamines were measured by the method of Blix (1948), with glucosamine hydrochloride as a standard. To test for possible interference by other substances in the hydrolysate

individual hexosamines were determined in a hydrolysate from a large batch of normal *Mycobacterium lepraemurium*, by using the modification by Crumpton (1959) of the ion-exchange separation of Gardell (1953).

Neutral sugars. Samples were hydrolysed in 2 N-H_2SO_4 for 3 hr at 105°. Total reducing sugars in the hydrolysed samples were measured by the phenol + sulphuric acid method (Dubois *et al.* 1956), with glucose and galactose as standards. Individual components were identified by thin-layer chromatography on cellulose (Vomhof & Tucker, 1965). The plates, dried at 35° and ambient humidity, effected satisfactory separations of sugars with the solvent advocated, formic acid + butan-2-one + *tert*-butanol + water (15 + 30 + 40 + 15 by vol.; Fink, Cline & Fink, 1963) as well as with ethyl acetate + pyridine + water (2 + 1 + 2, by vol.; Jermyn & Isherwood, 1949) and *n*-butanol + acetic acid + water (4 + 1 + 1, by vol.).

Sugars were detected with alkaline silver nitrate (Trevelyan, Procter & Harrison, 1950), aniline phthalate (Partridge, 1949) or periodate + benzidine (Cifonelli & Smith, 1954).

Amounts of various components were estimated by gas-liquid chromatography of trimethylsilyl ethers of the sugars, kindly done by Mr N. Gregory. Amounts of glucose in hydrolysates were measured with glucose oxidase (Bergmeyer & Bernt, 1963).

Chemicals. Sodium ribonucleate from yeast was supplied by British Drug Houses Ltd., glucose oxidase was from Koch-Light Ltd., Pronase from Calbiochem and MN-cellulose powder 300G from Macherey, Nagel and Co.

RESULTS

Length and weight of organisms. The mean weight of normal *Mycobacterium lepraemurium* was $1{\cdot}33 \pm 0{\cdot}23$ S.D. $\times 10^{-14}$ g. (5 batches), and of elongated bacteria $2{\cdot}65 \pm 0{\cdot}61$ S.D. $\times 10^{-14}$ g. (7 batches). The bacterial weights obtained directly and the bacterial lengths measured electron microscopically in a single experiment are compared in Table 1. Electron microscopy, according to the methods of Valentine (1962), also showed that the densities of normal and elongated bacteria were similar, so that weight increased proportionally to length.

Table 1. *Weights and lengths of Mycobacterium lepraemurium*

	Dry wt per organism (g.)	Length (μ)
Normal	$1{\cdot}7 \times 10^{-14}$	1·62 (range 1·0–2·6)
Elongated	$2{\cdot}8 \times 10^{-14}$	3·08 (range 1·2–5·6)
Relative increase	1·65	1·9

Dry weights were measured by drying known numbers of bacteria at 80° for 16 hr. Lengths were measured directly with an electron microscope. The results are for a single batch of bacteria.

Lipids. The lipid content of bacteria was large, and in the elongated bacteria variable. Material amounting to $27{\cdot}5 \pm 2{\cdot}5$ S.D. % (4 batches) of the dry weight of normal bacteria and $32 \pm 6{\cdot}2$ S.D. % (7 batches) of elongated bacteria was soluble in chloroform + methanol.

Phosphorus. Normal bacteria contained 1·3 % (3 batches) of phosphorus, elongated bacteria $1{\cdot}15 \pm 0{\cdot}06$ S.D. % (6 batches). Chloroform + methanol extraction removed 0·26 % (1 batch; 20 % of the total phosphorus) from normal bacteria and $0{\cdot}27 \pm 0{\cdot}02$

S.D. % (3 batches; 23 % of the total phosphorus) from elongated bacteria. Trichloroacetic acid extracts from normal bacteria contained phosphorus corresponding to 1·1 % (2 batches) of the dry weight of the organisms, from elongated bacteria to 0·9 % (4 batches). These amounts correspond to 11 and 9 % nucleic acid, respectively (calculated as RNA).

Nucleic acids. Normal bacteria contained 4·9 ± 0·3 S.D. % (3 batches) and elongated bacteria 4·1 ± 0·9 S.D. % (5 batches) of total nucleic acids, measured spectrophotometrically. Normal bacteria contained 0·74 ± 0·05 S.D. % (4 samples) of deoxyribose, corresponding to 3·4 % DNA and, by difference from the total nucleic acid, 1·5 % RNA. Elongated bacteria contained 0·44 ± 0·10 S.D. % (9 batches) of deoxyribose, corresponding to 2·0 % DNA and 2·1 % RNA by difference. The calculated amounts of DNA assume that it contains 22 % deoxyribose, which is correct only for DNA containing equal numbers of purine and pyrimidine bases.

Ribose, measured by the orcinol reaction, was apparently present in bacteria as follows: normal 8·0 ± 0·05 S.D. % (4 batches), elongated 6·3 ± 0·5 S.D. % (8 batches). These values correspond to 35 and 27 % of RNA, respectively, assuming RNA contains 23 % ribose. The orcinol reaction is not very specific, and it is probable that other carbohydrate (see below) was extracted from the bacilli together with the nucleic acids and contributed to the colour obtained in the orcinol reaction.

α-Amino nitrogen. Normal bacteria contained 5·0 % of nitrogen (2 batches), elongated bacteria 4·1 % (2 batches), measured as alanine.

Table 2. *Amounts of neutral sugars in Mycobacterium lepraemurium, relative to arabinose, measured by gas-liquid chromatography*

	Normal bacteria	Elongated bacteria
Arabinose	1·0	1·0
Galactose	0·6	0·5
Glucose	1·8	0·5
Inositol	0·3	0·1
Mannose	0·8	0·6

Table 3. *Amounts of various substances in normal and elongated Mycobacterium lepraemurium*

	Weight (units of 10^{-14} g. per organism)		
	Normal	Elongated	Increase
Whole bacillus (dry wt)	1·33	2·65	2·0
Lipid	0·36	0·86	2·4
Phosphorus	0·017	0·030	1·8
Total nucleic acid	0·065	0·11	1·7
Deoxyribose	0·01	0·013	1·3
α-Amino nitrogen	0·066	0·11	1·6
Hexosamine	0·028	0·037	1·3
Neutral sugar	0·20	0·52	2·6
Ribose	0·11	0·17	1·5

Hexosamines. Total hexosamines in normal bacteria were 2·1 % (2 batches) and in elongated bacteria 1·4 % (3 batches). Further analysis of one sample indicated that normal bacteria contained 0·82 % of glucosamine, 0·71 % of muramic acid, 0·49 %

of an unknown material eluted from the column more slowly than other components and 0·32 % of galactosamine.

Neutral sugars. Total neutral sugars were as follows: normal bacteria 14·8 ± 1·3 S.D. % (3 batches), elongated bacteria 19·6 ± 3·4 S.D. % (4 batches), as galactose. Glucose oxidase detected 4·8 % of glucose in normal and 4·4 % in elongated bacteria (1 batch each). Thin-layer and gas-liquid chromatography showed the following sugars to be present: glucose, galactose, arabinose, mannose and inositol. A fast-running spot was observed on thin-layer chromatograms, possibly a deoxyhexose, and small amounts of at least three unidentified components were detected by gas-liquid chromatography. The amounts of the various components relative to arabinose are shown in Table 2. No ribose or ribitol could be detected.

Summary of analyses. The amounts of the various bacterial components expressed in g. dry wt per organism, and the relative increases associated with elongation are summarized in Table 3.

DISCUSSION

At each stage of investigation of the elongation of *Mycobacterium lepraemurium* the question has arisen whether the process could be considered as near-multiplication, so offering a hopeful lead in attempts to cultivate the organism (and possibly *M. leprae*), or whether it was a passive swelling process, and of interest only as a means of identifying the organism. Earlier analyses by Drs J. Mandelstam and A. J. Garrett (reported by Hart, 1965) were hampered by very small numbers of bacteria available, and by contamination of the suspension with material from the liver of the host. About 100 times more bacteria were available for the present experiments, and the treatment with Pronase and density-gradient separation removed all host material as judged immunologically by the Ouchterlony technique.

Although the observed percentages of bacterial components depend only on measured weights, the calculated increases in bacterial weights and amounts of components depend on the recovery of the original inoculum. Counting of suspensions of elongated bacteria indicated recoveries of between 50 and 100 %, but the results were uncertain because some 'clumping' of bacteria occurred. Attempts to separate the bacteria by ultrasonic treatment led to lower counts, presumably because the bacteria were fragile. Recovery was therefore assumed to be complete in each case, and consequently it is possible that increases in bacterial dry weights and amounts of individual components were actually somewhat greater than reported.

If a normal cell of *Mycobacterium lepraemurium* is taken to be a cylindrical rod 1·5 μ long and 0·13 μ in radius, its volume is $8{\cdot}1 \times 10^{-14}$ ml. Undried *Mycobacterium tuberculosis* contains 86 % of water, and has a density 1·1 g./ml. (Luria, 1960). Assuming a similar water content for undried *M. lepraemurium*, a density of 1·1–1·2 g./ml., confirmed by its behaviour in the sucrose density gradients, and a volume of $8{\cdot}1 \times 10^{-14}$ ml., its wet weight should be $8{\cdot}9$–$9{\cdot}7 \times 10^{-14}$ g., and its dry weight $1{\cdot}25$–$1{\cdot}36 \times 10^{-14}$ g., a value which agrees well with the dry weight of normal bacteria measured here. Direct weighing of bacteria confirmed the evidence obtained with the electron microscope that an increase of bacterial weight occurred during elongation.

Each of the components measured increased in total amount in the elongated bacteria. Only small increases of DNA and hexosamines were found. A failure to synthesize sufficient new DNA might explain the failure of the bacteria to divide.

Hexosamines occur especially in the cell-wall mucopeptide, and provide a measure of the increase of the 'backbone' of the cell wall.

Increases rather less than proportional to the weight increase occurred in phosphorus, total nucleic acids (measured by u.v. absorption) and α-amino nitrogen (mainly derived from protein). RNA measured by the orcinol reaction also increased but was grossly interfered with by other material extracted by trichloroacetic acid, probably carbohydrate. An attempt to extract RNA selectively by the Schmidt–Thannhauser process (Munro & Fleck, 1966), which uses dilute alkali, did not yield any ultraviolet absorbing material. The RNA content calculated by subtracting DNA from total nucleic acids also increased. Although the possibility that there was some ultraviolet absorbing non-nucleic acid material in the extracts prevents great reliance being placed on this increase, it appears that more new RNA is synthesized than DNA by the elongated bacteria. The ratio E_{232}/E_{260} was greater for bacterial nucleic acid extract than for pure RNA, indicating that some protein was present. The extracts also contained more phosphorus than could be accounted for as nucleic acid.

Lipid increased on average more than proportionally to the weight, as did carbohydrate, but there were large variations in lipid content and increase in different batches. Agreement between duplicate samples of each batch indicated that the variations were not primarily due to loss of material in extraction. In one experiment a decrease in percentage (though not of absolute amount) occurred. Carbohydrate increased regularly, though different batches had differing initial contents. Of the various components mannose and inositol are probably derived from a phospholipid similar to that studied in *Mycobacterium phlei* by Lee & Ballou (1965), glucose may form a bacterial glycogen such as that described by Chargaff & Moore (1944) in *M. tuberculosis*, and galactose and arabinose may occur in an arabinogalactan such as that found by Misaki & Yukawa (1966) in *M. tuberculosis* strain BCG. Mr M. R. Young (see Hart, 1965) described an increase in numbers and size of bacterial granules which absorbed at 257 mμ and could be stained (in elongated bacteria only) by euchrysin, a dye which stains DNA. Since DNA and phosphate did not increase excessively in the bacteria, the suggested identification of the granules as DNA or as polyphosphate carrying DNA seems unlikely. It is more likely that they are of lipid or carbohydrate, possibly carrying absorbed DNA. The presence of DNA on or in the granules would suggest some disorganization of the elongated bacteria. Schaefer & Lewis (1965) showed lipid granules in *Mycobacterium kansasii*.

Kátó & Gözsy (1966) described a medium, rather different from elongation medium in which a limited multiplication of *Mycobacterium lepraemurium* occurred, with an optimal pH 8·5 (our elongation medium is at pH 6·0–6·5) and containing 2% of sodium chloride and a plant polysaccharide, gum guar. Multiplication could be maintained in the medium for six generations of bacteria. It is not clear what relation elongation at acid pH has to multiplication in Kátó's alkaline-galactomannan medium or what relation either has to multiplication of the bacteria *in vivo*. Neither elongation nor multiplication occurs in elongation medium at alkaline pH.

The conclusion of Hart (1965) that elongation was a 'low-grade form of living growth *in vitro*' has been confirmed by analysis; *Mycobacterium lepraemurium* was apparently able to synthesize at least a little of all the macromolecular substances needed for growth. It remains to be investigated which components of the medium provide the raw materials.

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The Occurrence of the Root-nodule Endophytes of *Alnus glutinosa* and *Myrica gale* in Soils

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SUMMARY

Twenty-one soil samples from sites in West Scotland where alder (*Alnus glutinosa*) has not grown for many years were examined for the presence of the alder root-nodule endophyte by observing the production of nodules on test plants which were grown (*a*) in water culture and their roots inoculated with an aqueous suspension of a soil sample, or (*b*) in the soil itself. Twelve of the samples gave positive results, most of the remaining samples were at pH < 4. Ten samples from sites free from bog myrtle (*Myrica gale*) were similarly tested for the corresponding endophyte; 8 of these gave positive results. To explain these results it seems necessary to assume either that infective bodies of the endophyte can survive in soils for long periods, though not grow; or that the endophytes can grow saprophytically in soils and thus are not near-obligate symbionts.

INTRODUCTION

Alnus glutinosa (L.) Gaertn. (alder) and *Myrica gale* L. (bog myrtle) are the commonest examples in Britain of non-leguminous plants with nitrogen-fixing root nodules. The regular occurrence of root nodules on these species in the field raises the question of the source of the micro-organisms whose infection of the roots is necessary for nodulation to occur. On the basis of previous studies in this Department (Bond, 1963) the nodules of these two species are believed to be tenanted by distinct, specific organisms. Long experience here confirms that seed collected directly from the parent plants of these species does not carry the nodule organism, since plants grown from such seed in sterile media remain free from nodules. Under field conditions infection of young plants must occur from the soil.

In considering how the presence of the appropriate endophyte near a germinating seed could regularly arise, there is the difficulty that we have very little knowledge of the life history and growth requirements of these endophytes. The continued failure of attempts to isolate the endophytes of these and other non-leguminous plants into pure culture encourages the belief that they are near-obligate symbionts. One well-established feature, important in the present connexion, is that infective forms of the endophytes are present in the soil immediately around a nodulated plant. This is clear from the fact that one way of raising nodulated plants of these non-legumes is to sow seed in soil collected from near nodulated plants of the appropriate species. Experience in this laboratory has shown that this infectivity of habitat soils persists for at least several months in the absence of the host plant.

Three possible explanations of the regular presence of nodules in the field suggest themselves.

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(1) Seed and endophyte may be disseminated from parent stands of host plants more or less simultaneously and by the same agents, of which wind would be the most important. Infective forms of the endophytes might be carried on soil dust or on leaves infected by contact with the soil.

(2) If the infective forms present around nodulated plants have considerable longevity, though they may be incapable of growth except in the vicinity of the host plant, then bearing in mind that prior to land drainage and clearance alder and bog myrtle were very widespread in Britain, a very broad distribution of the endophytes at the present time could be explained.

(3) If the belief, adopted in the two previous explanations, that the endophytes are near-obligate symbionts be set aside as unproved, then it may be supposed that they can grow saprophytically in soil and may have attained a wide distribution largely independent of that of the host plants, past or present.

It seemed possible that by a suitable examination of soil samples from sites where the host plants are not now growing, the choice between these suggestions could be narrowed down. Accordingly, soil samples were collected from various sites in the Glasgow region and tested for ability to induce nodulation in host seedlings growing in the greenhouse.

A difference between alder and bog myrtle is that while alder propagates by seed, under Scottish conditions bog myrtle chiefly spreads vegetatively by suckers and thus forms compact colonies. With alder the problem of the source of infection arises for every seedling except for those situated very close to the parent tree. With bog myrtle it is obviously possible that the soil near a colony becomes generally infected with the endophyte, for example as a result of water movement, so that roots of new suckers will be automatically infected. With bog myrtle it is chiefly at the initiation of a colony that the problem of the source of infection arises.

METHODS

General. Two methods were used: (1) The test plants were grown in water culture and inoculated by application to their roots of a water extract of a soil sample. This method has the advantages that standard and favourable conditions are provided for nodule development when the organism is present, while ready inspection of the root system is possible from day to day. (2) Young test plants were transplanted into the actual soil sample and allowed to grow there. It is possible that when the hyphae or resting bodies of the endophyte are present very sparsely in the soil, this might be a surer way of securing eventual contact between them and the roots. On the other hand, there is the risk that because of a very small pH value, or a richness in nitrogen, a soil might not be favourable to nodulation despite the presence of the endophyte. There is also the drawback with method (2) that the result of the experiment cannot be assessed before harvesting.

Collection of soil samples. Twenty-three soil samples were collected from sites within a 30-mile radius of Glasgow (Table 1). Two samples (nos. 22, 23) were from sites where alder trees were present and four (nos. 6–9) from sites carrying bog myrtle bushes; these habitat soils were included as a test of the efficacy of the methods for detecting the endophytes. With these exceptions the sites were free from both host plants and have been so for at least the periods indicated in Table 1. All samples were tested

for the presence of the alder endophyte, but only a proportion for the bog myrtle endophyte.

In collecting the samples sterile metal cans of 500 ml. capacity were used as digging implements, being held by the base so as to obviate contamination by the hands. The samples were taken from the top 10–15 cm. of soil after removal of any surface litter. Immediately after collection the samples were transferred to new plastic bags. The samples were used 2–4 days after collection.

Table 1. *Details of soils used and results of tests of their capacity to induce nodules in Alnus glutinosa and Myrica gale*

Soil sample no.	Ordnance map grid reference	Present description of site	Years of such use	pH value of sample at collection	No. of test plants forming nodules* *Alnus glutinosa* Water culture	*Alnus glutinosa* Soil culture†	*Myrica gale* Water culture	*Myrica gale* Soil culture†
1	NS 550705	Garden	50	6·6	0	0	—	—
2	NS 564679		50	6·7	0	0	—	—
3	NS 546716		100	7·3	0	4	—	1
4	NS 303828		100	6·5	0	11	1	2
5	NS 537674		90	6·8	0	0	—	0
6	NN 537956	Grouse moor, bog myrtle present	> 100	3·8	0	0	3	11
7	NN 537956		> 100	3·8	0	0	—	—
8	NN 533956		> 100	3·9	1	0	—	6
9	NN 533956		> 100	3·9	0	0	—	—
10	NN 534939	Conifer plantation	20	4·4	1	0	—	12
11	NN 535944		20	3·6	0	0	—	0
12	NN 499014		38	3·5	0	0	1	0
13	NN 499014		38	3·6	0	0	—	0
14	NN 547007		32	6·6	4	9	0	3
15	NN 547007		32	4·1	7	6	—	—
16	NN 532922	Arable field	> 50	5·8	0	9	—	—
17	NN 656004		> 50	6·3	0	4	—	—
18	NN 653954		> 50	5·7	1	0	—	—
19	NN 649954		> 50	5·7	0	3	0	2
20	NN 664993	Pasture	> 50	6·1	0	8	—	—
21	NN 662955		> 50	6·1	0	10	—	—
22	NS 548758	Amenity woodland, alder present	20	5·7	7	16	6	12
23	NS 561746		> 50	3·3	7	0	—	3

* In the water-culture method one jar containing seven plants of alder or six plants of bog myrtle was set up for each soil sample. In the soil-culture method two pots, each containing eight plants of alder or six plants of bog myrtle were set up for each soil sample; the total number of nodulated plants in the two pots together is given above.

† Statistical analysis of the data for the duplicate pots set up shows that the totals of plants forming nodules in any two soils should differ by at least 4 for significance at $P = 0{\cdot}05$ for alder, and by 5 for bog myrtle.

Germination of seed for test plants. Seed of the two host species was collected locally, and after storage at 2° for 2 months was surface-sterilized by being shaken for 45 min. in a 2 % (w/v) solution of calcium hypochlorite, followed by thorough washing with sterile water. The seed was then sown in Peralite (British Gypsum, Ltd., Cocklakes, Carlisle) moistened with nitrogen-free nutrient solution. The seedlings were used as indicated in the following section.

Setting up of test plants. For the water-culture method Crone's culture solution (nitrogen-free formula) was used, adjusted to pH 4·8 for alder and to pH 5·4 for bog myrtle. Containers consisted of sterilized 2 l. earthenware jars covered by teak squares previously dipped in molten paraffin wax, with seven holes for the plants. The seedlings were transplanted into water culture when two leaves had emerged, and 2·5 mg. ammonium nitrogen was then added to each jar to give slight support to the seedlings during the period normally required for nodulation. An inoculum was prepared from each of the soils by shaking 50 g. with 50 ml. sterile water in a flask for 10 min., standing the flask for 30 sec. and then filling a 20 ml. specimen tube with the supernatant suspension. The inoculation was effected by raising the tube so that the root systems of the plants in the teak top (now held in the hand) were immersed in succession, each for 3 sec. The inoculations were made a few days after transplanting, and from each soil sample one jar with seven alder plants was inoculated, while from a proportion of the soil samples one jar with six bog myrtle plants was similarly inoculated. The remainder of the soil–water mixture was used for pH determination by glass electrode. Some jars were left uninoculated to serve as controls, and were interspersed on the greenhouse bench among the inoculated jars. The plants were allowed to grow for 13 weeks after inoculation.

For the soil-culture method autoclaved clay pots (10 cm. wide at the top, 9 cm. deep) were used. Two pots were filled from each sample of soil for alder and, in some cases, another two for bog myrtle. Since many of the soil samples were from areas of low fertility, a fertilizer supplement was added to all pots to obviate the possibility of nodulation and plant growth being hindered by mineral deficiencies. This took the form of the addition to each pot of 0·14 g. $CaHPO_4$ + 0·1 g. K_2SO_4; test showed that this addition had negligible effect on the soil pH value. Eight seedlings of alder or six of bog myrtle were transplanted into each pot. Control pots containing autoclaved soil or in some cases Peralite were also set up, and were interspersed among the normal pots on the greenhouse bench. Distilled water was added to the pots at frequent intervals to restore them to their original weight. The plants were allowed to grow in the soils for 11 weeks.

RESULTS

Alder

No plant developed nodules in any of the eight uninoculated jars (total 56 plants) set up in water culture, nor in any of the 10 control pots (total 80 plants) set up in soil culture. Thus accidental infection of plants by air-borne dust or by contamination from adjacent jars or pots did not affect the outcome of the experiment as a whole.

The results obtained are included in Table 1. The two soils from alder sites (nos. 22, 23) both induced nodulation of all the test plants in the water-culture method, after only 15 days from inoculation. Nodulation was also complete with no. 22 in the soil-culture method, but did not occur with no. 23; this was very probably because the pH value of this soil, which had fallen considerably in recent years owing to deteriorating drainage, is now too low (pH 3·3) to permit nodulation. Ferguson & Bond (1953) found that no nodules developed in alders in water culture in response to inoculation at pH 3·3, and only a few at pH 4·2.

With the remaining 21 soils, all from sites where alders are not now growing, some degree of nodulation was shown in five samples by the water-culture method, although

the speed of nodulation was much below that with the habitat soils. By the soil-culture method nine soils gave positive results, the greater sensitivity being presumably due to the consideration mentioned in discussing this method on page 190. The disparity between the two culture methods was sometimes quite large, e.g. in soils nos. 4, 20 and 21. Taking both methods together, evidence of the presence of the alder endophyte was obtained in 12 of the soils, comprising two from gardens, one from acid moorland, three from conifer plantations, four from arable fields, and two from pasture fields.

In the water-culture method, where the medium was nitrogen-free, the plants which formed nodules showed superior growth evidently as a result of nitrogen-fixation, since the degree of growth depended on the number and time of formation of nodules. With the soil-culture method, however, the incidence of nodules in the different soils could not be determined by inspection of the tops, since some of the soils contained sufficient combined nitrogen to sustain healthy growth for the duration of the experiment.

Bog myrtle

In the more limited tests for the bog myrtle endophyte, the two soils (nos. 6, 8) from sites where the host plant was present both showed evidence of the presence of the endophyte. Bond (1951) found that a few bog myrtle plants succeeded in forming nodules in water culture at pH 3·3, and that many did at pH 4·2. Of the 10 other soils tested by one or both culture methods, eight showed evidence of the presence of the endophyte to various degrees. Again the soil-culture method gave more positive results than the water-culture method. As with alder, nodulated bog myrtle plants showed superior growth in the water-culture method, evidently as result of nitrogen-fixation. The same was true in the soil-culture method where fertility was sufficiently low.

DISCUSSION

It is evident that the alder and bog myrtle endophytes are fairly widely distributed in Scottish soils in which the host plants are not now growing. For alder it is probable that the results reported underestimate the prevalence of the endophyte, since in those soils of pH < 4 it is possible that the endophyte was present but was unable to induce nodulation in the soil-culture method because of the smallness of the pH value. It had been expected that in such circumstances the water-culture method would reveal the presence of the endophyte, but this method proved to be of low sensitivity.

The explanation of this widespread occurrence will now be considered in the light of the possibilities listed in the Introduction. With reference to the first of these the proximity of host plants to the sites where the endophytes proved to be present is important. A hilly terrain and limitation of access prevented a thorough examination of the area around a few of the sampling sites, but in the other instances it was established that the nearest host plants were too remote (800 m. or more away) for it to be supposed that the presence of the endophyte was due to its conveyance by wind or other factor from host stands.

The second suggested explanation supposes that infective parts of the endophyte can survive in the soil for long periods in the absence of the host. The data in Table 1 show that infective agents were present in soils which had not carried the host plants for at least 100 years. These were, however, garden soils, and there is a possibility that during that period the endophytes might have been introduced to the soils in added

leaf-mould, peat, etc. The same could apply to the agricultural soils, but this objection does not apply to soil samples nos. 14 and 15, from Forestry Commission conifer plantations near Aberfoyle, nor to no. 10. Thus according to this explanation, infective power must have survived in the soil for at least 32 years, since there is no certainty that host plants were growing on the sites up to the time of their conversion to their present uses.

The ability of micro-organisms to survive for long periods under conditions which preclude active multiplication was reviewed by Sussman & Halvorson (1966), who quoted instances of the survival of spores of various organisms including actinomycetes for periods of years. Jensen (1961) reported the survival of various bacteria, actinomycetes and fungi for 10–40 years when put into autoclaved air-dry soil; Sneath (1962) provided evidence of the persistence of bacteria in air-dry soil for at least 300 years, and of saprophytic actinomycetes for some 100 years. It appears possible that infective parts of the alder and bog myrtle endophytes might survive for long periods in the absence of host plants. If these infective parts originate in nodules it is possible that they are the so-called 'bacteroids' which persist in older parts of alder and bog myrtle nodule clusters from which other endophytic structures have disappeared.

The third possible explanation can also account for the findings of the present study but is open to objection on other evidence. The survey indicates that the usual reason why plants of alder and bog myrtle are regularly nodulated in the field in Scotland is that under existing conditions the soils already contain the requisite endophyte.

Pommer (1956) reported that pseudonodules can form in alder as a result of infection by common soil fungi, such as *Cylindrocarpon radicicola* or *Penicillium albidum*; such nodules were ephemeral and fixed no nitrogen. In the present study no such nodules were detected, and in all instances the presence of nodules was attended by obvious gain to the plant, except in a few cases in the soil-culture method where this could not be judged owing to the high fertility of the soil.

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The Metabolism of Formic Acid by *Sarcina ventriculi*

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SUMMARY

Sarcina ventriculi possesses a hydrogen-producing mechanism similar to that present in the coli-aerogenes group of bacteria. Formate is converted to hydrogen and carbon dioxide by formate hydrogenlyase and *S. ventriculi* is thus the only organism outside the coliform group known to possess this system. Formate dehydrogenase has also been demonstrated in the intact *S. ventriculi* organisms with both methylene blue and benzylviologen as hydrogen acceptors, but with extracts activity was only observed with benzylviologen. Hydrogenase activity was present in whole organisms and extracts of *S. ventriculi*. Hydrogenlyase, hydrogenase and, to a lesser extent, formate dehydrogenase activities in *S. ventriculi* are extremely sensitive to inhibition by oxygen. It is important support for the postulated mechanism of action of formate hydrogenlyase in the coliform bacteria that *S. ventriculi* also possesses the enzymic components deemed necessary for this activity, namely formate dehydrogenase (benzylviologen) and hydrogenase.

INTRODUCTION

The occurrence of a gastric fermentation caused by a sarcina was first recorded in 1842 by John Goodsir, an Edinburgh Surgeon, who proposed the name *Sarcina ventriculi* for the organism which he found in the gastric fluid of one of his patients. Although *S. ventriculi* subsequently was widely reported in the nineteenth century, early workers failed to cultivate the organism in artificial media. Falkenheim (1885) caused considerable confusion in the literature by describing easily grown aerobic sarcinas as *S. ventriculi*. This error was perpetuated by a number of workers and became accepted in the literature of microbiology by its incorporation in standard reference works (*Bergey's Manual of Determinative Bacteriology*, 1923–39; *Topley and Wilson's Principles of Bacteriology and Immunity*, 1st to 4th eds., 1929–55). Beijerinck (1905), during a series of soil enrichment experiments, was surprised to find *S. ventriculi* in certain of his cultures. This was the first isolation of the organism in pure culture and his work demonstrated the conditions necessary for the growth of *S. ventriculi*. Enrichment from soil was obtained under anaerobic conditions in a complex glucose-containing medium at acid pH. In 1911 Beijerinck succeeded in isolating *S. ventriculi* from the human stomach and was able to show that the gastric form was identical with that found in soil. Later, the organism was extensively studied by Smit (1930) who found that ethanol and carbon dioxide, together with smaller amounts of acetate and hydrogen, were the major products of glucose fermentation. Kluyver (1931) also

published a fermentation balance for *S. ventriculi* which agreed closely with the results of Smit. Milhaud, Aubert & van Niel (1956) showed that glucose was fermented by the Embden–Meyerhof route, while Bauchop & Dawes (1959) and Arbuthnott, Bauchop & Dawes (1960) produced evidence that the terminal fermentation mechanism in *S. ventriculi* consisted of a unique combination of two pathways of pyruvate catabolism, namely a yeast-type pyruvate decarboxylase and a coliform-like thioclastic fission of pyruvate. These reaction mechanisms adequately accounted for the fermentation products previously reported. The present report concerns the mechanism of hydrogen formation in *S. ventriculi*.

METHODS

Organism. Sarcina ventriculi was isolated from garden soil. Approximately 2 g. soil were placed in a 250 ml. Erlenmeyer flask filled with medium A; the flask was plugged with cotton-wool and incubated at 37°. After 16–20 hr a vigorous fermentation began and large numbers of sarcinas were present. The contents of the flask were stirred and 10 ml. inoculated into a second flask containing 200 ml. medium A which had been freshly boiled and cooled. This transfer procedure was repeated three or four times until *S. ventriculi* was established in pure culture. Inoculation from this culture into sterile medium N then enabled the pure culture to be maintained at pH 7·0.

Media. Medium A (for isolation). 2% (w/v) glucose; 1% (w/v) Bacto-peptone (Difco); 1% (w/v) yeast extract (Difco); 0·3% (v/v) conc. HCl. This medium was used without sterilization.

Medium N. Where bacteria were required for biochemical studies the above medium was used with the omission of HCl. This medium as made up was at pH 7·0 and no adjustment was made. The glucose was prepared as a separate solution and added to the remainder of the medium after each portion had been sterilized and cooled.

Conditions of growth. Volumes (400 ml.) of medium N were dispensed into 500 ml. Erlenmeyer flasks which were plugged and sterilized by autoclaving at 120° for 15 min. When inoculation was to be done immediately, the flask was removed from the autoclave, cooled quickly and given an inoculum (5%, v/v) of actively fermenting culture. When the medium had been stored for some time before use, it was boiled for a few minutes and cooled immediately before inoculation. Removal of oxygen from the medium was an essential condition for growth. *Sarcina ventriculi* was cultivated at 37°. In some experiments larger amounts of bacteria were obtained by scaling up the above procedures to 5 or 10 l. batches.

Harvesting of bacteria. This was simplified by the fact that *Sarcina ventriculi* grew as a sediment at the bottom of the culture vessel, which allowed most of the medium to be decanted and the bacteria to be collected by centrifuging the small volume which remained. In biochemical studies only actively fermenting bacteria were used. When large cultures (5 or 10 l.) were grown. the bulk of the medium was syphoned off and the bacteria obtained in a small volume which could be conveniently handled on the centrifuge.

Preparation of active washed bacterial suspensions. During the growth of *Sarcina ventriculi* large volumes of gas were produced. However, preliminary experiments to study the metabolism of glucose, pyruvate and formate by washed bacterial suspensions failed to show gas production. When bacterial suspensions were pipetted from the culture flask directly into Warburg flasks and incubated under N_2, it was found that H_2 and CO_2 were produced by *S. ventriculi* during glucose fermentation. It was sub-

sequently discovered that active washed bacterial suspensions could be obtained when precautions were taken to protect these preparations from atmospheric oxygen.

To obtain consistently active washed bacterial suspensions the following procedures were used. Warburg flasks were prepared with all additions except buffer solution and bacterial suspension, the side-arms stoppered and the manometer joints greased. With an actively fermenting culture the medium was decanted as previously described. The bacterial suspension remaining (20–30 ml.) was made up to 80 ml. with freshly boiled and quickly cooled distilled water. This suspension was transferred immediately to a centrifuge tube and centrifuged at 1500*g* for 2 min. During the centrifugation procedure some of the buffer to be used was boiled and quickly cooled, and the appropriate quantities pipetted into Warburg flasks. By this time the centrifuge had stopped and the bacteria were taken up in 10 ml. oxygen-free buffer solution. This bacterial suspension was pipetted into the Warburg flasks, which were quickly attached to manometers and gassed with N_2 for 4 min. This procedure enabled active washed bacterial preparations to be obtained consistently. Usually small amounts of glucose were carried over, because of insufficient washing; the fermentation of this glucose was allowed to proceed to completion before substrates were added. This procedure was preferable to further washing of the bacteria with possible loss of activity.

Cell-free extracts. These were prepared by grinding bacteria with powdered glass (Utter & Werkman, 1942).

Protein nitrogen was determined by the method of Stickland (1951).

Manometric methods. Standard manometric techniques were used. All experiments were done at 37·5°. The rate of shaking was 110–120 cyc./min.

Viologen dyes. Methylviologen was obtained from Jacobsen Van Den Berg and Co. (73 Cheapside, London, E.C. 2) and benzylviologen from British Drug Houses Ltd.

Powdered glass. This was purchased from Canadian Laboratory Supplies (8655 Delmeade Road, Mount Royal Box 2090, Station 'O', Province of Quebec, Canada) and was Pyrex glass powder passed through a 200-mesh sieve.

RESULTS

Formate hydrogenlyase

Washed suspensions of *Sarcina ventriculi* metabolized formate to equimolar amounts of H_2 and CO_2 (Fig. 1), indicative of the classical hydrogenlyase reaction. Initially the ratio of gases evolved in the absence and presence of alkali was not 2:1, presumably because the initial reaction occurred too rapidly for the CO_2 evolved to be absorbed by the alkali in the centre-well. As the reaction rate decreased the ratio approached 2:1.

The evolution of hydrogen from formate by whole organisms of *Sarcina ventriculi* had a broad pH optimum in the range pH 5·0–7·0, but activity fell to low levels at pH 4·0 and 8·0. The pH optimum of formate hydrogenlyase in whole *Escherichia coli* organisms was 7·0 (Stephenson & Stickland, 1932).

The evolution of hydrogen was sensitive to the inhibitors of the classical hydrogenlyase system (Table 1). Nitrate and nitrite were inhibitory, also extremely low concentrations of cyanide. Inhibition by somewhat higher concentrations of formaldehyde and sensitivity to oxygen and carbon monoxide were also typical of both the *Sarcina ventriculi* and *Escherichia coli* formate hydrogenlyase systems.

The effect of an atmosphere of hydrogen on hydrogen evolution from formate by

Sarcina ventriculi appeared to constitute an important difference from the *Escherichia coli* system. Stephenson & Stickland (1932) obtained 50% inhibition of hydrogen evolution with *E. coli* under these conditions; with *S. ventriculi* an atmosphere of hydrogen produced only 5% inhibition. The 50% inhibition observed with *E. coli* was attributed to reversal of the reaction with H_2 and dissolved CO_2 under the test conditions (Woods, 1936). However, under similar conditions it appears that acid produced from residual glucose in the *Sarcina ventriculi* suspensions causes rapid removal of the CO_2 which is essential for reversing the reaction, and consequently the inhibition observed under H_2 is slight (Table 1).

Table 1. *Inhibition of formate hydrogenlyase of S. ventriculi*

The system contained 20 μmoles of sodium formate (side arm); inhibitors at concentrations indicated; 105 μmoles of potassium phosphate; 11–17 mg. (dry weight) of cells; pH 5·0; total volume 2·8 ml.; 0·2 ml. of 20% (w/v) KOH in the centre-well; atmosphere N_2; temperature 37°. For inhibition with H_2 the pH was 7·0. For inhibition with CO, Warburg flasks were first flushed with N_2, then flushed with CO for 5 min. In the experiments with CO, flasks were enclosed in opaque bags to prevent possible photo-dissociation of CO–Fe complexes.

Inhibitor	Inhibitor concentration (mM)	H_2 produced (μl./hr./mg. dry wt)		Inhibition (%)
		Control	Inhibitor	
$NaNO_3$	1·0	60	22	63
$NaNO_2$	1·0	60	14	76
KCN	0·005	185	93	50
KCN	0·05	185	0	100
HCHO	1·0	222	186	16
HCHO	80·0	222	30	86
H_2	100%	240	228	5
O_2	20%	no specific experiment		100
CO	100%	104	0	100

Reversibility of the hydrogenlyase reaction was demonstrated by the inclusion of bicarbonate in the reaction mixture to neutralize the acid formed from residual glucose; the presence of hydrogen was essential for gas utilization to occur (Fig. 2).

The extreme sensitivity to inhibition by oxygen of the *Sarcina ventriculi* formate hydrogenlyase prevented the demonstration of activity in cell-free extracts. Glutathione and cysteine did not restore activity either to oxygen-inhibited washed suspensions or to extracts of *S. ventriculi*.

Stephenson & Stickland (1932), Yudkin (1932) and Pinsky & Stokes (1952) all found that oxygen had little effect on the preformed hydrogenlyase system in whole *Escherichia coli* organisms. In direct contrast, Lascelles (1948) found that hydrogenlyase activity of *E. coli* was strongly depressed by exposure to oxygen. The demonstration of hydrogenlyase activity in extracts of *E. coli* presented difficulties in the early work of Gest & Gibbs (1952). Later, active preparations were apparently more easily obtainable (Gest & Peck, 1955; Peck & Gest, 1957*a*). More recently Pichinoty (1962) found that hydrogenlyase in extracts of *E. coli* was inactivated irreversibly by contact with air.

Formate dehydrogenase

Escherichia coli possesses two types of formate dehydrogenase activity, with methylene blue and benzylviologen as the respective hydrogen acceptors. If grown with vigorous aeration, the organism displayed no hydrogenlyase activity but formate

dehydrogenase (methylene blue) was present (Stickland, 1929; Gale, 1939). However, Gest & Peck (1955) showed that benzylviologen can act as acceptor in the formate dehydrogenase system with extracts of *E. coli*. These authors and Peck & Gest (1957*a*) also demonstrated that the presence of formate dehydrogenase (benzylviologen) was essential for hydrogenlyase activity.

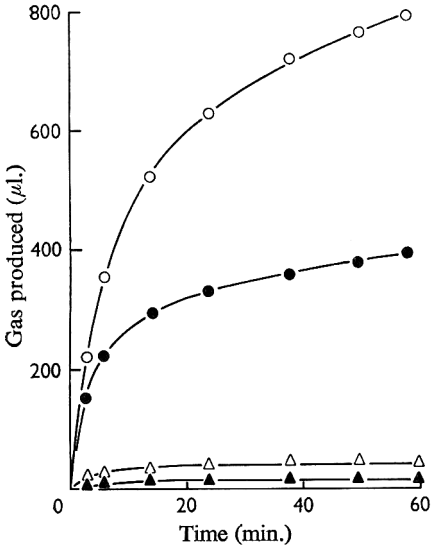

Fig. 1

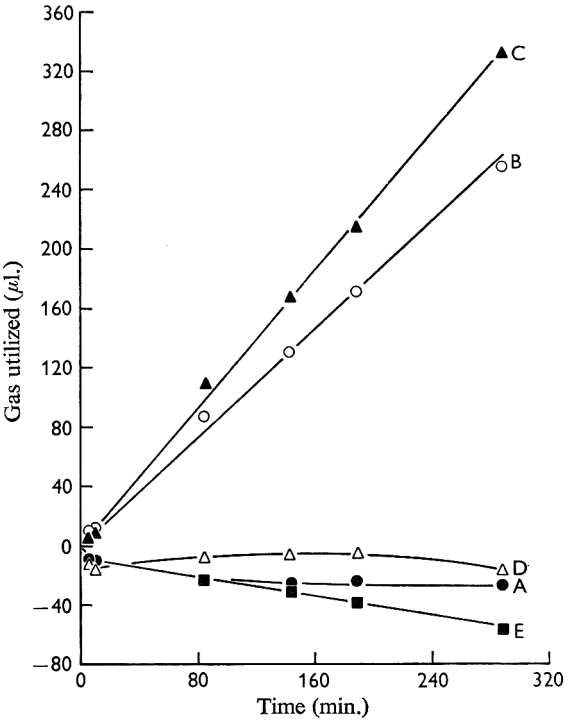

Fig. 2

Fig. 1. Formate hydrogenlyase of *S. ventriculi*. Warburg flasks contained: 20 μmoles of sodium formate (side arm); 57·5 μmoles of potassium phosphate; 11 mg. (dry wt) of cells; pH 5·0; total volume 2·8 ml.; 0·2 ml. of 20 % (w/v) KOH in the centre-well of the Warburg flasks where indicated; atmosphere N_2. H_2+CO_2 evolution, ○; H_2 evolution, ●; endogenous H_2+CO_2, △; endogenous H_2, ▲.

Fig. 2. Reversibility of formate hydrogenlyase in whole cells of *S. ventriculi*. Warburg flasks contained in the main compartment 67 μmoles of either potassium phosphate or $NaHCO_3$, and 16 mg. (dry wt) of cells; side arms contained either water or 25 μmoles of $NaHCO_3$; total volume 2·8 ml., atmosphere, H_2 or N_2 as indicated. All flasks were flushed with H_2 for 5 min. Flasks D and E were then flushed with N_2 for 5 min. Zero time readings were taken immediately and then, where applicable, $NaHCO_3$ was tipped from the side arms. A, 67 μmoles of phosphate buffer, pH 7·4 (main vessel), $NaHCO_3$ absent, H_2, ●; B, 67 μmoles of $NaHCO_3$ (main vessel), H_2, ○; C, 67 μmoles of $NaHCO_3$ (main vessel), 25 μmoles of $NaHCO_3$ (side arm), H_2, ▲; D, 67 μmoles of $NaHCO_3$ (main vessel), N_2, △; E, 67 μmoles of $NaHCO_3$ (main vessel), 25 μmoles of $NaHCO_3$ (side arm), N_2, ■.

Washed suspensions of *Sarcina ventriculi* possessed formate dehydrogenase activity with methylene blue (Table 2). When the bacteria were thoroughly washed, hydrogenlyase was characteristically absent, although high formate dehydrogenase activity remained. Formate dehydrogenase activity has also been successfully demonstrated in whole *S. ventriculi* organisms with benzylviologen as acceptor (Table 2). It was note-

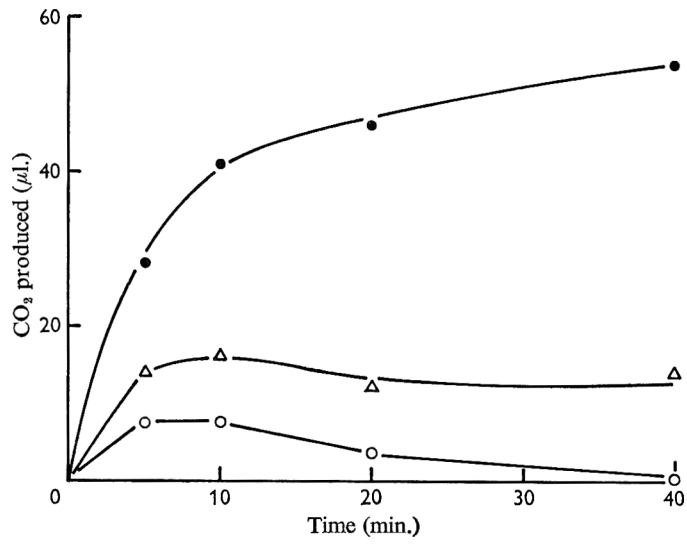

Fig. 3. Formate dehydrogenase (benzylviologen) in extracts of *S. ventriculi*. Cell-free extracts were prepared by grinding with powdered glass. Warburg flasks contained: 20 μmoles of sodium formate (side arm); 40 μmoles of potassium phosphate; 20 μmoles of benzylviologen; 1·0 ml. extract (0·35 mg. N/ml.); pH 7·0; total volume 3·0 ml.; atmosphere N_2. Benzylviologen, ●; benzylviologen absent, ○; endogenous with benzylviologen, △.

Table 2. *Formate dehydrogenase activities of S. ventriculi with methylene blue and benzylviologen as acceptors*

Harvested cells were washed twice with distilled water and resuspended in water. Warburg flasks contained: 100 μmoles of sodium formate (side arm); 40 μmoles of potassium phosphate; 14 mg. (dry wt) of cells; 20 μmoles of methylene blue or benzylviologen; pH 6·0; total volume 3·0 ml.; atmosphere N_2. Sodium formate was omitted for measurement of endogenous dye reduction.

	CO_2 produced (μl.)					
	Methylene blue			Benzylviologen		
Time (min.)	Endogenous with dye	Dye absent	Dye present	Endogenous with dye	Dye absent	Dye present
3	7	15	180	5	5	30
6	8	15	278	—	2	45
12	10	17	315	—	—	—
15	—	—	—	6	1	64
24	18	25	343	—	—	—
40	—	—	—	7	1	115
60	—	—	—	8	5	135

worthy that these cells were devoid of hydrogenlyase activity although formate dehydrogenase (benzylviologen) was still present. With extracts of *S. ventriculi* prepared by grinding with powdered glass, formate dehydrogenase was not detected with methylene blue as acceptor although activity was found with benzylviologen (Fig. 3). At pH 7·0, although only low activity was obtained as measured by CO_2 evolution, the benzylviologen was strongly reduced only in the presence of formate. Again hydrogenlyase activity was absent from these preparations.

Hydrogenase

Sarcina ventriculi, harvested and transferred to Warburg flasks with anaerobic precautions, possessed hydrogenase activity with methylene blue as hydrogen acceptor. Low activity was also observed with benzylviologen (Fig. 4). Both activities were increased by sodium dithionite. In experiments with the hydrogenase of *Clostridium butylicum* in cell-free extracts, Peck & Gest (1957*b*) found that an induction period preceded reduction of benzylviologen; this induction period was eliminated in the presence of small amounts of sodium dithionite.

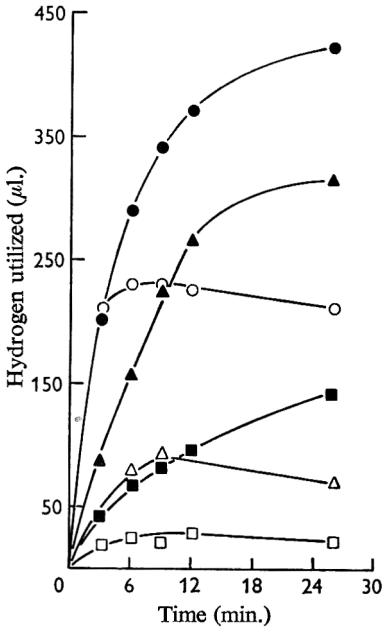

Fig. 4

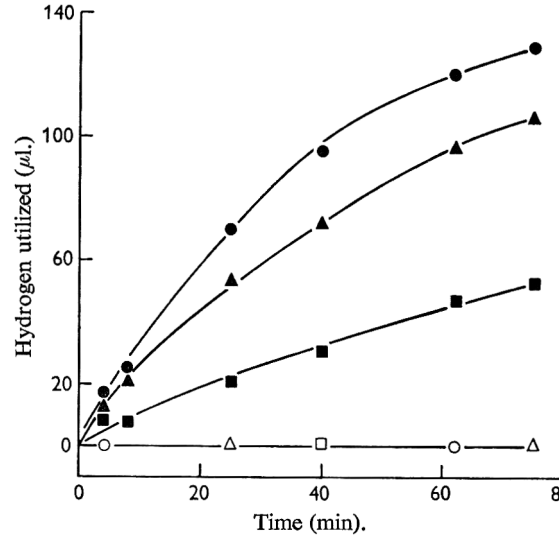

Fig. 5

Fig. 4. Hydrogenase of *S. ventriculi*. Warburg flasks contained: 20 μmoles of dye (side arm); 90 μmoles of potassium phosphate; 15 mg. (dry wt) of cells; 5 μmoles of sodium dithionite where indicated; pH 7·5; 0·2 ml. of 20 % (w/v) KOH in the centre-well; total volume 3·0 ml.; atmosphere H_2. Dyes used were methylene blue (MB), benzylviologen (BV) and methylviologen (MV). Control flasks with boiled cells possessed no activity. MB, ○; MB + dithionite, ●; BV, △; BV + dithionite, ▲; MV, □; MV + dithionite, ■.

Fig. 5. Hydrogenase in cell-free extracts of *S. ventriculi*. Details as for Fig. 4 except that cell extract (0·4 mg. N/flask) replaced the whole cell suspension. Boiled extract possessed no activity. MB, ○; MB + dithionite, ●; BV, △; BV + dithionite, ▲; MV, □; MV + dithionite, ■.

When *Sarcina ventriculi* organisms were washed thoroughly with distilled water, hydrogenase activity with benzylviologen or methylviologen was not obtained unless dithionite was present. With methylene blue high activity was obtained only in the presence of dithionite, whereas in its absence low activity was obtained and only after a lag period.

Extracts of *Sarcina ventriculi* were devoid of hydrogenase activity with any of the redox dyes tested. However, activity was restored to these preparations by the addition of small amounts of dithionite (Fig. 5). This is in agreement with the results of Fisher,

Krasna & Rittenberg (1954) who showed with the *Escherichia coli* system that oxygen inhibited hydrogenase by oxidation and reversible oxygenation. By using the evolution assay of Peck & Gest (1956) attempts to show hydrogenase activity in whole organisms and extracts of *S. ventriculi* were unsuccessful.

DISCUSSION

The present results show that *Sarcina ventriculi* possesses a mechanism for the production of hydrogen essentially similar to that found in the coli-aerogenes group of bacteria. This system involves the production of formate from pyruvate, followed by the catabolism of formate to H_2 and CO_2. The enzymic activities shown in other organisms to be associated with the formate pathway of hydrogen production, namely hydrogenlyase, formate dehydrogenase and hydrogenase, are all present in *S. ventriculi*.

Inhibition of hydrogenlyase by oxygen is known to occur in other organisms, but the extreme sensitivity of the *Sarcina ventriculi* system would seem to be particularly noteworthy. Unless adequate precautions are taken to prevent exposure to air, the hydrogenlyase of *S. ventriculi* is irreversibly inhibited, even in whole organisms. With cell extracts, presumably due to similar inactivation, hydrogenlyase activity could not be demonstrated. Reducing agents were ineffective in annulling this inhibition both with whole organisms and with extracts.

Formate dehydrogenase activity was more stable than hydrogenlyase activity and conditions which inactivated hydrogenlyase did not affect formate dehydrogenase (benzylviologen) in intact organisms. The dehydrogenase activity associated with methylene blue in whole organisms was not detected in extracts; extracts were active with benzylviologen.

Gest & Peck (1955) and Peck & Gest (1957*a*) showed with different strains of *Escherichia coli* that the presence of formate dehydrogenase (benzylviologen) is essential for hydrogenlyase activity. The presence of both these activities in *Sarcina ventriculi* means that hydrogen formation in this organism could also be explained by this hypothesis. To date, *S. ventriculi* is the only organism outside the coliform group which has been shown to possess hydrogenlyase activity. It is therefore of importance to the concept of Peck & Gest that enzymic components similar to those found in coliform organisms, and deemed by these authors to be essential for enzymic activity, are also present in *S. ventriculi*.

Hydrogenase of *Sarcina ventriculi* was also extremely sensitive to inhibition by oxygen, although dithionite effectively annulled this inhibition. It was, however, significant that conditions which produced inactivation of hydrogenase also inactivated hydrogenlyase. With *Escherichia coli* a lag period was frequently observed in hydrogenlyase activity which Peck & Gest (1957*a*) suggested was due to the combined effect of oxygen on formate dehydrogenase (benzylviologen) and hydrogenase. With *S. ventriculi* our results suggest that hydrogenase is the more likely site of hydrogenlyase inhibition. However, Peck & Gest (1957*a*) found that the electron carrier required for coupling formate dehydrogenase (benzylviologen) with hydrogenase was also inactivated by aeration. Determination of the exact site of oxygen inhibition of the formate hydrogenlyase system in *S. ventriculi* must await the production of an active cell-free preparation.

This work was done while the authors were members of the Biochemistry Department, University of Glasgow.

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Growth Rate Patterns of the So-called Osmophilic and Non-osmophilic Yeasts in Solutions of Polyethylene Glycol

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SUMMARY

The growth rates of nine yeasts, conventionally classified as 'osmophilic' and six yeasts, conventionally classified as 'non-osmophilic' were measured at 30° in media adjusted to known water activities with polyethylene glycol. The organisms were less tolerant of low water activities (a_w) in the presence of polyethylene glycol than in the presence of sugars. There was no significant difference between average minimal water activities supporting growth of the two groups of yeasts in polyethylene glycol, although differences in sugar tolerance were conspicuous. There was good correlation, however, between quantitative water relations of the organisms in polyethylene glycol and qualitative observations of ability to grow at specified sugar concentrations. The osmophilic organisms grew about half as fast as the non-osmophilic organisms at their respective optimal water activities. The osmophils had relatively broad water activity optima; the non-osmophils had sharp optima. Neither group of yeasts had a general requirement for a water activity lower than that of the basal medium ($0{\cdot}997\,a_w$) in order to grow, although there were two osmophilic strains which would not grow in the basal medium at 30°. The adjective 'osmophilic' is considered to be inaccurate; until the physiology of these yeasts is better understood we propose that they be designated simply as 'sugar tolerant'. The quantitative results have provided a basis for selecting sugar tolerant organisms for biochemical and physiological investigations.

INTRODUCTION

The ability of an organism to thrive in a relatively dry environment is potentially subject to determination by its response to high solute activity, low solvent activity and, less obviously, possible effects of osmotic pressure. The halophilic bacteria exemplify a group of organisms with a strict requirement for high solute activity, the dominant solutes in this case being the ions Na^+ and Cl^-. The requirement for Na^+ is related to some distinctive features of halophilic bacterial proteins (Brown, 1964). There are at least two other groups of micro-organisms which can thrive in a dry environment which need not necessarily have a high ionic strength. These organisms are the so-called 'osmophilic' yeasts and 'xerophilic' moulds (Scott, 1956).

The osmophilic yeasts are distinguished by their ability to grow in highly concentrated sugar solutions and it is a common practice to discuss the behaviour of such yeasts in terms of sugar tolerance; occasionally they will tolerate relatively concentrated solutions of NaCl. The osmophilic yeasts are the subject of quite a substantial

literature of which the reviews by Scott (1956), Ingram (1957) and Onishi (1963) are recent and useful. In spite of a substantial literature, however, there is currently no information about the physiological or biochemical basis of their tolerance of concentrated sugar solutions. The designation 'osmophilic', which was proposed by Richter (1912), is itself unfortunate because there is nothing at present to justify the implication that osmotic pressure is a factor in determining the peculiar physiology of these organisms. Scott (1956) has also commented on the disadvantages of interpreting the water relations of osmophilic yeasts in terms of osmotic pressure.

The current state of information is such that it is difficult, if not impossible, to describe the condition of 'osmophilia' in other than qualitative or loose quantitative terms. There is no systematic information relating growth rates to water activity as distinct from sugar concentration. This is not necessarily a disadvantage in seeking a definition, but it is no help in understanding the biological phenomenon involved. The literature leaves little doubt, however, that 'osmophilia' is a genuine phenomenon which warrants investigation as a means of understanding the ability of organisms to thrive in dry environments. The present paper describes the first stage of such an investigation and, in part, is an attempt to define osmophilic yeasts in terms of growth rates and water activities. Growth rates of fifteen yeasts were determined in media adjusted to known water activities with polyethylene glycol. This solute was selected in order to minimize the complication of large and varying concentrations of ionizing metabolites which accumulate when water activity is adjusted with sugars and also, it was found, with the monomer ethylene glycol. The classification of the yeasts as osmophilic or otherwise based on growth rate measurements agreed with the classification based on sugar tolerance. The growth rate patterns showed that, in general, osmophilia is a tolerance of, rather than a requirement for, dry conditions and allow some tentative generalizations to be made.

METHODS

Organisms. Nine yeasts, previously classified as osmophilic and six, previously classified as non-osmophilic, were obtained from sources shown in Table 1. Yeasts, other than those which were obtained from the Centraalbureau voor Schimmelcultures, were identified by conventional diagnostic methods (Lodder & Kreger-van Rij, 1952). The sugar fermentation and assimilation characteristics of Centraalbureau cultures were checked and confirmed in each case. The sugar tolerance of each yeast culture was ascertained by incubating the organisms in a series of sugar media at 30° for up to 2 months (see Scarr & Rose, 1966). Stock cultures of the osmophilic organisms were maintained on 'Synthetic Honey Agar' (yeast-extract agar plus 48 % (w/v) glucose) at 4°. The non-osmophils were maintained on malt agar at 4°.

Growth medium. The organisms were grown, for experimental purposes, in a basal medium supplemented with polyethylene glycol as specified. The basal medium had the following composition: Peptone (Difco), 5·0 g.; yeast extract (Difco), 2·5 g.; KH_2PO_4, 1·0 g.; $MgSO_4.7H_2O$, 0·25 g.; $CaCl_2$, 0·1 g.; glucose, 1·9 g.; water, 1 l. The water activity (a_w) of this medium is approximately 0·997. Polyethylene glycol with an average molecular weight of 200 (Carbowax–200) was de-ionized on a column of mixed-bed ion exchange resin (Elgastat) and added to the basal medium to give the required water activity. The water activity was interpolated from a water sorption

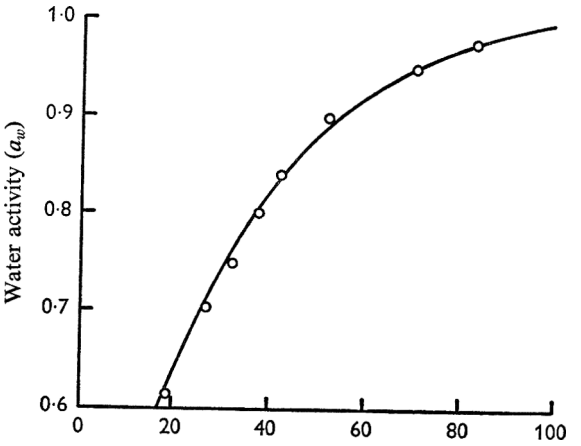

Fig. 1. The composition of aqueous polyethylene glycol solutions of known water activity at 30°.

Table 1. *Description and origin of yeasts used*

Code number	Name	Source
	Osmophilic strains	
YA	*Saccharomyces rouxii*	Indian conserves
YB	*Zygosaccharomyces rugosus* (CBS 741)	C.B.S.*
YC	*S. rouxii* Boutroux var. *polymorphus* (CBS 1091)	C.B.S.
YD	*S. mellis* (CBS 736)	C.B.S.
YE	*Z. nectarophilus* (CBS 740)	C.B.S.
YF	*Z. nussbaumeri* (CBS 738)	C.B.S.
YG	*S. rosei* (CBS 1090)	C.B.S.
YH	*Torulopsis halonitratophila* (CBS 5240)	C.B.S.
YK	*S. acidifaciens*	University of New South Wales
	Non-osmophilic strains	
Y14	*S. fragilis*	University of New South Wales
Y31	*S. cerevisiae*	University of New South Wales
Y34	*S. cerevisiae*	Brewery
Y40	*S. cerevisiae*	Royal North Shore Hospital
Y41	*S. cerevisiae*	Baker's yeast
Y43	*S. cerevisiae*	Brewer's Bottom Yeast

* Centraalbureau voor Schimmelcultures, Delft, Holland.

isotherm determined by equilibration against saturated salt solutions of known water activities between 0·62 and 0·98 (Robinson & Stokes, 1949). This isotherm is shown in Fig. 1.

Determination of growth rates. The organisms were grown in 40 ml. medium con-

tained in Erlenmeyer flasks (capacity 250 ml.) fitted with cylindrical side arms, and plugged with cotton wool. Under conditions causing very low growth rates, flasks were also covered with polyethylene sheeting to minimize evaporation. The flasks were incubated in a horizontal platform shaker (70 oscillations/min.) at 30°. Growth rates were determined turbidimetrically at 700 mμ with reference in each case to a sterile solution of the same composition as the growth medium. In preliminary determinations, turbidity was calibrated against total count of organisms and found, during exponential growth, to maintain a constant relation with count at all values of water activity down to 0·92. The average standard deviation of growth rate measurements was 0·054 generations/hr (or, for mean generation time, 0·36 hr).

Table 2. *Growth of yeasts at specified sugar concentrations*

	Concentration of sugar in medium (%, w/v)				
		Sucrose			
Yeast	Fructose 70 (0·765a_w)	65 (0·865a_w)	60 (0·895a_w)	55 (0·917a_w)	50 (0·935a_w)
Osmophilic					
YA	+	+	+		
YB	+	+	+		
YC	+	+	+		
YD	+	+	+		
YE	+	+	+		
YF	+	+	+		
YG	−	+	+		
YH	+	+	+*		
YK	+	+	+		
Non-osmophilic					
Y14		−	−	−	+
Y31		−	−	+	+
Y34		−	+	+	+
Y40		−	−	+	+
Y41		−	−	−	+
Y43		−	−	+	+

Negative results were recorded after incubation had continued for at least 2 months at 30°. A blank space implies that the observation was not made.

* Yeast YH did not grow at 0·997a_w, nor at 0·990 in the presence of glycerol or sucrose; see Table 3 and compare Onishi (1960).

RESULTS AND DISCUSSION

Table 2 lists the ability of the organisms to grow in the basal medium plus sucrose or fructose at the specified concentration. The table enables the organisms to be classified into two groups, osmophilic and non-osmophilic, according to the criteria used by Scarr & Rose (1966). Only one of the osmophils (YG) would not grow in sugar media at a water activity lower than 0·865 (equivalent to 65° 'Brix', see Scarr & Rose, 1966). None of the osmophils grew in 75 % fructose (0·700a_w). None of the non-osmophils grew in media more concentrated than 60 % (w/w) sucrose (0·895a_w) and only one (Y34) grew above 55 % (w/w) sucrose (0·917a_w).

Figures 2 and 3 show, for all the organisms, the relations between growth rate and water activity in media containing polyethylene glycol. Under these conditions the minimal water activities at which growth occurred were higher than in media adjusted

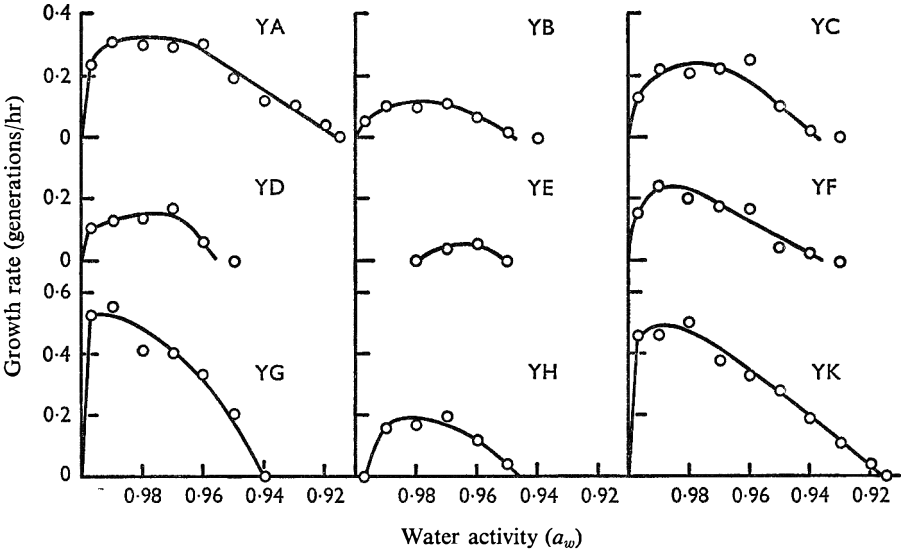

Fig. 2. Growth rate against water-activity relations; osmophilic yeasts in polyethylene glycol. The average standard deviation of the growth rate measurement was 0·054 generation/hr.

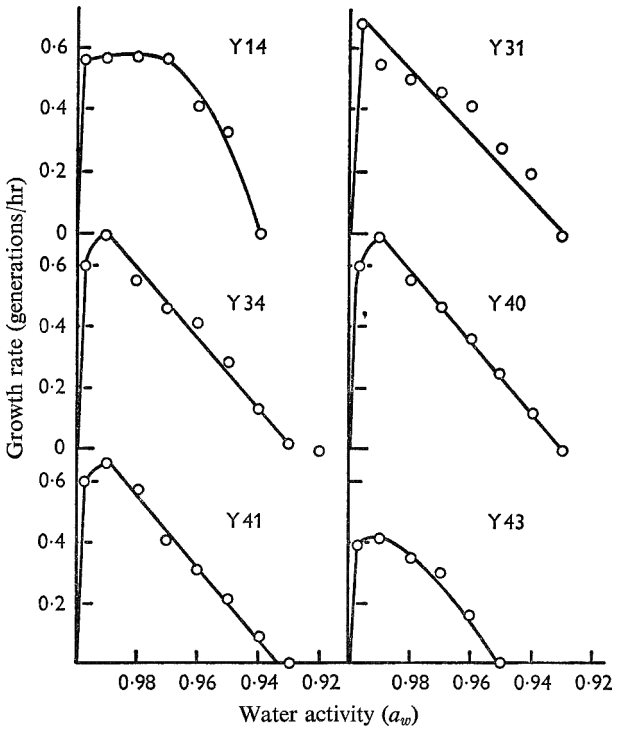

Fig. 3. Growth rate against water-activity relations; non-osmophilic yeasts in polyethylene glycol. The average standard deviation of the growth rate measurement was 0·054 generation/hr.

with sugars. There was no significant difference between the average minimal water activity tolerated by the two groups of organisms, although the yeasts which grew at the lowest activity (0·920) were osmophils (YA, YK). The maximal growth rates of the non-osmophils were more than twice as great as those of all but two of the osmophils. The two exceptional organisms were YG and YK. Yeast YG was less sugar-tolerant than the other members of its group and is presumed to be an intermediate type; yeast YK was distinguished by a low minimal water activity. The relatively low maximal growth rate of the osmophils is evidently an intrinsic property of the organisms since it was not caused specifically by polyethylene glycol. Table 3 lists mean generation times at 0·990a_w in media adjusted with polyethylene glycol, glycerol or sucrose. There was close agreement with all three media.

Table 3. *Mean generation time of yeasts in media adjusted to 0·990a_w separately by three different solutes*

	Mean generation time (hr) in		
Yeast	PEG*	Glycerol	Sucrose
Osmophils			
YA	3·27	4·60	3·25
YB	9·00	8·60	9·40
YC	4·63	4·50	3·60
YD	7·80	7·30	5·40
YF	4·00	5·70	4·50
YG†	1·80	2·10	1·40
YH	6·00	— ‡	—‡
YK	2·13	2·60§	—§
Non-osmophils			
Y14	1·82	1·33	1·60
Y34	1·35	1·30	1·40
Y40	1·30	1·40	1·43
Y41	1·50	1·50	1·70
Y43	2·42	1·70	1·55

* Polyethylene glycol.
† Probably an intermediate type; see text.
‡ No growth at this water activity in glycerol or sucrose.
§ Organisms clumped making turbidimetric measurement difficult in glycerol and impossible in sucrose.

The optimal water activity of the non-osmophils (with one exception, Y14) was confined to the narrow limits of 0·990–0·998; growth rates decreased sharply when water activity was decreased below the optimum. The exceptional yeast, Y14, was distinguished also by poor tolerance of decreased water activity, both in polyethylene glycol and sugar solutions (Table 1). The osmophils (except YG), on the other hand, had a broad optimum which extended in some cases down to 0·960a_w. Decreasing water activity below the optimal value caused a decline in growth rate which was appreciably less steep than that of the non-osmophilic yeasts. With two exceptions, the osmophils did not require a decreased water activity and grew well on the basal medium (0·997a_w). The exceptions were YE and YH. YE grew at measurable rates only within the range 0·97c–0·960a_w. The shape of this curve does not necessarily imply that the organism specifically requires a decreased water activity; the curve shows what is evidently the optimal water activity in a poor medium. A more suitable

medium (which was not sought) might be expected to raise the general values of growth rate to give a relation more closely resembling those of the other osmophilic organisms.

Yeast YH was a more interesting exception. It did not grow in the basal medium at $0{\cdot}997\,a_w$ nor in the presence of sucrose or glycerol at $0{\cdot}990\,a_w$; it did grow at the latter water activity, however, in the presence of polyethylene glycol. The organism, *Torulopsis halonitratophila* was described by Onishi (1960) as obligately halophilic (*sic*) at 30°, but facultatively so at 20°. The solute effects at $0{\cdot}990\,a_w$, as a first approximation, seem likely to be related to differences in solute penetration with associated effects on intracellular ionic strength. This suggestion can be tested experimentally; we hope to discuss it in due course.

There is thus no evidence that the osmophilic yeasts examined have a general requirement for decreased water activity, although there is limited evidence that some might grow optimally under drier conditions than do non-osmophilic yeasts. This finding supports Onishi's (1963) doubts about the existence of obligately osmophilic organisms. The designation '-philic' is inaccurate and would be better substituted by '-tolerant'.

Table 4. *Mean generation time of yeast YA in media adjusted with polyethylene glycols of three molecular weights*

Water activity (a_w)	Mean generation time (hr) in PEG-200*	PEG-1000*	PEG-4000*
0·980	3·50	3·70	4·40–5·80
0·970	3·40	4·20	—†
0·960	3·40	3·80	—†

* Polyethylene glycol of average molecular weight, 200, 1000 or 4000.
† Stated water activity not obtainable with PEG-4000.

The present results provide no information about the role of osmotic pressure in the physiology of these yeasts. Increased size and, therefore presumably, decreased penetration of polyethylene glycol molecules, however, decreased the growth rate of one yeast at high water activities (Table 4). Whether this effect was caused by differences is osmotic pressure or, as we believe, other physico-chemical consequences of solute penetration can be ascertained experimentally; it is the subject of another investigation. In the meantime we agree with Scott's (1956) comment that the osmophilic yeasts are better considered in terms of their water relations. We believe the designation 'osmophilic' to be misleading and we propose to refer to these organisms as 'sugar tolerant'.

Although simple measurements of growth rate give no information about physiological mechanisms they have allowed a phenomenon to be described quantitatively in terms which correlate satisfactorily with conventional qualitative statements of whether or not growth occurs at a specified sugar concentration. The quantitative description has also provided a rational basis for the selection of organisms for biochemical and physiological studies which are now in progress.

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Some Aspects of the Biochemical Differentiation of Pathogenic Fungi: a Review

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SUMMARY

Biochemical procedures for the differentiation of fungi in general and pathogenic fungi in particular have been slow to develop because many such organisms possess a sufficiently varied morphology to allow of considerable subdivision by that means alone. When, however, one considers organisms with a very reduced morphology, such as yeasts, the picture is very different and biochemical procedures have long been standard. Dependence on morphology for the identification of filamentous fungi has several disadvantages, of which slowness and lack of precision are the two most important. It is not desirable in medicine to have to wait several months till a particular identifiable structure is produced and there are a few pathogenic fungi which remain permanently nondescript. The lack of precision is best shown by the plethora of synonyms and ill described 'species' that have long plagued mycology.

The capacity of fungi to utilize various compounds and the chemical nature of their structure have both been used as methods of differentiation. The former method has been successfully used to show differences between morphologically similar organisms in several genera such as *Microsporum*, *Trichophyton* and *Madurella*. This method has also proved of the utmost value in distinguishing those aerobic Actinomycetes which are responsible for mycetoma from one another. Tests commonly used are the ability to degrade compounds such as casein, tyrosine, xanthine and urea or the lack of ability to grow in the absence of certain amino acids or vitamins or the ability to assimilate a particular compound such as dulcitol, erythritol, lactose or sucrose. Chemical analysis of fungi has not yet progressed so far but differences have been demonstrated, and the detection of arabinose and diaminopimelic acid in the cell walls of Actinomycetes appears to be on the verge of becoming standard technique.

INTRODUCTION

In a sense biochemical differentiation of pathogenic fungi has been with us since the beginning of medical mycology. Sabouraud (1910), in *Les Teignes*, noted the effects of certain variations of culture medium on the growth and appearance of dermatophytes. Today, in the absence of the crude ingredients then used, it is difficult or impossible accurately to reproduce those early results. That the appearances of fungi differ when grown on different substrates is well known and the importance of the quality of peptone was forcibly brought home to us by the import restrictions of 1946 (Carlier, 1948). But even before the publication of *Les Teignes* some work involving the nutrition of pathogenic fungi had been done by Verujsky (1887) and Bodin (1899, 1902)

The pressure to develop techniques of identification for filamentous pathogenic fungi has not been great because these organisms do possess a sufficient variety of structure to allow of a fair degree of separation by this method alone. Morphology rightly still plays an important part in the identification of pathogens but there are occasions when it proves inadequate. Increasingly, what the eye can see is being supplemented by other tests, biochemical and serological, which often allow an earlier or more precise diagnosis to be made. Because of the lack of pressure to develop alternatives to identification by form alone, research has been rather patchy and sporadic. There is not yet a coherent pattern of biochemical tests applicable to pathogenic fungi. Nevertheless, the many isolated ventures have at least shown that fungi do exhibit biochemical differences and there is no fundamental objection to the use of biochemical methods as supplements to other methods.

Biochemical tests currently in use to assist in the differentiation of pathogenic fungi can broadly be divided into two categories:

(1) Tests to assess what particular compounds fungi assimilate or degrade in their growth processes. Alternatively, the identification of metabolites produced under controlled conditions may serve as a test.

(2) Tests to assess what fungi are actually made of.

Assimilation and degradation tests

Assimilation tests are extremely important in the taxonomy of yeasts. This is an example of how a group of organisms of reduced morphology has given rise to a coherent pattern of biochemical tests; but such tests are of much less value in the study of filamentous fungi. This is no doubt in part due to lack of fundamental work but, also, such fungi are such extraordinarily catholic feeders that it is difficult to find compounds of sufficient selectivity. Nevertheless, several valuable tests have been described.

The nutrition of dermatophytes was reviewed by Stockdale (1953). Nutritional factors capable of differentiating dermatophytes were observed by Goddard (1934) who noted that *Trichophyton interdigitale* assimilated galactose but that *Microsporum canis* did not; neither of these fungi assimilated lactose. Earlier, Tate (1929) failed to detect invertase or lactase in *T. tonsurans*, *M. audouinii*, *M. canis* and *T. schoenleinii* but all contained maltase and diastase. Mallinckrodt-Haupt (1927), working with *T. gypseum*, observed that animal fats, but not cod liver oil, supported growth whereas vegetable oils other than olive oil, almond oil and cocoa butter did not. These observations are interesting in themselves but do not further the cause of differentiation much.

Georg (1949, 1950, 1951, 1952) studied the nutritional requirements of several dermatophytes and Georg & Camp (1956) described methods for differentiating some rather difficult fungi from one another. Simple basal media to which single nutrients (vitamins, amino acids) could be added were used as the bases of these tests. Table 1 shows some of their results; it is of particular interest to note that their findings tend to validate *Trichophyton equinum* and *T. megninii* as genuine species. These organisms had been fighting a losing battle to avoid being classed as *T. mentagrophytes* and *T. rubrum*, respectively, but the readily demonstrable absolute requirement for nicotinic acid or histidine clearly separates them. Other useful differences are shown in Table 1; *T. verrucosum* and *T. schoenleinii* are morphologically similar but one grows well in the absence of thiamine and inositol and the other demands them. *T. tonsurans* can also be readily differentiated from *T. rubrum* and *T. mentagrophytes*.

The requirements of single amino acids by dermatophytes were studied by Pinetti, Bonomi & Lostia (1959). In general, any amino acid serves dermatophytes well enough as a sole source of nitrogen, but there are exceptions. As examples one can quote *Epidermophyton floccosum* which cannot utilize lysine, and *Trichophyton megninii*, which is really happy only in the presence of histidine. The latter fungus may also show some growth in the presence of threonine, alanine and lysine but apparently cannot utilize glycine, leucine, asparagine, cystine, valine or phenylalanine. Rosenthal & Vanbreuseghem (1962) studied the nutritional requirements of several dermatophytes, and Table 2 shows that *T. soudanense* cannot utilize ammonium nitrate or histidine as nitrogen sources whereas *T. yaoundei* and *Microsporum langeronii* can.

Table 1. *Nutritional requirements of several Trichophyton species*

Recommended for routine use to assist in identification (Georg & Camp, 1956).

Species	Growth on basal medium alone	Growth on basal medium with added: Inositol	Thiamine	Thiamine and Inositol	Histidine	Nicotinic acid
T. verrucosum	o	±	±	+	.	.
T. schoenleinii	+	+	+	+	.	.
T. concentricum	+	+	+	+	.	.
T. tonsurans	±	.	+	.	.	.
T. mentagrophytes	+	.	+	.	.	+
T. equinum	o	.	.	.	.	+
T. rubrum	+	.	+	.	+	.
T. megninii	o	.	.	.	+	.
T. gallinae	+	.	.	.	+	.
M. ferrugineum	+	.	+	.	.	.
T. violaceum	±	.	+	.	.	.

o = no growth; ± = weak growth; + = good growth; . = no test.

Table 2. *Histidine or Ammonium nitrate as sole nitrogen sources for three dermatophytes*

Rosenthal & Vanbreuseghem (1962).

Species	Nitrogen source: NH_4NO_3	Histidine
Trichophyton soudanense	o	o
T. yaoundei	+	+
Microsporum langeronii	+	+

o = no growth; + = growth.

Koehne (1962) studied the nutrition of three species of the genus *Microsporum* and showed that while a very large number of compounds provided adequate carbon sources there were several small differences. He noted that *Microsporum cookei*, *M. distortum* and *M. nanum* differed in their abilities to utilize starch, erythritol and dulcitol (Table 3). These species are as a rule perfectly easy to distinguish morphologically, but the fact that differences exist shows that an extension of the findings might easily lead to knowledge of taxonomic significance. For example, can the different members of the *M. gypseum* complex be so distinguished or is there a true and acceptable difference between *M. audouinii* and *M. langeronii*?

A problem that may arise in any laboratory is to decide whether an isolated fungus is a dermatophyte or not, and a neat presumptive test has been suggested by Baxter (1965) and amplified by Quaife (1967); the test depends on the fact that dermatophytes, unlike many other fungi, tend to increase the pH value of the supporting medium. Ink blue changes from blue to colourless between pH 6·5 and 7·2 and can safely be incorporated in glucose peptone agar to a concentration of 0·05 %. Dermatophytes and other keratinophilic fungi such as *Trichophyton terrestre* discharge the blue colour, whereas the wide range of other fungi which Baxter and Quaife tested had no such effect.

Table 3. *Differences between three Microsporum species with respect to dulcitol, erythritol and starch*

Koehne (1962).

	Carbon source		
Microsporum spp.	Dulcitol	Erythritol	Starch
	Growth		
M. cookei	o	+++	++
M. distortum	+	o	+
M. nanum	+	++	o

o = no growth; + = weak growth; ++ = moderate growth; +++ = vigorous growth.

Table 4. *Hydrolysis of casein, tyrosine, gelatine and urea by dermatophytes*

Philpot (1967), Rosenthal & Sokolsky (1965) and unpublished observations at the Mycological Reference Laboratory.

	Hydrolysis of			
Organism	Casein	Tyrosine	Gelatine	Urea
Trichophyton mentagrophytes	6/6*	6/6	6/6	6/6
T. rubrum	7/7	7/7	7/7	0/7
T. gallinae	1/1	1/1	1/1	0/1
T. megninii	2/2	2/2	2/2	2/2
T. sulphureum	6/6	6/6	6/6	6/6
T. tonsurans	4/4	4/4	4/4	4/4
T. soudanense	6/6	6/6	6/6	0/6
Microsporum ferrugineum	1/4	4/4	1/4	0/4
T. schoenleinii	6/6	6/6	6/6	1/6
T. verrucosum	6/6	0/6	6/6	5/5

* Number of strains positive/number of strains tested.

We and others have made a few observations about the ability of certain dermatophytes to hydrolyse casein, tyrosine, gelatine and urea, and some quite useful differences have come to light (Philpot, 1967; Rosenthal & Sokolsky, 1965); see Table 4. Urease production seems to be a useful way of distinguishing *Trichophyton rubrum* and *T. gallinae* from *T. megninii* and *T. mentagrophytes*, as well as *T. tonsurans* from *T. soudanense*. At first we thought that *T. soudanense* and *Microsporum ferrugineum* could be readily distinguished from one another by their proteolytic activities but, unless one of our strains of *M. ferrugineum* was incorrectly identified, this is only partly true.

Another group of filamentous fungi in which there has been some progress in biochemical differentiation is the group responsible for mycetoma. This is true of both fungi, agents of maduromycetoma, and aerobic actinomycetes, agents of the clinically similar actinomycetoma. It is again to be noted that the reduced morphology of actinomycetes has led to the use of a better array of biochemical tests for their differentiation. Table 5 lists some of the fungi known or thought to be capable of causing mycetoma, together with some details of their chemical behaviour. These studies have been largely made by Mackinnon, Ferrada & Montemayor (1949*a*, *b*), Mackinnon (1951*a*, *b*, 1954), Segretain (1957) and Segretain & Segretain (1960). It is perhaps of interest to note that *Madurella mycetomi* has a long list of synonyms including *M. ikedae* and probably also *M. americana*, and it is largely the work of Mackinnon which has brought this difficult group into a single species. Mackinnon and his colleagues, together with Segretain, found simple nitrogen compounds of no value for differentiation of mycetoma-causing organisms, but an examination of Table 5 at once reveals that a fair degree of separation can be made by maltose, sucrose and lactose. Opinions on the proteolytic powers of these fungi vary but the results of sugar assimilations have been constant.

Table 5. *The action of mycetoma-causing fungi on carbohydrates and proteins*

This table is compiled from data published by: Mackinnon *et al.* (1949*a*, *b*); Mackinnon (1951*a*, *b*, 1954); Segretain (1957); Segretain & Segretain (1960).

	Assimilation of				Hydrolysis of		
Organism	Maltose	Sucrose	Galactose	Lactose	Coagulated serum	Milk	Gelatine
Madurella mycetomi	+	o	+	+	+	+	+
M. ikedae	+	o	+	+	+	+	+
M. americana	+	o	+	+	+	+	+
M. grisea	+	+	+	o	±	+	+
Allescheria boydii	o	±	±	o	±	+	+
Cephalosporium recifei	+	+	+	o	o	+	o
C. falciforme	+	+	+	o	±	±	+
C. acremonium	+	+	+	+	+	+	+
P. jeanselmei	+	+	+	o	o	o	o

o = no growth; ± = variable growth; + = good growth.

The organisms so far considered possess a fair variety of structures which make differentiation by morphology a not impossible task. Those aerobic actinomycetes which cause mycetoma possess very little morphological variation and the laboratory becomes almost totally dependent on biochemical features. The organisms most often encountered in human pathology are *Nocardia asteroides*, *N. brasiliensis*, *N. caviae*, *Streptomyces madurae*, *S. pelletierii* and *S. somaliensis*. The tests for them have become fairly standard; some are outlined in Table 6. Many workers have contributed the data in Table 6, notably Gordon & Mihm (1957, 1959, 1962*a*, *b*), Mariat (1957, 1958, 1962, 1963), Georg, Ajello, McDurmont & Hosty (1961) and Gonzalez-Mendoza & Mariat (1964). The use of this table of biochemical features, together

with morphology, especially of parasitic forms in the tissues when available, not only makes identification of the pathogenic species relatively easy but usually permits the numerous saprobic but similar organisms to be recognized.

Tests based on the chemical analysis of fungi

A good deal of work has been done on the chemical constitution of dermatophytes, though in a manner unlikely to help the taxonomist much. In general all dermatophytes seem to be composed of the same lipids, carbohydrates and amino acids but some differences are known to exist. Chattaway, Toothill & Barlow (1961) showed that strains of *Trichophyton mentagrophytes* did not contain aspartic acid, serine and proline in the free state; in contrast, *T. rubrum*, *Microsporum canis* and *Epidermophyton floccosum* did. There are also differences in sterol content, ergosterol being characteristic of most and brassicasterol of others. There are some contradictions in the

Table 6. *Biochemical differentiation of pathogenic Nocardia and Streptomyces species*

No single test is completely reliable but the use of several gives excellent results. These tests are used routinely in many laboratories and are based on the works of Mariat (1957, 1958, 1962, 1963) and Gordon & Mihm (1957, 1959, 1962 *a*, *b*).

Species	Acid fastness	Hydrolysis of: Gelatine	Casein	Tyrosine	Xanthine	Egg Albumin	Assimilation of: Maltose	Mannitol	Production of urease
Nocardia asteroides	±	±	o	o	o	o	o	o	+
N. brasiliensis	±	+	+	+	o	o	.	+	+
N. caviae	±	o	o	o	+	.	±	+	±
Streptomyces madurae	−	+	+	.	.	o	.	+	o
S. pelletierii	−	+	+	.	.	+	o	o	o
S. somaliensis	−	+	+	.	.	+	+	o	o

o = no response; + = positive response; ± = variable response; . = uncertain or untried.

literature. According to Wirth, Beesley & Miller (1961) and Wirth & Anand (1964), *T. rubrum* contains both ergosterol and brassicasterol, the latter predominating in most strains; but Blank & Shortland (1962) affirmed that brassicasterol was dominant in *T. violaceum*, *T. verrucosum* and *T. megninii* and that ergosterol was dominant in *T. tonsurans*, *T. rubrum*, *T. mentagrophytes* (and its varieties), *M. audouinii*, *M. canis* and *M. gypseum*. Were Wirth and colleagues perhaps mistakenly working with the rather similar *T. megninii*? Audette, Baxter & Walker (1961) reported that ergosterol was the more abundant sterol in *T. mentagrophytes*.

Analysis of cell walls has proved of particular value in the separation of species of the genus *Nocardia* from saprobic species of the genus *Streptomyces*. Theoretically this can be done by a simple stain for acid-fastness but this is far from reliable. Two suitable methods, both involving paper chromatography, have been described. Cummins & Harris (1956, 1958) showed that the cell walls of species of the genera *Mycobacterium* and *Nocardia* were rich in arabinose but species of the genera *Actinomyces* and *Streptomyces* were deficient. Murray & Proctor (1965) described a simple method of paper chromatography that allows this criterion to be used as a laboratory routine. By this means, certain pathogenic, aerobic actinomycetes were grouped as follows.

(1) Those containing a great deal of arabinose: *Nocardia asteroides, N. brasiliensis, N. caviae*, several Mycobacterium species.

(2) Those containing less but a still detectable amount of arabinose: *Streptomyces madurae, S. pelletierii.*

(3) Those containing no arabinose: *Streptomyces somaliensis*, saprobic Streptomyces species.

It is interesting to compare this separation based on arabinose content with that found by Gonzalez-Ochoa & Vazquez-Hoyos (1953) who used serological methods. Their groups were as follows.

(1) 'Bovis' group: anaerobic Actinomyces species, *Nocardia asteroides, N. brasiliensis.*

(2) 'Madurae' group: *Streptomyces madurae, S. pelletierii.*

(3) 'Somaliensis' group: only *Streptomyces somaliensis.*

(4) 'Paraguayensis' group: saprobic Streptomyces species.

The agreement of the two methods is of a high order, the main differences being that anaerobic Actinomyces species lack arabinose in the cell wall and *S. somaliensis* goes with the saprobic species by the chromatographic method.

A somewhat similar method which uses diaminopimelic acid as a marker was described by Becker, Lechevalier, Gordon & Lechevalier (1964). This method gives results in accord with those given by the arabinose method but simple sugars are easier to handle than amino acids and related compounds. Extended studies are likely to show that this type of analysis for arabinose, diaminopimelic acid and other compounds is of great taxonomic value.

General considerations

How valuable is biochemistry in differentiating pathogenic fungi? In clinical practice a mixture of cultural morphology, histology and serology seldom leave the diagnostician in doubt. However, at a more fundamental level the degree of precision hitherto available has certainly not been sufficient. The fact that fungi exhibit great variety of form has probably retarded the development of alternative differential criteria. But it is well known that wide form variations occur in any given species from time to time, and mycology has always been plagued by a great plenitude of 'species', many of which have been created on quite insufficient morphological data. Considerable space has been given here to those fungi which cause ringworm and mycetoma because in these two groups traditional methods of classification have created a great array of 'species' whose true status in many cases has still to be correctly assessed. One feels that the study of form is reaching its limit and that future differentiations must include other criteria; biochemical and serological methods are the obvious candidates in this field and both have already proved useful.

It would be too much to say that biochemical methods have now been developed to the point where they can serve as routine tools. The published results are patchy but they are sufficient to show the earnest seeker that biochemical differences do exist and that diligent research ought in time to create a useful and usable pattern of methods. Assimilation tests should be sought before tests involving chemical analysis of the fungus for the very simple reason that they are easier to perform and generally require no specialized apparatus.

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The Inhibitory Action of α-Methylmethionine on *Escherichia coli*

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SUMMARY

α-Methylmethionine is a potent growth-inhibitory analogue of methionine producing an immediate effect on the growth rate of *Escherichia coli* at very low concentrations. Under certain conditions inhibition was transient, apparently due to an adaptation by the organisms. The analogue did not repress the synthesis of the methionine-forming enzymes but mimicked methionine as a feedback inhibitor of homoserine-*O*-trans-succinylase, acting on the enzyme at even lower concentrations than did methionine itself. Comparison of the effects on enzyme activity and growth rate leads to the conclusion that such enzyme inhibition is the basis of the effect of analogue on growth. Certain other compounds related to methionine also inhibited homoserine-*O*-trans-succinylase and of these D-methionine, DL-homocysteine and *N*-acetylmethionine probably caused inhibition after conversion to L-methionine.

α-Methylmethionine markedly inhibited the formation of infective phage after irradiation of *E. coli* HfrH(λ); methionine annulled the effect and allowed phage development to occur. The analogue was not incorporated into proteins in place of methionine, nor did it interfere with the incorporation of added methionine. Protein synthesis was inhibited by the analogue only when the process was dependent on methionine formation.

INTRODUCTION

Amino acid analogues produce inhibitory effects in a number of different ways. Competition with the normal amino acids either for incorporation into protein, or in some other key metabolic reaction, frequently occurs. Thus the phenylalanine analogue, *p*-fluorophenylalanine, the methionine analogues norleucine and ethionine and the histidine analogue triazolealanine all readily replace the natural amino acids in proteins (Richmond, 1960; Munier & Cohen, 1959; Gross & Tarver, 1955; Levin & Hartman, 1963), although the methionine analogues may also influence other key reactions such as transmethylations (Lewis, 1963). Analogues may also mimic a natural metabolite in a regulatory feedback reaction and therefore interfere with metabolite synthesis. Thus the histidine analogue thiazolealanine (Moyed, 1961) mimics histidine as a feedback inhibitor of phosphoribosyl-ATP pyrophosphorylase, and 8-azaguanine mimics guanine as a repressor of the synthesis of the purine-forming enzymes (Levin & Magasanik, 1959).

Much work has been described in which the methionine analogues norleucine and ethionine were used; the present work describes the inhibitory effect of another analogue α-methylmethionine which appears to mimic the feedback effect of methio-

nine. These observations form part of a wider study on the mechanism of analogue inhibition and resistance to inhibition (Lawrence, Smith & Rowbury, 1967).

METHODS

Organisms. The auxotrophic strains of *Escherichia coli* used were maintained and subcultured as described previously (Rowbury & Woods, 1966). Strain PA 15 requires serine or glycine for growth and strain 7/9 responds to methionine, homocysteine or cystathionine. Strains HfrH(λ) (obtained from Dr W. Hayes) and CLA (obtained from Dr G. Bertani) are prototrophic.

Organisms for growth experiments and for the preparation of enzymic extracts were grown on medium GL (Rowbury & Woods, 1964) supplemented with L-serine (mM) for strain PA 15 or DL-homocysteine (0·2 mM) for strain 7/9. Growth conditions and the preparation of enzymic extracts were as described previously (Rowbury & Woods, 1964).

Growth tests. For testing the effect of α-methylmethionine and other analogues on the growth of *Escherichia coli* strain PA 15 from small inocula, tubes containing 4 ml. medium GL with L-serine (mM) and appropriate amounts of analogue were sterilized and after seeding with 0·1 ml. of organism suspension (equiv. 0·3 mg. dry wt/ml.) incubated in a sloped position at 37° for 16 or 40 hr. Growth was assessed by using the EEL photoelectric colorimeter (Evans Electroselenium Ltd., Halstead, Essex).

For studying the growth of cell suspensions, washed organisms equiv. 0·05 mg. dry wt/ml. were incubated with shaking (Rowbury & Woods, 1964) at 37°. Samples were removed at 30 min. intervals and growth assessed with a Unicam SP 600 at 620 mμ.

Enzyme assays. Homocysteine methylase and homoserine-*O*-trans-succinylase were assayed in intact organisms as described previously (Rowbury & Woods, 1964, 1966); cystathionase and cystathionine synthetase were measured in ultrasonic extracts (Rowbury & Woods, 1966).

Induction of λ phage and phage assay. Escherichia coli HfrH carrying prophage λ was grown in medium GL overnight, harvested, washed, resuspended in fresh medium GL and grown with shaking at 37° for 2 hr to obtain logarithmically growing organisms. Batches (5 ml.) of such cultures were then irradiated for 50 sec. (Phillips 6 Watt TUV lamp 95 % emission at 2537 Å at 12 in. from culture) to induce the prophage. Irradiated organisms were incubated with shaking for a further 2 hr to allow phage development, chloroform (10 %, v/v) being then added to bring about lysis. After removal of the chloroform by bubbling with air, suitable dilutions were plated with indicator bacteria (*E. coli* strain CLA) in a soft agar overlay to estimate the number of effective phage particles.

Uptake of phenylalanine by organisms. Organisms of *Escherichia coli* strain PA 15 or strain 7/9 grown as previously described were incubated (equiv. 1 mg. dry wt organisms/ml.) in 2 ml. of medium previously used for methionine assays with *Streptococcus equinus* (see Gibson & Woods, 1960) diluted 1/4. ^{14}C-phenylalanine (0·5 μc/ml., uniformly labelled, obtained from the Radiochemical Centre, Amersham, Buckinghamshire), was also added together with appropriate amounts of α-methylmethionine. Incubation was for 1·5 hr, after which period organisms were heated in a boiling water bath for 10 min. Organisms were harvested by centrifugation, washed twice on the centrifuge, taken up in 2 ml. water and the radioactivity assessed by spotting samples

on ground-glass discs. A mica end-window Geiger-Müller tube (General Electric type 2B2) was used in conjunction with a Scaler 1700 of Isotope Developments Ltd., Reading, Berks.

Chemicals. The chemicals used were as described previously (Rowbury & Woods, 1966).

RESULTS

The effect of α-methylmethionine on the growth of Escherichia coli

When tested on the growth of *Escherichia coli* strain PA 15 from small inocula, α-methylmethionine was far more potent an inhibitor than ethionine or norleucine. Growth was completely inhibited by 3 μM α-methylmethionine, whereas 0·1–1·0 mM was needed for full inhibition by the other analogues. The effect of α-methylmethionine was completely annulled by equimolar amounts of methionine or cystathionine, but greater amounts of homocysteine were required to restore normal growth. Homoserine, an earlier methionine precursor, also annulled analogue inhibition.

The effect of α-methylmethionine was also tested with *Escherichia coli* strain 7/9 which is blocked in cystathionine formation. When these organisms were grown on

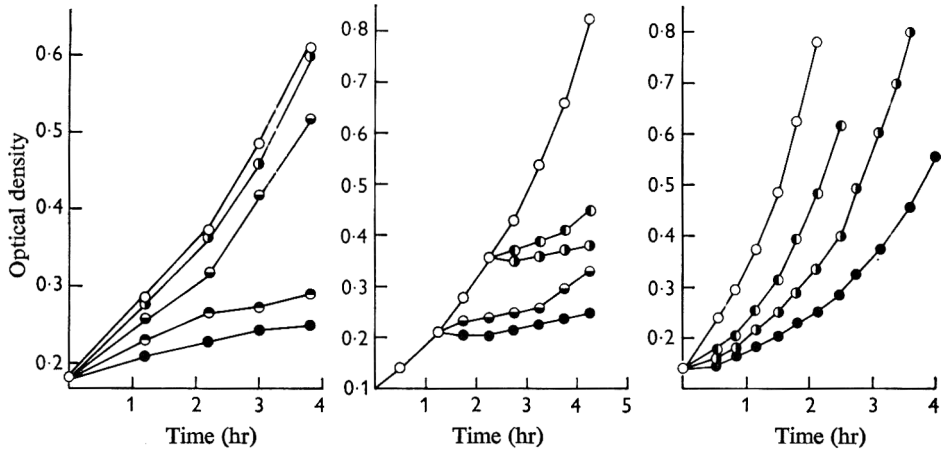

Fig. 1 Fig. 2 Fig. 3

Fig. 1. The effect of α-methylmethionine on the growth of washed suspensions of *Escherichia coli* strain PA 15. Organisms grown on GL medium + serine (mM) were resuspended in fresh medium (+serine) with shaking at 37°. Growth was measured as described in Methods. ○, Control culture, no analogue added; ●, 20 μM-α-methylmethionine added at zero time; ◑, 20 μM-α-methylmethionine and 20 μM-DL-methionine added at zero; ◒, 20 μM-α-methylmethionine + 20 μM-DL-cystathionine added at zero; ◓, 20 μM-α-methylmethionine + 20 μM-DL-homocysteine added at zero.

Fig. 2. The effect of α-methylmethionine added during growth of *Escherichia coli* strain PA 15. Organisms grown as for Fig. 1 were resuspended in fresh medium (+serine) and grown with shaking at 37°. ○, Control culture, no analogue added; ●, 20 μM-α-methylmethionine added at 1·25 hr; ◒, μM-α-methylmethionine added at 1·25 hr; ◑, 20 μM-α-methylmethionine added at 2·25 hr; ◐, μM-α-methylmethionine added at 2·25 hr.

Fig. 3. The effect of low concentrations of analogue on the growth of washed suspensions. Organisms (*Escherichia coli* strain PA 15) grown in medium GL + serine, were resuspended in fresh medium (+serine) and grown at 37° as for Figs. 1 and 2. ○, control culture, no analogue added; ◐, 0·4 μM-α-methylmethionine added at zero; ◑, 0·7 μM-α-methylmethionine added at zero; ●, μM-α-methylmethionine added at zero.

growth-limiting amounts of methionine, the analogue had only a slight inhibitory effect.

The effect of α-methylmethionine was studied more closely with washed suspensions of *Escherichia coli* strain PA 15. Analogue (20 μM) added at zero time produced marked inhibition which again was readily annulled by methionine; cystathionine also restored growth, but homocysteine was ineffective unless higher concentrations (0·3 μM) were used (Fig. 1). α-Methylmethionine was not only more potent than ethionine and norleucine but it acted more quickly; norleucine and ethionine produce gradual growth inhibition (Lawrence *et al.* 1967), but α-methylmethionine stopped the growth of *E. coli* PA 15 immediately it was added (Fig. 2). When lower concentrations of analogue were tested (either by addition initially or during exponential growth) it was observed that above μM the inhibition was almost complete and lasting but that between 0·1 μM and 1 μM there was only a transient effect and the growth rate recovered almost to that of the control culture (Fig. 3). After growth in the presence of α-methylmethionine organisms became partially adapted. Thus after growing with 1·2 μM analogue, washed suspensions grew better on low amounts of analogue than did control suspensions (Table 1), although partial growth inhibition occurred.

Table 1. *Adaptation of Escherichia coli strain PA 15 to growth in presence of α-methylmethionine*

Organisms (*E. coli* strain PA 15) were grown on minimal medium (+serine) with α-methylmethionine (1·2 μM) present (adapted culture) or absent (control culture). Washed suspensions of each culture were then suspended in minimal medium with the stated amount of analogue.

	Mean generation time (min.)		
	In minimal medium	In minimal +analogue (0·6 μM)	In minimal +analogue (0·9 μM)
Control culture	115	240	330
Adapted culture	115	143	207

The effect of α-methylmethionine on the synthesis and activity of the methionine-forming enzymes of Escherichia coli

α-Methylmethionine did not act by repressing the synthesis of the methionine-forming enzymes because at growth inhibitory concentrations the amounts of the enzymes were somewhat increased (1·5 to 2-fold); such increases may be responsible for the partial adaptation to the analogue (Table 1). These observations confirm the earlier view (Rowbury & Woods, 1961) that the analogue was a poor repressor as compared to methionine even at 1 mM. When the analogue was tested for its effect on the activity of the methionine forming enzymes, it was observed that the first enzyme of the pathway (homoserine-*O*-succinylase) was very sensitive to inhibition; activity was decreased to 10% at 2 μM analogue. The later methionine-forming enzymes were however quite unaffected by analogue even at 1 mM.

The effects of the analogue on growth and enzyme activity were compared by examining the initial growth rates of suspensions (*Escherichia coli* strain PA 15) and the homoserine-*O*-trans-succinylase activities of organisms (*E. coli* strain 7/9) in the presence of a range of analogue concentrations. The analogue influenced the two processes very similarly (Table 2). The effect of α-methylmethionine on the enzyme was

in fact more marked than that of methionine itself. Thus methionine decreased activity to 50% at 8 μM, whereas analogue produced the same inhibition at 0·3 μM. Several other compounds structurally related to methionine were tested on homoserine-*O*-trans-succinylase. Ethionine and norleucine produced inhibitory effects only at high concentrations. Activity was very sensitive to the addition of D-methionine, DL-homocysteine and *N*-acetylmethionine, all of which produced 80–90% inhibition at 0·1 mM. Two growth-inhibitory compounds, norvaline and *S*-methylcysteine were also strong inhibitors, although much less so than α-methylmethionine (Table 3).

Table 2. *The effect of α-methylmethionine on growth and on the activity of homoserine-O-trans-succinylase in Escherichia coli*

For growth experiments, *E. coli* strain PA 15 was grown as described in Methods, harvested and resuspended (equiv: 0·05 mg. dry wt/ml.) in GL medium with the stated concentration of analogue. Growth was measured every 30 min. For enzyme experiments *E. coli* strain 7/9 was grown in GL medium, harvested and resuspended (equiv. 0·5–1·0 mg. dry wt/ml.) in fresh medium. Homoserine-*O*-trans-succinylase was assayed as described in Methods.

Concentration of analogue (μM)	Percentage of control	
	Enzyme activity	Growth
0	100	100
0·3	51	55
0·6	30	36
0·9	23	23
1·2	17	16

Table 3. *The effect of methionine and related compounds on the activity of homoserine-O-trans-succinylase of Escherichia coli strain 7/9*

Organisms of *E. coli* strain 7/9 were grown in GL medium and homoserine-*O*-trans-succinylase assayed in the whole organisms as described in Methods with the stated additions.

Additions (mM)	Enzyme activity (% of control)
None	100
DL-α-methylmethionine (0·001)	20
DL-Ethionine (0·5)	51
DL-Norleucine (0·5)	70
DL-Homocysteine (0·1)	8
D-Methionine (0·1)	15
N-Acetylmethionine (0·1)	11
DL-Norvaline (0·025)	42
DL-Norvaline (0·1)	19
DL-*S*-Methyl-cysteine (0·1)	65
DL-*S*-Methyl-cysteine (0·5)	30
L-Methionine (0·05)	10

The effect of α-methylmethionine on bacteriophage induction

Following ultraviolet irradiation of *Escherichia coli* HfrH(λ), incubation for about 2 hr with shaking at 37° allows development of the induced phage in minimal medium. α-Methylmethionine 0·2 mM added during this period decreased the formation of infective phage by a thousand-fold. Methionine (mM) almost completely annulled the effect (Table 4).

Analogue inhibition of phenylalanine incorporation

Escherichia coli strain PA 15 incubated in a medium containing all the protein amino acids except methionine incorporated ^{14}C-phenylalanine into the organisms. Such incorporation was inhibited by α-methylmethionine; μM analogue led to 20% inhibition, 10 μM to 65% inhibition, and 100 μM to 80% inhibition. Inhibition was annulled by equimolar methionine. These results suggested that not only did α-methylmethionine inhibit methionine formation but also that the analogue did not replace methionine in protein. This was further investigated by using a methionine-requiring strain (*E. coli* strain 7/9). As expected this strain incorporated very little phenylalanine unless methionine was added. α-Methylmethionine did not stimulate incorporation in the absence of methionine but ethionine, norleucine, homocysteine and cystathionine were all effective in place of methionine (Table 5).

Table 4. *The effect of α-methylmethionine on the induction of bacteriophage λ in Escherichia coli*

Logarithmically growing *Escherichia coli* HfrH (λ) was ultraviolet irradiated (see Methods) and incubated with shaking (with the stated additions) for 2 hr at 37°. After lysis with chloroform, phage counts were done.

Additions	Infective phage produced particles/ml. culture
None	$3{\cdot}2 \times 10^9$
α-Methylmethionine (0·2 mM)	$1{\cdot}6 \times 10^6$
α-Methylmethionine (10 mM)	$2{\cdot}5 \times 10^4$
α-Methylmethionine (0·2 mM)+DL-methionine (mM)	$2{\cdot}7 \times 10^9$

Table 5. *The effect of various methionine analogues on phenylalanine incorporation by Escherichia coli strain 7/9*

Organisms of *Escherichia coli strain* 7/9 were incubated in 2 ml. of *Streptococcus equinus* medium (see Methods) with ^{14}C-phenylalanine (0·5 μc/ml) and the stated additions. Incubation was for 1·5 hr, after which the organisms were killed, washed and counted as described in Methods.

Additions to culture fluid	Phenylalanine incorporated counts/100 sec./2 ml. fluid
None	2,010
α-Methylmethionine (0·1 mM)	2,010
DL-Methionine (0·1 mM)	16,110
DL-Homocysteine (0·1 mM)	16,050
DL-Cystathionine (0·1 mM)	13,020
L-Ethionine (0·1 mM)	11,160
DL-Norleucine (0·1 mM)	6,090

DISCUSSION

The ability of methionine and biosynthetically related compounds to overcome inhibition by α-methylmethionine shows that the latter is a methionine analogue. The immediate effect of the analogue on the growth of appropriate strains of *Escherichia coli* and its extreme potency as a growth inhibitor implied that it might influence

processes quite different from those affected by ethionine and norleucine. It is attractive to consider that the inhibitory effect of the analogue is due to its similarity to methionine which causes blockage of homoserine-*O*-trans-succinylase. Concentrations of analogue which inhibit enzyme activity also inhibit or prevent growth. Feedback inhibition of this sort would be expected to produce an immediate effect on growth rate (due to an immediate limitation of methionine) as observed (Fig. 2), while at low concentrations of analogue, limitation of methionine would lead to enzyme derepression with gradual restoration of methionine synthesis, and therefore inhibition would be transient (Fig. 3). Such transient growth inhibition has been observed with the histidine analogue thiazolealanine which is a feedback inhibitor of histidine synthesis (Moyed, 1961). The observation that *E. coli* strain 7/9 (which cannot form cystathionine) was almost insensitive to inhibition is consistent with an effect of the analogue on cystathionine synthesis.

A parallel study with *Salmonella typhimurium* (Lawrence, *et al.* 1967) provides further evidence that α-methylmethionine acts on growth by inhibiting homoserine-*O*-trans-succinylase .Many mutants of *Salmonella typhimurium* which excrete methionine and are resistant to α-methylmethionine do not show cross-resistance to ethionine and norleucine and such mutations map very close to the *met*-A gene (the structural gene for homoserine-*O*-trans-succinylase (Smith & Childs, 1966) and it is proposed that they contain an altered enzyme insensitive to both methionine and α-methylmethionine.

α-Methylmethionine may have other effects. Thus the inability of small concentrations of homocysteine to annul inhibition of growth suggests that α-methylmethionine may influence the uptake of homocysteine. Cystathionine, an earlier methionine precursor, is more effective than homocysteine and possibly gets into organisms without interference from the analogue.

The formation of methionine from D-methionine, DL-homocysteine, and *N*-acetylmethionine probably accounts for the effect of these compounds on homoserine-*O*-trans-succinylase. Homocysteine is readily methylated to methionine by intact organisms of *Escherichia coli* (Gibson & Woods, 1960), methionine-requiring mutants of *E. coli* will utilize D-methionine for growth (Cooper, 1966), while methionine (identified by paper chromatography) is readily produced from *N*-acetylmethionine by intact organisms of *E. coli* strain PA 15 (unpublished observations).

It has been previously observed that methionine is much more effective as an inhibitor of homoserine-*O*-trans-succinylase when intact organisms of *Escherichia coli* are tested than when ultrasonic extracts are used. It is probable that intact organisms convert methionine to *S*-adenosylmethionine which is a co-inhibitor of the enzyme (Lee, Ravel & Shive, 1966). The inhibition of growth and enzyme activity by very low concentrations of α-methylmethionine implies that this compound also may form an *S*-adenosyl derivative active as a co-inhibitor.

Limitation of methionine by the feedback effect of the α-methylmethionine was probably responsible for the inhibition of (*a*) bacteriophage formation in *Escherichia coli* HfrH, and (*b*) phenylalanine incorporation in *E. coli* PA 15. Apart from its effect on methionine formation the analogue did not apparently influence protein synthesis. Thus it did not replace methionine in protein synthesis (Table 5) nor did it interfere with incorporation of labelled methionine (unpublished observations).

The author is grateful to Dr D. A. Smith and Mr D. A. Lawrence for helpful discussions during this work and to Miss Elaine Warden for expert technical assistance.

Since this work was completed Schlesinger (1967) has published results and conclusions very similar to those in the present paper.

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Deoxyribonucleic Acid Base Composition of Some Species within the Genus *Candida*

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SUMMARY

The base composition of purified DNA from 18 species of the genus *Candida* was determined from the denaturation temperature, *Tm*. A great genetic heterogeneity was found with a mean molar guanine+cytosine (% GC) composition ranging from 35·1–57·6 %.

Candida albicans, *C. tropicalis*, *C. clausenii* and *C. stellatoidea* showed essentially identical base composition 35·1–35·7 % GC. The highest values (54·1–57·6 % GC) were noted for *C. catenulata*, *C. brumptii* and *C. zeylanoides*. These three species also showed a compositional distribution of their DNA distinctly higher than the other species examined. The taxonomic and phylogenetic affinity between *C. catenulata*, *C. brumptii* and *C. zeylanoides* and the *C. albicans* group must on the basis of these investigations be seriously reconsidered. The rest of the species examined showed a wide range of mean % GC between the two clusters already mentioned. A variable degree of genetic affinity between these species and the *C. albicans* group cannot be excluded on the basis of the present examinations. Knowledge of DNA base compositions would appear to be of definite significance in the taxonomy of yeasts.

INTRODUCTION

Recent progress in molecular biology has rendered it possible to compare some properties of the deoxyribonucleic acid (DNA) molecules in different micro-organisms and has in this way given a better foundation of taxonomy, the latter being theoretically based on similarities within genomes (Marmur, Falkow & Mandel, 1963). The methods most widely used are: (1) determination of DNA base composition, and (2) assessment of base-sequence homology by means of DNA-DNA or DNA-RNA hybrid experiments. A determination of base composition will most naturally be the first approach to the problem, because great similarity in base composition is a prerequisite for extensive base-sequence homology and thus also for similarity in phenotype. Determination of base composition has been shown to be of considerable value in bacterial taxonomy. The main reason for this is the wide variation in mean base composition (most frequently expressed as the mean molar percentages (guanine + cytosine) (% GC)) found between the different members of the bacterial world (25–75 % GC) as opposed to the narrow compositional distribution of DNA molecules within the individual bacterial cell (Sueoka, 1961). Whether similar conditions are valid as regards yeasts cannot be assessed, as only scarce information of base composition in these organisms is available. However, by determining the base composition in species from different genera of fungi including a few yeast species, Storck (1966) found a fairly wide variation of mean % GC. On the basis of these

determinations and a very few others, culled from the literature (referred to in his paper), he suggested that knowledge of base composition might be of value in the taxonomy of fungi.

The present paper deals with the base composition in different species of the genus *Candida* and gives an assessment of the value of the determinations in yeast taxonomy. We have chosen this genus for study because of its heterogeneity. The main characteristic of the genus is the ability of its members to form a pseudomycelium. This feature may be difficult to observe because the ability to produce this character varies within and between species and is dependent on the conditions of cultivation. The genus comprises species of very different morphology and ecological status. Some species are strong fermenters; others ferment only weakly or fermentation ability may be absent. Some species assimilate nitrate; most do not. Their ability to assimilate carbon compounds is also varied.

METHODS

Organisms. All species investigated except *Candida atmosphaerica* and *C. clausenii*, obtained from the Czechoslovak Collection of Micro-organisms (CSAV), were obtained from the Centraalbureau voor Schimmelcultures, Baarn, Netherlands (CBS). Their names and species numbers are listed in Table 1. All species were reclassified according to the principles given by Lodder & Kreger-van Rij (1952).

Cultivation of organisms. All species were grown in 7·5 l. quantities of a liquid medium containing (%, w/v): glucose 5·0, peptone (Orthana) 1·0, yeast extract (Oxoid) 0·5, K_2HPO_4 0·1; adjusted to pH 5·6. Growth took place at room temperature (about 23°) in 10 l. Pyrex flasks, vigourously aerated. After growth for 1–3 days, the yeasts were harvested by centrifugation in a Sorvall continuous flow system at 4°, 10,000 *g*, 500 ml./min. Tests for bacterial contamination were made by direct microscopy and plating on blood agar (incubated at 25° and 37°) immediately before centrifugation.

Preparation of pure DNA. The organisms (at least 50 g. wet weight) were washed three times in cold saline EDTA (0·15 M-NaCl + 0·1 M-ethylenediaminetetra-acetate (EDTA), pH 8) and disrupted with a Branson sonifier, Model S 75, operating at maximal energy. The sonic treatment was done on 50 ml. portions of a 10 % suspension of the yeast paste in saline EDTA to which were added glass beads (Ballotini, 0·1 mm.). Each portion was treated for 5 min. on an ice-bath. Sonic treatment was chosen as the most convenient method for routine use. Judging from an experiment by Marmur (1961) on sonic treatment of pneumococci a molecular weight of the DNA of about 1×10^6 might be anticipated.

For extraction of pure DNA from the partially broken organism suspension the method of Marmur (1961) was followed throughout except for the introduction of an additional ribonuclease treatment and isopropanol precipitation early in the procedure to remove the bulk of the RNA and polysaccharide. The final fibrous precipitate of pure DNA was dissolved in saline citrate (0·15 M-NaCl + 0·015 M-trisodium citrate; pH 7·0) and stored at 4° over a few drops of chloroform.

Determination of DNA base composition by thermal denaturation. An automatically recording thermospectrophotometer was set up according to the principles given by Szybalski & Mennigmann (1962) and De Ley & van Muylem (1963). The arrangement consisted of an Unicam SP 800 ultraviolet spectrophotometer, equipped with a thermostatted cuvette holder (Unicam, SP 874). The cuvette holder was heated from a

circulating water bath, the temperature of which could be raised at a constant rate. The temperature was measured in a control cuvette containing the platinum resistance of a Gilford linear thermosensor. Temperature and extinction were recorded on the x and y axes, respectively, of a Moseley x–y autograph, Model 2D-4M. The temperature was increased by 0·2° min., and the extinction recorded in the temperature interval from 60 to 100°. One sample containing pure DNA in saline citrate and adjusted to give an extinction of about 0·7 (approximately 28 μg. DNA/ml.) was heated in each experiment. The blank contained adenine of the same extinction in the same buffer to correct for thermal expansion. The temperature standards on the thermosensor was checked against a calibrated thermometer, and the reliability of the method was checked by comparing the measured *Tm* values for several specimens of bacterial DNA with those recorded in the literature.

The *Tm* values were determined as the mid-point of the extinction–temperature curves between extinction at 69° and extinction at maximal hyperchromicity (Rogul, McGee, Wittler & Falkow, 1965). The mean base composition, expressed as % GC was calculated from *Tm* by the formula: % GC = $(Tm - 69{\cdot}3)/0{\cdot}41$ (Marmur & Doty, 1962).

Table 1. *'Melting point' Tm, with standard deviation, and the mean base composition expressed as % GC of pure DNA from species of the genus Candida*

For methods, see text.

Species	Source	*Tm* (° C) ± S.D.	% GC
C. albicans	CBS 2712	83·7 ± 0·05	35·1
C. tropicalis	CBS 120/5	83·6 ± 0·10	34·9
C. clausenii	CSAV 29-31-1	83·6 ± 0·10	34·9
C. stellatoidea	CBS 1905	83·9 ± 0·15	35·7
C. pelliculosa	CBS 605	84·4 ± 0·15	36·8
C. truncata	CBS 1899	84·4 ± 0·15	36·9
C. krusei	CBS 573	85·5 ± 0·05	39·6
C. atmosphaerica	CSAV 29-50-1	85·6 ± 0·10	39·7
C. parapsilosis	CBS 604	86·0 ± 0·15	40·8
C. melinii	CBS 661	86·1 ± 0·10	40·9
C. pseudotropicalis	CBS 607	86·2 ± 0·10	41·3
C. tenuis	CBS 615	87·4 ± 0·15	44·0
C. utilis	CBS 621	88·1 ± 0·10	45·8
C. pulcherrima	CBS 610	89·0 ± 0·15	48·0
C. lipolytica	CBS 599	89·6 ± 0·00	49·6
C. brumptii	CBS 564	91·5 ± 0·10	54·1
C. catenulata	CBS 565	91·7 ± 0·05	54·5
C. zeylanoides	CBS 619	92·9 ± 0·15	57·6

RESULTS

The species of *Candida* examined, together with the thermal denaturation temperature *Tm*, and the mean base composition % GC, are presented in Table 1. Figure 1 gives examples of denaturation curves for three species. The *Tm* values are averages of at least four determinations. The standard deviation of the mean *Tm* was in most cases 0·10–0·15°, corresponding to a deviation in % GC of 0·25–0·40.

In most cases the denaturation curves showed a regular S shape indicating an unimodal Gaussian or nearly Gaussian distribution of the DNA molecules. However, a skewness to the left or even a small shoulder at the initial part of the denaturation

curves was seen for some species. The two most pronounced examples are given in Fig. 2. This irregularity may suggest that a smaller DNA fraction is present in the DNA preparations from these species. The *Tm* values of this possibly satellite DNA are very low (below 80°). A similar pronounced AT-type DNA in *Saccharomyces cere-*

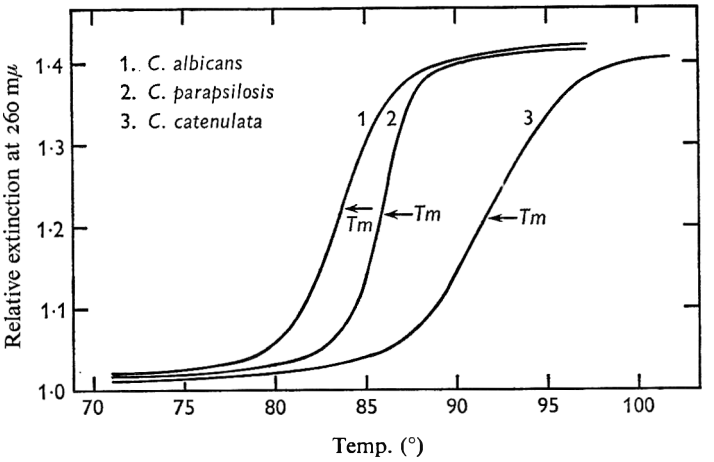

Fig. 1. Examples of thermal denaturation curves of DNA of species selected from each of the three groups in Table 1. The results are expressed as the relative absorbance at 260 mμ (see text) versus temperature. The curves are displaced vertically to allow better resolution.

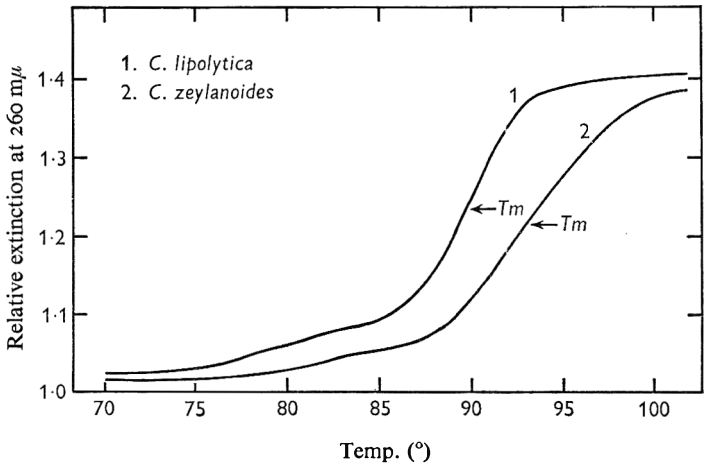

Fig. 2. Thermal denaturation curves of DNA fo two species showing two components, a major 'nuclear' DNA, and a minor presumably mitochondrial DNA. The results are expressed as in Fig. 1.

visiae and *S. carlsbergensis* has been found to correspond to a double helical mitochondrial DNA by Tewari, Jayaraman & Mahler (1965) and Corneo, Moore, Sanadi, Grossman & Marmur (1966).

The *Tm* values in Table 1 are corrected to correspond to the main 'nuclear' DNA. The correction was lower than 0·5° in all instances, usually 0·2°.

DISCUSSION

The mean base compositions are seen to cover a wide range (35·1–57·6 % GC) indicating a pronounced genetic heterogeneity among the species investigated. The lowest % GC values are seen for *Candida albicans*, *C. tropicalis*, *C. clausenii* and *C. stellatoidea*. These four species which, from a conventional point of view, must be regarded as very closely related, show essentially identical base composition (35·1–35·7 % GC). At the other extreme is found a relatively narrow cluster (54°1–57·6 % GC) composed of *C. catenulata*, *C. brumptii* and *C. zeylanoides*. These three species also differ from the other species in having a distinctly greater heterogeneity among their DNA molecules. When the standard deviation σ of the compositional distribution of DNA molecules around the mean % GC was calculated by using the equation of Doty, Marmur & Sueoka (1959), a value greater than 5 % GC was found for these species. For the other species the σ values varied between 0·5–3·0 % GC, comparable with the intermolecular heterogeneity in many bacteria. However, the taxonomic significance of this parameter should be evaluated with great caution. While *Tm* is not influenced by a decrease in molecular weight down to at least 600,000 (Marmur & Doty, 1962), this need not be so as regards the σ value. A greater intramolecular than intermolecular heterogeneity will thus increase the value of σ when the DNA molecules are degraded.

The variation in σ among the yeasts studied here may result from a difference in molecular weight of the DNA caused by the differences in the ease with which the different species were disrupted during ultrasonic treatment. If this be so, the different σ values must be an indication of a pronounced non-random distribution of bases in the DNA molecules. However, the high values of σ within the high *Tm* group were reproducible and constantly at a higher value than was the case within the rest of the species examined. On the basis of these two factors, the high mean % GC and the great compositional distribution of the DNA molecules, the placing of *Candida catenulata*, *C. brumptii* and *C. zeylanoides* in the same genus as the other species must be seriously reconsidered. These species may have a different phylogenetic origin.

The rest of the species examined cover a wide range (36·8–49·6 % GC) between the two clusters already mentioned. On the basis of our present knowledge it seems premature to discuss the relation of these species to the *Candida albicans* group. However, on account of the presumed non-random distribution of bases and the fact that the σ values calculated by the equation of Doty *et al.* (1959) presumably underestimate the actual degree of possible common DNA molecules (De Ley, Park Tijtgat & van Ermengen, 1966), a certain degree of genetic affinity between the different members of these two groups cannot be excluded.

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J. gen. Microbiol. (1968), **52**, 237–241
With 1 *plate*
Printed in Great Britain

Growth Retardation, Colonial Changes and Nutritional Deficiency in *Mycobacterium phlei* Due to Lysogeny

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SUMMARY

Changes in *Mycobacterium phlei* due to B2h lysogeny were studied by the replica plating method. The changes could be divided into three categories: mutation to slow growth and small colony size; mutation to relative but complex nutritional requirements; and finally mutation to thiamine deficiency (thi^-). A segregant clone obtained in the thi^- mutant showed the characteristics of slow-growing small colony variants. Change in the bacterial host could be related to the type of integrated phage.

INTRODUCTION

Genetic changes in bacteria due to host–phage interaction have been repeatedly reported. Toxin production in *Corynebacterium diphtheriae* (Freeman, 1951; Barksdale, 1959), altered colonial morphology in *Bacillus megaterium* (Ionesco, 1953), changes in the antigenic composition of Salmonella (Uetake, Nagakawa & Akiba 1955) are the best known instances of phage and lysogenic conversion.

Lysogenic conversion in *Mycobacterium phlei* affects colonial morphology, growth characteristics and nutritional requirements, as reported briefly by Juhasz & Bönicke (1966) and Juhasz (1967*a*). It has also been found instrumental in the conversion of *M. phlei* to *M. smegmatis* (Juhasz, 1967*b*). In this paper the effects of lysogeny by phage B2h in *M. phlei* are described in detail.

METHODS

Bacterial strains. Mycobacterium phlei F89 (hereafter designated F89) was obtained from Dr S. Froman, Olive View Sanatorium, Olive View, California. F89(B2h) strains 1–5 were isolated from lysogenic complexes established between F89 and phages B2 and B2h, respectively. Both their isolation and purification by serial single colony transfers were described earlier (Juhasz & Bönicke, 1966; Juhasz, 1967*b*). Since each of the five highly purified lysogenic strains showed B2h immunity and released, under certain conditions, infectious B2h particles, all of them will be referred to as B2h lysogens irrespective of whether they were infected originally with B2 or B2h. F89(B2h) strains 1–3 were originally designated F89(B2h), F89(B2)′ and F89(B2)′′′′ (Juhasz & Bönicke, 1966). The bacterial strains were maintained on Loewenstein–Jensen

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medium. Immediately before use they were transferred and grown on nutrient agar (Difco).

Phages. Phage phlei B2 (hereafter designated B2) was isolated from dung (Juhasz & Bönicke, 1965). Phage B2h. F89 (hereafter designated B2h) was obtained on reisolation of phage from lysogenic strain F89(B2) (Juhasz & Bönicke, 1966). Propagation and purification of phages as well as their use in the lysogenization of strain F89 were described elsewhere (Juhasz & Bönicke, 1965; Juhasz & Bönicke, 1966).

Persistence of lysogeny was tested repeatedly during the experiments by testing for (*a*) the release of infectious particles able to form plaques on the susceptible strain F89 and (*b*) the superinfection immunity of strains to phages B2 and B2h.

Replica plating. Tenfold dilutions of each strain were spread over nutrient agar plates and the plate which showed 50–100 colonies was chosen as masterplate. The replica plating technique of Lederberg & Lederberg (1952) was used to transfer the colonies to complete medium, minimal medium and minimal media supplemented with complex nutrients such as yeast extract, yeast RNA and casein hydrolysate + tryptophan. Subsequently, minimal media were supplemented with single growth factors: vitamins, amino acids, nucleosides, nucleotides, purine and pyrimidine bases, or, if necessary, combinations thereof.

Media. Nutrient agar was used for complete medium. The minimal medium used was a modified Davis minimal medium (Lederberg, 1950); 0·5 % glycerol replaced glucose as carbon source. Yeast extract was used in 0·3 %, casamino acids and nucleic acid derivatives in 50 μg. per ml. amounts. With the exception of the complete medium, media were solidified by 1·5 % (w/v) purified agar. The ingredients of media and chemicals were obtained from the following manufacturers: nutrient agar, yeast extract and purified agar from Difco; amino acids, thiamine and ascorbic acid from the British Drug Houses Ltd., other vitamins and yeast RNA from Mann Research Laboratories, Inc. In addition to the latter, yeast RNA prepared by Nutritional Biochemical Corp., and Worthington Chemicals were used in special studies with the thi$^-$ mutant.

RESULTS

(i) *Growth characteristics of lysogens.* Three main categories could be distinguished among the investigated lysogens. (1) Slow-growing small colony variants; F89(B2h) strains 1 and 2; (2) strains with complex but partial nutritional requirements; F89(B2h) strains 3 and 4; (3) thi$^-$ mutant; F89(B2h) strain 5.

Plate 1 shows the growth of these categories on replica plates after 7- to 8-day growth. Failure to grow on minimal medium distinguishes the thi$^-$ class from the other categories. The slow-growing small colony class only reach $\frac{1}{2}$–$\frac{1}{3}$ the size of wild-type colonies, even when grown on complete medium. The strains with complex but partial nutritional requirements only reach full size when grown on complete medium or media supplemented with yeast extract. It is obvious from Pl. 1 that the thiamine requirement of thi$^-$ can be replaced to a considerable extent by yeast RNA. This effect is not due to contamination of yeast RNA with thiamine, since RNA obtained from different sources, including sRNA, had a similar effect while yeast DNA had no effect. More important, the pyrimidine moiety of thiamine (2-methyl-4-amino-5-hydroxymethyl pyrimidine provided by the courtesy of Merck, Sharp and Dohme of Canada Ltd., Montreal) restored full growth to the thi$^-$ mutant.

Figure 1 shows the growth curves for colonies in each category as indicated by colony size on replica plates. It is clear that the initial growth rate of wild-type colonies on minimal medium is about half that on complete medium. The growth rate of the slow-growing small colony variant is about 2–4 times slower than that of the other two categories on complete medium and it produces colonies 1·8–2·4 mm. diam. after 14 days, while colonies of the other two classes measure 4·0–5·0 mm. diam. In addition to slow growth and small colony size this group shows other heritable changes such as decrease in pigmentation and a tendency to grow embedded in the medium in the form of smooth colonies. In contrast to the thiamine-requiring mutant the other categories can grow to varying degrees on minimal medium.

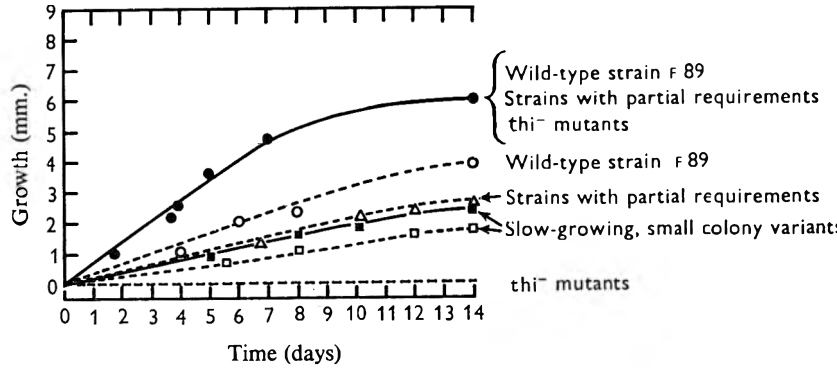


Fig. 1. Differences in growth rate between *Mycobacterium phlei* F 89 and its lysogenic derivatives as indicated by colony size on complete and minimal media. —, Complete medium; – – –, minimal medium.

Table 1. *Changes in Mycobacterium phlei F89 relative to the type of integrated phage genome*

Strain	Nutritional requirement	Growth rate	Size of colony	Pigment of colony	Carried phage	Definition of strain
F89(B2h) no. 1 F89(B2h) no. 2	None	Slow (2- to 4-fold decrease)	Small (1·8–2·4 mm.)	Pale yellow to colourless	Incomplete B2h	Slow-growing, small colony variant. Lysogenic
F89(B2h) no. 3 F89(B2h) no. 4	Partial	Slightly retarded	As wild type	Pale yellow (whitish)	B2h	Partial requirement. Lysogenic
F89(B2h) no. 5	Thiamine	As wild type	As wild type	As wild type	Defective B2h?	Thiamine-less. Defective lysogen?
Wild F89	None	Fast	3–5 mm.	Yellow	None	Prototrophic. Phage sensitive

The number of mutant classes among B2h lysogens may have been limited by the number of randomly selected strains, therefore F89 was freshly infected with B2h at a multiplicity of 3 and the effect of phage was studied before and after purification of lysogenic clones. From 23 single colonies examined 18 became stably lysogenic; 15 of

these were slow-growing small colony variants and three showed complex partial nutritional requirements. thi⁻ mutants were not recovered in this experiment.

Among the rare segregants encountered from these groups, one in the thi⁻ category revealed the characteristics of the slow-growing small colony variant.

(ii) *Phage carried by different categories of B2h lysogens.* A correlation has been found between changes in F89 host and the type of integrated B2h genome (Table 1). Complete B2h genome could be demonstrated only in strains with complex but partial nutritional requirements. The slow-growing small colony variants harboured a B2h genome, the h region of which could not be recovered; none the less they produced infectious particles and carried B2h immunity. Finally, no infectious particles could be demonstrated in the thi⁻ category; however, F89(B2h) strain 5 was immune to superinfection by phage B2h.

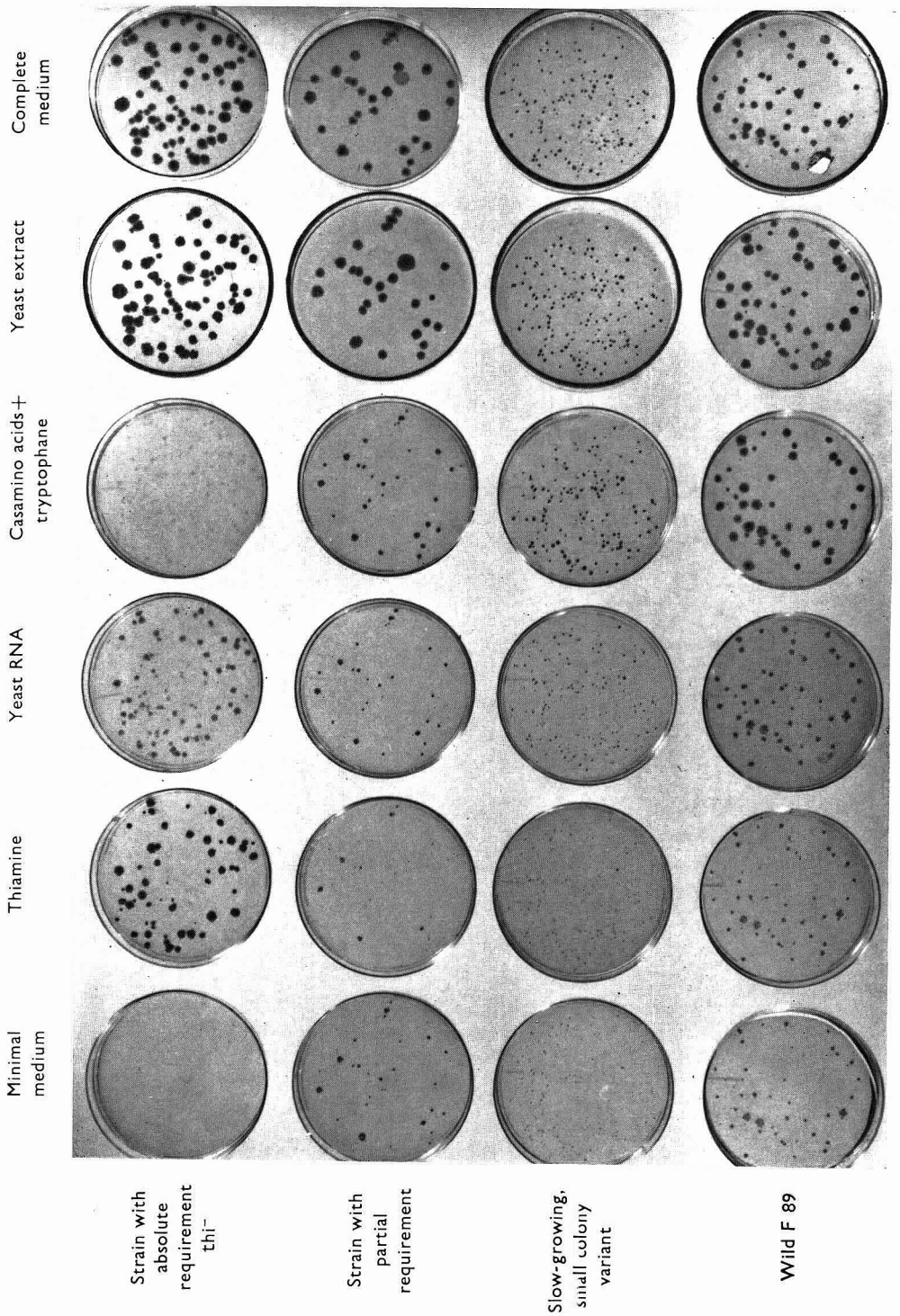
DISCUSSION

The experiments reported here were carried out with purified lysogens in order to analyse hereditary changes in *Mycobacterium phlei* F89 following B2h infection. The results obtained when F89 was relysogenized with B2h indicated, however, that the original 5 strains of this study were not a selected sample but probably rather represented the whole spectrum of change in F89 host following B2h integration.

The most characteristic type of change associated with B2h lysogeny is slow-growing small colony variation. Such changes were never observed in control F89 cultures although phenotypic fluctuation in colony size occurred frequently. Attempts to isolate small colony mutants from wild type failed since subsequent transfers of small colonies yielded repeatedly large colonies along with small ones. In addition, while small and large wild-type colonies differed only in size as a result of random fluctuation, the colony size of slow-growing small colony variants was heritable together with other stable hereditary changes, such as considerable loss of pigmentation and a novel and strong tendency to grow embedded in the medium.

The conditions under which quantitative conversion to slow growth and small colony size occurred were earlier established. At a multiplicity of 1, a 5×10^{-5} fraction of the F89 population survived B2h.F89 infection, more than half of which became stably lysogenic as well as slow-growing small colony variants (Juhasz, 1967*b*). On the other hand, from among several hundred colonies of similarly diluted control F89 suspensions none showed these characteristic hereditary changes.

In contrast to the former type of change the conditions for quantitative conversion to either absolute or partial nutritional requirements have not yet been sufficiently studied. Conversion to partial nutritional requirements upon B2h lysogeny occurred frequently; but conversion to thiamine deficiency was not observed beyond the already available thi⁻ strain. This particular strain was immune to superinfection by B2h. Despite lack of any other evidence for the presence of a defective prophage at the time when thiamine deficiency was detected, mutation to phage resistance was not seriously considered for the following reasons. (1) F89(B2h) strain 5 was obtained upon lysogenization of F89 with B2h, (2) this strain was found to release infectious B2h particles in studies prior to the present one. Therefore it was hypothesized that thiamine deficiency resulted from additional changes in F89(B2h) host. Persistent diploidy over the thi locus of F89 and a reversible exchange between this and the immunity region of B2h could explain defective lysogeny, thiamine deficiency and segregation to slow-growing small colony variants. Nevertheless, so long as



S. E. JUHASZ

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quantitative recovery of thi⁻ mutants cannot be achieved, it will be impossible to rule out a spontaneous mutational origin for thiamine deficiency in this strain.

Phage-induced mutation to auxotrophy was earlier reported in *Escherichia coli* (Taylor, 1963). The circumstances under which MU 1 and B2h were isolated as well as their respective role in producing mutants bore much resemblance. However, in contrast to B2h, Taylor's mutator phage elicited a wide range of mutant classes in *E. coli*. Henceforth his conclusion: 'The overall effect of MU 1 on bacteria is superficially indistinguishable from that of a mutagen.' On the other hand, the following observations indicate the possibility of a fixed chromosomal location of B2h on the F89 chromosome: (*a*) the consistently small number of mutant classes; (*b*) the link between the thi⁻ mutant and the slow-growing small colony variants; (*c*) the correlation between the type (length) of integrated phage and the nature of change in the bacterial host.

Finally, it must be emphasized that it was noticed with great interest that yeast RNA could replace thiamine to a considerable degree in the thi⁻ group. Results of a study in progress on the biosynthesis of the pyrimidine moiety of thiamine will be published elsewhere.

This work was carried out at the Research Station, Canada Department of Agriculture, Vancouver, thanks to the generous hospitality of Dr. M. Weintraub, Chief, Section of Virus Chemistry and Physiology. Continuous interest and innumerable stimulating discussions with Dr. H. Ragetli of the Research Station are gratefully acknowledged. The experiments were carried out with the able assistance of Mr. N. Cvorkov.

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EXPLANATION OF PLATE

PLATE 1

Comparison of growth of wild *Mycobacterium phlei* F 89 and its lysogenic derivatives on replica plates. Incubated at 37° for 7–8 days. ×0·28.

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With 2 plates
Printed in Great Britain

A Mutant of *Chlamydomonas reinhardii* with Abnormal Cell Division

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SUMMARY

A mutant of *Chlamydomonas reinhardii* with abnormal cell division is described. Cultures of the mutant consist of cells with between one and about seven nuclei. The number of pairs of flagella per cell is the same as the number of nuclei, except in large moribund cells which lack flagella. Pyrenoid division lags behind nuclear division in some cells. The expression of the mutation is modified by the age of the culture, pH of the environment or by the presence of certain other mutations. Genetic analysis is slightly complicated by the presence of multinucleate gametes, but it is shown that cytokinesis deficiency is inherited as a single gene mutation which maps close to the *ac* 29 locus on linkage group VI.

INTRODUCTION

Studies on the effect of mutation on cell division may be fruitful since by blocking the processes of division at a particular stage they may help to clarify the sequence of events in the normal division process. Studies on such mutants should also provide considerable information concerning the genetic control of cell division.

Chlamydomonas reinhardii, a unicellular biflagellate alga, is well suited to a study of the effect of mutation on cytokinesis, although the small size of the chromosomes would be a hindrance to studies on mitosis. The ultrastructure of the organism has been studied in detail (Cavalier-Smith, 1967; Ringo, 1967), genetical techniques are well established (Ebersold & Levine, 1959; Ebersold, Levine, Levine & Olmsted, 1962) and large-scale cultures for biochemical experiments may be easily grown in simple inorganic media.

Buffaloe (1958) has shown that cytokinesis in *Chlamydomonas reinhardii* is by furrowing and two or three rounds of division can occur within the original cell wall, which then bursts to liberate four or eight daughter cells. The second cytokinetic furrow occurs at right angles to the first. More recently, Cavalier-Smith (1967) has studied cytokinesis in *C. reinhardii* in the electron microscope. He showed that the chloroplast starts to divide by furrowing before the commencement of cytokinesis proper and that some of the chloroplast disc membranes are actually cut in two by the advancing furrow. Before the chloroplast division is completed, the cytokinetic furrow starts near the basal bodies and develops in association with cytokinetic microtubules. The flagella regress before division and are regenerated after the completion of cytokinesis.

Lewin has briefly described a mutant of *Chlamydomonas moewusii* apparently

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having a deficiency in cytokinesis which leads to multinucleate cells (M 470 in Lewin, 1952). However, relatively little is known of the linkage relationships and genetic techniques of *C. moewusii*. A mutant deficient in cytokinesis has now been isolated in *C. reinhardii* and is the subject of the present paper.

METHODS

Strains. The mutation for cytokinesis deficiency (*cyt* 1) was isolated in this laboratory by Miss Anne McVittie in a wild type, mating type plus strain of *Chlamydomonas reinhardii* which was obtained from the Cambridge Collection of Algae and originally from Professor R. P. Levine's Laboratory at Harvard. Cells were grown for 2 days on solid medium containing 0·5 μg./ml. *N*-methyl-*N*-nitroso-*N*-nitroguanidine (Koch–Light, Colnbrook, Bucks) and then replated on semi-solid medium at 100 per dish. The cytokinesis-deficient mutant was selected on the basis of small colony size and subsequent microscopic examination.

The acetate-requiring and paralysed flagella strains, *ac* 17, *ac* 29A, *pf* 2 and *pf* 20 were kindly provided by Professor R. P. Levine's laboratory and are described in Ebersold *et al.* (1962). The origin and phenotype of *pf* 19B is described in Randall, Warr, Hopkins & McVittie (1964) and Warr, McVittie, Randall & Hopkins (1966).

General techniques. Culture conditions and crossing techniques were as described in Warr *et al.* (1966).

Estimation of flagella number. Cells from liquid cultures were fixed in osmium tetroxide vapour and the flagella on each cell counted with phase optics. Over 200 cells were examined in each sample. A very small proportion of cells (0–3 %) were sometimes enclosed in palmella envelopes (thus obscuring the flagella) and are not included in the data.

Staining methods. Nuclear staining was usually by Azure A according to Levine & Folsome (1959) or less frequently by Feulgen according to Bernstein (1964) after fixation in Carnoy's fixative (15 min.) or in osmium tetroxide vapour. Nuclei appeared less granular and better preserved and flagella are intact with osmium, but this fixative left more basophilic material in the cytoplasm than Carnoy's. Pyrenoids were stained with 0·3 % (w/v) iodine in 1 % (w/v) potassium iodide. Examination and photography of stained material was in bright-field illumination with apochromatic objectives with either a Zeiss photomicroscope using Ilford Pan F film or a Vickers microscope and camera attachment using Kodak P 300 plates.

Electron microscopy. Cells from log. phase liquid cultures were fixed in 1 % (w/v) osmium tetroxide (pH 7·3) or in 3 % (v/v) glutaraldehyde in 0·2 M-cacodylate buffer, pH 7·3. Fixed specimens were dehydrated in acetone and embedded in Araldite. Sections were double stained in uranyl acetate and lead citrate and examined in an A.E.I. 6B electron microscope.

RESULTS

General description of mutant. Cultures of the cytokinesis-deficient mutant (*cyt* 1) have some apparently normal, oval cells (about $7 \times 12\,\mu$) with one pair of flagella, but other cells are of unusual shape bearing several pairs of flagella. Cells with 4, 6 or 8 flagella are common and some cells with 10 to 12 flagella are nearly always present (Fig. 1). Cells with up to 20 flagella have been seen occasionally.

The shape of a cell is related to its number of flagella. Quadriflagellate cells are typically V-shaped with a pair of flagella at the end of each arm (Pl. 1, fig. 1). Cells which have 6 or 8 flagella are sometimes Y- or X-shaped respectively, with a pair of flagella on each arm, although a variety of other shapes is also seen (Pl. 1, fig. 2). Cells with 10 or 12 flagella are typically of grotesque, irregular shapes (Pl. 1, fig. 3). Cells with very high numbers of flagella are usually roughly oval or spherical and about 20–30 μ in diameter.

There are also a few cells present which have no flagella and appear moribund (Pl. 1, fig. 4). In unfixed material these cells are frequently observed to burst, permitting the cell contents to slowly ooze into the surrounding medium. Consequently, the medium in which this mutant is growing always contains a considerable amount of cell debris.

Azure A or Feulgen staining reveals that the normal-shaped cells are uninucleate, whereas V-, Y- or X-shaped cells have a nucleus in each of their 2, 3 or 4 arms (i.e. one nucleus is present for each pair of flagella). Up to seven nuclei may be counted in larger cells (Pl. 1, fig. 5–8). The proportion of cells with a given number of nuclei always closely corresponds with the proportion of cells in unstained samples of the same material with an equal number of pairs of flagella. It seems reasonable to conclude that the number of nuclei in a particular cell equals the number of flagella pairs on that cell. Occasional very large cells certainly have more than seven nuclei but overlapping makes reliable counting difficult.

The nuclear size in multinucleate cells appears similar to that of wild-type cells, although quantitative measurements of nuclear diameter or microspectrophotometric determination of DNA content have not yet been performed. Occasionally large cells appear to have large diffuse nuclei, but this may reflect the moribund nature of such cells.

Uninucleate cells have a single pyrenoid. In V-shaped binucleate cells there are usually two pyrenoids, but in about 15 % of these cells there is a single round pyrenoid at the base of the V (Pl. 1, fig. 9). In larger cells it is sometimes difficult to count numbers of pyrenoids due to overlapping.

The mutant has been examined in the electron microscope by J. M. Hopkins in this laboratory with particular attention being given to the bridge region between two cell parts of binucleate cells. In some cells the chloroplast division is incomplete as well as cytokinetic furrow formation (Pl. 2, fig. 10), whereas in other cells it appears that the chloroplast division has been completed with only cytokinetic furrow development blocked in its final stages (Pl. 2, Fig. 11). Cytokinetic microtubules have not so far been detected in this strain (although flagellar root microtubules have been seen).

It is necessary to consider whether cytokinesis in *cyt* 1 multinucleate cells has been halted and will not go to completion or whether it is merely proceeding slowly. An approach to this problem has been made by dissection of some of the largest cells of a *cyt* 1 strain on to solid medium using the zygote isolation technique of Ebersold & Levine (1959). The very large size of the dissected cells suggested eight or more flagella per cell, but in the absence of phase optics it was not possible to count the flagellar number precisely. Nine out of 18 such cells gave rise to colonies containing the characteristic range of *cyt* 1 cell types. Thus, approximately 50 % of large multinucleate cells are capable of eventually completing cytokinesis.

Lewin (1952) observed that a multiflagellate mutant of *Chlamydomonas moewusii*

also differed from wild type in its level of resistance to sodium citrate. *cyt* 1 strains, however, have the same level of citrate resistance as wild-type *C. reinhardii*.

Effect of age of culture on cyt 1. During the course of preliminary studies with the mutant, it appeared that the number of multiflagellate cells decreased with the age of the culture. To investigate this more fully a liquid culture was sampled from early log. phase well into stationary phase and the distribution of cell types in each sample determined. The results are summarized in Fig. 2, which shows that the mean number of flagella per cell rises during log. phase from about three to nearly four. With the

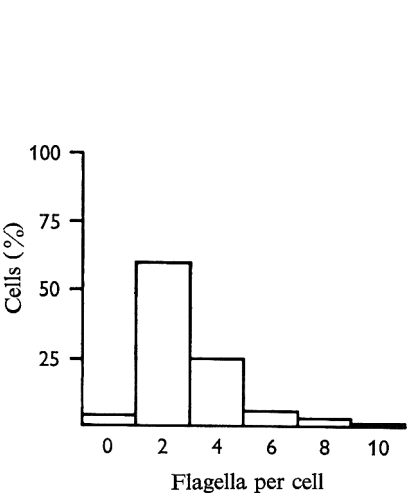

Fig. 1

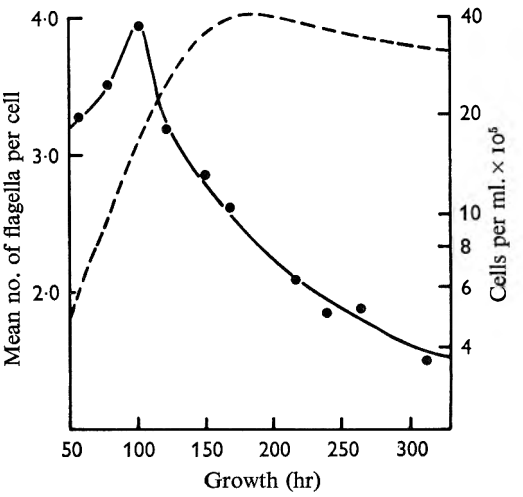

Fig. 2

Fig. 1. Proportions of cells with various numbers of flagella in a log phase culture of *cyt* 1.

Fig. 2. Effect of age of culture on the mean flagella number per cell of *cyt* 1. ●——●, Mean number of flagella per cell. – –, Total number of cells per ml. plotted on a log. scale.

onset of stationary phase, the mean flagella number falls sharply. Figure 3 shows more detailed data from the same experiment. It can be seen that the proportion of biflagellate, uninucleate cells falls to 40 % in log. phase but rises to about 70 % once stationary phase has been attained. Cells with four or more flagella reach a maximum of over 50 % by mid log. phase but decline to about 10 % after a time in stationary phase. Cells without any flagella (i.e. mostly moribund cells) increase steadily during stationary phase.

Effect of pH on cyt 1. The effect of pH on *cyt* 1 was studied by growing a series of cultures buffered with phosphate between pH 6 and pH 8. As shown in Fig. 4, the mean number of flagella per cell rises from 2·6 to 3·9 over this pH range. More detailed data from the same experiment is shown in Fig. 5.

Interaction of cyt 1 with other mutations. In strains carrying *cyt* 1 together with certain mutations causing paralysis of flagella (*pf* 2, *pf* 19B or *pf* 20) the proportion of multiflagellate cells is consistently lower than in strains carrying *cyt* 1 alone. The distribution of cell types in such a double mutant is shown in Fig. 6, which may be compared with the distribution observed in cultures of *cyt* 1 alone shown in Fig. 1. (These two cultures were examined at a similar stage of growth and are taken from a single experiment).

Strains carrying paralysed flagella mutations grow at the bottom of the culture vessel and it was considered possible that bottom growth could alter the cell environment in such a way as to affect the expression of *cyt* 1. However, this was discounted by growing cultures under conditions of gentle agitation to prevent settling of cells. Under these conditions the double mutants still had a consistently lower proportion of multiflagellate cells than strains carrying *cyt* 1 alone.

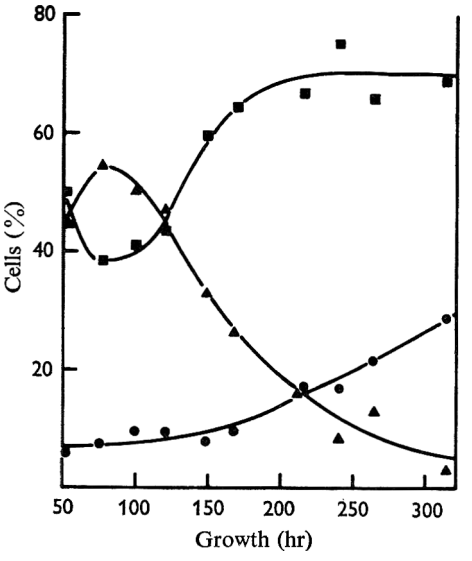

Fig. 3

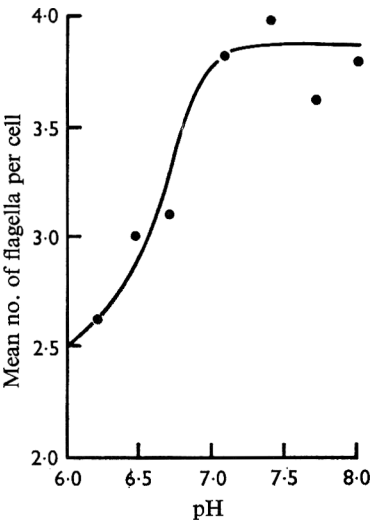

Fig. 4

Fig. 3. Effect of age of culture on the proportions of biflagellate, multiflagellate and aflagellate cells of *cyt* 1. ■——■, Cells with two flagella. ▲——▲, Cells with four or more flagella. ●——●, Cells with no flagella (mostly moribund cells).

Fig. 4. Effect of pH on the mean flagella number per cell in a log phase culture of *cyt* 1. Cells grown in $1{\cdot}5 \times 10^{-2}$ M-phosphate buffer plus normal nutrient salts except phosphate. pH of medium measured at time of taking cell samples.

Genetic analysis. Gametes of *Chlamydomonas reinhardii* differentiate directly from vegetative cells on transfer to nitrogen-free medium (Sager & Granick, 1954). Consequently, a high proportion of gametes of this mutant are multinucleate, which would seem *a priori* likely to complicate genetic analysis. It is therefore not unexpected that in the nine crosses so far performed between *cyt* 1 strains and wild type the germination of zygotes has been rather low (range 60–97 %, mean 86 %) and that other anomalies have been observed. On germination each Chlamydomonas zygote normally liberates 4 or 8 spores which each give rise to a progeny clone (Ebersold & Levine, 1959). In *cyt* 1 crosses, however, approximately one-third (range 15–47 %, mean 32 %) of the germinating zygotes give rise to abnormal numbers of spores or to progeny clones of pale granular cells which form very small colonies on solid medium. These colonies probably arise from matings involving multinucleate gametes of *cyt* 1 which lead to irregular meiosis in some zygotes with consequent formation of zoospores with abnormal chromosome complements.

Allowing for these discrepancies, it may be shown that *cyt* 1 is inherited as a single

gene difference. In crosses with normal strains, microscopic examination of progeny clones from 231 zygotes having normal germination showed 222 (96·1 %) with 2:2 segregation of cytokinesis deficient to normal types. The nine exceptional zygotes are thought to arise by a process similar to that discussed in the previous paragraph. This hypothesis is supported by the finding that a similar small proportion of zygotes from these crosses show irregular segregation ratios for well-established chromosomal genes.

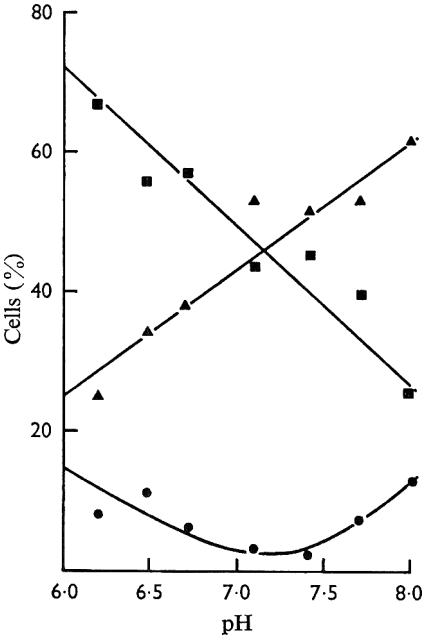

Fig. 5

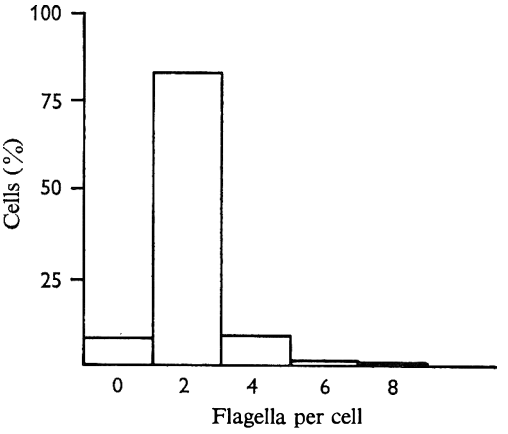

Fig. 6

Fig. 5. Effect of pH on the proportions of flagellate, multiflagellate and aflagellate cells in a log. phase culture of *cyt* 1. ■——■, Cells with two flagella. ▲——▲, Cells with four or more flagella. ●——●, Cells with no flagella (mostly moribund cells).

Fig. 6. Proportions of cells with various numbers of flagella in a log. phase culture of the double mutant *pf* 2; *cyt* 1. This culture was grown under the same conditions as the culture carrying *cyt* 1 alone represented in Fig. 1. The double mutant has more biflagellate cells but fewer cells with four or more flagella.

Preliminary mapping experiments indicate free recombination between *cyt* 1 and the *ac* 17, *pf* 2, *pf* 19 or *pf* 20 loci but demonstrates close linkage to the *ac* 29 locus, which is on the left arm of linkage group VI, close to the mating-type locus, (Ebersold *et al.* 1962). In the cross *cyt* 1, *ac* 29A$^+$, *mt*$^+$ × *cyt* 1$^+$, *ac* 29A, *mt*$^-$ germination was 85 % with 24 % of germinating zygotes giving abnormal progeny types (see above). Classification of 17 normal tetrads for *cyt* 1 and *ac* 29A showed 17 parental ditype, no non-parental ditype and no tetratype tetrads, indicating close linkage between these two loci (Perkins, 1953). A more detailed analysis of the linkage relationships between *cyt* 1 and markers on linkage group VI is now in progress.

DISCUSSION

In order to understand the mode of expression of this mutation we may first consider the timing of organelle duplication in the mutant and the wild type. The most important feature of this in the mutant is that there is good correlation between the number of nuclei and pairs of flagella per cell. Cavalier-Smith (1967) described a fibrous band, devoid of ribosomes, passing between the interphase nucleus and the basal bodies of *Chlamydomonas reinhardii* which may provide the physical basis for this observation. Buffaloe (1958) has shown that in wild-type *C. reinhardii*, pyrenoid division is always completed before cytokinesis occurs. Consequently, the finding that 15 % of V-shaped binucleate cells in the mutant have only a single pyrenoid clearly indicates that pyrenoid division tends to be delayed, as does completion of the chloroplast furrow. Thus it may be that division of nuclei and flagella on one hand and chloroplast and pyrenoid on the other are under separate systems of control. In this context it is of interest that the mutation has been shown to be in a chromosomal gene.

It may also be noted that in the stable diploid strains of *Chalmydomonas reinhardii* recently described by Ebersold (1967) there is apparently a single pyrenoid with each large diploid nucleus, showing that in these strains the number of pyrenoids is related to the number of nuclei and not the number of chromosomes in the cell.

A second approach to the problem of gene action is through an examination of the effects of environmental factors on the expression of the mutation. Since flagellar number can be readily determined in unstained material and is related to nuclear number, it provides an easily obtained quantitative measure of cytokinesis deficiency and hence of gene expression. This criterion has been used to show that pH alters gene expression and it may now be possible to demonstrate further changes in the presence of specific chemical substances, thus providing clues to the nature of the deficiency in the mutant. Stevens (1966*a*, *b*) has shown that the proportion of multinucleate cells in cultures from hamster ascites tumours is reduced by thymidine triphosphate or a combination of estrone and cancer serum.

It has been shown that the proportion of binucleate cells rises in log. phase cultures of the mutant then falls when stationary phase is attained. The increase in the proportion of uninucleate cells during stationary phase suggests the cytokinesis can eventually catch up with nuclear division in some cells at least. The proportion of binucleate cells in mouse liver increases with age although subsequently there is an increase in polyploid cells (Epstein, 1967). It would be important to investigate by quantitative microspectrophotometry whether polyploid nuclei are formed in stationary phase cultures of the mutant.

Before further genetic analysis is undertaken with this or other similar mutants, it would be preferable to eliminate multinucleate gametes since they inevitably complicate such analysis. Preliminary attempts to remove these larger cells from cultures by filtration or centrifugation have not been successful. More progress may come from investigation of environmental conditions which mitigate the deficiency in cytokinesis and maximize the proportion of uninucleate cells in a culture.

I wish to thank Professor Sir John Randall, F.R.S., for support and advice during this work and Miss Anne McVittie for many valuable suggestions and for making available the original isolate of the mutant. Thanks are also due to Mr J. M. Hopkins

for taking the electron micrographs, to Mr T. C. Appleton for advice on photography and especially to Miss Penny Wright for excellent technical assistance.

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EXPLANATION OF PLATES

PLATE 1

Photograph of cells from log. phase culture of *cyt* 1.

Figs. 1–4. Phase contrast, × 1115.

Fig. 1. V-shaped cell with 4 flagella adjacent to a normal oval cell with two flagella. The light regions near the base of the cells are pyrenoids.

Fig. 2. Cell with eight flagella. Note the arrangement of flagella in pairs.

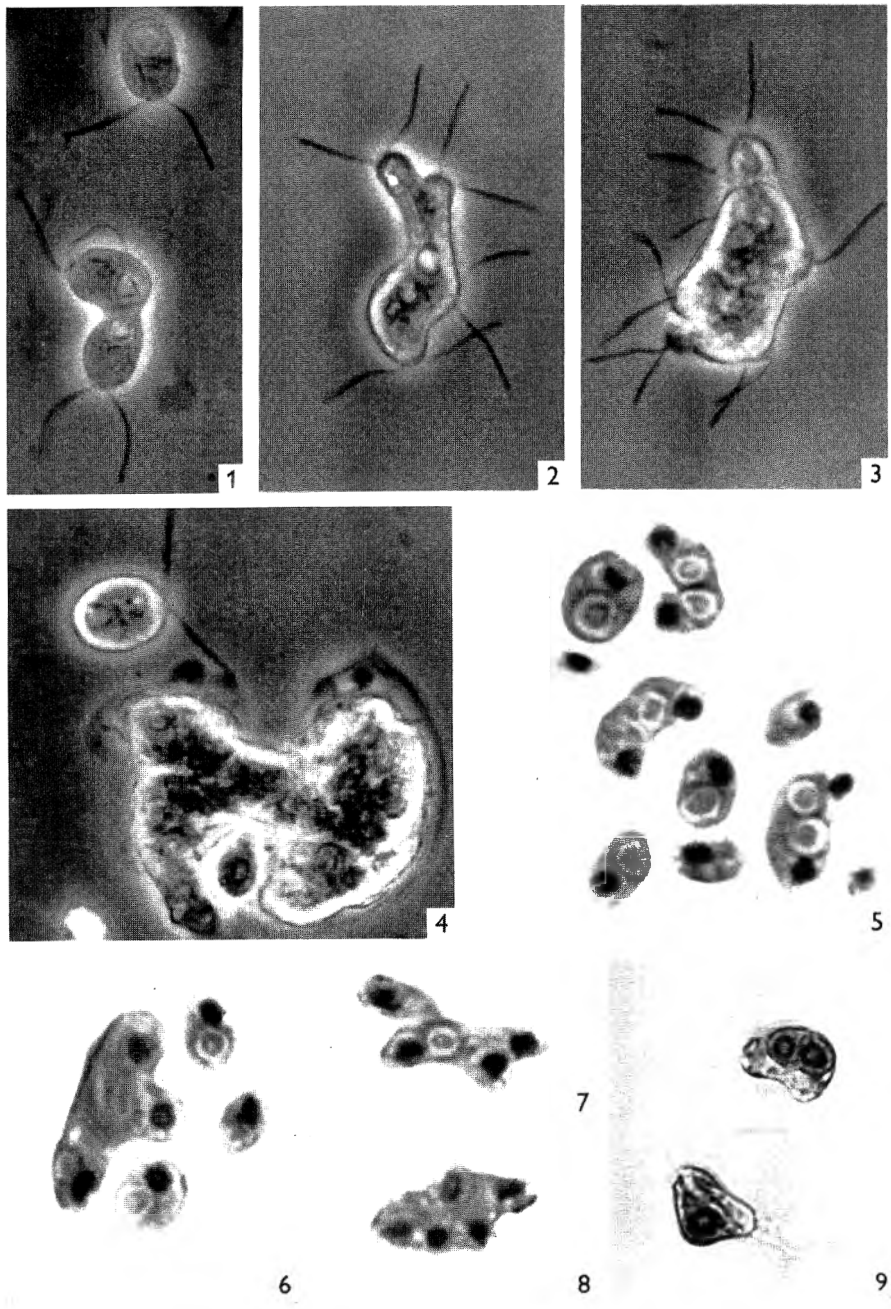
Fig. 3. Large cell with 11 visible flagella. At least 1 other flagellum is out of focus or behind the cell.

Fig. 4. Very large, moribund cell lacking flagella close to a normal biflagellate cell.

Figs. 5–8. Azure A stained. Bright field, × 1125.

Fig. 5. Group of V-shaped binucleate cells and oval uninucleate cells. Darkly staining regions are nuclei, light circles are pyrenoids.

Fig. 6. Y-shaped cells with three nuclei, adjacent to uninucleate cells.



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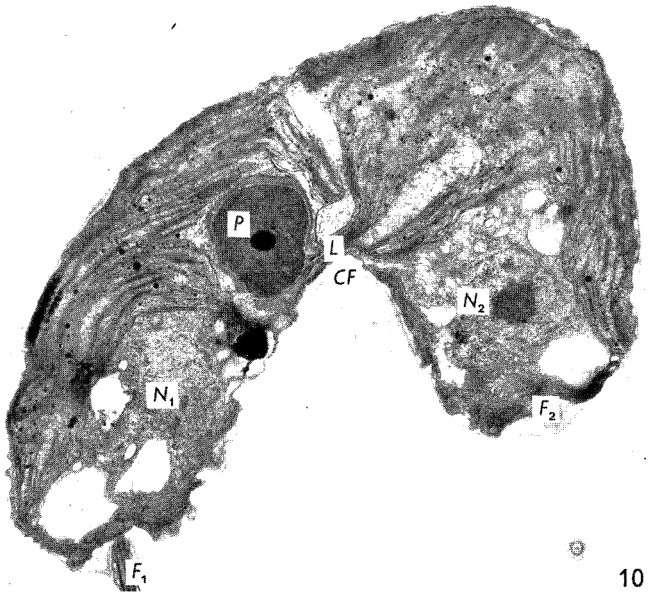

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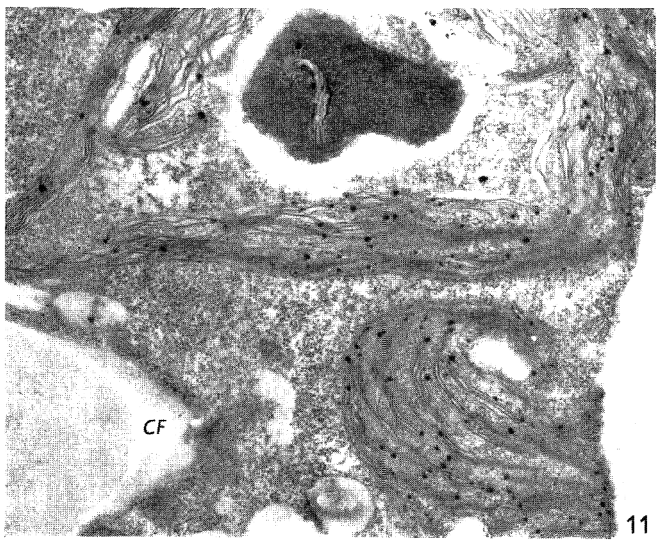

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Fig. 7. X-shaped cell with four nuclei.

Fig. 8. Cell with five nuclei. Only one nucleus is sharply in focus.

Fig. 9. V-shaped cells with four flagella (more clearly visible with phase optics) stained with iodine to show the pyrenoids. One quadriflagellate cell has two pyrenoids, the other has only one. Bright field, × 560.

PLATE 2

Fig. 10. Electron micrograph of a V-shaped cell of *cyt* 1. The section passes through the two nuclei (N_1, N_2), parts of the flagellar apparatus (F_1, F_2) and a single pyrenoid (*P*). Cytokinetic furrow formation (*CF*) and chloroplast division are incomplete; note the continuous chloroplast lamellae (*L*) running between the two cell portions. Osmium tetroxide fixation × 9400.

Fig. 11. Higher magnification electron micrograph of the bridge region of a different V-shaped cell showing complete division of the chloroplast. The cytokinetic furrow (*CF*) is still incomplete. Osmium tetroxide fixation, × 18,750.

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Carbohydrate Metabolism and Production of Diffusible Active Substances by *Staphylococcus aureus* Grown in Serum at Iron Levels in Excess of Siderophilin Iron-saturation and Below

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SUMMARY

Results of studies are reported on the carbohydrate metabolic capabilities of cells of *Staphylococcus aureus* grown in human serum at varied percentage iron-saturations of its contained siderophilin; on the production by such cells of coagulase, hyaluronidase, staphylokinase, and haemolysins; and on the effectiveness of some antibiotics as growth inhibitors under similar *in vitro* growth conditions. Cocci grown in serum at a normal serum iron level and percentage of iron-saturation show a significant reduction in their endogenous respiration, in their capacity to attack lactate, a complete loss of ability to oxidize formate, and a several-fold increase in anaerobic glycolytic capacity as compared to those cocci grown in serum containing unchelated free ionic iron. These metabolic changes, in conjunction with an observed increased rate of acid production and rate of glucose utilization by the growing culture of 'low-iron' cells *versus* the 'high-iron' cells, indicate an increased dependence upon glycolytic rather than oxidative energy production processes by the iron-restricted cells. The strain of *S. aureus* employed in these studies, although characterized by conventional methods as a producer of coagulase, hyaluronidase, haemolysins, and staphylokinase, did not elaborate any of these pathogenesis-linked factors at detectable levels when grown in normal serum. Coagulase, alone, was found at 'high-iron' levels. The amount of dimethoxyphenyl penicillin required to inhibit growth of our *S. aureus* strain grown in serum increased with increasing percentage of iron saturation of the siderophilin while the effectiveness of a given inhibitory concentration of kanamycin and chloramphenicol was indifferent to any variation in the iron-saturation value.

INTRODUCTION

When *Staphylococcus aureus* is grown in human serum under 5 % CO_2–air, the growth rate is a function of the percentage iron-saturation of its contained plasma siderophilin (Schade, 1963). If trypticase plus conalbumin of eggwhite is substituted for serum, similar growth-rate control is exercised by the percentage iron-saturation of the added conalbumin (Theodore & Schade, 1965*a*). A study of the relative oxidative capabilities and carbohydrate metabolism of resting *S. aureus* grown in glucose–trypticase–conalbumin media with and without iron restriction showed that iron-restricted cells were severely limited in their oxidative ability to attack glucose and not

at all capable of metabolizing L-lactate, D-lactate, pyruvate, or formate as compared with the unrestricted cells (Theodore & Schade, 1965*b*).

As iron is added to sera at physiological pH values and at levels below the plasma siderophilin saturation value, the metal is rapidly and firmly bound. No free iron can be detected by addition of α,α-dipyridyl or bathophenanthroline to such sera (Schade, 1961). The magnitude of the binding constants is very high, the estimates for the pK_1 and pK_2 at pH 7·4 being 27·7 and 30·3 respectively (Davis, Saltman & Benson, 1962). The iron in the complex is trivalent and bound with essentially ionic bonds (Ehrenberg & Laurell, 1955).

The present investigation was undertaken to determine the carbohydrate metabolic capabilities of cells of *Staphylococcus aureus* grown in serum at low and high iron concentrations; the production by such cells of coagulase, hyaluronidase, staphylokinase, and haemolysins; and the effectiveness of some antibiotics as growth inhibitors under similar *in vitro* growth conditions.

METHODS

Organism. A penicillin-resistant strain of *Staphylococcus aureus*, phage type 80/81, was used throughout. Stock cultures on nutrient agar slopes were kept in the cold and subcultured every 2 months.

Media. Pooled, blood-bank serum was employed. In any given experiment in which varied percentage iron-saturations of the serum's siderophilin were required, the same serum was used throughout. For the growth of bacteria employed in the metabolic studies, glucose was added to the serum to a final concentration of about 0·30 % (w/v); otherwise, the sera were glucose-fortified by the addition of 1 mg./ml. serum. To obtain conditions of restricted and unrestricted iron availability, iron as ferrous ammonium sulphate was added to the serum as required. All sera were equilibrated with 5 % CO_2 in air at 37° before incubation and maintained in this gas atmosphere during culturing. Seed cultures were grown in iron-restricted serum or in iron-depleted trypticase medium (Theodore & Schade, 1965*a*) for 16 hr at 37°.

Analytical procedures. Serum iron and iron-binding capacity of the serum were determined by the methods of Schade, Oyama, Reinhart & Miller (1954). Standard Warburg manometric techniques were used for measuring O_2 uptake and CO_2 production by resting cell suspensions. At harvest, the cultures, grown for 14 hr on a rotary shaker at 37°, were centrifuged at 4° at 4000 *g* for 20 min. and washed twice in 0·05 M-phosphate buffer (pH 7·4). For catalase (Schade, 1963) and O_2 uptake measurements, the washed cocci were resuspended in the same 0·05 M-phosphate buffer; for the measurement of glycolytic activity they were taken up in 0·03 M-bicarbonate buffer (pH 7·4). All suspensions of cocci in their respective salt solutions were diluted to an extinction at 600 mμ appropriate to their intended use. Preliminary analytical studies established that an $E_{600\ m\mu}^{1\cdot0\ cm}$ of 1·0 was equivalent to about 320 μg. dry weight organisms/ml. and 2×10^9 cocci/ml. of the suspension measured.

Glucose was determined by glucostat (Worthington Biochemical Corp., Freehold, New Jersey); pyruvate as the 2,4-dinitrophenylhydrazone, with benzene extraction (Friedemann & Haugen, 1943); lactate by the method of Barker & Summerson (1941); acetoin by the method of Westerfeld (1945); and acetate by the method of Soodak (1957).

For the determination of the production of soluble coagulase, we employed the qualitative method of Fisk (1940) and the quantitative procedure of Rogers (1954); for hyaluronidase estimation, the quantitative method of Swyer & Emmens (1947). The plate method of Elek & Levy (1950) served to define the haemolysins produced by our *Staphylococcus aureus* strain, while Jackson & Little's (1957) procedure was employed for quantitative estimation of haemolysin present in culture supernatants. The qualitative method of Christie & Wilson (1941) and the quantitative procedure of Tillet & Garner (1933) were used for staphylokinase determinations.

Antibiotic sensitivity discs on brain–heart infusion agar served preliminarily to determine growth inhibition of the cocci. More quantitative data with effective antibiotics were obtained through growth studies using serum as medium at given antibiotic concentrations.

EXPERIMENTAL

Carbohydrate metabolic activities of Staphylococcus aureus grown in high and low iron sera

The observation that the aerobic growth rate of *Staphylococcus aureus* in serum is governed by the serum iron concentration and the percentage iron saturation of the iron-binding protein, plasma siderophilin (Schade, 1963), suggests a restriction upon the energy-yielding oxidative metabolic systems of the organism involving iron as a functional constitutive element such as occurs, for example, in the cytochromes. To determine some relative oxidative capacities of cocci grown in serum at an iron level in excess of its binding capacity and at a percentage iron-saturation level found in normal serum, we cultured *S. aureus* in serum under both conditions in 5 % CO_2–air on a rotatory shaker at 37°. The harvested cocci were then examined for their oxidative

Table 1. *Rates of oxidation of various substrates, anaerobic glycolysis, and catalase activity of Staphylococcus aureus grown in serum at 105 % and 23 % iron saturation*

For oxidation rates, Warburg vessels contained 3·2 mg. dry wt cocci in 2·5 ml. 0·05 M-phosphate buffer (pH 7·4) in air at 37° plus 50–100 μmoles substrate; for glycolysis, 1·6 mg. cocci in 0·03 M-sodium bicarbonate buffer (pH 7·4) in 90 % N_2 + 10 % CO_2 at 37° plus 7 μmoles glucose; for catalase determinations with the high-iron and low-iron cocci, 0·05–0·1 mg. and 0·8–1·6 mg. cocci, respectively, in 0·055 M-phosphate buffer (pH 6·6) in air at 16° plus 36 μmoles H_2O_2. Q_{O_2} values represent μl. O_2 consumed or produced/hr/mg. dry wt. cocci; Q_{CO_2} values signify μl. CO_2 similarly produced. Q values were calculated for the maximum rate of gas consumption or production following a 10 min. initial equilibration period uncorrected for the endogenous rate where applicable.

	Serum iron saturation			
	105 % sat.	23 % sat.	105 % sat.	23 % sat.
	Q_{O_2} values		Q_{CO_2} values	
Substrate				
Endogenous	19	3	—	—
Glucose	44	38	22	83
L-Lactate	136	63	—	—
Pyruvate	0	0	—	—
Acetate	0	0	—	—
Succinate	0	0	—	—
Formate	85	0	—	—
H_2O_2 (catalase)	13,500	1,150		

activity on selected substrates as well as their glycolytic and catalase activities. Table 1 summarizes typical data from such an experiment.

The cocci used here were from a culture (low-iron) in pooled fresh normal human serum with a serum iron value of 80 μg. % and a total iron-binding capacity of 346 μg. %, or 23·1 % saturation, and from a culture (high-iron) in the same serum made 105 % iron-saturated by addition of ferrous ammonium sulphate. Both media were brought initially to 0·27 % (w/v) glucose concentration level and equilibrated at pH 7·4–7·5 with 5 % CO_2 in air. The inocula were cocci grown through 2 overnight transfers in the glucose-fortified 23 % iron-saturated serum on a shaker at 37° in 5 % CO_2–air. One ml. of such a culture was used to inoculate 200 ml. of the 23 % iron-saturated serum; 0·1 ml. served for the inoculum of 200 ml. of 105 % iron-saturated medium. Both sera were put in tightly screw-capped 1 l. Erlenmeyer flasks gassed with 5 % CO_2–air and shaken at moderate speed on a rotatory shaker at 37°. Since the pH of the low-iron serum had previously been observed to fall below 7·0 towards the end of the 14 hr growth period and the glucose concentration to fall more precipitously than in the high-iron culture, precautions were taken to maintain the pH above 7·2 at all times through the growth period by addition of appropriate amounts of 10 % $NaHCO_3$ solution to the low-iron medium. Such lowering of the pH must be avoided if the siderophilin–iron–chelate is expected to maintain its integrity and keep the ionic iron level in the medium at its low value. Further, the cocci must be harvested before the glucose has been completely removed from the medium, since the metabolic capacities of *Staphylococcus aureus* suspensions are significantly affected by the presence or absence of glucose in their growth environment (Theodore & Schade, 1965*b*). The initial pH of both media was 7·4–7·6; at harvest the high-iron culture had a pH of 7·0 and the low-iron culture 7·28. A total of 5 ml. 10 % $NaHCO_3$ solution (w/v) had been added to the low-iron culture and 5 ml. physiological saline to the high-iron culture over the growth period. The initial glucose concentration in both media was 2·7 mg./ml.; at harvest, the low-iron culture contained 0·6 mg./ml. and the high-iron culture 1·3 mg./ml. The final $d_{600\ m\mu}^{1.0\ cm}$ value of the high-iron 14 hr. culture was 4·0, while that of the low-iron culture was 2·0. The lower turbidity of the culture grown in the normal 23 % iron saturated serum compared to that grown in the same serum at 105 % saturation, despite the ten-fold greater inoculum, is illustrative of the depressed growth rate due to iron chelation by the plasma siderophilin.

The summarized results (Table 1) of oxidative studies with the cocci grown in serum at a normal serum iron level show a significant reduction in their endogenous respiration, in their capacity to attack lactate and a complete loss of ability to oxidize formate as compared with those cocci grown in serum containing unchelated free ionic iron. The rates of oxidation of glucose by both populations, however, are comparable as are their common failure to attack acetate and succinate. The latter deficiency apparently is due to the glucose repressive effect on acetate and tricarboxylic acid cycle intermediates oxidation previously observed in cocci cultured in glucose-containing semi-synthetic media (Collins & Lascelles, 1962; Theodore & Schade, 1965*b*). That pyruvate is not oxidized by the high-iron serum grown cocci was unexpected since trypticase/glucose/high-iron-grown cocci possess this capability (Theodore & Schade, 1965*b*). When analyses of the end-products of oxidation of glucose and lactate were made for acetate, lactate, pyruvate, acetoin, and CO_2, the

main products found were pyruvate and small amounts of CO_2. Traces of acetate were found in all cases. Acetoin was detected only in the supernatants of the glucose oxidation experiments.

The reduction in the oxidative capacity of *Staphylococcus aureus* grown at normal serum iron levels is accompanied by an approximately fourfold increase in the anaerobic glycolytic capacity of the cocci. These concomitant metabolic shifts, in conjunction with the observed increased rate of acid production and rate of glucose utilization by the growing culture of low-iron cocci *versus* the high-iron cocci, indicate an increased dependence upon glycolytic rather than oxidative energy production processes by the iron-restricted cocci. The nearly 12-fold differences in catalase activity of the high-iron cocci over the low-iron cocci is impressive evidence in itself of the effectiveness of the plasma siderophilin to restrict iron availability to the growing cocci.

Diffusible products of Staphylococcus aureus grown in serum at various iron saturation levels

Investigations were made of the production by our strain of *Staphylococcus aureus* of diffusible factors commonly associated with the pathogenicity of this organism, namely: coagulase, hyaluronidase, haemolysins, and staphylokinase. Both qualitative and quantitative estimations were made, where possible, of these products in human sera which served as test-tube growth media at different levels of iron saturation of their normally occurring plasma siderophilin. The serum iron levels chosen were

Table 2. *Coagulase activity of supernatants of Staphylococcus aureus grown in serum at different serum iron levels*

Fe sat. (%)	Fe conc. (μg./ml.)	Growth period (hr)		Growth ($d^{1\,cm}_{\lambda 600}$)		Coagulation time (Rogers, 1954)	
		*a**	*b*	*a*	*b*	*a*	*b*
16	0·56	30	41	0·240	0·336	> 15 min. (undil.)	> 15 min. (undil.)
32	1·12	24	—	0·336	—	> 15 min. (undil.)	—
48	1·68	20	—	0·360	—	> 15 min. (undil.)	—
64	2·24	19	19	0·380	0·424	> 15 min. (undil.), clot-2 hr.	9·45 min. (undil.)
80	2·80	16	—	0·328	—	> 15 min. (undil.), clot-2 hr.	—
110	3·85	16	17	0·548	0·460	3·06 min. (1/1 dil.)	2·43 min. (1/1 dil.)

* *a* and *b* signify two experiments.

those obtaining in iron-deficiency states, in normal subjects, and on conditions of haemochromatosis and transfusion haemosiderosis, i.e. 5–15 %, 20–60 %, and 80–100 % siderophilin iron-saturations, respectively. Serum at 110 % iron-saturation was included to provide a comparable medium in which free ionic iron was available to the growing organism. Appropriate controls were obtained from cultures grown in trypticase or brain–heart infusion media. All media were inoculated with 200–1000 colony-forming units and kept under 5 % CO_2 + air at 37°.

Coagulase. Through use of both the qualitative method of Fisk (1940) and the quantitative method of Rogers (1954), we determined that the soluble coagulase activity of culture supernatants from cultures of comparably sized populations

increased with increasing iron-saturation of the serum medium when its siderophilin was at least half saturated with iron. In all experiments the coagulase activity of supernatants from cultures in serum 110 % iron-saturated was readily demonstrable by both methods and at a relatively high level. Data from two illustrative experiments are summarized in Table 2. Comparable results were obtained with unheated or heated (56° for 30 min.) sera as media. In general, the data indicate that human sera at normal serum iron levels and below and at normal percentages of plasma siderophilin iron-saturation and below, fail to provide media productive of detectable levels of coagulase. Whether this fact is a consequence of the slower growth rate and altered metabolism of *Staphylococcus aureus* growing in a nutrient iron-restricted medium or reflects some destruction or inhibition of coagulase normally produced is not known.

Hyaluronidase. Applying the quantitative viscosimetric method of Swyer & Emmens (1947) for estimation of hyaluronidase in culture supernatants, we found none to be active, whether from unheated or heat-treated serum media at any percentage iron-saturation. Addition of potassium hyaluronate to the serum media (0·5 mg./ml.) did not effect hyaluronidase production by the growing bacteria. Supernatants of cultures of similar cell populations produced in brain–heart infusion broth or in trypticase with 1·5 % (v/v) egg white supplement at 5 %, 50 % and 110 % iron saturation of its conalbumin component, however, possessed hyaluronidase activity in rough proportion to the population and indifferently to the availability of iron in the medium.

Haemolysins. Using the plate method of Elek & Levy (1950) with rabbit, sheep, and human red blood cells in conjunction with an anti-α and minimal anti-β-hemolysin antiserum of Connaught Laboratories (Toronto, Canada), we determined that our *Staphylococcus aureus* strain produced α, β and δ hemolysins. With the Jackson & Little (1957) quantitative haemolysin method, it was found that no haemolytic activity was present in serum sypernatants of cultures at any iron-saturation level. The determined anti-α-haemolytic activity of the serum, naturally and initially present (1 ml. heated serum neutralized 2 units of α-haemolysin), was in no case decreased by the bacterial growth. Mere presence of anti-haemolyins in a medium is not responsible for failure of the organism to produce haemolysin, since trypticase plus egg-white medium to which antiserum was added had considerable haemolytic activity following growth of *S. aureus* at all levels of iron-saturation. Addition of 0·1 % iron-free agar to the serum media to provide some suspended solids did not effect a change in the failure of the bacteria to produce haemolysins in these cultures.

Staphylokinase. Through use of rabbit plasma plus nutrient agar media and the qualitative method of Christie & Wilson (1941), it was determined that our *Staphylococcus aureus* strain produced staphylokinase. However, no serum or nutrient-broth supernatants of the bacterial cultures applied to such plates gave positive reactions for kinase. Additionally, the tube method of Tillet & Garner (1933) for the quantitative estimation of kinase gave negative results for all supernatants; even overnight incubation at 37° did not give evidence of clot lysis. The positive results with the plate method involving large growing colonies probably reflect a higher concentration of kinase present in the agar at the colony site. It is also possible that this strain produces little or no kinase in the liquid media employed.

In summary, the strain of *Staphylococcus aureus* employed in these studies, although characterized by conventional methods as a producer of coagulase, hyaluronidase, haemolysins, and staphylokinase, did not elaborate any of these pathogenesis-linked

factors at detectable levels when grown in normal human blood serum. When iron was added to such serum at levels approaching saturation and beyond, then coagulase was found in increasing concentration in the culture supernatants. None of the other factors, however, were produced in response to changes in the serum iron values of normal serum. The fact of the absence of such products from growth of this pathogen in an approximation of one of its natural environments, serum, should be weighed in any assessments of their importance for its pathogenicity.

Antibiotic activities and percentage iron-saturation of serum

Preliminary tests with antibiotic sensitivity discs on brain–heart infusion agar using penicillin G, colistin, streptomycin, oxytetracycline, sulphamethizole, dimethoxyphenyl penicillin, kanamycin, and chloramphenicol indicated that our *Staphylococcus aureus* strain was resistant at high concentrations to all but the last three agents. Dimethoxyphenyl penicillin was effective at the 20 μg./disc. level, while both kanamycin and chloramphenicol were active at the 5 μg./disc level.

To determine the growth-inhibitory effectiveness of dimethoxyphenyl penicillin, kanamycin, and chloramphenicol against *Staphylococcus aureus* when grown in media of normal human serum at different levels of serum iron, we inoculated such sera with 500–1000 colony-forming units/ml. medium at measured antibiotic levels. The results established that with dimethoxyphenyl penicillin the amount of antibiotic required to inhibit growth completely after 40 hr incubation at 37° increased with increasing percentages of iron-saturation of the serum's siderophilin. For example, at 15 % iron-saturation, 0·4 μg. dimethoxyphenyl penicillin/ml. serum was sufficient to inhibit completely, while at 30 %, 60 % and 90 % saturation, 0·8, 1·6 and 3·2 μg., respectively, were required. With both kanamycin and chloramphenicol, on the other hand, the amount of antibiotic needed to suppress growth under similar conditions was independent of percentage iron-saturation of the siderophilin. Thus, 10 μg. kanamycin/ml. serum was sufficient at 18 %, 50 % and 110 % iron-saturation levels to effect complete growth inhibition; 7·5 μg. chloramphenicol/ml. serum was equally effective at 16 % and 110 % iron-saturation. From these results, it is evident that the *in vitro* effectiveness of an antibiotic against *S. aureus* grown in human serum may be determined by the iron saturation of the serum. The applicability of this conclusion is peculiar to the particular antibiotic under consideration.

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Modification of Lethality and Mutagenesis by Growth Inhibition of Ultraviolet-irradiated *Escherichia coli* strain B/R

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SUMMARY

The influence of post-irradiation growth inhibition on survival and absolute yields of prototrophic mutants has been studied in *Escherichia coli* B/R, u.v.-irradiated in the lag and logarithmic phases of the growth cycle. Without post-irradiation growth inhibition, survival and yields of mutants were higher for logarithmic phase cells. Post-irradiation growth inhibition reduced yields of mutants from both types of cells to similar extents and markedly increased the survival of logarithmic phase cells, both after small and large u.v. doses. In the case of lag phase cells, a survival increase mediated by post-irradiation growth inhibition, could only be observed by giving a large u.v. dose or by inhibiting repair of damage occurring after plating for colony formation. Post-irradiation growth inhibition was observed to have two effects—one tending to increase survival and another tending to enhance lethality. The results indicate that observable effects of post-irradiation growth inhibition on survival and induced mutation in *E. coli* B/R and whether or not such effects are correlated depend upon a number of factors. These include: (1) relative amounts of repair occurring during growth inhibition and during subsequent growth; (2) heterogeneity of the population with respect to growth phase, intrinsic repair ability and amount of damage; and (3) relative contributions of increased repair and increased killing—both mediated by post-irradiation growth inhibition.

INTRODUCTION

Inhibition of growth after ultraviolet (u.v.) irradiation has been used frequently to elicit information about the nature of primary damages causing lethality and about the possibility that the same primary damages also cause mutation. In *Escherichia coli* B, post-irradiation growth inhibition increases survival (Roberts & Aldous, 1949; Alper & Gillies, 1960). The type of damage repaired is also photoreactivable, i.e. consists of u.v.-induced dimers in DNA (Castellani, Jagger & Setlow, 1964; Cook, 1967). It is also known that in strains which are genetically defective in the ability to repair dimers by excision, u.v.-induced lethality is increased and post-irradiation growth inhibition is not very effective in improving survival (Setlow & Carrier, 1964; Boyce & Howard-Flanders, 1964; Harm, 1966). Thus it appears that post-irradiation growth inhibition increases survival by increasing the number of dimers repaired by the excision mechanism.

Genetic reduction of the ability to excise dimers has another effect—increased sensitivity to the u.v. induction of mutations in *Escherichia coli* B/R (Hill, 1965; Witkin,

1966*a*, *b*; Kondo & Kato, 1966). If u.v.-induced dimers are responsible for lethality in this strain as well as in *E. coli* B, and also responsible for mutation, it might be expected that post-irradiation growth inhibition of excising strains of B/R should increase survival and decrease mutation. In some cases, these expectations have been confirmed. Thus in one case where survival effects only were studied, post-irradiation growth inhibition did increase survival, although not as markedly as in *E. coli* B (Harm, 1966). In two cases where both survival and mutation were determined, survival was increased and mutation decreased (Hill, 1963; Kondo & Jagger, 1966). However, other reports have not conformed to expectation. Post-irradiation growth inhibition has failed to influence survival, although it has reduced the yield of one kind of mutation. The yield of another type of mutation in the same strain was unaffected (Witkin & Theil, 1960). Post-irradiation growth inhibition of prototrophic *E. coli* B/R has even reduced survival (Alper & Gillies, 1960).

The present report concerns evidence which indicates how most of these diverse reports may be reconciled. The effects of post-irradiation growth inhibition on survival and prototrophic mutation in auxotrophic *Escherichia coli* B/R were studied. The study compares results for cells irradiated in the lag and logarithmic phases of growth. Experiments were also designed to see whether post-irradiation growth inhibition might have dual effects on the same population.

METHODS

Bacterial strain. The strain employed was *Escherichia coli* WU 36, a tyrosine-requiring mutant of B/R. It was supplied by Dr E. M. Witkin.

Treatment of bacteria. Cultures were grown to the stationary phase by aeration in nutrient broth. To obtain cells in lag phase a 1/10 dilution in broth was aerated for 45 min.; to obtain cells in logarithmic phase, a 1/2000 broth dilution was aerated until the viable count reached $2–3 \times 10^7$ per ml. (5–6 divisions). After chilling in an ice bath for 10 min., the cultures were washed free of broth by two centrifugations and resuspended in the original volume of minimal medium E containing glucose (Vogel & Bonner, 1956). Cultures were kept chilled during irradiation. For growth inhibition in liquid, irradiated cultures were rapidly diluted appropriately in this medium and held in a 37° water bath. The absence of amino acids inhibits growth. One tenth ml. samples were removed periodically and plated for viability or yields of prototrophic mutants. Experimental error was reduced by this procedure of diluting before the period of holding at 37° and also by using the same 0·1 ml. pipette for all aliquots removed from the same dilution. For growth inhibition on agar, irradiated cells were plated on minimal agar (see below). For photoreactivation, the same procedure as for growth inhibition in liquid was followed. In order to dissociate the effects of photoreactivation from those of growth inhibition in liquid, the water-bath temperature was reduced to 20°. As will be seen, the time for maximum photoreactivation was 5–10 min., whereas an hour was required for the maximum effect of growth inhibition in liquid.

Plating media. The standard agar medium for colony formation was SEM agar containing minimal medium E, 0·4 % glucose and 5 % nutrient broth. When inhibition of excision on the plate was required, acriflavine was added at a concentration of 1 μg./ml. (Setlow, 1964). Minimal medium agar (MM) consisted of minimal medium E,

glucose and 1 μg. tyrosine/ml. When the results of using different agar media were required, the same irradiated sample was plated on three plates of each of the different media (using the same 0·1 ml. pipette) and these platings were done in random order. Counts of auxotrophic parental colonies on SEM agar (with or without acriflavin present) were made after 24 hr of incubation at 37°. Because of growth-slowing on MM agar, counts of parental colonies on this agar were made after 48 hr of incubation. All counts of prototrophic mutant colonies were also made after 48 hr of incubation.

Ultraviolet-irradiation and photoreactivation. The apparatus used for u.v.-irradiation, for measurement of u.v. dose and for photoreactivation (p.r.), has been described previously (Hill & Simson, 1961; Hill, 1965). In the present experiments, white light from the G.E. A-H 5 lamp was used for photoreactivation.

RESULTS

Figure 1 shows complete dose–survival and dose–mutation curves for cells irradiated in the lag and in the logarithmic phases but not subjected to post-irradiation growth inhibition, i.e. irradiation was followed by immediate plating on SEM agar. Logarithmic phase cells were slightly more resistant to u.v.-induced lethality than lag phase cells and yielded 6–10 times more mutants. When both types of cells were irradiated with similar doses and this was followed by growth inhibition in liquid prior to plating on SEM agar, the yields of mutants were reduced at about the same rate and to about the same extent (Fig. 2).

Post-irradiation growth inhibition in liquid did not, however, have similar effects on the survival of both types of cells. In the case of lag phase cells, previous reports were confirmed in that the survival after low u.v. doses was not noticeably increased (Fig. 3) (Witkin, 1964). A survival increase—but only by a factor of two—was obtained by increasing the u.v. dose. More marked survival increases required plating on acriflavine-supplemented SEM agar (Fig. 3). In the case of logarithmic phase cells, post-irradiation growth inhibition in liquid was much more effective in increasing survival. This was true for all doses and could be observed even without acriflavine supplementation of SEM agar (Fig. 4).

In the above experiments, growth inhibition was in liquid and was presumably terminated once the cells were plated on SEM agar. The marked survival increase obtained for logarithmic phase cells did not preclude the possibility that effects tending to decrease survival might also have been occurring, i.e. that the survival increase was in fact limited by such effects. Since decreased survival upon post-irradiation growth inhibition of *Escherichia coli* B/R had been reported, it was decided to see whether lethal effects were also operating in the present experiments (Alper & Gillies, 1960). For this purpose, growth inhibition in liquid of irradiated logarithmic phase cells was prolonged and the effects of subsequent plating on MM agar were compared with those obtained by plating on SEM agar.

Figure 5 shows the results of these experiments. Since incubation on MM agar slows growth, it was not surprising that when irradiated cells were immediately plated on this medium and allowed to form colonies on it, survival was greater than when immediate plating and colony formation were on SEM agar. The increase in survival obtained in this way was equal to the maximum increase obtained in the usual way—by growth-inhibition in liquid before plating on SEM agar. When growth-inhibition

in liquid was prolonged before plating on SEM agar, the survival decreased again so that the gain was eventually lost. This can also be seen in Fig. 4. Thus lethal effects of growth inhibition in liquid were present. When growth inhibition was in liquid and this was followed by plating on growth-inhibiting MM agar, survival also eventually decreased. Since this manner of treating the cells amounted to even greater prolongation of growth inhibition, survival was eventually reduced below the survival obtained when

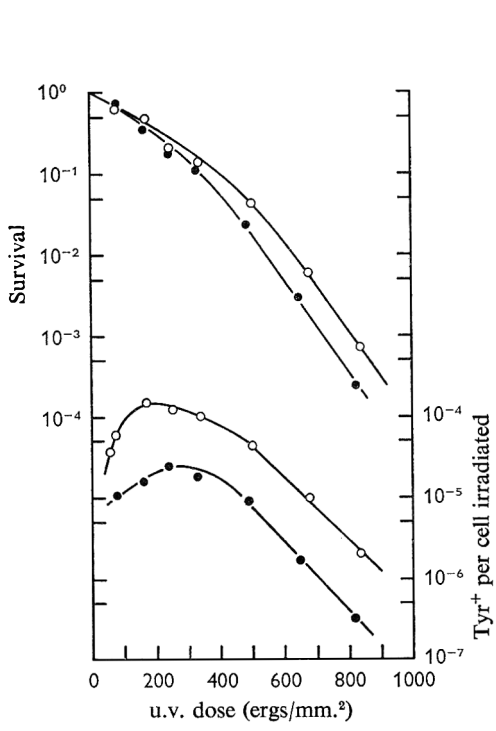

Fig. 1

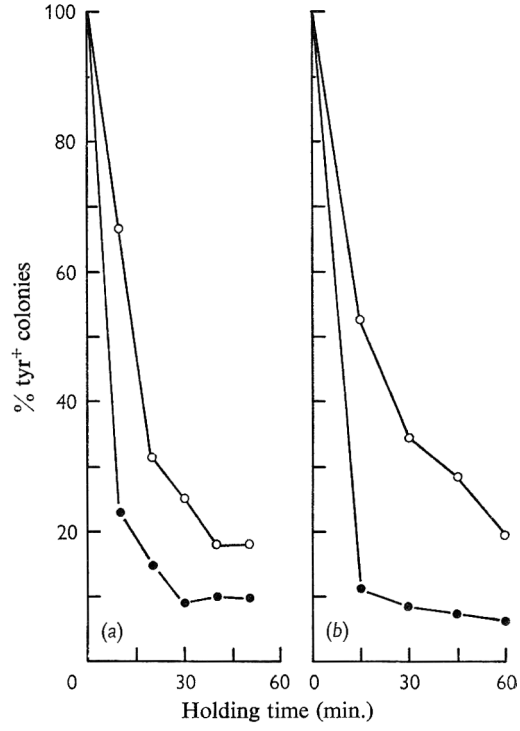

Fig. 2

Fig. 1. Survival curves (above) and curves for absolute yield of tyrosine-independent mutants (below) for *E. coli* WU 36, u.v.-irradiated in lag phase (●) and in logarithmic phase (○). Irradiation followed by immediate plating on SEM agar.

Fig. 2. Effect of post-irradiation holding of cells in minimal liquid medium at 37° on yields of tyr+ mutants: (*a*) cells irradiated in lag phase; (*b*) cells irradiated in logarithmic phase. Ultraviolet doses for lag phase cells were 112 ergs/mm.² (●) and 430 ergs/mm.² (○); for logarithmic phase cells, 112 ergs/mm.² (●) and 530 ergs/mm.² (○). Plating on SEM agar.

prolonged growth inhibition in liquid preceded plating on SEM agar. Thus lethal effects of growth inhibition occurred also on MM agar. It should be mentioned that these lethal effects were not observed in control experiments using unirradiated cells.

Lethal effects of growth inhibition in liquid have been reported to reduce photoreactivability (Castellani *et al.* 1964; Terry, Kilbey & Howe, 1967). This was also observed in the present system. Irradiated logarithmic phase cells were exposed to photoreactivating light immediately after irradiation and after growth inhibition in liquid for the time required to obtain maximum survival without photoreactivation. Plating for colony formation on MM agar was compared with plating on SEM agar.

The survival level obtained on SEM agar by growth inhibition in liquid followed by exposure to photoreactivating light was less than that obtained by photoreactivation immediately after u.v. irradiation (Fig. 6). In addition, survival after exposure to photoreactivating light was reduced if the cells were plated on MM agar rather than on SEM agar. Thus photoreactivation did not prevent the lethal effect of growth inhibition on agar.

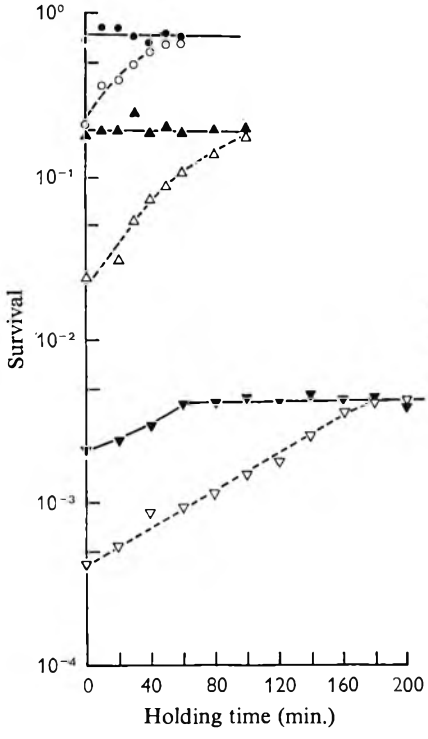

Fig. 3

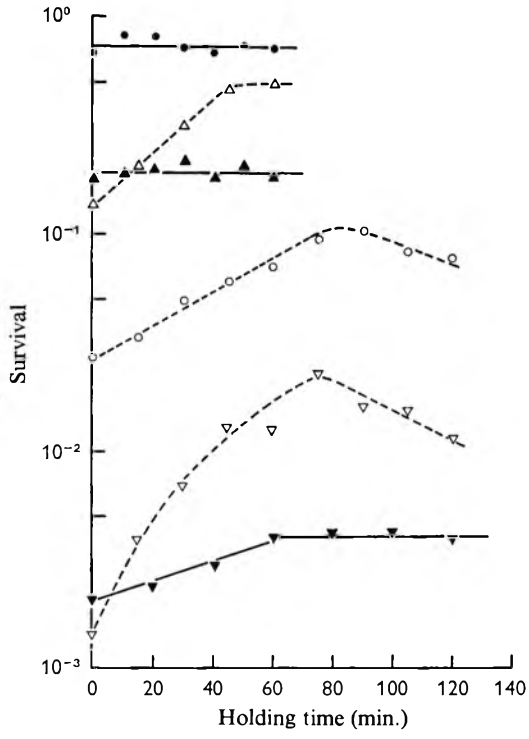

Fig. 4

Fig. 3. Effect of post-irradiation holding of cells in minimal liquid medium at 37° on survival of cells irradiated in lag phase. Plating on SEM agar, —; plating on acriflavine-SEM agar, - - -. Doses were 120 ergs/mm.^{2} (● ○), 280 ergs/mm.^{2} (▲ △) and 660 ergs/mm.^{2} (▼ ▽).

Fig. 4. Effect of post-irradiation holding of cells in minimal liquid medium at 37° on survival of cells irradiated in logarithmic phase (- - -). Data from Fig. 3 for cells irradiated in lag phase (—) are repeated for comparison. Doses to logarithmic phase cells were 300 ergs/mm.^{2} (△), 520 ergs/mm.^{2} (○) amd 760 ergs/mm.^{2} (▽).

DISCUSSION

When DNA has been damaged by radiation, the survival of the cell should depend at least partly upon the repair of the damage and also upon delaying replication until the damage has been repaired. The re-initiation of DNA replication after u.v. irradiation requires prior protein synthesis but excision of u.v.-induced dimers from DNA can occur in the absence of protein synthesis (Harold & Ziporin, 1958; Doudney, 1959; Drakulic & Errera, 1959; Setlow, 1964). Therefore delaying the onset of protein synthesis should increase the probability of DNA repair before replication and con-

sequently increase the probability of survival, provided that the delay does not also have adverse effects. The present results for logarithmic phase cells show that survival was indeed markedly increased, but that post-irradiation growth inhibition did have adverse effects as well. Prolongation of the inhibition revealed the existence of a lethal effect.

The rate of dimer excision in lag phase cells is poor compared with the rate for logarithmic phase cells (Setlow, 1966). Hence it is not surprising that post-irradiation growth inhibition of lag phase cells did not have the same marked effect on survival.

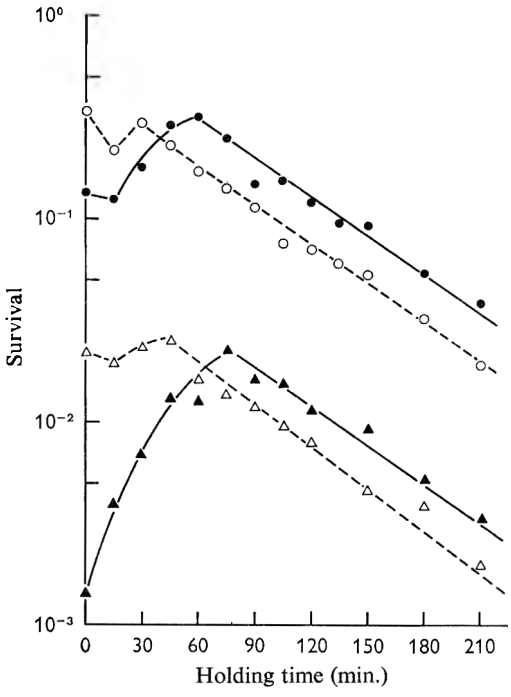

Fig. 5

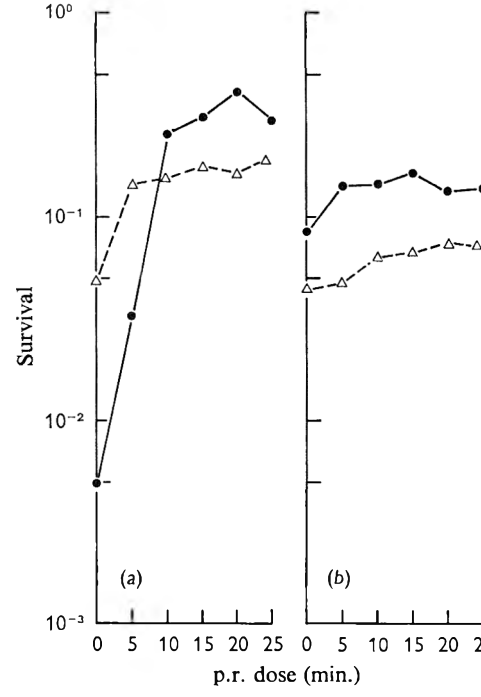

Fig. 6

Fig. 5. Effect of prolonged post-irradiation holding of cells in minimal liquid medium at 37° and of subsequent plating agar on survival of logarithmic phase cells. Plating on SEM agar, —; plating on MM agar, – – –. Doses were 340 ergs/mm.² (upper set of curves) and 760 ergs/mm.² (lower set).

Fig. 6. Effect of plating agar on survival of logarithmic phase cells exposed to photoreactivating light in minimal liquid medium after u.v.-irradiation. Plating on SEM agar, —; plating on MM agar, – – –. Exposure to photoreactivating light immediately after u.v.-irradiation (*a*) and after 1 hr of holding in minimal liquid medium at 37° (*b*). Temperature during exposure to photoreactivating light = 20°. Dose was 660 ergs/mm.².

However, the inhibition greatly reduced the yield of mutants from both types of cells. One reasonable explanation for this is that most of the mutants obtained from lag phase cultures may have originated in cells already in logarithmic phase, i.e. that the lag phase populations were heterogeneous in this respect. According to Fig. 2, when the same u.v. dose of 112 ergs/mm.² was given to both types of populations, post-irradiation growth inhibition caused very nearly the same rate of loss of mutants. At this dose plating immediately after irradiation gave a tenfold greater yield of mutants from logarithmic phase cells (Fig. 1). Simple calculation shows that if the presumptive

logarithmic phase fraction in the lag phase population had amounted to only 10 %, it would account for nearly 100 % of the mutants and thus for the rapid loss upon post-irradiation growth inhibition. On the other hand, the effect of this presumptive logarithmic phase component on survival would not be nearly as great. At low doses, the increase in survival would be so small as to easily escape detection whereas at high doses, the increase would become larger and more likely to be detected (Table 1).

Table 1. *Calculation of effect of a logarithmic phase fraction in increasing survival of a lag phase population exposed to post-irradiation growth inhibition*

It is assumed that (*a*) post-irradiation growth inhibition increases the survival of logarithmic phase cells but not lag phase cells,* and (*b*) the presumptive logarithmic phase fraction is 10 % (see text).

s = survival of logarithmic phase cells when plated immediately after irradiation;
S = survival of the lag phase population when plated immediately after irradiation;
F = factor for maximum increase in survival of logarithmic phase cells;
F′ = factor for maximum increase in survival of the overall 'lag' phase population

$$= \frac{10^{-1}\,s\,(F-1)+S}{S}.$$

Dose (ergs/mm.²)	120	280	660
s (Fig. 1)	$6{\cdot}0\times10^{-1}$	$2{\cdot}8\times10^{-1}$	$7{\cdot}0\times10^{-3}$
S (Fig. 1)	$5{\cdot}0\times10^{-1}$	$2{\cdot}0\times10^{-1}$	$2{\cdot}5\times10^{-3}$
F (Fig. 4)†	1·7‡	3·3	7·5
F′ (calc.)	1·1	1·3	2·8
F′ (obs.) (Fig. 3)	1·0	1·0	2·0

* Lag phase cells show poor dimer excision (Setlow, 1966).

† Since the doses of Fig. 3 are not exactly the same as in Fig. 4, values for F are approximations from Fig. 4.

‡ This assumes that at this low dose, growth inhibition rescues all logarithmic phase cells.

Another possible explanation for the marked loss of mutants, but an unimpressive survival increase upon post-irradiation growth inhibition of lag phase cells, is suggested by the lethal effect of this post-treatment. Although this was clearly demonstrated for logarithmic phase cells, it may also occur in lag phase cells. In either case, the lethal effect would limit a survival increase but would cause an even greater loss of mutants than would result from efficient dimer repair alone. It may be noted that these opposite effects of post-irradiation growth inhibition probably explain why different authors have reported different results. The *net* effect on survival probably varies with strain as well as with other particular experimental conditions (Kos, Drakulic & Brdar, 1965).

Although post-irradiation growth inhibition of lag phase cells given small doses did not increase the survival measured on SEM agar, a large survival increase occurred if survival was measured on acriflavine-supplemented SEM agar. Without prior growth inhibition in liquid, survival on acriflavine-SEM was considerably lower than on SEM alone. Since acriflavine reduces excision repair, the increased lethality probably reflects loss of cells which had either more dimers to be repaired or less excision ability than cells which managed to survive in the presence of acriflavine. When plating on acriflavine-SEM was preceded by growth inhibition, the excision taking place during this inhibition was presumably sufficient to prevent loss of cells with many dimers or too little excision ability. According to this interpretation, the distribution of dimers in the population and/or the distribution of excision ability are among the

factors which determine modification of lethality by post-irradiation treatments which modify excision repair (Hill, 1965).

The fact that post-irradiation growth inhibition may have little or no detectable effect on survival but will reduce yields of prototrophic mutants has been taken as evidence that the primary u.v. lesions causing this mutation in dimer-excising strains include a lesion which is not an ordinary dimer (Witkin, 1966*a*). In view of the above discussion relating to other interpretations of non-correlation between effects on survival and mutant yields, it seems worthwhile to consider additional evidence which has been used to support the idea of a unique u.v. lesion for prototrophic mutation.

(1) *Effect of photoreactivating light*

When auxotrophic strains lacking photoreactivating enzyme were u.v.-irradiated and then exposed to photoreactivating light, the yield of prototrophic mutations was reduced, although such strains cannot split dimers (Witkin, Sicurella & Bennett, 1963). Since the effective wavelengths of the light are known to cause growth inhibition, it would appear that this situation is covered by the previous discussion (Jagger, Wise & Stafford, 1964). Other objections to this evidence have been given (Kondo & Jagger, 1966; Kondo & Kato, 1966).

(2) *Effect of growth inhibition on u.v.-induced mutation to streptomycin resistance*

When u.v.-irradiated cells of a streptomycin sensitive, auxotrophic strain were kept on chloramphenicol-supplemented nutrient broth agar prior to transfer to media for scoring mutants, only the yield of prototrophic mutants was reduced (Witkin & Theil, 1960). The scoring conditions were not, however, the same. Prototrophic mutants were scored after plating on the surface of SEM agar, streptomycin-resistant (and dependent) mutants were scored by incorporating cells within nutrient broth agar and adding streptomycin after allowing time for phenotypic expression. Therefore before this evidence can be evaluated properly it would seem necessary to know how much growth inhibition actually occurred during the exposure to chloramphenicol (stationary phase cells were used) and how much dimer repair occurred during this period relative to that occurring during the later periods of growth in different conditions. It is possible that very little dimer repair occurred during the actual period on chloramphenicol agar. The different results for the two types of mutations could then be due to a difference in the rate of chloramphenicol elimination from cells under the different scoring conditions, i.e. a slower rate on SEM agar would allow more repair on this medium and hence a much more pronounced loss of mutants.

(3) *Action spectra for lethality and for prototrophic mutation*

Evidence obtained from determinations of action spectra has also been quoted in support of a unique mutagenic lesion for prototrophy. The action spectrum for mutation measured on acriflavine-SEM agar has been reported to match the action spectrum for lethality but the action spectrum for mutation measured on SEM agar without acriflavine is said to differ (Witkin, 1966*b*). It was suggested that on SEM agar where repair of dimers is more efficient than on acriflavine-SEM agar, most of the observable yield of mutants must come from the hypothetical second mutagenic lesion so that the mutational action spectrum as measured on SEM agar will not match the spectrum for dimer formation. This line of evidence is difficult to evaluate

since the actual spectral data have not been published. It would be interesting to know in what way the spectrum for mutation measured on SEM agar differs from the spectrum for dimer formation and whether the discrepancy does in fact reflect a non-dimer mutagenic lesion or whether it indicates a non-mutagenic, lethal damage.

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With 2 plates
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Stromatal Rind Formation in the Brown Rot Fungi

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SUMMARY

The development in culture of a stromatal rind of *Sclerotinia fructicola* was studied with a light microscope and the surfaces of stromata of the brown rot fungi grown on agar cultures and on fruits were examined with a Stereoscan electron microscope. The hyphae on the surface of some stromata retained their shape, while on other stromata a crust of thickened, collapsed hyphae developed over the outside. In some places rounded cells were seen below the gaps in the crust. The deposit of a melanin-like pigment around and in the walls of exposed stromatal hyphae may possibly increase the resistance of these hyphae to drying and be of importance in survival of the brown rot fungi.

Whetzel (1945) attributed the dark colour of the rind cells of sclerotial stromata to impregnated oxidation products of the dead protoplasmic contents. Willetts (1968*a*) suggested that a dark rind forms on all exposed parts of the stromata of the brown rot fungi in contact with air following drying or autolysis of the surface hyphae and that the colour is due to the deposition and accumulation of dark brown melanin-like pigments, around and in the walls of the outer hyphae. Since the latter paper was submitted for publication, the surfaces of young and mature stromata formed in culture and on fruits have been examined with both the ordinary light microscope and the Stereoscan electron microscope. The observations made during the investigation are outlined below.

Sclerotinia fructicola (Wint.) Rehm. was cultured on potato dextrose agar; *S. fructigena* (Aderh. & Ruhl.), *S. laxa* (Aderh. & Ruhl.) and *S. laxa* forma *mali* (Wormald) Harrison were grown on malt agar; also apples, pears, cherries, apricots and plums were inoculated with the brown rot species of the genus *Sclerotinia* found in England. Small pieces (about 6×5 mm and about 1 mm. deep) of the stromata that were produced in culture or in the fruits were attached to aluminium stubs with a proprietary adhesive, coated under a high vacuum with a thin layer of gold–palladium alloy and examined in a Stereoscan electron microscope. Similar material sectioned with a freezing microtome was used for light microscope studies.

Plate 1, fig. 1–4, shows stages in the development in culture of a stromatal rind of *Sclerotinia fructicola* as seen with a light microscope. The amount of pigmentation increased as the stroma matured and in Pl. 1, fig. 4, the outermost surface of the rind is a black layer with two rows of cells beneath. These cells have dark walls and fit closely together to form a pseudoparenchymatous tissue. Enclosed within the rind is a medulla of loosely interwoven hyaline hyphae.

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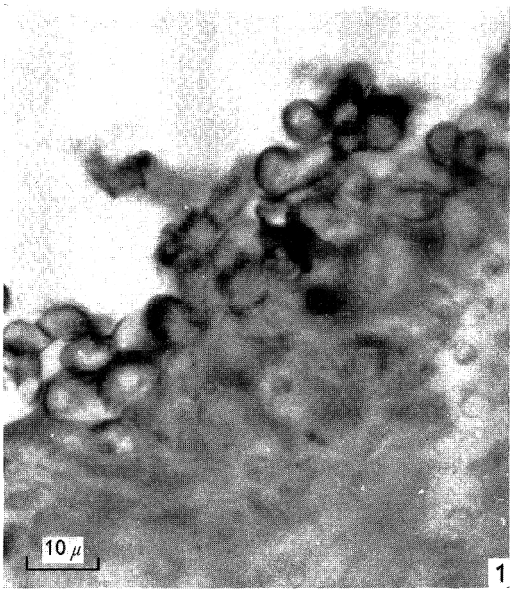
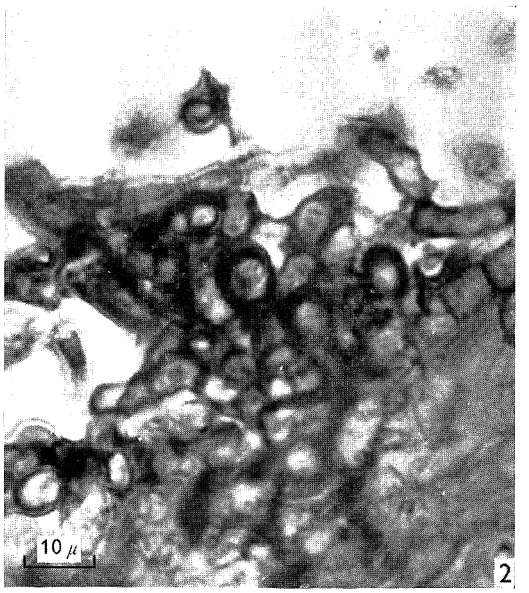
The appearances of the surfaces of old stromata produced in agar cultures when examined with the Steroscan electron microscope are shown in Pl. 2, fig. 5–7. The individual hyphae on the surface of the stroma of *Sclerotinia laxa* (Pl. 2, fig. 5) retained their shape, even after being subjected to the high vacuum essential for the gold–palladium coating. When young vegetative hyphae and conidiophores were treated in the same way they lost their structure and collapsed. The retention of the shape of the stromatal surface hyphae is probably due to the deposit of the pigment which gives the dark colour to the hyphae. In some areas of the surface of the stromata the hyphae were dried out and formed a crust (Pl. 2, figs. 6, 7), although their walls were thickened. It is probably such areas which, when transversely sectioned, give the black layer shown in Pl. 1, fig. 4. The whole of the stroma of *S. fructigena* (Pl. 2, fig. 7) was covered by an incomplete crust of collapsed, thickened hyphae, which may have been a sparse covering of aerial hyphae over the surface of the stroma. Possibly as a result of natural drying, the hyphae had lost their shapes, but had produced a great deal of pigment. Rounded cells can be seen in some of the gaps in the crust and these probably correspond to the outermost pseudoparenchymatous layer of cells beneath the dark outer layer.

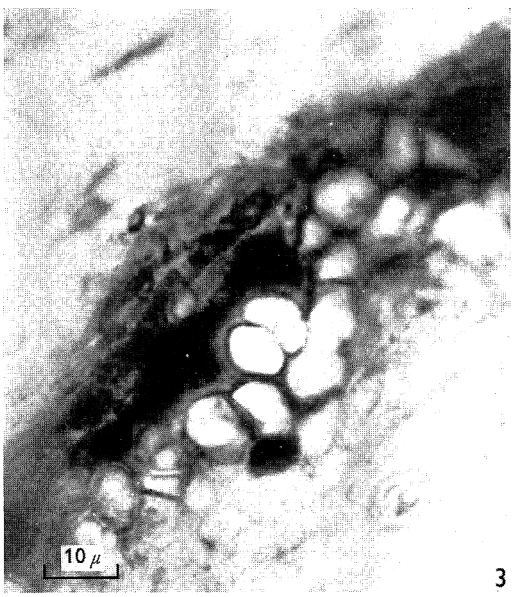
The surface of stromata of the brown rot fungi that form inside fruits is similar in appearance to that of stromata that develop in agar cultures. Cherries infected with the fruit-rotting Sclerotinias do not normally develop much aerial mycelium (Willetts, 1968*b*) and the rind of the stroma in areas where it ruptures the surface of the cherry is not usually obscured by thin-walled aerial hyphae, since these dry-out and collapse when the stroma matures or when the material is prepared for viewing with the Stereoscan electron microscope. Plate 2, fig. 8, is a Stereoscan electron micrograph of the surface of a cherry infected by *Sclerotinia laxa* forma *mali*, showing an exposed surface of the stroma, and Pl. 2, fig. 9, is a higher magnification of one area of this preparation. Numerous hyphae which have retained their shape are seen protruding from the surface. No areas of collapsed hyphae were found in this material.

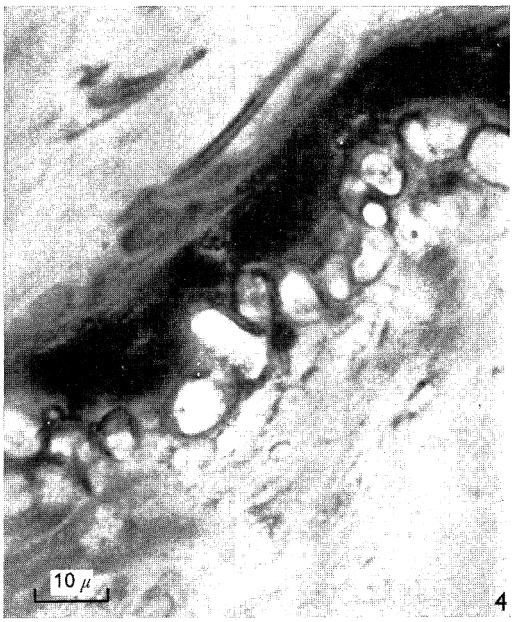
Stromata are able to survive conditions that are too severe for the ordinary vegetative mycelium, and this is due to the compact structure and chemical composition of the hyphae, particularly those of the outer layers that form the rind. The melanin-like pigment that develops in the hyphae on the surface of the stroma may reduce desiccation of the structure and, under extreme conditions, be of importance in the survival of the fungus. Chet & Mitchell (1967) found that sclerotial walls of *Sclerotium rolfsii* Sacc. contain a melanin-like pigment which is absent from hyphal walls and they suggested that the pigment may play some role in the resistance of sclerotia to biological degradation.

Thanks are due to Professor H. E. Hinton, F.R.S., for granting permission to use the Stereoscan electron microscope, which was purchased by the Science Research Council, and to Professor L. E. Hawker for her encouragement and advice. Mr J. W. Heavens operated the instrument and Mr B. Gray assisted in the preparation of the material.









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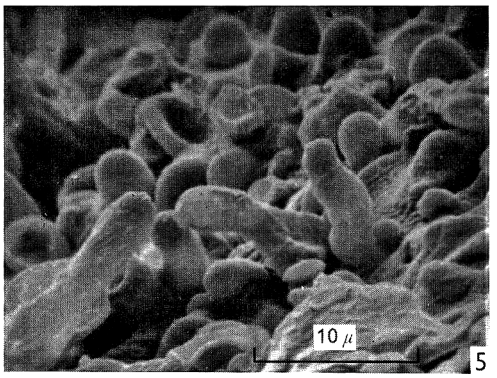
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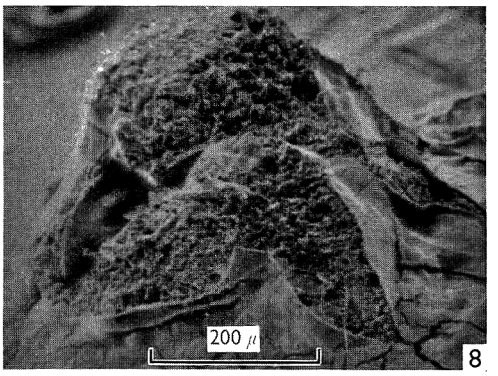
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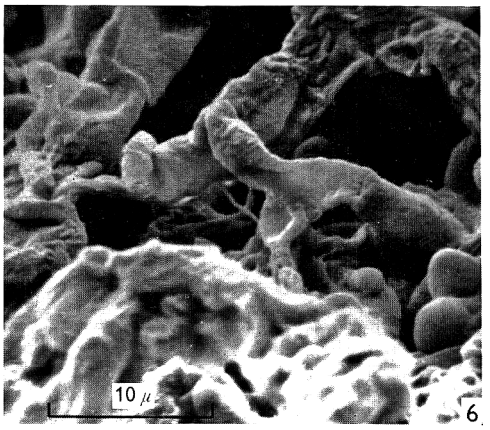
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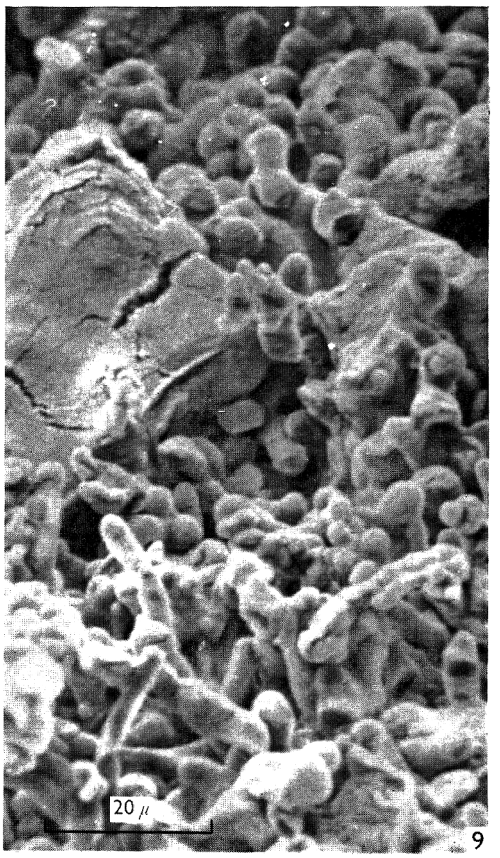
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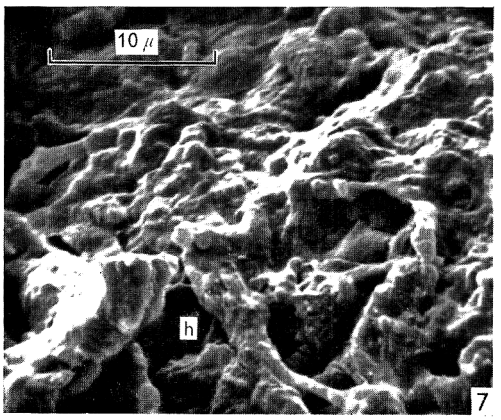
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EXPLANATION OF PLATES

PLATE 1

Figs. 1–4. Stages in the development in culture of a stromatal rind of *Sclerotinia fructicola* as seen with a light microscope. Note the increase in the amount of pigmentation from the least mature (fig. 1) to the most mature stroma (fig. 4) and the presence of a dark layer on the outside surface of the latter.

PLATE 2

Stereoscan electron micrographs of the surfaces of mature stromata

Fig. 5. The surface of the stroma of *Sclerotinia laxa*, showing hyphae that have kept their shapes despite being subject to a high vacuum.

Fig. 6. Another area of the stroma shown in Fig. 5. Note the crust of thickened, collapsed hyphae and the rounded cells in some of the gaps in this crust.

Fig. 7. The surface of a stroma of *Sclerotinia fructigena* with an extensive crust and several rounded hyphal tips (h), showing through gaps in the crust.

Fig. 8. The surface of a cherry infected by *Sclerotinia laxa* forma *mali*, showing an area where the stroma is exposed by a break in the skin of the cherry.

Fig. 9. An area of the same exposed stroma of *Sclerotinia laxa* forma *mali* on cherry. Note the absence of a crust and the numerous rounded hyphae protruding from the surface where cherry skin has been ruptured.

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An X-factor Requiring Haemophilus Species

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SUMMARY

The isolation from a perianal abscess of a Haemophilus species requiring for growth the presence of X-factor but not V-factor is recorded. Two other similar strains were isolated from normal faeces. The properties of these isolates are given and compared with those of previously described species of the genus *Haemophilus* having the same growth requirements. The isolates form a homogeneous group resembling *Haemophilus influenzae-murium*.

INTRODUCTION

All members of the genus *Haemophilus* have in common the characteristic of requiring for growth certain accessory factors normally present in blood. Two of these essential growth substances have been identified, haematin or the X-factor and nicotinamide adenine dinucleotide (diphosphopyridine nucleotide) or the V-factor. The genus has been subdivided according to growth-factor requirements. Thus Pittman (1957) listed 15 species of which five require both X- and V-factors, two require V-factor only, and five require X-factor only. The remaining three species require factors as yet unidentified. The species listed as requiring X-factor only are *Haemophilus aphrophilus*, *H. influenzae-murium*, *H. ovis*, *H. ducreyi* and *H. haemoglobinophilus*. Only two of these species have been found in human infections, *H. aphrophilus* and *H. ducreyi*. The present paper describes a member of this genus which requires X-factor only, isolated from a perianal abscess in a woman aged 45 years.

METHODS

Morphology. Morphology was routinely investigated in smears stained by Gram method. Motility was determined in hanging-drop preparations of young cultures in suitable liquid media. The wet indian-ink method (Duguid, 1951) was used for demonstrating capsules.

Physiology

Gas-phase conditions. The optimal gas-phase conditions for growth were determined by incubation of inoculated blood-agar plates in air, in a candle-jar for increased CO_2 tension, and anaerobically in a McIntosh & Fildes jar.

Temperature. The optimum temperature for growth was found by incubation of inoculated blood-agar plates at 37°, 30° and 25°.

Optimum pH value. Gradient plates, prepared according to the method of Watson & Bennett (1957), were used to determine the optimum pH value for growth and the pH range. Peptone water agar containing the X-factor was the basal medium used.

Nutritional requirements. All experiments on growth requirements were done under

optimal gas-phase, temperature and pH conditions. Small inocula were used throughout. Bacterial suspensions were made, in quarter-strength Ringer solution, of sufficient concentration to give semi-confluent growth or less when a 3 mm. loopful was inoculated on a segment of a plate. Serial subcultures were done when growth occurred. The following media were used: Koser citrate medium (Gillies, 1960), 1 % (w/v) Oxoid peptone water, peptone water medium solidified with 1 % (w/v) Davis powdered agar, peptone water agar enriched with X-factor, with V-factor, and with X-factor + V-factor together, Lemco nutrient broth, Lemco nutrient agar, Lemco nutrient broth and agar enriched with 10 % (v/v) horse serum, Lemco nutrient agar enriched with 10 % (v/v) horse blood, and chocolate agar prepared from Lemco nutrient agar containing 10 % (v/v) horse blood heated at 70° for 10–15 min.

X- and V-factors were prepared from haematin and from brewer's yeast, respectively, as described by Stokes (1960). The final concentration of haematin in the medium was 50 μg./ml.; yeast extract was added to a final concentration of 10 % (v/v).

Action on carbohydrates. Peptone water containing X-factor and 1 % (w/v) of the appropriate carbohydrate was used with Andrade indicator. Cultures were incubated for 3 weeks at 37° before results were recorded as negative. The method of Hugh & Leifson (1953) was used to determine whether acid production from glucose was the result of a fermentative or oxidative process. Starch hydrolysis was tested by incorporating 0·02 % (w/v) soluble starch in peptone water agar containing X-factor. After 3 days of incubation at 37° the starch plates were flooded with Gram iodine; clear zones were an indication of hydrolysis.

Oxidase. A few drops of a freshly prepared 1 % (w/v) solution of tetramethyl-*p*-phenylenediamine hydrochloride were poured over a 24 hr culture on blood agar and rapid development of a purple colour noted.

Catalase. A small amount of growth from peptone water X-factor agar was suspended in a drop of '20 vol.' hydrogen peroxide on a glass slide and production of effervescence noted.

Urease. A heavy inoculum was placed on the centre of a slope of Christensen medium (Christensen, 1946) and incubated for 24 hr at 37°.

Nitrate reduction. A strip of filter paper impregnated with potassium nitrate was laid across the surface of a blood agar plate and stab inoculations made into the medium adjacent to the strip (Cook, 1950). Browning of the medium around the inoculum indicated reduction of nitrate. Readings were taken after incubation for 24 and 48 hr. The conventional Griess–Ilosvay method was not used as satisfactory growth could not be obtained in nitrate-containing broth.

Egg-yolk reaction. Organisms were grown on Lemco nutrient agar containing X-factor and 10 % (v/v) egg-yolk saline. The egg-yolk saline was prepared by emulsifying the yolk of one egg in 250 ml. physiological saline and sterilizing by Seitz filtration. Readings were taken after incubation for 48 hr.

Indole. Organisms were grown for 48 hr at 37° in peptone water containing X-factor. Ehrlich reagent was used to test for indole production after extraction of cultures with ether.

Serum liquefaction. Liquefaction of coagulated serum was tested on Loeffler serum slopes. Readings were taken after 24 and 48 hr.

Gelatin liquefaction. Nutrient gelatin containing X-factor was used. Incubation was at 37° and was, when necessary, prolonged for 2 weeks.

Casein hydrolysis. Organisms were grown on Lemco nutrient agar containing X-factor and 10 % (v/v) skim milk. A positive reaction was indicated by clear zones around colonies after 2–3 days of incubation.

Fibrinolysis. Fresh human plasma 10 % (v/v) was added to melted Lemco nutrient agar containing X-factor and the fibrinogen precipitated by heat at 56° (Christie & Wilson, 1941). Plates were poured, dried and inoculated. Clear zones around colonies after 2–3 days of incubation indicated fibrinolysin production.

Litmus milk. X-factor was incorporated in ordinary litmus milk. Cultures were incubated, when necessary, for 3 weeks.

Amino acid breakdown and sodium malonate utilization. Decarboxylation of lysine, deamination of phenylalanine and utilization of sodium malonate were investigated by micro-methods (Shaw & Clarke, 1955). Positive and negative controls were included with all tests.

Haemolysis. Lemco nutrient agar plates containing X-factor and 2 % (v/v) washed red blood cells were used. Horse, human, rabbit, sheep and chicken red cells were tested, by using layered plates. Results were read after incubation for 24 and 48 hr.

Haemagglutination. A drop of a heavy bacterial suspension in saline was added to a drop of 10 % (v/v) washed red blood cell suspension on a slide and rocked gently for 2–3 min. Human group O, rabbit and chicken red cells were used; the test was done at room temperature (about 18°) and at 4°.

Sodium chloride tolerance. Sodium chloride was incorporated in Lemco nutrient agar containing X-factor in the following concentrations (% w/v): 5·5, 4·5, 3·5, 2·5, 1·5, 0·5. Plates were inoculated with a drop of a light bacterial suspension, sufficient to give semi-confluent growth on control plates containing 0·5 % NaCl. Readings were taken after 3–4 days of incubation.

Heat-resistance. Heavy bacterial suspensions in peptone water were placed in small screw-capped bottles and immersed in water baths at 56° and 100°. At intervals a large loopful was removed and plated on blood agar.

Viability. Survival at 37°, at room temperature, and at 4° was determined by storing fully grown blood agar cultures at these temperatures in the dark and subculturing at intervals to fresh blood agar plates.

Resistance to inhibitory agents. The effect of potassium tellurite, sodium azide and crystal violet was examined by incorporating these substances in blood agar in the following final concentrations (w/v): potassium tellurite 1/15,000; sodium azide 1/2,500, 1/5,000; crystal violet 1/125,000, 1/250,000, 1/500,000. A drop of bacterial suspension sufficient to give semi-confluent growth on control plates was used as inoculum. Results were read after 3–4 days.

Antibiotics and chemotherapeutic agents. Sensitivity tests were done on blood agar plates by using commercialy available discs. In addition, sensitivity to penicillin and to neomycin was investigated by the tube dilution method, using as basal medium Lemco nutrient broth containing X-factor. Results were recorded after 2 days.

Antigenic structure

Suspensions of organisms in formol saline (physiological saline containing 0·5 % (v/v) formalin), made up to a concentration of about 3000 million organisms/ml. were used to immunize rabbits. Sera taken before beginning the injections contained no agglutinins against the immunizing organisms. The dosage schedule used was that

described by Boyd (1947), 1·0 ml. doses being used with 3 intravenous injections on the first 3 days of the first week, followed by 2 intravenous and one intraperitoneal injection on the first 3 days of the 3 succeeding weeks. The animals were bled 1 week after completing the course, provided that a trial bleeding showed satisfactory results.

Agglutination tests were done in Dreyer tubes in a 52° water bath using as antigens suspensions containing about 300 million organisms/ml. Both formolized suspensions and suspensions heated at 100° for 15 min. were used as antigens.

RESULTS

A specimen of pus from a perineal abscess of 5 weeks duration in a woman aged 45 years was submitted for bacteriological examination. Aerobic blood agar plates showed a moderate growth of *Escherichia coli* and a profuse growth of a small Gram-negative cocco-bacillus producing minute colonies about 0·5 mm. diam. Anaerobic cultures yielded in addition a scanty growth of *Clostridium welchii*. The cocco-bacillus showed no growth on unenriched nutrient media and did not, in early subcultures, show satellitism around colonies of *Staphylococcus aureus*; moreover growth was not improved by substituting chocolate agar for blood agar. It was decided that this organism might be an unusual member of the genus *Haemophilus*. It was given the laboratory reference number S 3105.

Morphology, colonial appearance and growth in liquid media

The organism S 3105 was a small Gram-negative cocco-bacillus with occasional filamentous forms, resembling some strains of *Haemophilus influenzae*. It was non-capsulated, non-sporing, and non-motile after growth at 25° and 37°. Colonies on horse blood agar in 24 hr were small and pin-point, 0·5 mm. or less in diam. In 48 hr they were slightly larger, round, smooth, convex, greyish, butyrous in consistency and easily emulsifiable. In 3–4 days the colonies had enlarged to a diameter of about 1·0 mm. but no increase in size occurred on further incubation. There was no haemolysis or other alteration of the horse blood. In suitable liquid media growth occurred as a moderate uniform turbidity with slight deposit and no pellicle or surface ring.

Optimum gas-phase conditions, temperature and pH value

The organism S 3105 was aerobic and facultatively anaerobic. Growth under anaerobic conditions was almost as good as that in air; carbon dioxide had no appreciable effect. The optimum temperature was 37°; growth at 30° was poor and at 25° very poor or absent. The optimum pH value was 7–7·5; no growth occurred below pH 6 or above pH 9.

Nutritional requirements

Large inocula gave erratic results; growth occurred sometimes on simple media. For consistent and reproducible results it was found essential to use small inocula. Under these conditions no growth was apparent in Koser citrate medium, peptone water or Lemco nutrient broth, or on peptone water agar or Lemco nutrient agar when incubated aerobically. Slight growth occurred on anaerobic Lemco nutrient agar plates. The organism grew well aerobically on Lemco nutrient agar enriched with 10% (v/v) horse blood, and almost equally well on the same medium base enriched with 10% (v/v) horse serum; heating the blood (chocolate agar) did not enhance growth. Good

growth was obtained on peptone water agar containing both X- and V-factors or X-factor alone, but no growth occurred when this basal medium was enriched with V-factor only. The adequacy for growth of peptone water agar enriched with X-factor was tested by subculture on this medium. Eight serial transfers of the organism were made with ease from small inocula, the experiment then being discontinued.

Similar findings were obtained with liquid media. There was minimal or no growth in simple media but good growth in peptone water and in Lemco broth enriched with horse serum or with X-factor. No growth occurred in these media containing V-factor only, nor in Koser citrate medium enriched with X-factor. The growth-promoting effect of serum was probably due to contamination with X-factor; the batch of serum used gave a strongly positive benzidine reaction.

After repeated subculture on enriched medium the strict requirement for X-factor might be lost and slight growth occur even on peptone water media. This, however, was never as good as in the presence of X-factor.

When freshly isolated the organism did not show satellitism near staphylococci on any medium. After repeated laboratory subculture slight growth occurred in the neighbourhood of staphylococcal colonies on peptone water agar with or without V-factor; this satellite growth could be propagated through several subcultures. On X-factor containing media there was never any evidence of satellitism. Further experiments were done using nicotinamide adenine dinucleotide (NAD) instead of yeast extract to supply V-factor; the final concentration of this substance in the medium was 10 μg./ml. The present isolate was tested in parallel with a strain of *Haemophilus influenzae* freshly isolated in this laboratory and with a strain of *H. aphrophilus* obtained from the National Collection of Type Cultures, London (NCTC 5886). The results are shown in Table 1.

Table 1. *Effect of nicotinamide adenine di-nucleotide (NAD) on growth of strain S 3105 and of Haemophilus influenzae*

Strains	Peptone water agar	Peptone water agar + X-factor	Peptone water agar + NAD (10 μg./ml.)	Peptone water agar + X-factor + NAD	Blood agar
S 3105	−	++	−	++	+++
Haemophilus influenzae	−	−	−	+++	++
H. aphrophilus NCTC 5886	−	++	−	++	+++

+++ = Good growth; ++ = moderately good growth; − = no growth.

No growth of the present isolate occurred in the absence of X-factor, indicating that the staphylococcal factor causing satellitism was not NAD and was probably related to haematin. Heating experiments showed that the staphylococcal factor was destroyed by autoclaving at 121° for 15 min. but not by heating at 70–80° for 10 min. It is perhaps worth noting here a possible fallacy in the common practice of relying on satellitism for the demonstration of V-factor requirement.

Action on carbohydrates

Acid without gas was produced in 2–3 days from xylose, glucose, galactose, lactose, maltose and salicin, in 5 days from rhamnose, sucrose, raffinose and inulin, and in

15 days from arabinose. Mannitol, dulcitol, adonitol, sorbitol and inositol were not attacked. Glucose was fermented, not oxidized. Starch plates showed a small zone of hydrolysis.

Other biochemical reactions

Tests for oxidase, urease, catalase, indole, serum liquefaction and gelatin liquefaction were all negative. Nitrates were not reduced. Litmus milk enriched with X-factor showed acid and clot production in 3–5 days. There was no decarboxylation of lysine, deamination of phenylalanine or utilization of sodium malonate. There was no opacity on egg-yolk plates, no hydrolysis of casein and no fibrinolysin production.

Haemolysis and haemagglutination

There was no haemolysis in plates containing horse, human, rabbit, sheep or chicken red blood cells. There was no agglutination of human group O, rabbit or chicken red cells at room temperature or at 4°. No attempt was made to demonstrate a soluble haemolysin.

Tolerance to sodium chloride and inhibitory agents

Good growth occurred in the presence of 0·5 % and 1·5 % sodium chloride but partial inhibition was caused by 2·5 % and complete inhibition by 3·5 %. There was no growth on plates containing 1/15,000 potassium tellurite, 1/2,500 sodium azide or 1/125,000 crystal violet, but the organism grew moderately well in the presence of 1/5000 sodium azide and 1/250,000 and 1/500,000 crystal violet. By the disc method, the organism was sensitive to novobiocin, chloramphenicol, ampicillin, tetracycline and nitrofurazone, and resistant to penicillin, streptomycin, neomycin and erythromycin. In tube dilution tests the organism was resistant to 25 units penicillin/ml. but sensitive to 50 units/ml.; it was resistant to at least 100 μg. neomycin/ml.

Viability, and resistance to heat

The organism was very sensitive to heating under the conditions of the test. Death occurred in seconds at 100° and in less than 5 min. at 56°. Viability tests showed survival for 2 weeks (when the experiment was discontinued) at room temperature and at 37° and for at least 4 weeks at 4°.

Pathogenicity for laboratory animals

A suspension in peptone water from a 48 hr blood agar plate diluted to the opacity of a No. 1 Brown's opacity tube, was injected intramuscularly into a guinea-pig (0·5 ml.) and intraperitoneally into a mouse (0·2 ml.). No local lesions developed and the animals remained well. After 7 days both animals were killed. Post-mortem examination showed no lesions.

Possible natural habitat

An attempt was made to isolate further strains of this organism from laboratory specimens. The specimens were plated on a selective medium based on the resistance of the organism to various inhibitory agents. The medium used was Lemco nutrient agar containing horse blood 10 % (v/v), crystal violet 1/500,000 final concentration, penicillin 1 unit/ml. and neomycin 10 μg./ml. This medium proved very satisfactory and yielded pure cultures even from faeces. It may, however, have suppressed the

growth of strains sensitive to one or other of the selective agents used, if such strains exist. In all, 364 specimens were examined including 130 nose and throat swabs, 110 sputa, 100 faeces, 14 vaginal swabs and 10 miscellaneous specimens. The only positive finding was the isolation from 2/100 faeces specimens of a heavy pure growth of an organism resembling the original isolate. Both positives were from normal children aged 6 months. The isolations were made at different times and there was no known contact between the children or between them and the original patient. The two new strains were given the laboratory reference numbers V 196 and V 231. Detailed examination of these isolates gave virtually identical results to those obtained with the original strain S 3105, except that both fermented arabinose more quickly and strain V 196 did not ferment inulin.

Antigenic structure

Antisera were prepared in rabbits against the original isolate S 3105 and against strain V 231. Each antiserum agglutinated to high titre suspensions of the homologous organism. Formolized and heated suspensions gave similar titres but formolized suspensions were agglutinated in 3–4 hr whereas heated suspensions required overnight incubation at 52°. Antiserum S 3105 gave no agglutination with antigens of strains V 196 and V 231. Similarly, antiserum V 231 did not agglutinate strains V 196 and S 3105. Thus no antigenic relationship was demonstrated between the three cultures.

DISCUSSION

X-factor is associated with the formation of various cytochrome haematins and catalase. The latter is a relatively heat-stable enzyme which apparently can replace X-factor with some strains of *Haemophilus influenzae* (Brumfitt, 1959). White & Granick (1963) showed that the haematin biosynthetic pathway of haemin-independent Haemophilus is similar to that used in animals, plants and other micro-organisms, and proceeds through the steps

δ-aminolaevulic acid → porphobilinogen → uroporphyrinogen III → coproporphyrinogen → protoporphyrinogen → protoporphyrin IX → haematin.

They confirmed Brumfitt's (1959) finding that protoporphyrin IX, but not earlier precursors, could substitute for haematin in the case of *H. influenzae*, but found that this was not so with the strain of *H. aegyptius* tested, which required fully formed haematin. Mannheim (1965) showed that the cell-free culture fluid of a strain of *Achromobacter metalcaligenes* supplied the X-factor requirements of *H. influenzae* but not of *H. aegyptius*. This substance was sensitive to light, was relatively heat-labile, and was thought to be a porphyrinogen, a haematin-precursor. Mannheim (1966) showed that a similar diffusible substance was produced by a wide variety of organisms including a strain of *Staphylococcus epidermidis* (*S. albus*). Satellitism of staphylococcal colonies by the present isolate S 3105 could be due to the production by the staphylococci of such a substance; the original strict requirement for haematin itself may be lost on subculture and growth may become possible in the presence of haematin precursors.

The two X-factor requiring Haemophilus species known to cause infection in man are *H. ducreyi* and *H. aphrophilus*. Lwoff & Pirosky (1937) examined 21 laboratory strains of *H. ducreyi* which over a period of years had become accustomed to grow on ordinary blood agar media, although none of these grew in simple peptone water.

They found that haematin (X-factor) was an adequate substitute for blood, but V-factor was ineffective. Pittman's (1957) classification of this organism as an X-factor requiring species is based on Lwoff's findings. Beeson (1946) and Ajella, Deacon, Paul & Walls (1956) were unable to obtain growth in the presence of X- and V-factors alone or in combination, and most workers agree that primary isolation is difficult even on enriched media, special media being required. It seems evident that the growth requirements of recently isolated strains of *H. ducreyi* are not satisfied by enrichment with X-factor alone. *Haemophilus aphrophilus* was isolated by Khairat (1940) from a case of endocarditis, this organism had the characteristic property of failing to grow in air on primary isolation, the presence of CO_2, preferably in concentration of 5 % (v/v), being necessary; peptone water enriched with X-factor provided all the essentials for growth. On repeated subculture, however, both CO_2 and X-factor might become unnecessary, growth occurring in air on peptone water media. King & Tatum (1962) found that even recently isolated strains of *H. aphrophilus* grew on unenriched nutrient media in air without added CO_2, and had no requirement for X- and V-factors. Biochemically *H. aphrophilus* does not ferment arabinose, xylose, rhamnose or salicin but reduces nitrates to nitrites (Khairat, 1940; King & Tatum, 1962). *Haemophilus ducreyi* ferments no sugars (Reymann, 1949; Ajella *et al.* 1956). Both organisms therefore differ in growth requirements and in biochemical reactions from the present isolates.

Haemophilus haemoglobinophilus, *H. ovis* and *H. influenzaemurium* have been isolated from animals but not from man. *H. haemoglobinophilus* is found in the prepuce of normal dogs. Freshly isolated strains grow adequately on peptone water media enriched only with X-factor, but the organism differs from the present isolates in fermenting mannitol but not lactose, maltose or arabinose and in reducing nitrates to nitrites (Rivers, 1922). *H. ovis* was isolated by Mitchell (1926) from haemorrhagic bronchopneumonia of sheep. An autoclave-stable factor present in blood, presumably X-factor, was necessary for primary isolation, but for optimal growth an autoclave-labile factor was also required. The species differs from the present strains in fermenting mannitol and sorbitol but not arabinose, rhamnose or salicin, in reducing nitrates to nitrites and in being highly virulent for the guinea-pig. *H. influenzaemurium* was first isolated by Kairies & Schwartzer (1936) from mice with bronchopneumonia and also from the upper respiratory tract of normal mice. Its requirement of X-factor was demonstrated by Ivanovics & Ivanovics (1937). Information regarding biochemical reactions is scanty, but this organism differs from the present isolates in failing to produce acid and clot in litmus milk and in its pathogenicity for mice.

A further organism to be considered is the bacillus first isolated by Leopold (1953) and named *Haemophilus vaginalis* by Gardner & Dukes (1955). The X- and V-factor requirements of this organism are not clear. Edmunds (1960) and Dukes & Gardner (1961) showed that X-factor was ineffective in a basal medium. Moreover, all authors are agreed that this is an exacting species and special media are recommended for isolation and subculture. Zinnemann & Turner (1963) consider that *H. vaginalis* is in fact a Gram-positive bacillus and should be re-named *Corynebacterium vaginale*. It appears to bear no relationship to the present isolates.

Table 2 shows the growth requirements and Table 3 the biochemical reactions of the present isolates together with those of named species.

The three strains (S 105, V 196, V 231) under study form a homogeneous bio-

Table 2. *Growth requirements of Haemophilus species*

	Enriched media	Peptone water media	Peptone water media + V-factor	Peptone water media + X-factor	Peptone water media + X- and V-factor	Require CO_2 for optimum growth
Present strains	+ + +	−	−	+ + +	+ + +	No
H. ducreyi						
Old laboratory strains	+ + +	−	−	+ + +	+ + +	?
Recently isolated strains	+ + +	−	−	−	−	Probably yes
H. aphrophilus	+ + +	−	−	+ + +	+ + +	Yes
H. haemoglobinophilus	+ + +	−	−	+ + +	+ + +	?
H. ovis	+ + +	−	Probably	Probably	Probably	?
H. influenzaemurium	+ + +	−	—	+ + +	+ + +	?

+ + + = Good growth; + + = moderate growth; − = no growth; ? = not known. Results from the named species compiled from the literature.

chemical and cultural group although antigenically they are heterogeneous. Their biochemical reactions distinguish them from all hitherto described X-factor requiring Haemophilus species, although with *H. influenzaemurium* the distinction must be made solely on acid and clot production in litmus milk. This, however, was a very constant and characteristic feature of these strains which, moreover, showed no pathogenicity for mice. Their lack of requirement for CO_2 further differentiates them from *H. aphrophilus* as even the aerobic strains of King & Tatum showed enhanced growth under increased CO_2 tension. Growth requirements alone are sufficient to distinguish them from *H. ducreyi* and *H. vaginalis*; biochemical reactions confirm this distinction. The guinea-pig pathogenicity of *H. ovis* is characteristic and the biochemistry of this organism and of *H. haemoglobinophilus* quite distinctive.

Table 3. *Biochemical reactions of Haemophilus species*

	Present strains	*H. ducreyi*	*H. aphrophilus*	*H. haemoglobinophilus*	*H. ovis*	*H. influenzaemurium*
Arabinose	A	–	–	–	–	.
Rhamnose	A	–	–	.	–	.
Xylose	A	–	–	A	A	.
Glucose	A	–	A	A	A	A
Galactose	A	–	V	A	A	.
Lactose	A	–	A	–	A	.
Maltose	A	–	A	–	A	A
Sucrose	A	–	A	A	A	A
Raffinose	A	–	V	.	A	.
Adonitol	–	–	–	.	.	.
Dulcitol	–	–	–	–	.	.
Mannitol	–	–	–	A	A	–
Sorbitol	–	–	–	.	A	.
Inositol	–	–	–	.	–	.
Salicin	A	–	–	.	–	.
Starch	Hydrolysed	–	A	.	.	.
Oxidase	–	–	–	.	.	.
Urease	–	.	Usually –	.	.	.
Catalase	–	.	–	.	.	.
Nitrate reduction	–	–	+	+	+	.
Indole	–	–	–	+	–	–
Gelatin liquefaction	–	–	–	.	.	–
Coagulated serum liquefaction	–	–	.	.	.	.
Litmus milk	AC	.	Weak A	–	–	–

A = acid; AC = acid and clot; + = positive reaction; – = negative reaction; V = variable results. Reactions of the named species compiled from the literature.

On the available evidence the present isolates are most appropriately considered as a variety of *H. influenzaemurium*. Unfortunately the recorded findings on this species are incomplete and a strain was not available for comparison.

It does seem questionable whether any of the X-factor requiring Haemophilus species, with the exception of Ducrey's bacillus, merit specific status; they might perhaps be more suitably described as varieties of *H. haemoglobinophilus*.

Haemophilus has seldom been reported in the gut or gut contents, perhaps because of the difficulty of isolation. Rogers, Zinnemann & Foster (1960) reported the finding

of *Haemophilus influenzae* in perianal abscesses and in the lumen of appendices removed at operation. Bishop & Allcock (1960) recorded the isolation of *H. aphrophilus* from the proximal loop of small intestine in a case of acute intestinal obstruction; its presence was thought to be due to ingestion and a causal role was not suggested. A small Gram-negative bacillus thought to be a Haemophilus species was isolated by Kok, Dybkaer & Rostgaard (1964) from jejunal biopsies in a patient with Whipple's disease. Its pathogenic significance was not definitely determined.

The pathogenic role of the present original isolate, strain S 3105, remains doubtful. Obviously the lesion was exposed to faecal contamination as evidenced by the presence of *Escherichia coli* and *Clostridium welchii*. The Haemophilus species was, however, the predominant organism; indeed its presence would otherwise hardly have been detected. Unfortunately serum from the patient was not available for antibody titration. After treatment with tetracycline, scanty flora of normal faecal type was isolated and haemophilic bacilli were no longer found. The patient's faeces were not examined for Haemophilus as a suitable selective medium had not then been developed.

Strain S 3105 has been deposited with the National Collection of Type Cultures, Central Public Health Laboratory, Colindale, London, as NCTC 10555.

I am indebted to the National Collection of Type Cultures, London, for the culture of *Haemophilus aphrophilus* NCTC 5886.

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Pectin Methyl-*trans*-eliminase as the Maceration Factor of *Sclerotinia fructigena* and Its Significance in Brown Rot of Apple

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SUMMARY

Good correlation between pectin methyl-*trans*-eliminase (PTE) activity and the ability to macerate plant tissue slices was found in fractions obtained from culture filtrates of the fungus *Sclerotinia fructigena* by gel filtration on dextran gel, or by ion-exchange chromatography. The presence of two isoenzymes was indicated, one of which was markedly activated by sodium polypectate or potato extract. Endo-polygalacturonase and α-L-arabinofuranosidase activities were not correlated with macerating ability. Negligible amounts of PTE were, however, detected in extracts of apple fruitlets rotted by *S. fructigena*; the pH optima of the enzyme components in the culture filtrates differed greatly from the pH of apple tissue. For this and other reasons PTE would appear to have little, and at most very localized, significance in the infection of apple fruits by *S. fructigena*.

INTRODUCTION

The fungus *Sclerotinia fructigena* Aderh. & Ruhl. causes a brown rot disease of apple fruits characterized by the comparative firmness of the invaded tissue, which is a result of the relatively small degradation of pectic components during fungal attack (Cole, 1956; Cole & Wood, 1961). Culture filtrates of this fungus are known to contain polygalacturonase (PG; Cole, 1956) and to be able to macerate plant tissue. It has been suggested that generally such maceration of plant tissue by fungal enzymes can be related to PG, with or without pectin methylesterase (Demain & Phaff, 1957), and that it is not necessary to postulate a specific 'protopectinase'. Bateman (1963) subsequently identified the macerating enzyme of *Rhizoctonia solani* as a polygalacturonase. However, by the use of gel filtration and cellulose chromatography it has been found possible to resolve the PG and macerating activities of a culture filtrate of *S. fructigena*, and to obtain preparations devoid of PG but still capable of tissue maceration (Byrde & Fielding, 1962). This action was attributed to a 'maceration factor', still biochemically undefined. Subsequently the presence of an enzyme capable of liberating L-arabinose from potato fibre was demonstrated in such macerating fractions and the enzyme was found to be an α-L-arabinofuranosidase (Byrde & Fielding, 1965).

Albersheim, Neukom & Deuel (1960) showed the existence of an enzyme which cleaved the polygalacturonate chain by an eliminative instead of by a hydrolytic mechanism. The role of enzymes of this general type (*trans*-eliminases) in tissue maceration has subsequently been shown in several instances (e.g. Bateman & Millar,

1966; Dean & Wood, 1967). The present paper identifies the 'maceration factor' of *Sclerotinia fructigena* as a pectin methyl-*trans*-eliminase, and not as an α-L-arabinofuranosidase. The possible significance of the former enzyme in the host/parasite relationship of brown rot of apple has also been examined.

METHODS

Organism. The culture of *Sclerotinia fructigena* used was initially isolated from a naturally infected apple, and stock cultures were maintained on potato+carrot agar (Dade, 1960). For conidial production, the fungus was grown in boiling tubes on slopes (20 ml.) of 15 % (v/v) 'V. 8' mixed vegetable juice (Campbell's Soups Ltd., King's Lynn, Norfolk), containing 3 % (w/v) agar. After 3 days at 25°, the cultures were transferred to daylight (but not direct sunlight) at room temperature to promote sporulation, which is light-induced in this species (Hall, 1933). Spores were normally taken from 7-day cultures.

Routine production of enzymes in vitro. The fungus was grown on the following medium (pH 5·7), similar to that used by Cole (1956), dispensed in 200 ml. lots in penicillin-culture flasks: sodium polypectate (Exchange Lemon Products Co., California), 10 g.; ammonium tartrate, 15 g.; KH_2PO_4, 1 g.; $MgSO_4.7H_2O$, 0·5 g.; water to 1 l. After autoclaving, the medium was inoculated with a dense conidial suspension and the culture incubated for 11 days at 25°. The culture filtrate (about pH 7·0) was harvested by filtration, stored at 0° to −4° and concentrated by partial 'freezing out' (Dixon & Webb, 1958, p. 57).

Time-course of enzyme induction. For a more critical evaluation of enzyme induction the fungus was obtained in pellet form in liquid culture by inoculating a medium of 1·5 % (w/v) glucose and 3·5 % (w/v) malt extract (dispensed in 50 ml. lots in 250 ml. conical flasks) with a dense spore suspension of *Sclerotinia fructigena.* This medium, as used by McCallan & Miller (1957) for *S. fructicola*, was the only one tested in which satisfactory pellets could be grown. Flasks were incubated overnight at 25° without shaking (on the shaker the germinating spores were liable to clump at this stage). The following morning the flasks were transferred to a reciprocating shaker (100 strokes/min., stroke 4 cm.) at 25° and shaken for a further 30 hr before harvesting the mycelial pellets by centrifugation in sterile tubes and washing the mycelial deposit with sterile water.

The test system for induction was as follows: 0·5 ml. thrice-washed mycelial pellets, with 3·0 ml. sterile basal medium, in a 50 ml. flask. The basal medium comprised: malic acid, 10 g.; KH_2PO_4, 2 g.; $MgSO_4.7H_2O$, 0·5 g.; NH_4Cl, 5 g.; water to 1 l.; adjusted to pH 4·0 with 2·5 N-NaOH.

After incubation overnight, with the object of depleting excess polysaccharide in the mycelial pellets (otherwise found to delay enzyme induction), the inducing substrates were added, usually as 1 ml. sterilized solution in basal medium. The cultures were replaced on the shaker for a further period before finally being harvested by filtration. The culture filtrates were retained for enzyme assay, while some of the mycelial pellets were inoculated into wounds in apple fruitlets, as described below.

Enzyme production in vivo. Fruitlets of apple var. Laxton's Superb were harvested in early July, and infected by wounding with a sterile scalpel followed by the insertion of a mycelial inoculum of *Sclerotinia fructigena.* For a comparison with enzyme in-

duction *in vitro* on a time basis, wounds were made with a special instrument resembling a cork borer, the depth of its cut being limited to 3 mm. by a flange; the disc cut (5 mm. in diam.) was then removed with a needle. The inoculum comprised mycelial pellets of *S. fructigena*, grown in liquid medium as described, and washed in sterile water. Lesion development was measured at intervals along two predetermined diameters at right-angles to each other.

Host tissue, both infected and healthy, was extracted with sodium chloride (5 g./100 ml. in water or in McIlvaine buffer, pH 8 (w/v)) or with 0·5 M-sodium carbonate containing 0·1 g. sodium dithionite/100 ml. at the rate of 10 ml. extractant to 5 g. host tissue. When sodium carbonate solution was used, the extract was mixed immediately after filtration with twice its volume of McIlvaine buffer (pH 6) to decrease the pH value and minimise enzyme inactivation.

Resolution of enzyme components. Gel filtration was done on Sephadex G 75 dextran gel (bead form), in a column approximately 80 × 4·0 cm. Concentrated culture filtrate (25 ml.) was applied, followed by elution with water. Fractions (10 ml.) were collected and their enzyme activities assayed.

For ion-exchange chromatography, CM-Sephadex (C-50) was used. This was taken up in 0·05 M-acetate buffer (pH 5·8), and to a column approximately 25 × 3·0 cm. was added 10 ml. concentrated culture filtrate which had been dialysed for 6 hr against distilled water and then overnight against the buffer. Gradient elution, achieved by using a mixing chamber and constant pressure by means of a Mariotte flask, was done with increasing concentrations of NaCl (from zero to 0·1 M in the acetate buffer); 10 ml. fractions were collected and assayed as before.

Enzyme estimations

Polygalacturonase (PG) was estimated by the cup-plate assay of Dingle, Reid & Solomons (1953) using sodium polypectate as substrate. The diameter of the white ring obtained on the agar plate was proportional to the logarithm of enzyme concentration over a range of dilutions. Activities were expressed relative to an aqueous solution (1 mg./ml.) of the commercial enzyme 'Pectinol 10 M' (Rohm & Haas Ltd.), defined arbitrarily as having 100 units activity/ml. The white rings are thought to be due to the presence of oligo-uronides insoluble at pH 1, which are intermediate products of hydrolytic degradation (Dingle *et al.* 1953). Whilst it is possible that the unsaturated products of a *trans*-eliminative breakdown might react similarly, this mechanism of degradation of polypectate by *Sclerotinia fructigena* preparations was discounted by means of u.v. spectrophotometry (Table 3).

α-L-Arabinofuranosidase (AF). The method used (see below) was based on the liberation of *p*-nitrophenol from the chromogenic substrate *p*-nitrophenyl α-L-arabinofuranoside, prepared by the method of Fielding & Hough (1965).

β-D-Galactopyranosidase (GP) was estimated by a similar method with *o*-nitrophenyl β-D-galactopyranoside (Koch–Light) as substrate (1·0 mg./ml.). The test system comprised: enzyme solution (diluted when desired), 1·0 ml.; 0·1 M-acetate buffer (pH 4·7), 5·0 ml. After equilibration at 30°, substrate solution (0·5 ml., 0·5 mg./ml.) was added, and the mixture incubated at 30°. The reaction was stopped by adding saturated Na_2CO_3 solution (1·0 ml.), which also developed the yellow colour of the *p*-nitrophenate ion. This was read on a Spekker absorptiometer with an Ilford no. 601 violet filter, against an appropriate substrate blank. Necessary enzyme blanks were

also included. One unit was defined as that amount of enzyme which catalysed the hydrolysis of 1 μmole of substrate per min. at 30° (*Enzyme Nomenclature*, 1965).

The low substrate concentration (0·5 mg./ml.), which imposes limits on the AF assay, was used because it was in short supply.

Pectin methyl-trans-eliminase (PTE) was estimated by a method based on that of Albersheim & Killias (1962). A solution of pectin (2 g./100 ml.; Brown Ribbon brand, Union Crystalex Gelatine Ltd., London) was first centrifuged at 34,000 *g* to remove colloidal particles (Mr R. C. Codner, private communication) and one volume mixed with 3 volumes of McIlvaine buffer or 0·05 M-tris HCl buffer of appropriate pH value. To 4 ml. of this 0·5 % solution of pectin, equilibrated at 30°, was added 0·2 ml. of the test enzyme solution, and the extinction was read on a spectrophotometer immediately after mixing and again after an appropriate incubation time. Activity was expressed arbitrarily as the increase/100 min. in extinction at 240 mμ, the wavelength at which maximum absorption of the products of the enzyme reaction was found to occur (Fig. 1). Generally, a wavelength of 235 mμ has been used by other workers, but Cole (1967) also recorded maximum absorption at higher wavelengths under certain conditions, using the corresponding enzyme from *Penicillium digitatum*.

Macerating activity was estimated by following the loss of coherence at 25° of discs of potato tissue (Brown, 1915). Plugs 1 cm. diam. were cut from potato tubers, and immersed in water for 20 min. under reduced pressure. Discs 0·35 mm. thick were then cut by using a hand microtome, and sets of four discs transferred to 2 ml. test solution in watch-glasses. Samples of fractions to be tested were arranged in a random order. Maceration activity was expressed as $100/t$, where t was the time (min.) for the discs to lose coherence under the gentle pull of dissecting needles. One maceration test was done on apple tissue; discs were 0·40 mm. thick and the tissue did not receive the reduced pressure treatment.

Inactivation experiments. For temperature inactivation experiments, enzyme preparations were held at the appropriate temperature for 20 min. before being cooled rapidly by immersing the tubes in ice-water.

For pH inactivation, 1 ml. of a suitable gel-filtration fraction free from low molecular weight material was added to 4 ml. citrate phosphate borate buffer (of required pH; Teorell & Stenhagen, 1938) and the pH value of the mixture recorded. After 20 min. at room temperature 0·2 ml. was added to 2 ml. McIlvaine citrate phosphate buffer (pH 5·2) for pectin methyl-*trans*-eliminase assay following the addition of pectin (1·0 %, 2 ml.). For assay of macerating activity 1 ml. of the first-named mixture was added to 4 ml. McIlvaine buffer (pH 5·2) and 2 ml. of this mixture used for assay.

RESULTS

Concentrated culture filtrate (25 ml.) was partially resolved by gel filtration through Sephadex G 75, and fractions assayed for the enzymes PG, AF and PTE and maceration of potato discs (at pH 5·2 for PTE estimation). The elution pattern took the form of two peaks. Figure 2 shows the relationship between PTE and macerating activity for the fractions tested (every third one): the partial correlation coefficient for these data was +0·883 ($P < 0·001$). By contrast, the partial correlation coefficient between PG and macerating activity was +0·271 (not significant), and between AF and macerating activity −0·190 (not significant). A similar relationship was evident in

seven other gel-filtration experiments, though no statistical correlation was attempted. On no occasion were anomalous results obtained.

A dialysed concentrated culture filtrate (pH 6·5, 10 ml.) was applied to a column of CM-Sephadex which was then eluted, and fractions (10 ml.) tested for PG, AF, PTE and macerating activity on potato and apple discs (Fig. 3, 4). Figure 4 also shows that the two PTE components differed in their pH/activity relationships. However, even taking pH effects into account, the macerating activity of the second component

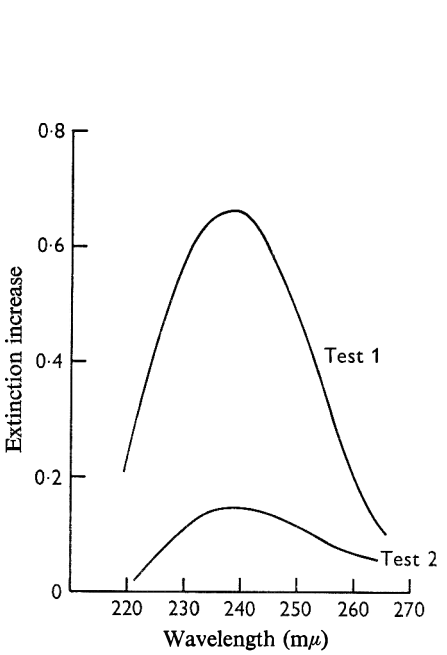

Fig. 1

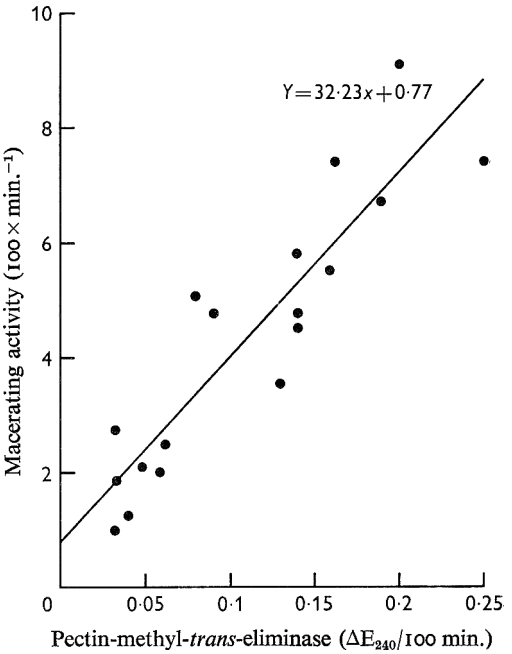

Fig. 2

Fig. 1. Ultraviolet absorption of products of pectin methyl-*trans*-eliminase action. Test 1: pH 8·0 in tris HCl buffer; pectin concentration 0·5 %; enzyme from dialysed culture filtrate concentrate; incubation time, 30 min. Test 2: pH 5·2 in half-strength McIlvaine buffer; pectin concentration 0·25 %; enzyme from gel filtration fractions; incubation time, 100 min.

Fig. 2. Relationship between macerating activity and pectin methyl-*trans*-eliminase (at pH 5·2) in fractions from gel filtration.

was greater than that expected on the basis of its PTE activity. This result could however be explained if the second component were activated in the maceration assay. In a subsequent experiment the effect of an aqueous extract of potato tuber (both dialysed and undialysed) and of Ca^{2+}, Mg^{2+} and K^{+} on the PTE activity of the second component was therefore examined. Activation by both potato preparations was observed, but the dialysate from the potato extract, and also the inorganic cations, had negligible effect. Table 1 shows that only the second PTE component was thus activated. Similar results were obtained in subsequent tests when an extract of cucumber fruit was substituted for potato extract. Table 1 shows that similar activation could also be effected by a boiled culture filtrate of *Sclerotinia fructigena* grown on the usual polypectate medium, or by an aqueous solution of sodium polypectate, itself subject to negligible PTE attack, or to a lesser degree, by polygalacturonic acid.

In a subsequent fractionation with CM-Sephadex, sodium polypectate was added to the assay mixture for PTE for each fraction tested, at pH 6·0 (about that of the potato tissue). The pattern of the results obtained (Fig. 5, 6) shows the much closer relationship between PTE and macerating activity than in the previous fractionation when sodium polypectate was not included in the assay. Figure 5 also shows the presence of a β-galactosidase at the second PTE peak.

The pH/activity relationships of the two PTE isoenzymes were examined in the presence of sodium polypectate, with the results shown in Fig. 7.

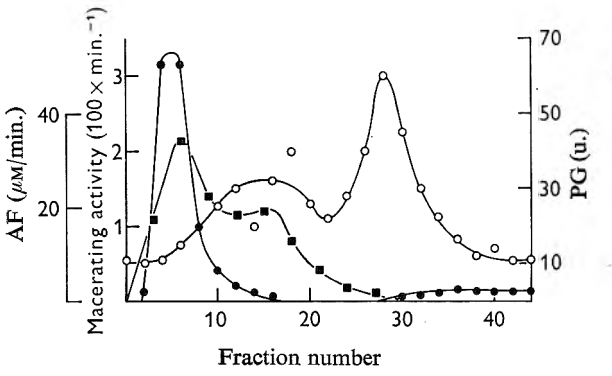

Fig. 3

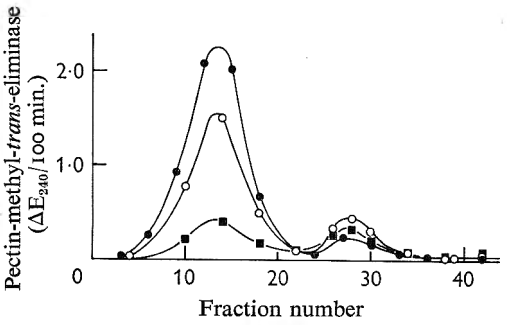

Fig. 4

Fig. 3. Polygalacturonase (●), α-L-arabinofuranosidase (■) and macerating (○) activities of fractions from gradient elution of CM-Sephadex. Maceration of apple discs (not shown) was maximal at fractions 14 and 28 (activity 0·75).

Fig. 4. Pectin methyl-*trans*-eliminase activities of CM-Sephadex fractions corresponding to Fig. 3 at pH 6·0 (■), 7·0 (○) and 8·0 (●).

Table 1. *Activation of pectin methyl-trans-eliminase components from Sclerotinia fructigena culture filtrate obtained by CM-Sephadex fractionation*

Test	Activator*	pH value of assay	ΔE_{240}/100 min. First PTE component	ΔE_{240}/100 min. Second PTE component
1	Potato extract	5·3	0·029	0·246
	Water	5·3	0·008	0·023
2	Boiled culture filtrate	5·3	—	0·113
		8·0	—	0·155
	Sodium polypectate (2 %)	5·3	—	0·093
		8·0	—	0·132
	Water	5·3	—	0·000
		8·0	—	0·060
3	Sodium polypectate (2 %)	8·0	—	0·133
	Polygalacturonic acid (2 %)	8·0	—	0·064
	Water	8·0	—	0·048

* 0·2 ml. added to 4·2 ml. of usual assay system. Appropriate activator/enzyme blanks were included and gave readings less than 0·02.

— signifies no test.

Effects of exposure to high temperature and extreme pH values

From plots of residual activity against temperature of exposure (20 min.) in three experiments the following mean values for 50 % decrease of activity were obtained: macerating activity, 49·4°; PTE, 48·0°.

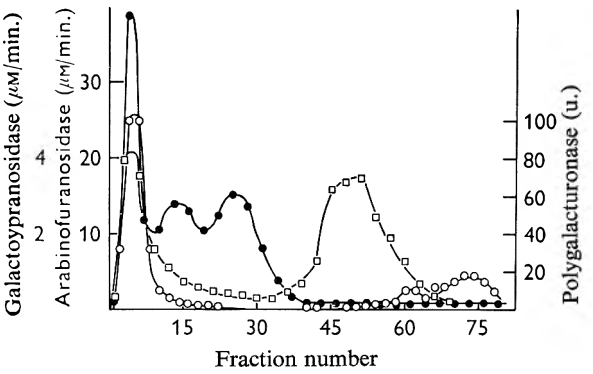

Fig. 5

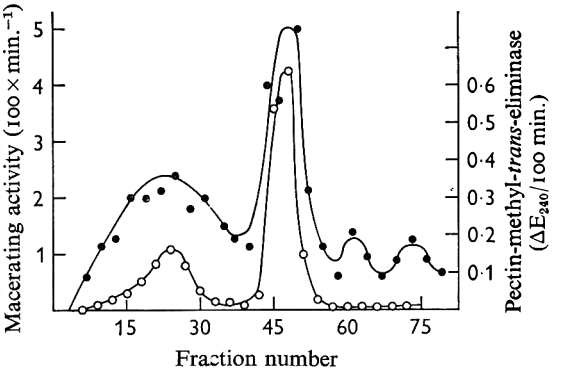

Fig. 6

Fig. 5. Polygalacturonase (○), α-L-arabinofuranosidase (●) and β-galactopyranosidase (□) activities of fractions from gradient elution of CM-Sephadex.

Fig. 6. Macerating activity (●) and pectin methyl-*trans*-eliminase activity in the presence of sodium polypectate at pH 6 (○) of fractions corresponding to Fig. 5.

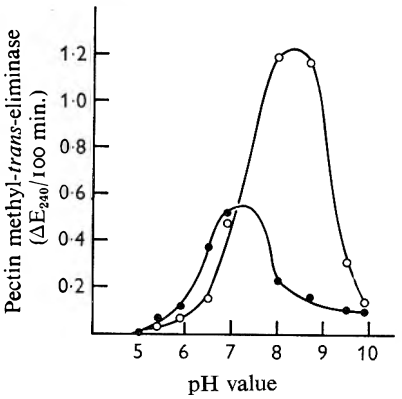

Fig. 7

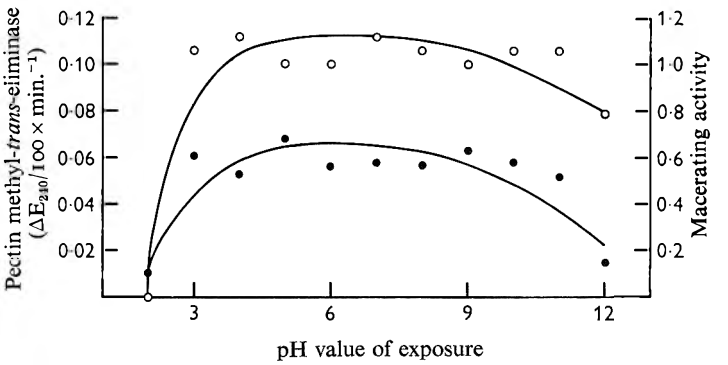

Fig. 8

Fig. 7. pH/activity relationship for pectin mcthyl-*trans*-eliminase isoenzymes in fractions 24 (○) and 48 (●).

Fig. 8. Residual pectin methyl-*trans*-eliminase (●) and macerating (○) activities following exposure (20 min.) to different pH values.

The effect of exposure (20 min.) to different pH values is shown in Fig. 8, which shows the comparatively high, and similar, tolerance of both activities towards extremes of pH.

Extraction of host tissue

Polygalacturonase was readily detected in all extracts of apple fruitlets freshly rotted by *Sclerotinia fructigena*, but not in healthy tissue (see Akinrefon, 1967). Repeated attempts to detect quantities of PTE above the value of experimental error were unsuccessful, at pH 4, 6 or 8. However, the extract of rotted tissue showed no inhibitory effect on PTE when mixed with a gel filtration fraction of high activity.

Table 2. *Polygalacturonase and pectin methyl-trans-eliminase in filtrates of young Sclerotinia fructigena cultures*

Test no.	Substrate	Final conc. in basal medium (%, w/v)	PG (u.). Incubation time 8 hr	PG (u.). Incubation time 24 hr	PTE*. Incubation time 8 hr	PTE*. Incubation time 24 hr
1	Pectin	1·0	15·8	—	0·04	—
	Apple fibre†	1·0	8·0	—	0·01	—
	Nil	—	4·0	—	0·04	—
2	Apple fibre†	0·5	6·3	25·1	0·00	0·05
	Nil	—	1·3	2·3	0·01	0·01
Comparable figures for routine 11-day cultures...			400		4·13 (pH 8)	

* Assayed at pH 5 in test 1; at pH 8 in test 2 (ΔE_{340}/100 min.).
† Prepared by the method of Cole & Wood (1961); not sterilized.
— signifies no test.

Induction of pectolytic enzymes in vitro

Table 2 summarizes the results of two experiments in which mycelial pellets of *Sclerotinia fructigena* were exposed to different substrates. After harvesting, the culture filtrates were assayed for enzyme activities. Similar pellets harvested 6 hr after the substrates were added were used as inoculum for standard wounds (5 mm. diam.) made in apple fruitlets. Rots developed rapidly, reaching mean diameters of 9 mm. and 13·5 mm. after 16 and 23 hr respectively. There were statistically significant differences in rot diameter following different induction treatments: mycelial pellets from apple juice gave the largest diameters. Control wounds, which received no inoculum, did not become infected.

Enzymes not involved in maceration

During experiments on the biochemical identity of the 'maceration factor', a number of hypotheses were tested and subsequently discarded: these are summarized in Table 3.

DISCUSSION

The results presented identify the 'maceration factor' in culture filtrates of *Sclerotinia fructigena* as a pectin methyl-*trans*-eliminase (PTE) enzyme. The gel filtration pattern showed a highly significant correlation between these two activities, but no correlation between rate of maceration and the activities of polygalacturonase and α-L-arabinofuranosidase. In earlier work, preparations with macerating activity but devoid of polygalacturonase were obtained (Byrde & Fielding, 1962); purified polygalacturonase preparations have subsequently been found to have negligible macerat-

Table 3. *Hypotheses for action of 'maceration factor' which were subsequently discarded*

Chemical group or linkage involved	Reference to role in plant structure	Substrate used in tests	Assay of incubation mixture (normally pH 5)	Result*
Cellulose	Wood (1960)	(*a*) Cellulose	Paper chromatography	A
		(*b*) Carboxymethyl-cellulose	Viscometric estimation	A
Protein	Ginzburg (1961)	(*a*) Gelatin-charcoal discs	Charcoal liberation (Kohn, 1953)	A
		(*b*) Potato fibre	Absorption at 280 mμ wavelength	A
Hydroxyproline-rich protein	Lamport (1965)	Hydroxyprolylglycine and glycylhydroxy-proline	Paper chromatography and hydroxyproline assay	A
Xylan	Sørensen (1957), Hancock & Millar (1965)	(*a*) Xylan	Paper chromatography	A
		(*b*) *o*-Nitrophenyl-β-D-xyloside (Fisher *et al.* 1966)†	Colorimetric estimation	A
Methoxyl groups in pectin	Joslyn (1962)	High-methoxyl pectin	Methanol estimation (pectin methylesterase assay)	B
Glycosidic linkages in polygalacturonate chain	Wood (1960)	Sodium polypectate	Absorption at 230 mμ wavelength (polygalacturonate *trans*-eliminase assay)	A
Aldobiuronic acid units in polygalacturonate chain	Barrett & Northcote (1965)	Pectinic acid	Column chromatography of partial acid hydrolysate (Barrett & Northcote, 1965, fig. 7)	A
Phosphate ester linkages between pectin chains	Henglein (1958)	(*a*) Potato fibre or pectin	Inorganic phosphate estimation	A
		(*b*) Bis (*p*-nitrophenyl) phosphoric acid, Na salt	Colorimetric estimation	A
Calcium and other di-valent ions as 'bridges' between pectin chains	Joslyn (1962), Bateman & Millar (1966)	(*a*) Potato fibre	Calcium estimation	A
		(*b*) Potato discs	Effect of 0·1 M-Ca $(NO_3)_2$ and 0·01 M-EDTA on rate of maceration	A

* A, no activity in crude culture filtrate; B, culture filtrate active but no activity in purified 'maceration factor preparations.

† Kindly supplied by Dr P. W. Kent.

ing activity (Byrde & Fielding, unpublished). A similar close relationship was apparent in the elution pattern from a CM-Sephadex ion-exchange dextran column, but only when PTE was assayed at pH 6 in the presence of sodium polypectate (in addition to the pectin substrate). The results showed the unexpected existence of two PTE isoenzymes: that which eluted first had an activity optimum at pH 8·3, and was not activated by potato extract. The second isoenzyme had an activity optimum at pH 7·3 and was strongly activated by potato extract or sodium polypectate. Account should thus be taken of such activation effects when assessing the role of purified enzyme preparations in tissue maceration: it is likely that the activator known to be present in the crude culture filtrate became separated from the enzyme during the CM-Sephadex fractionation.

The pH optima recorded for these isoenzymes are considerably higher than those

quoted for other pectin methyl-*trans*-eliminase enzymes (as distinct from polygalacturonate *trans*-eliminases), which are generally about pH 5 (see e.g. Albersheim & Killias, 1962), although a second peak at pH 8·5 in the presence of Ca^{2+} was reported by Edstrom & Phaff (1964). Further biochemical investigation of the enzyme, such as the mechanism of its activation and whether it attacks by a random or terminal mechanism, is clearly required.

The effects of pH value on the stability of the maceration factor and of PTE also showed good agreement, and the slight discrepancy between the temperature inactivation data may be due to departures from linearity in the use of an inverse time function as a measure of maceration activity (see McClendon & Somers, 1960; Cole, 1967).

The results summarized in Table 3 show that a number of other enzymes, which include polygalacturonate *trans*-eliminase, could not be identified with tissue maceration by *Sclerotinia fructigena*. The absence of cellulase activity is probably a factor in the long survival of mummified fruits following infection: in any event, cellulases do not generally appear to play a part in maceration (Bateman & Millar, 1966).

The pH optima for both the PTE components are considerably higher than those normally encountered by *Sclerotinia fructigena* in host tissue. The pattern of maceration of potato shown in Fig. 6 appears to be the expression of PTE activity at the pH value of the tissue (6·5) and the same is probably true of apple fruitlet tissue, which has a lower pH value (3·5) and macerated more slowly than did potato tissue. This raises the question of the significance of the PTE enzyme in the brown rot disease of apples, where the host may be lower than pH 3·0. In addition, two other results cast doubt on the role of the enzyme. First, negligible amounts of PTE activity were recovered from infected tissue, and this lack of enzyme activity did not appear to be due solely to inactivation. Similarly, Cole & Wood (1961) detected no macerating activity in extracts of apple tissue rotted by *S. fructigena*. However, appreciable quantities of polygalacturonase can be extracted, particularly from the oldest part of the rot (Akinrefon, 1967). Secondly, negligible amounts of PTE were secreted by mycelial pellets of *S. fructigena* during the first 24 hr of incubation *in vitro* under conditions in which polygalacturonase was rapidly formed. These pellets were, however, capable of rapid invasion of host tissue within 16 hr. In the invasion of apple tissue the possibility of the local secretion of a small, but temporarily effective, quantity of enzyme, which is subsequently inactivated cannot be finally excluded (see Cole & Wood, 1961). However, the weight of evidence indicates that the genetic ability of *S. fructigena* to secrete pectin methyl-*trans*-eliminase is not utilized, and the relatively firm nature of the resultant rot is in accordance with this view.

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The Fine Structure of *Bacillus fastidiosus*

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SUMMARY

Bacillus fastidiosus, an aerobic spore-forming bacterium able to utilize uric acid or allantoin as sole source of carbon and nitrogen, has been re-isolated and its fine structure described. Anatomical features of vegetative cells are similar to those of other members of the genus *Bacillus*. The mature endospore has typical core and cortical regions. The spore coat is highly laminated and surrounded by a tight-fitting exosporium to which conspicuous exosporial hairs are attached.

INTRODUCTION

Den Dooren de Jong (1929) isolated and described an aerobic spore-forming bacterium able to utilize an unusually limited range of substrates for growth. Reflected in its name, *Bacillus fastidiosus*, is its inability to utilize common sugars, amino acids, peptones, etc., as growth substrates; of the wide variety of organic compounds tested, only uric acid and allantoin were so utilized. Also recognized were the hair-like or rhizoidal extensions of colonies which, although not so pronounced as those of *B. mycoides*, were quite distinctive.

Although *Bacillus fastidiosus* was a well-described and recognized Bacillus in the 5th edition of *Bergey's Manual of Determinative Bacteriology*, more recent editions either relegate this organism to an uncertain status (6th edition) or do not even recognize its existence (7th edition). This change undoubtedly reflects, albeit incorrectly, the results of Smith, Gordon & Clark's (1952) comparative study of Bacillus species in which *B. fastidiosus* was not listed as an organism extensively studied and classified. These investigators clearly recognized that the culture received by them under the name *B. fastidiosus* 'bore no resemblance to the original organism' as described by den Dooren de Jong.

This paper reports the re-isolation of *Bacillus fastidiosus* and an examination of its fine structure.

METHODS

Isolation. The organism studied was isolated from Florida Everglades soil by enrichment culture in a medium containing 1·0% (w/v) uric acid and 0·1% (w/v) K_2HPO_4 in distilled water. Approximately 0·2 g. soil was added to 50 ml. of this medium in a 250 ml. Erlenmeyer flask; incubation was at 30° on a reciprocating shaker. After 48 hr incubation, a small sample of the enrichment culture was pasteurized at 80° for 15 min. and then plated on to the same medium solidified with 2% (w/v) Bacto-agar. A small portion of a well-isolated colony which appeared after 4 days

incubation was emulsified, pasteurized, and replated to obtain a pure culture. Additional strains were isolated from a variety of other soil samples.

Growth. In addition to growth on solid medium, the organism was also grown in liquid, using biphasic conditions. One hundred ml. of 2% (w/v) Bacto-agar in distilled water was allowed to solidify on the bottom of a 1 l. Erlenmeyer flask. One hundred ml. uric acid–agar medium was then added aseptically. This was allowed to solidify and then 200 ml. uric acid (0·1%, w/v)–K_2HPO_4 (0·1%, w/v) broth was aseptically added. All cultures were incubated at 30°; biphasic cultures were aerated by shaking on a reciprocating shaker.

Phase-contrast microscopy. Both plate-grown and biphasic cultures were used for phase-contrast microscopy; cells grown on solid medium were scraped from the surface and dispersed in distilled water; cells from liquid biphasic cultures were examined directly. Samples for light photomicroscopy were layered on agar-coated glass slides, covered with coverslips and photographed with a Zeiss GFL phase-contrast photomicroscope, using Adox KB-14 photographic film.

Electron microscopy. Samples for electron microscopy were fixed in osmium tetroxide (1%, w/v), embedded in Epon 812 (Holt & Leadbetter, 1967), and thin sections stained with lead hydroxide (Karnovsky, 1961).

For phosphotungstic acid (PTA) negative staining, 1% or 3% PTA (adjusted to pH 6·85 with NaOH pellets) was used. Samples were either diluted with the stain until faintly turbid and then placed on 300-mesh carbon-coated grids, or stained on the grids.

Shadow casting was performed in a Denton DV-502 vacuum evaporator. The sample to be shadowed was placed on 300-mesh carbon-coated grids, dried for 60 min., and shadowed with platinum-carbon at an angle of 30° at a distance of 8 cm. Carbon replicas were prepared by the procedure of Holt & Canale-Parola (1967). All samples for electron microscopy were examined in a Phillips EM-200 electron microscope operating at 60 kV., equipped with either a 10 or 30 μ objective aperture. Contamination of the specimen was avoided by employing a liquid nitrogen cold finger at the level of the specimen.

RESULTS

Organism. The organisms which grew on uric acid + agar medium appeared identical to those observed by den Dooren de Jong (1929), as judged by their distinctive colonial characteristics, the size and shape of spores and vegetative cells, motility, dissolution of uric acid, and inability to grow on a variety of other carbon or carbon and nitrogen sources, including complex media such as yeast extract and nutrient agar. The one apparent difference between den Dooren de Jong's isolate and ours was in the Gram reaction; young, motile cells of our isolate were Gram-positive. Den Dooren de Jong's report of Gram-negative cells may reflect the physiological age of the cells examined.

Cultures growing on uric acid liberated ammonia, with a concomitant rise in pH to between 8 and 9. Attempts to obtain growth at pH 8·0 and 8·5 on phosphate-buffered complex media containing NH_4Cl, but lacking uric acid, were unsuccessful.

Phase-contrast microscopy. The vegetative bacilli of *Bacillus fastidiosus* are long rods, about 5 $\mu \times$ 1·5 μ (Pl. 1, fig. 1). The cytoplasm is uniform in appearance, indicating the absence of a multinucleate condition or visible storage material. The spores

of *B. fastidiosus* are highly refractile structures (Pl. 1, fig. 2), $3\,\mu \times 1{\cdot}5\,\mu$, and are predominantly ellipsoidal, although a few tend toward a nearly spherical shape (Pl. 1, figs. 2, 3). The prespores are located in an essentially terminal position within the mother cell.

Flagella. Growth in liquid culture was dispersed, and the chains of cells that predominate on agar medium were rarely found. Motility was readily observed only in very young cultures; when biphasic cultures reached densities greater than 10^8 cells/ml., or when organisms were grown on solid medium for more than 24 hr, very few bacilli appeared motile. Occasionally, when young vegetative bacilli were examined by light microscopy, a helical structure approx. $0{\cdot}4\,\mu$ diam. could be detected on one side of non-motile vegetative organisms or could be seen lying free in the microscopic field. Electron microscopic examination of such cultures after negative staining revealed these helical structures to be bunches or aggregates of flagella (Pl. 1, fig. 4; Pl. 2, fig. 5).

Flagella were arranged peritrichously on vegetative bacilli and each flagellum was *c.* $0{\cdot}02\,\mu$ in diam. As seen in Pl. 2, fig. 5, the flagella terminated in a typical 'hook' (Abram, Koffler & Vatter, 1965; Abram, Vatter & Koffler, 1966).

The cell wall had a mesh-like or woven fine structure similar to that reported for other bacteria (Salton, 1964) (Pl. 2, figs. 6, 7).

Cell surface. As judged by shadowed preparations, the vegetative bacillus surface was smooth and the organism rather flat (Pl. 3, fig. 9), while the spore surface (Pl. 4, fig. 10) was quite irregular and threw a very long shadow, thus indicating that it had considerable height. The cell wall fine structure seen in negatively stained preparations was not visible.

Treatment with chromic acid after shadowing and carbon deposition (replication) caused the vegetative bacilli and spores to lose their opacity, owing to the dissolution and extrusion of cellular contents. After this treatment, it became possible to observe on replicas taken from vegetative bacilli (Pl. 4, fig. 11) a textured surface similar to that observed in both sectioned and negatively stained cells (Pl. 2, fig. 5–7; Pl. 3, fig. 8). Again, replicas revealed the contrast between the regular surface of the vegetative bacillus and the multiple ridges and irregularities of the spore surface (Pl. 4, fig. 12). The fine structure observed on the vegetative bacillus surface was not seen in the spore integument. The spore surface, including terminal knobs, appeared similar to that reported by Fitz-James & Young (1959) and Lechtman, Bartholomew, Phillips & Russo (1965).

Electron microscopy of thin sections

Vegetative organisms

The vegetative bacilli of *Bacillus fastidiosus* were encased in what appeared to be a typical Gram-positive cell wall (Pl. 5, fig. 13–16), approx. 500 Å in width. The border of the cell has a regular shape. Although they are both clearly defined in Pl. 5, fig. 13, and Pl. 6, fig. 17, it was often difficult to observe a demarcation between the cell wall and the plasma membrane, probably because of adhesion of the plasma membrane to the innermost layer of the wall. This adhesion of cell wall and plasma membrane was suggested by Mitchell & Moyle (1956) to be characteristic of Gram-positive bacteria. The cell wall appeared slightly more dense than the enclosed cytoplasm. This difference in density may reflect a difference in chemical composition, may depend on the capability of the fixative (osmic acid) to penetrate into the cytoplasm through the cell

wall (where it may accumulate) or may depend on the quantity of ribonucleoprotein in the cytoplasm (Edwards & Stevens, 1963). Adsorption, on the wall, of material of foreign origin sometimes gave the wall an irregular shape (Pl. 5, fig. 13) (Glauert, 1962).

The multi-layered wall (Pl. 5, fig. 13; Pl. 6, fig. 17) had dense outer and inner layers, about 120 and 250 Å in thickness, and a middle layer, of lower density, about 180 Å thick. This middle component varied in width and was not always clearly observed. In Pl. 5, fig. 14, the cell wall was separated from the plasma membrane by a 120 Å space of low density, and possible cross-bridging (Edwards & Stevens, 1963; Ghosh & Murray, 1967) between the cell wall and the outer portion of the plasma membrane can be observed.

Plasma membrane

Where the plasma membrane was clearly defined, it showed typical unit membrane structure (Robertson, 1959), with a total thickness of 60–75 Å (Pl. 5, fig. 14, 15). In some portions of the membrane, the outer and inner layers appeared to have the same thickness but this was not evident in many sections because of the similar density of the inner layer and adjacent cytoplasm.

Intracytoplasmic membranes

A system of membranes, variable in size and shape, are located in the cytoplasm of *Bacillus fastidiosus*. These mesosomes (Fitz-James, 1960) or plasmalemmosomes (Edwards & Stevens, 1963) appeared (Pl. 5, figs. 14, 16) to be developed by intrusions of the plasma membrane, as has already been established by many workers, although their attachment to the plasma membrane was not always seen in the plane of sectioning. They may be simple invaginations or complex organelles of various appearances (Pl. 5, fig. 14–16).

Cytoplasm

The cytoplasm of *Bacillus fastidiosus* vegetative cells is packed with small granules, approx. 120 Å in diam. and are here referred to as ribosomes. The cytoplasm appeared free of other inclusions, usually conspicuous in Bacillus, such as metaphosphate, lipid or glycogen bodies.

Nuclear apparatus

The nuclear apparatus of vegetative bacilli of *Bacillus fastidiosus* shows the same general features as that found in other bacteria. The nuclear region, which had a lower density than the surrounding cytoplasm and contained fine fibrils, was found either dispersed (Pl. 5, fig. 14) or localized in one central region of the bacillus (Pl. 5, fig. 15).

Spores

The ultrastructural changes accompanying spore formation in *Bacillus fastidiosus* appeared rather similar to those observed in other Bacillus species (Fitz-James, 1960; Ellar & Lundgren, 1966).

The spore is formed directly within the vegetative mother cell and at a specific position. In Pl. 5, fig. 16, and Pl. 7, fig. 19, prespores can be seen at the cell pole. The double forespore membranes, each 50 Å thick, are separated by a 15 Å single mem-

brane component which, however, does not appear to have typical 'unit structure'. The forespore membranes appeared structurally similar to, and continuous with, the plasma membrane of the vegetative bacillus. Elements of plasmalemmosome structure still appeared attached to the plasma membrane (Pl. 5, fig. 16). The nuclear material appeared diffuse and fibrous in this prespore condition.

In later stages in spore formation (Pl. 6, fig. 17, 18), an inner membrane surrounds the diffuse spore core, while the developing electron transparent cortex is bordered by an outer membrane. A highly membranous spore coat developing outside the outer membrane and the beginning of an exosporium, partially surrounding the prespore, was also observed. In a transverse section of a still later stage (Pl. 7, fig. 20), an accentuated cortex, and what appears to be a completed spore coat and exosporium, could be seen. The diffuse, densely staining areas surrounding the exosporium may be developing exosporial hairs (Gerhardt & Ribi, 1964) and may connect with the electron dense vegetative wall (sporangium).

After spore liberation, the highly complex nature of this sporangial wall became apparent, possibly because of the loss of intracellular materials (Pl. 8, fig. 21, 22). This structure, 650 Å thick, may be the vegetative wall with the plasma membrane attached by transverse bridges (see Discussion).

The free, mature spore had a diffuse spore core and accentuated cortex (Pl. 9, fig. 23), a highly developed spore coat completely surrounding the cortical region, and a well-developed exosporium with an outer covering of exosporial hairs.

The high magnification micrograph (Pl. 9, fig. 24) shows a portion of the complex spore coat. Some layers of the membranous outer spore coat completely enclose the cortical region, while exterior layers form rather regular projections at intervals of 0·62 μ. At each of these apices or ridges, the separation of the membranes is apparent where they enclose an electron dense material which appears tubular in cross section. The ridge between the outer spore coat and exosporium is filled with a heavily staining ground substance. The multi-layered exosporium is cross-bridged (Pl. 6, fig. 17: Pl. 9, fig. 24) and its fine structure appears similar to that of the vegetative cell wall (Pl. 6, fig. 17; Pl. 8, fig. 21), observations which are consistent with the fine structural similarities of the exosporium (Gerhardt & Ribi, 1964) and the cell wall (Pl. 2, fig. 6) as seen in negatively stained preparations.

DISCUSSION

The ultrastructural components of *Bacillus fastidiosus* spores are not markedly different from those found in various other bacterial endospores. Rarely, however, has examination of spore fine structure revealed, in a given bacterial species, the variety and arrangement of ultrastructural features observed here.

The outer exosporial surface seen (Young & Fitz-James, 1962) in *Bacillus cereus* var. *alesti* has been described as a nap or hair-like projection in *B. cereus* strain TERMINALIS (Gerhardt, & Ribi, 1964), in *B. anthracis* (Hachisuka, Kojima & Sato, 1966), and probably exists in *B. subtilis* (Giesbrecht, 1964). This feature of the exosporium is clearly visible in free endospores of *B. fastidiosus*, although there are only faint indications of its presence on spores not yet released from the sporangium. Underneath this nap the exosporium has a lamellar structure similar to that reported in the exosporial 'basal membrane' in *B. cereus* strain TERMINALIS (Gerhardt & Ribi, 1964). In

contrast, another strain of *B. cereus* (Ellar & Lundgren, 1966) has only a single dense layer visible in the exosporium of spores still within the sporangium.

The apices or projections formed by the laminated outer spore coat are similar to those in *Bacillus megaterium* 350 (Robinow, 1960). The distinct spines or ridges in *B. polymyxa* (Holbert, 1960) and *Bacillus* sp. strain 636 (Warth, Ohye & Murrell, 1963), however, did not seem to be formed by such an outer coat. Both the inner and outer spore coats of *B. fastidiosus* appear to be more highly laminated structures than did those of *B. megaterium* 350 (Robinow, 1960) and *B. cereus* (Ellar & Lundgren, 1966) and considerably different from those of *B. coagulans* and strain 636 where, although the inner spore coat was highly laminate, the outer coat was uniformly electron-opaque (Warth *et al.* 1963).

The position and striking structural similarity of the spore coats of *Bacillus fastidiosus* and the so-called parasporal inclusion bodies reported in *B. cereus* strain TERMINALIS and *B. anthracis* (Gerhardt & Ribi, 1964; Hachisuka, Kojima & Sato, 1966) suggests to us that these 'bodies' are not discontinuous entities but may represent, in fact, portions of otherwise unpreserved or disrupted coat components. The term parasporal bodies refers to inclusions formed 'alongside the spore' (Hannay, 1956) and not within it. Obviously, then, a different term for the intra-spore structures should be used if our interpretation of their nature proves incorrect.

Robinow's (1960) observation that 'spores of different species of bacteria...differ in the degree of elaboration of their *outer* envelopes' is again confirmed by this study, for the areas inside the spore coats of *Bacillus fastidiosus* appear indistinguishable from their counterparts in other species. It is difficult to ascertain critically the extent to which the surface structures of *B. fastidiosus* endospores are identical to or different from those of other aerobic species, for an extensive comparative study of spore ultrastructure is not available. Although it is, then, somewhat hazardous to attempt to relate *B. fastidiosus* to one or another group of aerobic spore-formers on morphological considerations, we believe that the presence of a rather tight-fitting exosporium and reasonably prominent apices or spines indicates a closer relationship to *B. megaterium* than to, for example, *B. cereus*.

Apart from considerations of spore ultrastructure, one additional feature of especial interest relates to the ultrastructure of the Gram-positive cell wall and plasma membrane as seen in *Bacillus fastidiosus*. We believe that the structure in Pl. 8, fig. 21 and 22, is the vegetative cell wall and attached plasma membrane from which some chemical components may have been removed as a result of lysis accompanying spore liberation and by extraction during the fixing and dehydration preparations for electron microscopy. The dimensions and the position of the components of the 'emptied' structure agree well with those of the wall-membrane complex of the vegetative cell (Pl. 5, fig. 13–16). The bridging seen between the plasma membrane and the cell wall of *B. fastidiosus* is not localized in one specific region of the cell. It thus differs from the localized membrane-to-membrane bridging in the polar regions of *Spirillum serpens* (Murray & Birch-Anderson, 1963) but may be similar to the wall-membrane bridging reported in *Listeria monocytogenes* (Edwards & Stevens, 1963; Ghosh & Murray, 1967).

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EXPLANATION OF PLATES

(Unless otherwise indicated, the bar in each photograph represents 0·25 μ.)

PLATE 1

Fig. 1. *Bacillus fastidiosus*. Vegetative cell. Phase contrast.

Fig. 2. *B. fastidiosus*. Mature endospores. Phase contrast.

Fig. 3. *B. fastidiosus*. Chains of vegetative cells with mature endospores. Note cross-walls (arrows). Phase contrast.

ELECTRON MICROGRAPHS

Fig. 4. *Bacillus fastidiosus*. Vegetative cell. PTA-negatively stained preparation.

PLATE 2

Fig. 5. *Bacillus fastidiosus*. Vegetative cell, negatively stained (PTA). Note flagellar 'hook' (arrow) and fine structure of cell wall (CW).

Fig. 6. *B. fastidiosus*. Vegetative cell envelope after partial autolysis. PTA-negatively stained preparation.

Fig. 7. *B. fastidiosus*. Spore emerging from sporangium. Note (arrows) adhering vegetative cell envelope with periodic, apparently linear, fine structure. PTA-negatively stained preparation.

PLATE 3

Fig. 8. *Bacillus fastidiosus*. Vegetative cell. Tangential thin section through outer cell surface.

Fig. 9. *B. fastidiosus*. Vegetative cell. Platinum–carbon shadowed preparation, 20° angle.

PLATE 4

Fig. 10. *Bacillus fastidiosus*. Free spore. Platinum–carbon shadowed preparation, 20° angle.

Fig. 11. *B. fastidiosus*. Vegetative cell. Surface replica. Note fine structure (dotted lines).

Fig. 12. *B. fastidiosus*. Free spore. Surface replica. Polar knobs (single arrows) and possibly an equatorial ridge (double arrows) are seen.

PLATE 5

Fig. 13. *Bacillus fastidiosus*. Vegetative cell. Thin section. N = nuclear material; R = ribosomes; PM = plasma membrane. Note layering in cell wall (SW).

Fig. 14. *B. fastidiosus*. Vegetative cells. Thin section. N = nuclear material; M = mesosome; CW = cell wall. Arrows indicate areas where possible cross-bridging is visible. The inset shows a connexion (arrow) between a mesosome (M) and plasma membrane.

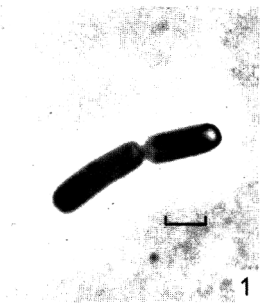
Fig. 15. *B. fastidiosus*. Vegetative cell. Thin section.

Fig. 16. *B. fastidiosus*. Longitudinal thin section of vegetative cell with a developing endospore. M = mesosome; N = nuclear material; CW = cell wall; Sp.m. = spore membranes; IM = inner forespore membrane; OM = outer forespore membrane; IL = intermediate layer; Ex. = exosporium; PM = plasma membrane. Arrows indicate continuity between plasma membrane and mesosomes.

PLATE 6

Fig. 17. *Bacillus fastidiosus*. Developing spore. Longitudinal thin-section. Inner (IM) and outer spore membranes (SM) may be present. C2 = cortex; PM = plasma membrane; VCW = vegetative cell wall; SC = spore coats; IM = inner spore membrane; OM = outer spore membrane. Double arrows indicate developing exosporium.

Fig. 18. *B. fastidiosus*. Developing endospore. Transverse thin section. SB = spore body or core; N = nuclear region; IM = inner membrane; C1 = inner cortex; C2 = outer, less dense cortex; OM = outer membrane; SC = developing spore coats; Ex. = developing exosporium; Sp.G. = vegetative cell wall or sporangium.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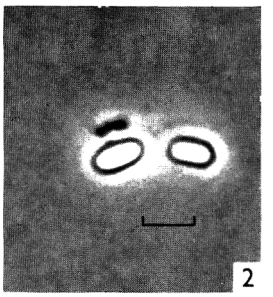
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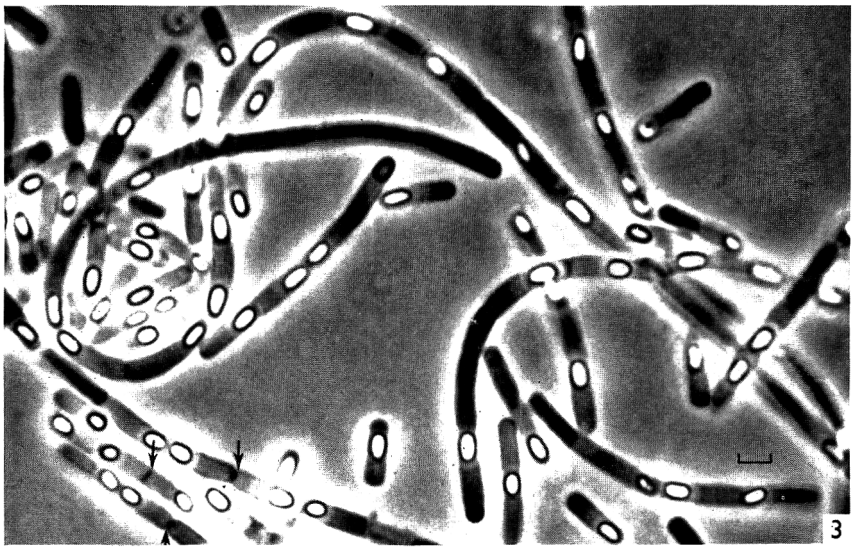
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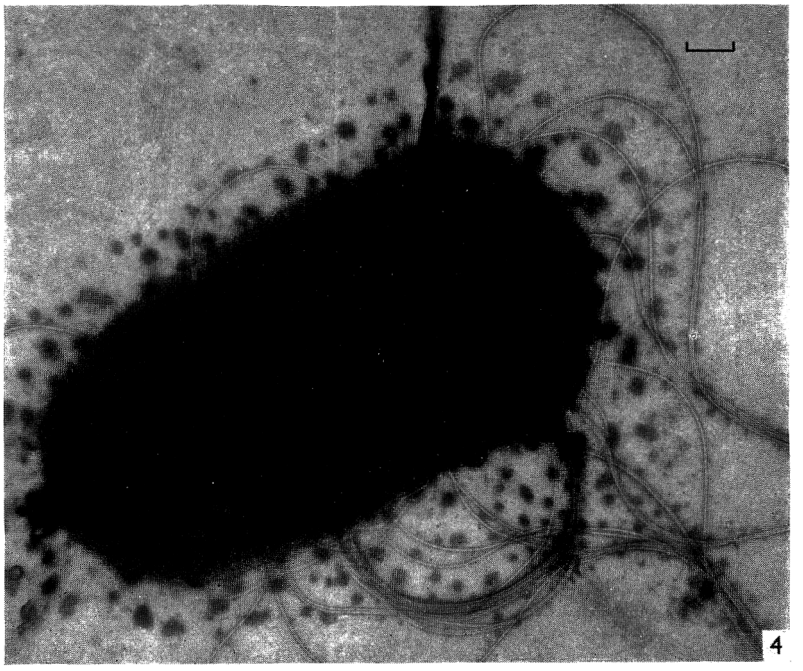
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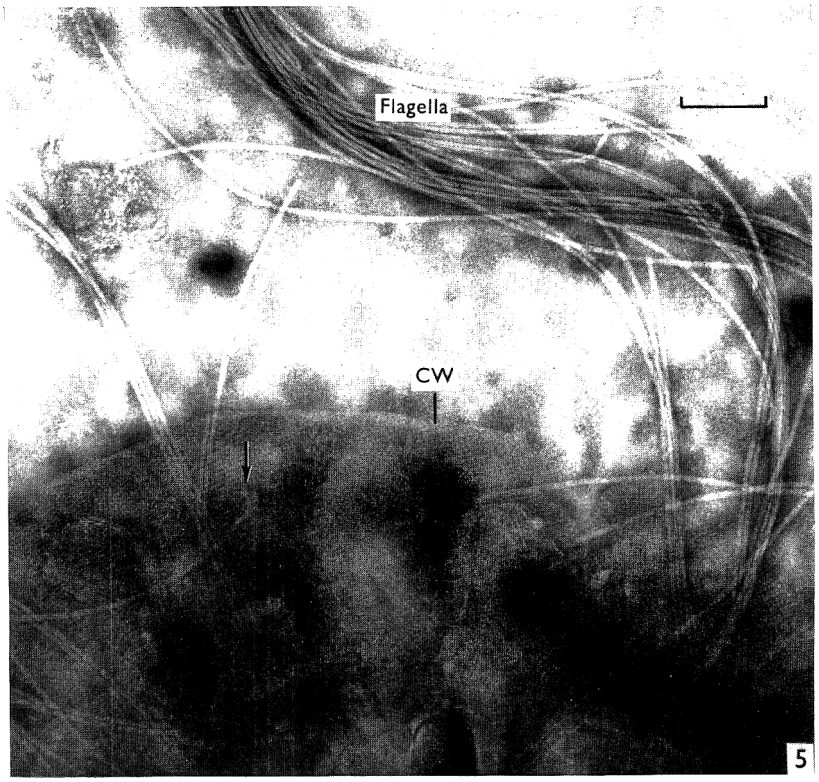
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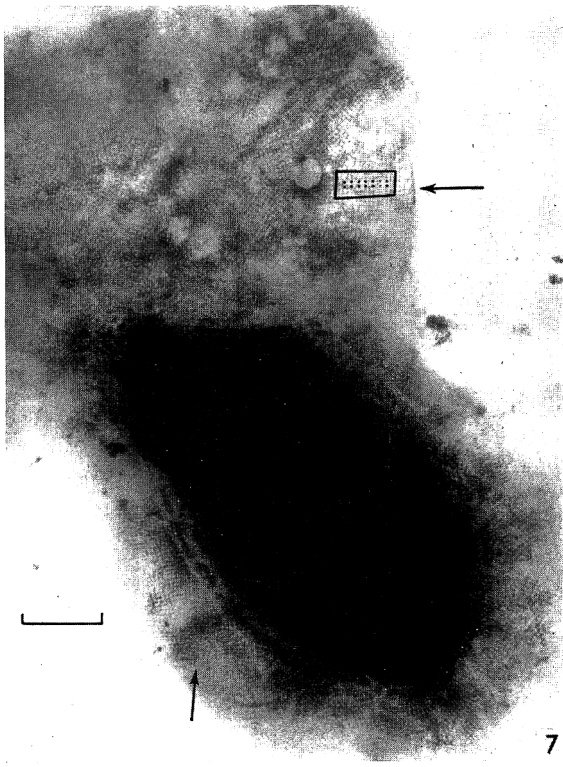
CW
5


6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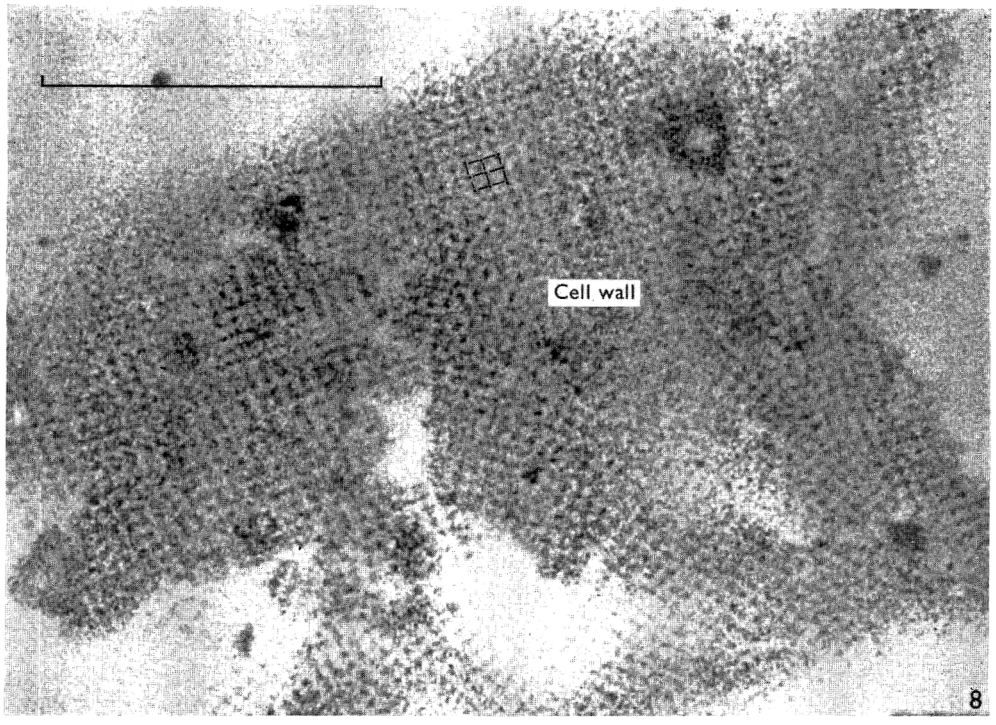
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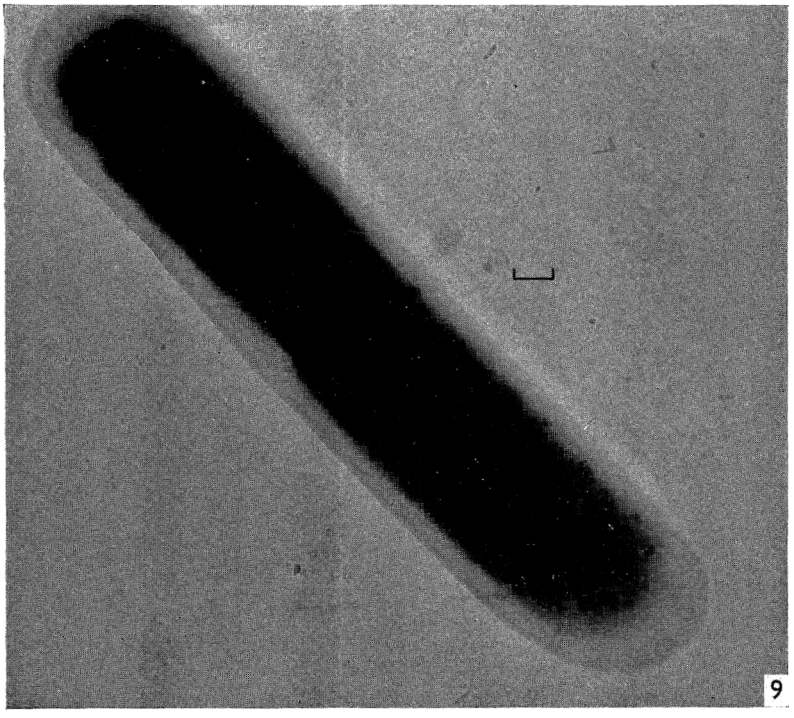
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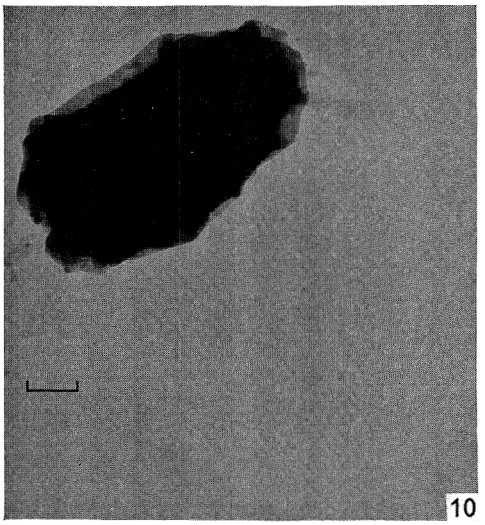

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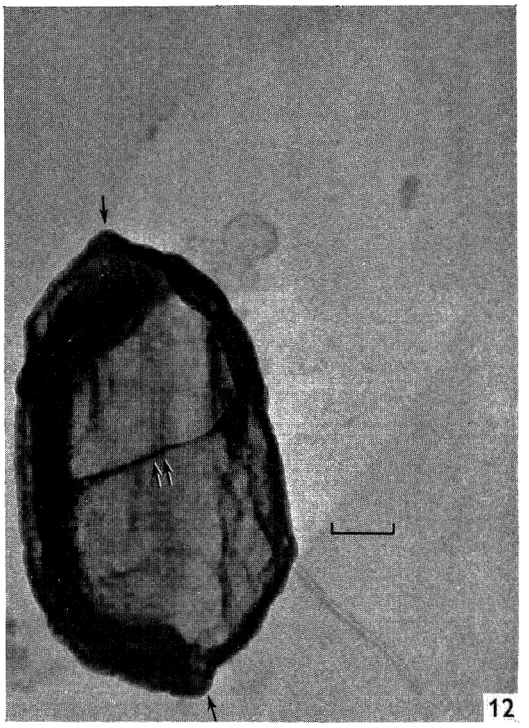

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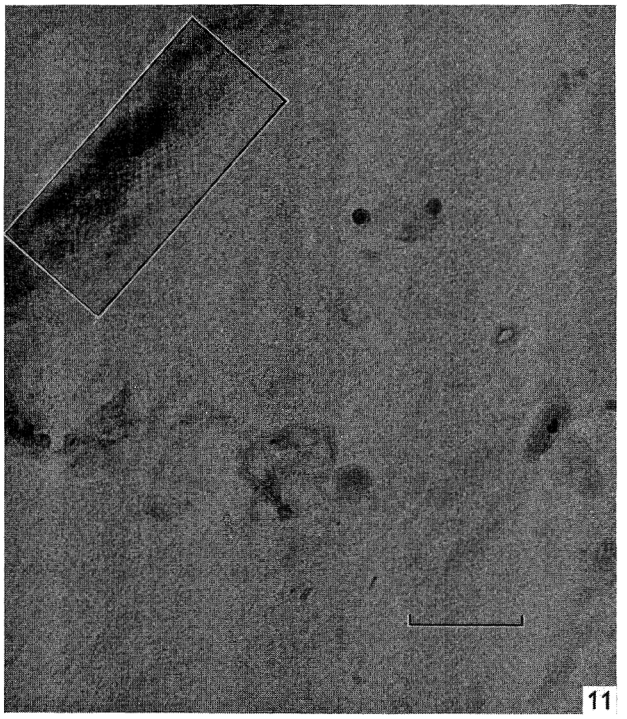

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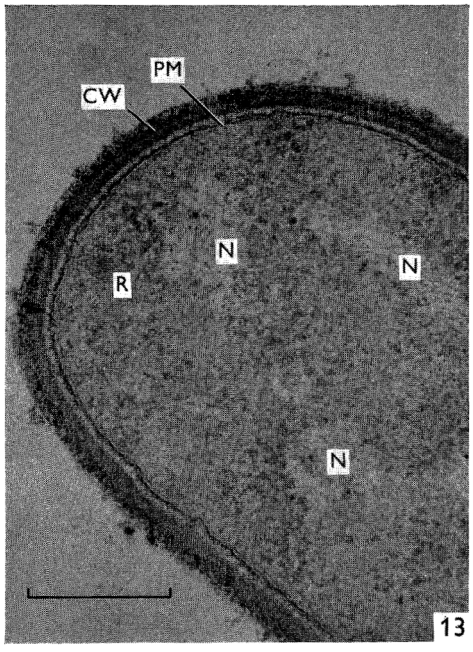
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R
N
N
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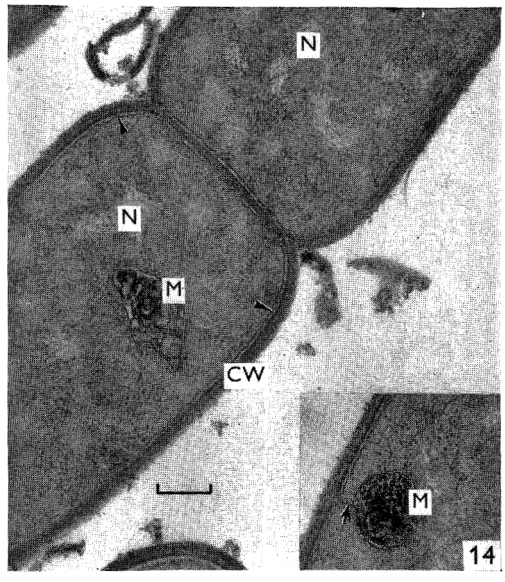
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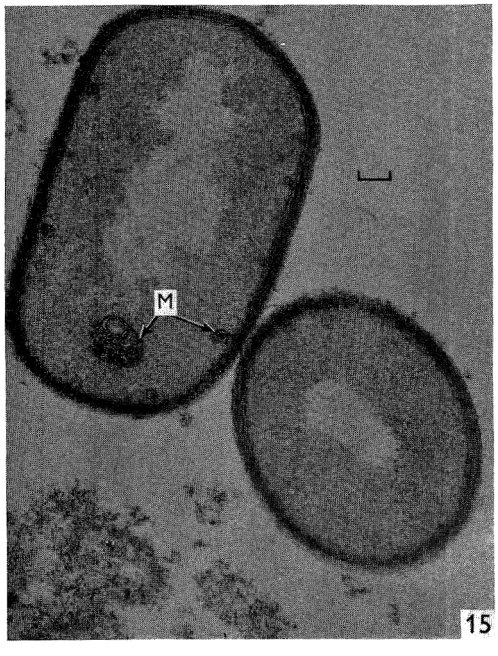
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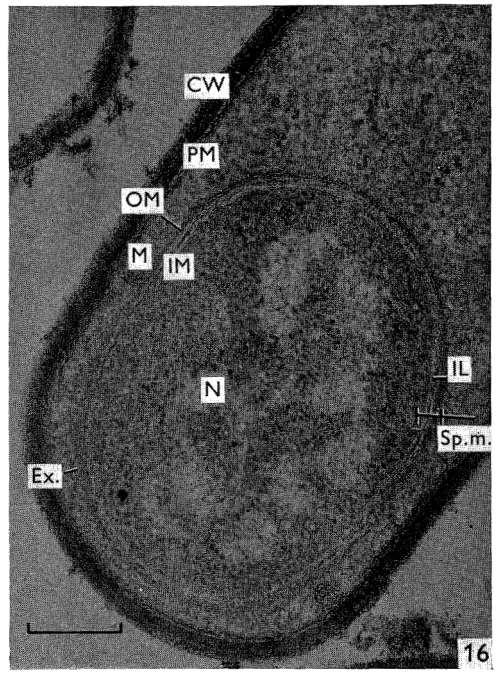
CW
PM
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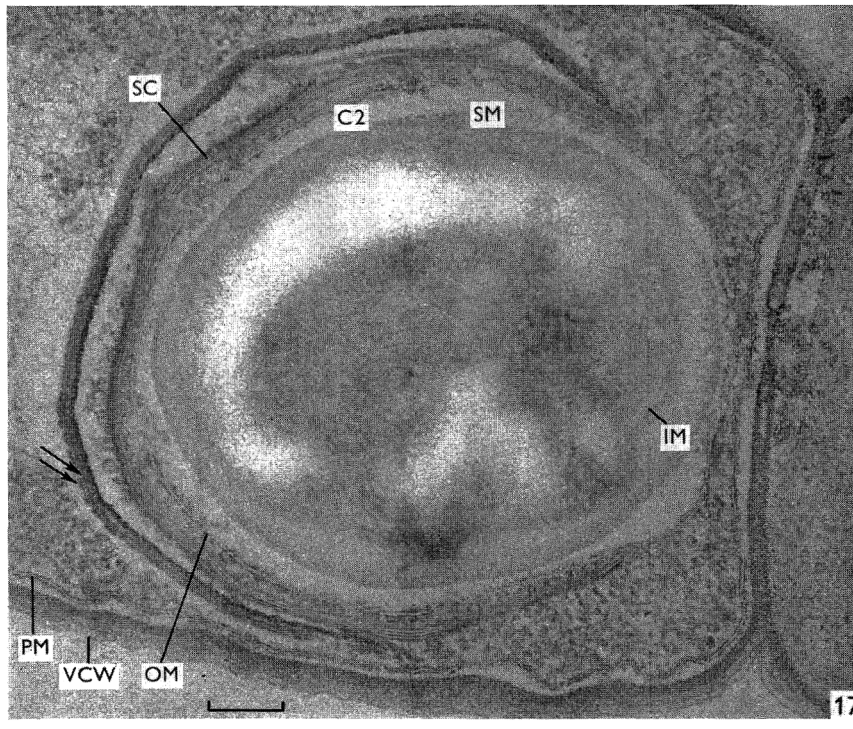
Sp.m.
Ex.
16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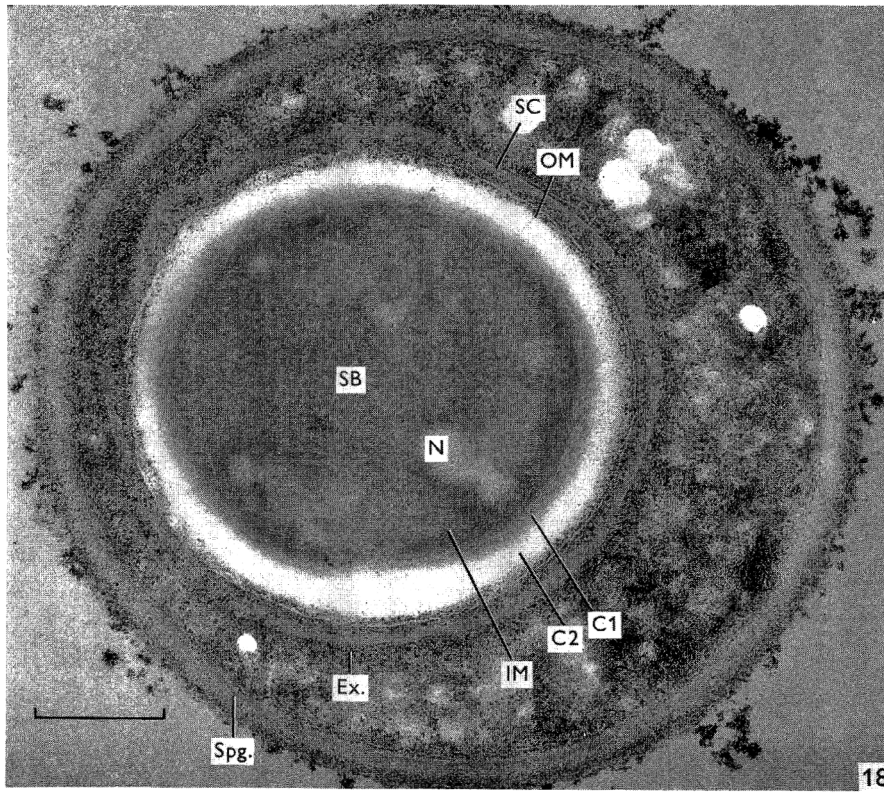
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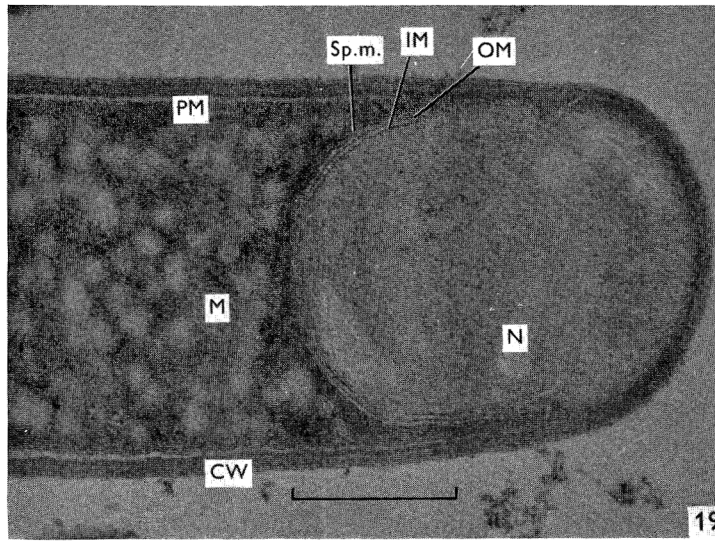
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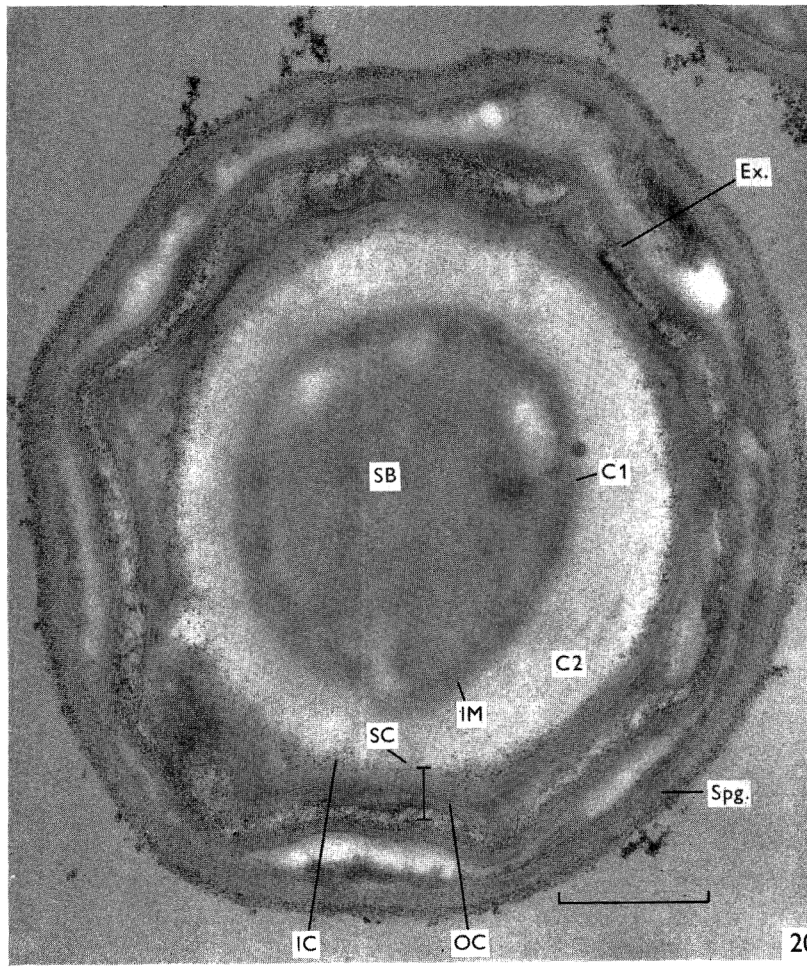
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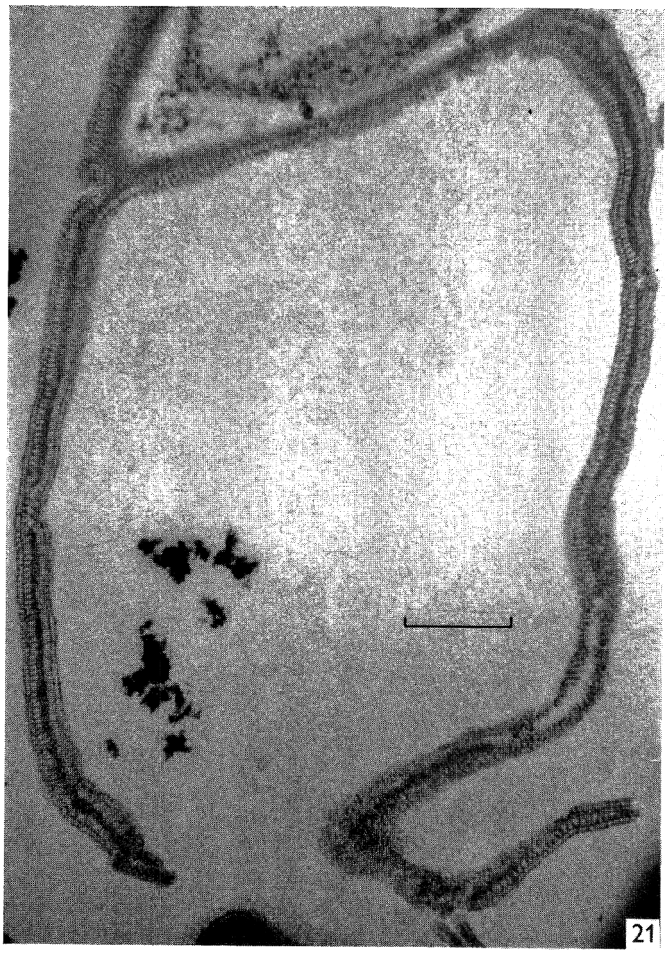
20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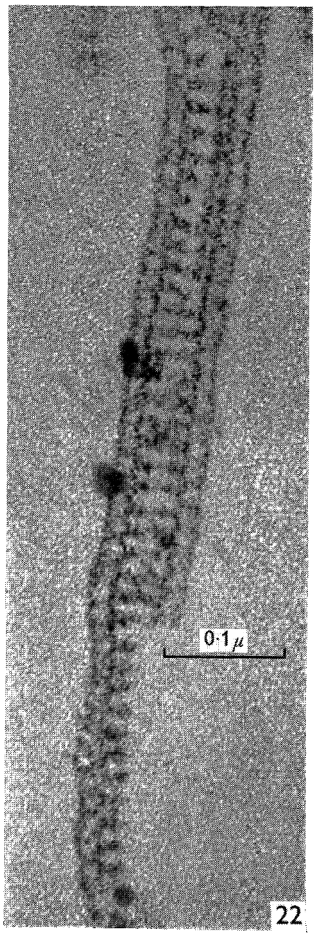
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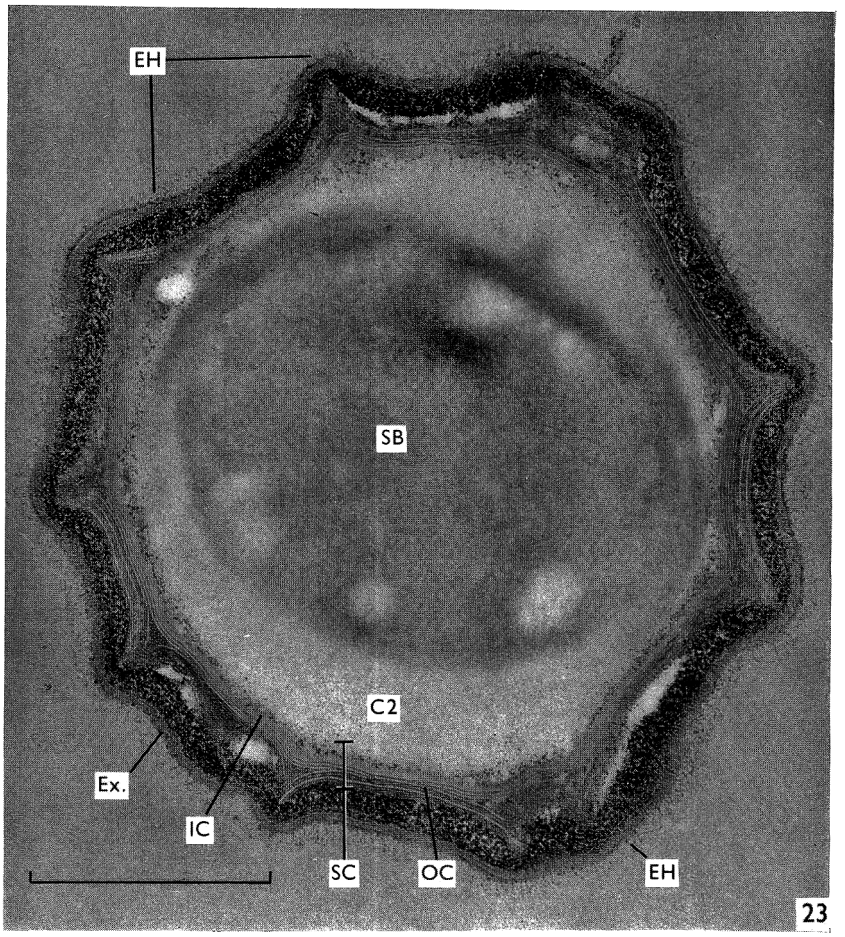
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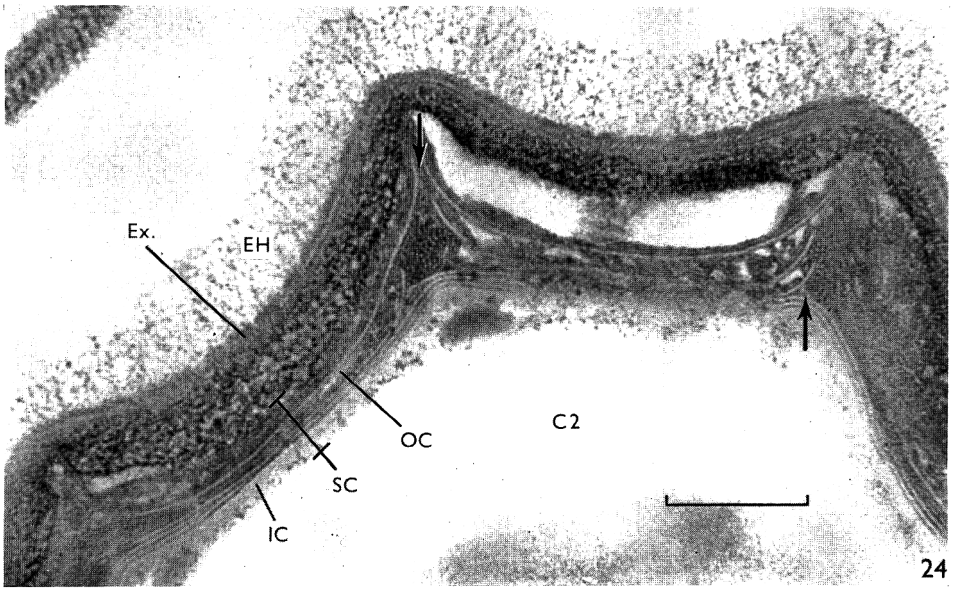
Ex.
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C2
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24

PLATE 7

Fig. 19. *Bacillus fastidiosus*. Developing endospore. Longitudinal thin section. IM = inner spore membrane; OM = outer spore membrane; Sp.m. = spore membranes; M = mesosome; PM = plasma membrane; N = nuclear material; CW = vegetative cell wall. Arrow indicates continuity between plasma membrane and spore membranes.

Fig. 20. *B. fastidiosus*. Developing spore. Transverse thin section. SB = spore body or core; IM = inner membrane; C1 = dense cortical area; C2 = less dense cortical area; SC = spore coats, composed of inner (IC) and outer (OC) layers; Ex. = exosporium; Sp.g. = vegetative cell wall or sporangium.

PLATE 8

Fig. 21. *Bacillus fastidiosus*. Vegetative cell wall and attached plasma membrane. Longitudinal thin section.

Fig. 22. *B. fastidiosus*. Portion of a fragmented vegetative cell wall and attached membranes.

PLATE 9

Fig. 23. *Bacillus fastidiosus*. Free, mature spore. Transverse thin section. SB = spore body or core; C2 = diffuse outer cortex; SC = spore coats, composed of inner (IC) and outer (OC) layers; Ex. = exosporium; EH = exosporial hairs.

Fig. 24. *B. fastidiosus*. Free, mature spore. Transverse thin section. C2 = cortex; SC = spore coats, consisting of inner (IC) and outer (OC) layers; Ex. = exosporium; EH = exosporial hairs. Arrows indicate apices or projections formed by the spore coats.

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The Influence of Extracellular Products on the Behaviour of Mixed Microbial Populations in Magnesium-limited Chemostat Cultures

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

SUMMARY

Magnesium-limited mixed cultures, each containing two microbial species, were produced by exchange of small numbers of organisms between magnesium-limited chemostat cultures of *Aerobacter aerogenes*, *Pseudomonas fluorescens*, *Bacillus subtilis*, *B. megaterium*, *Staphylococcus epidermis* and *Torula utilis*. The fate of each species in these mixed cultures was determined following cross-inoculation. Gram-negative organisms invariably outgrew the Gram-positive ones, but the ability of *B. subtilis* and *B. megaterium* to outgrow each other, or to outgrow the yeast, depended on the inoculum size. This dependence resulted from the presence of specific extracellular products in the Bacillus cultures which stimulated their growth and uptake of magnesium. The concentration of these extracellular growth-promoting substance(s) in magnesium-limited cultures of *B. subtilis* varied with population density. Thus, when the magnesium concentration in the medium was decreased from 0·9 to 0·15 μg./ml. (thereby causing the steady-state population density to be decreased to one-eightieth the initial value), *B. subtilis* could no longer maintain itself against proportionately low concentrations of *T. utilis*. But addition of extracellular fluids from dense magnesium-limited *B. subtilis* cultures to magnesium-limited *T. utilis* cultures enabled small numbers of the Bacillus now to outgrow the yeast. From these two observations it is concluded that magnesium assimilation by *B. subtilis* is more dependent on extracellular substance(s) than is magnesium assimilation by either *T. utilis* or the Gram-negative organisms. Modification of the basic theory of microbial growth in a chemostat culture, to take account of product-stimulated substrate-assimilation, is suggested.

INTRODUCTION

It is generally assumed (Monod, 1950; Herbert, Elsworth & Telling, 1956; Powell, 1965) that the relationship between the growth rate of organisms and the concentration of growth-limiting nutrient, in a chemostat culture, can be represented adequately by a Michaelis–Menten type of equation:

$$[D =]\ \mu = \mu_{\max}\left(\frac{s}{K_s+s}\right), \tag{1}$$

where μ is the specific growth rate, $\mu_{\max}$ the maximum rate of growth of the organisms in the medium, s the extracellular concentration of growth-limiting substrate in the culture (Mg^{2+} in most of the experiments to be described here) and K_s a saturation constant (numerically equal to the extracellular concentration of growth-limiting

nutrient at 0·5 μ_{max}). Under steady-state conditions in a chemostat, μ is equal to the dilution rate (D).

In the above equation there are two variables (μ and s) and when these are plotted against one another for a pure culture of an organism (A), a curve is obtained as shown in Fig. 1. With cultures of a different organism (B) a plot of similar shape would be obtained, but it would not be identical to the curve for organism A unless both of the constants (μ_{max} and K_s) were also identical. Now, assuming the saturation curves for two organisms to be exactly as shown in Fig. 1, and that these were grown in separate chemostats under identical (magnesium-limited) conditions at a dilution rate of D_1 hr^{-1}, then the extracellular concentration of Mg^{2+} in the culture of A-type organisms would be s_A, whereas that in the culture of B-type organisms would be s_B. If a small number of A-type organisms were now transferred to the chemostat containing a steady-state population of B-type organisms, then (assuming no lag period) A-type organisms would begin growing at a rate μ_A (since the growth-limiting substrate concentration would be s_B; Fig. 1). Because μ_A is greater than D_1, the concentration of A-type organisms in the culture would increase. Their growth would cause the growth-limiting substrate concentration to decrease towards the value s_A; at this latter concentration B-type organisms could grow only at a rate μ_B which, being less than D_1, would result in them being washed completely from the culture. Thus, organism A would totally displace organism B from the chemostat culture and this should occur irrespective of the initial concentration of each organism in the mixed culture and, in this case, the dilution rate.

We described previously (Tempest, Dicks & Meers, 1967) how *Aerobacter aerogenes* outgrew several Gram-positive organisms in Mg^{2+}-limited mixed culture experiments in a manner predicted by the above theory. But mixed cultures of *Torula utilis* and *Bacillus subtilis* behaved paradoxically: neither organism outgrew the other species unless the inoculum population exceeded a certain minimum concentration. The present paper reports the results of experiments designed to explain the unexpected behaviour of these mixed cultures. A preliminary report has been published (Meers & Tempest, 1968).

METHODS

Organisms. Aerobacter aerogenes (NCTC 418); *Pseudomonas fluorescens* (strain KB 1, from the University of Sheffield); *Bacillus subtilis* var. *niger* (ATCC 9372); *B. megaterium* (strain KM, from the University of Edinburgh); *Staphylococcus epidermidis* (isolated by Mr F. A. Dark, Porton). Each organism was maintained on tryptic meat digest agar slopes. *Torula utilis* (NCYC 321) was maintained on yeast extract peptone glucose agar slopes.

Growth conditions. Continuous cultures of organisms were maintained in 0·25 l. chemostats (designed by Dr D. Herbert) without pH control. The magnesium-limited medium was basically that described previously (Tempest *et al.*, 1967), but the magnesium content was varied as indicated in the Results section. The potassium-limited medium was that described by Tempest, Dicks & Hunter (1966), but with the potassium concentration decreased to 0·15 mM; the carbon-limited medium was that of Tempest, Hunter & Sykes (1965) but with glucose (1 g./l.) replacing the glycerol. The same magnesium-limited medium, with the magnesium concentration adjusted to 0·02 mM, was used for batch culture experiments.

In some experiments the medium was supplemented with supernatant fluids from previous chemostat cultures. These fluids were obtained by rapid filtration of the culture through a sintered glass filter (no. E5), to eliminate most of the organisms, and then through a membrane filter (Oxoid; standard grade) to effect their complete removal. Where larger volumes of culture were to be treated, the bulk of organisms were removed by centrifugation (5000*g* for 5 min.), before sterilization by filtration as above.

The technique for growing mixed cultures of organisms in the chemostat was that described by Tempest *et al.* (1967). For batch culture experiments, 20 ml. volumes of medium (contained in 350 ml. conical flasks) were inoculated with organisms from magnesium-limited chemostat cultures and aerated by shaking, on a reciprocating shaker, at 37°. At appropriate intervals of time, 3 ml. samples were withdrawn from each culture and measurements made of the extinction at 540 mμ (Bausch and Lomb 'Spectronic 20' spectrophotometer with tube of 0·5 in. (1·2 cm.) internal diameter) and culture population density (by counting directly the number of organisms in a known volume of culture, by using a Thoma Haemocytomer of 0·02 mm. depth). The results obtained by both methods correlated closely.

Magnesium determinations were made using an EEL (model 140) atomic absorption spectrophotometer on samples prepared as described previously (Tempest *et al.* 1967).

RESULTS

The magnesium-limited mixed culture experiments reported previously (Tempest *et al.* 1967) were extended to include most the possible combinations of two species from the following group of organisms: *Aerobacter aerogenes*, *Pseudomonas fluorescens*, *Bacillus subtilis*, *B. megaterium*, *Staphylococcus epidermidis* and *Torula utilis*. It was found that the Gram-negative bacteria invariably outgrew the Gram-positive organisms rapidly, irrespective of the initial population densities of the two competing species in the mixed cultures. Thus it can be concluded that both *A. aerogenes* and *P. fluorescens* have more efficient mechanisms for the uptake of magnesium from their environment than the Gram-positive organisms listed above. It is important to point out that *S. epidermidis* would grow only in simple salts media that had been supplemented with Casamino acids (1 %, w/v) and yeast extract (0·001 %, w/v), each of which contain substances that stimulated growth and the uptake of magnesium by *B. subtilis* (Tempest *et al.* 1967) and presumably also by the Staphylococcus organisms. Nevertheless, *S. epidermidis* cultures still were rapidly outgrown by either *A. aerogenes* or *P. fluorescens* organisms.

The ability of *Bacillus megaterium* and *B. subtilis* to outgrow each other or to outgrow *Torula utilis* in magnesium-limited mixed cultures depended on the relative amounts of each species in the culture immediately after mixing the two populations. Thus, when magnesium-limited mixed cultures of either *B. megaterium* and *T. utilis* or *B. megaterium* and *B. subtilis* were prepared, the ultimate fate of each species in each mixed culture depended on their concentrations immediately after cross-inoculation (Fig. 2). These results, which were identical to that with *B. subtilis* and *T. utilis* (Tempest *et al.* 1967), cannot be predicted from the theory outlined in the Introduction, since equation (1) contains no factor for population density.

The most obvious explanation for these paradoxical take-over patterns is that the

Gram-positive organisms produced substances that accumulated in the culture extracellular fluids and inhibited the growth of the competing species. To investigate this possibility, batch cultures of *Bacillus subtilis*, *B. megaterium* and *Torula utilis* were grown in low-magnesium simple salts media which had been supplemented with equal volumes of the supernatant fluids from magnesium-limited chemostat cultures of the three species. The resulting growth curves (Fig. 3) showed no evidence of growth inhibition. On the contrary, the growth curves indicated clearly the presence

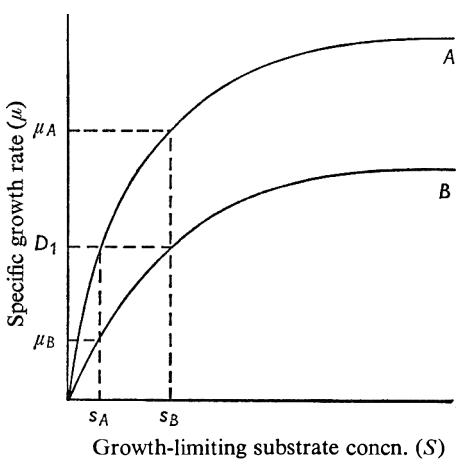

Fig. 1

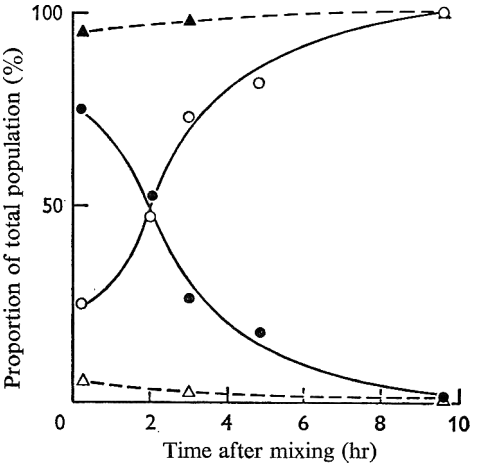

Fig. 2

Fig. 1. Hypothetical saturation curves for two microbial species (*A* and *B*) growing in magnesium-limited chemostat cultures. At a fixed dilution rate (D_1) the growth-limiting substrate concentration in a culture of *A* organisms would be s_A and of *B* organisms s_B. It is assumed that the maximum growth rate (μ_{max}) for *A* organisms is greater than for *B* organisms and that the saturation constants (K_s values) bear a similar relationship.

Fig. 2. Growth of *Bacillus megaterium* and *Torula utilis* in magnesium-limited simple salts medium, in a chemostat. In the experiment represented by the solid lines the initial concentration of *B. megaterium* (●) was 9×10^7 orgs/ml. and *T. utilis* (○) 3×10^7 orgs/ml., i.e. 75% and 25% of the initial mixed population, respectively. Also recorded (broken lines) are the results of a similar experiment in which the initial concentration of *B. megaterium* (▲) was increased to $1{\cdot}8 \times 10^8$ orgs/ml. and that of *T. utilis* (△) lowered to 1×10^7 orgs/ml., i.e. 95% and 5% of the total population, respectively. In both experiments the dilution rate was 0·3 hr^{-1} and the temperature 33°. The pH value was not controlled but did not vary beyond the range 6·1 to 6·5 in each experiment.

of growth-promoting substances in the culture extracellular fluids. Some specificities were apparent since the growth of each of the *Bacillus* species was promoted to the greatest extent by its own extracellular fluid (Fig. 3). It seemed possible, therefore, that the effect of population density on the growth patterns of Gram-positive organisms in magnesium-limited mixed cultures resulted from the presence of specific substances, secreted into the culture fluids, which facilitated magnesium assimilation.

If the above hypothesis is correct, then addition of extracellular fluid from a magnesium-limited Bacillus culture to the medium supplying a mixed culture of that *Bacillus* species and, for example, yeast organisms, possibly could enable small numbers of the bacilli now to outgrow the yeast. When sterile supernatant fluid from a magnesium-limited *Bacillus subtilis* culture which had contained 5×10^8 bacilli/ml.

was mixed (50 %, v/v) with a magnesium-limited simple salts medium (0·9 μg. Mg^{2+}/ml., final concentration) and used as the medium supplying a magnesium-limited chemostat culture containing *Torula utilis* ($4{\cdot}5 \times 10^7$ organisms/ml.) and *B. subtilis* (2×10^6 bacilli/ml., initial concentration), the bacilli now outgrew the yeast organisms (Table 1).

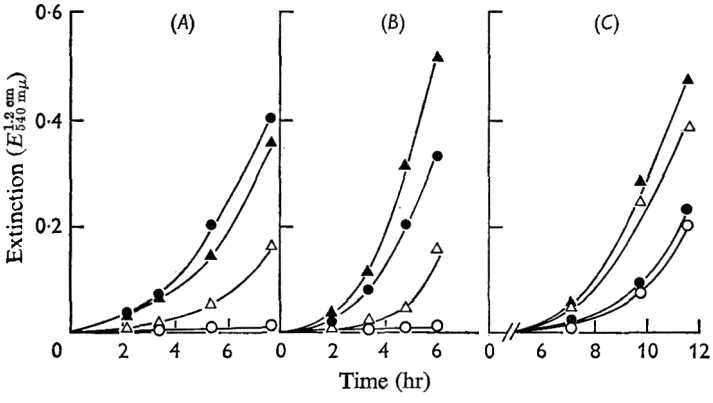

Fig. 3

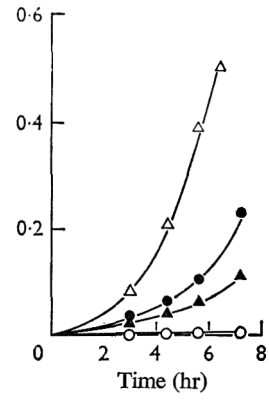

Fig. 4

Fig. 3. Growth of (*A*) *Bacillus subtilis*, (*B*) *Bacillus megaterium* and (*C*) *Torula utilis* in simple salts media that were supplemented (50 %, v/v) with cell-free fluid from magnesium-limited cultures of *B. subtilis* (●), *B. megaterium* (▲) and *T. utilis* (△); growth of organisms in an unsupplemented medium is shown by the open circles. The cultures were grown in 350 ml. conical flasks, incubated at 37° on a reciprocating shaker. All the media contained 0·5 μg. Mg^{2+}/ml. and in each case the inoculum was from a magnesium-limited chemostat culture of the appropriate organism.

Fig. 4. Growth of *Bacillus subtilis* in a simple salts medium containing 0·5 μg. Mg^{2+}/ml. that had been (△) supplemented (50 %, v/v) with cell-free fluid from a magnesium-limited culture of *B. subtilis* which had contained about 5×10^8 orgs/ml., (▲) supplemented (5 %, v/v) with the same cell-free fluid, and (●) supplemented (50 %, v/v) with cell-free fluid from a magnesium-limited *B. subtilis* culture that had contained about 1×10^8 orgs/ml. The open circles refer to growth of *B. subtilis* in an unsupplemented medium. The cultures were grown in 350 ml. conical flasks, incubated at 37° on a reciprocating shaker. The inoculum was from a magnesium-limited chemostat culture (D = 0·3 hr^{-1}, 33°, pH 6·5).

Table 1. *Growth of Bacillus subtilis and Torula utilis in a magnesium-limited chemostat culture supplemented with magnesium-limited B. subtilis extracellular culture fluid*

The culture temperature was controlled at 33°; the pH value was not controlled but varied only from 6·1 to 6·4 during the experiment. The magnesium-limited simple salts medium was mixed (50 %, v/v) with cell-free fluid from a magnesium-limited *B. subtilis* culture which had contained about 5×10^8 bacilli/ml.; the total magnesium concentration was adjusted to 0·9 μg./ml. The dilution rate was fixed at 0·3 hr^{-1}.

Time after mixing (hr)	No. of orgs/ml. ($\times 10^{-7}$)		Proportion of total no. of orgs (%)	
	B. subtilis	*T. utilis*	*B. subtilis*	*T. utilis*
0	0·2	4·5	5	95
3	0·3	5·6	5	95
8	0·7	5·0	12	88
13	4·6	5·0	48	52
21	13·2	2·2	86	14
26	20·0	1·8	92	8
29	39	1·6	96	4

The nature of these growth-promoting substances has not been established, but their concentration in a magnesium-limited culture of *Bacillus subtiis* varied with population density. Thus, when the concentration of magnesium in a culture of *B. subtilis* was decreased to a value (0·3 μg. Mg^{2+}/ml.) such that the steady-state culture population density was about 1×10^8 bacilli/ml., the growth-promoting capacity of the resulting extracellular fluid was decreased correspondingly (Fig. 4). At this lower bacterial concentration small numbers of yeast organisms still could not outgrow the bacilli, but when the culture bacterial population density was decreased further, to 6×10^6 bacilli/ml. (culture magnesium concentration, 0·15 μg./ml.), proportionately small numbers of the yeast organisms (about 1×10^7 orgs/ml., initially) now outgrew the bacilli (Table 2) but only slowly. Presumably the very small population of *B. subtilis* organisms could not produce a sufficient concentration of extracellular growth promoting substance(s) to facilitate magnesium uptake by the bacilli to an extent that would allow them to compete effectively with the yeast organisms for the available magnesium.

Table 2. *Growth of Bacillus subtilis and Torula utilis at low population densities in a magnesium-limited culture*

The magnesium-limited simple salts medium contained 0·15 μg. Mg^{2+}/ml.; 0·1 ml. of a *T. utilis* chemostat culture, growing in this low-magnesium medium, was transferred to a *B. subtilis* culture also growing in this medium. The dilution rate was maintained throughout at 0·3 hr^{-1} and the temperature controlled at 33°. The pH value (uncontrolled) was 6·4 and did not vary.

Time after mixing (hr)	No. orgs/ml. ($\times 10^{-6}$)		Proportion of total no. orgs (%)	
	B. subtilis	*T. utilis*	*B. subtilis*	*T. utilis*
0	6	< 0·1	99	1
3	6	< 0·1	99	1
6	6	< 0·1	99	1
23	6	0·3	95	5
30	2·2	3·6	38	62
49	< 0·1	9·4	1	99

The inability of small numbers of *Bacillus subtilis* or *B. megaterium* organisms to outgrow *Torula utilis* in a simple salts medium did not obtain when magnesium was present in excess of requirement and growth was limited either by the availability of potassium or glucose. In both these cases the bacilli rapidly outgrew the yeast, irrespective of the initial population densities. This finding suggests that the growth-promoting substances were associated specifically with magnesium assimilation and not with cation uptake in general.

DISCUSSION

The above results provide an explanation for the unpredictable behaviour of some magnesium-limited mixed cultures described here and previously (Tempest *et al.* 1967); clearly magnesium uptake by both *Bacillus subtilis* and *B. megaterium* was facilitated by substances that were secreted into the extracellular fluids when the bacilli were growing under conditions where availability of magnesium limited growth. Since the concentration of these growth-promoting substances varied with population density, so did the ability of the bacilli to compete with other organisms

in magnesium-limited chemostat mixed cultures. As yet we have no precise knowledge of the nature of these growth-promoting substances but the active material present in a magnesium-limited culture of *B. subtilis* was found to survive heating at 100° for 30 min., diffuse through a dialysis sac and separate on a Sephadex column (G-10) with material of molecular weight greater than 700. Since both Casamino acids and yeast extract promoted growth of *B. subtilis* and *B. megaterium* in low-magnesium environments (but had no specificity), the growth-promoting substances possibly may be polypeptides or polynucleotides; they do not appear to be teichoic acids. It may be significant that products of RNA degradation are usually, if not invariably, found in extracellular fluids from non-growing suspensions of bacteria (Strange, Dark & Ness, 1961; Postgate & Hunter, 1962; Strange, Wade & Ness, 1963; Dawes & Ribbons, 1964), particularly when the extracellular magnesium concentration is low (Strange & Hunter, 1967), and nucleotides have been found to be secreted by cultures of *B. subtilis* growing in defined media (Demain, Burg & Hendlin, 1965). Also of interest in this connexion is the observation of Byers, Powell & Lankford (1967) that *B. megaterium* could secrete a substance (Schizokinen) that actively initiated cell division by chelating iron and facilitating its transport into the cell.

Several workers have observed an effect of population density on the growth of bacteria in both 'batch' and 'continuous' cultures. Bail (1929) proposed the concept of a bacterial space requirement to account for inhibition of growth at high population densities, and Contois (1959) provided evidence that assimilation of NH_4^+, by *Aerobacter aerogenes* growing in an NH_4^+-limited chemostat culture, was less efficient at high population densities than at low ones. On the other hand, Jannasch (1962, 1963) showed with carbon-limited chemostat cultures of *Spirillum serpens* that assimilation of growth-limiting substrate (lactate) was inefficient at low population densities; this he ascribed in part to lack of reducing power in cultures of low population density. Our results suggest, like Jannasch's, that assimilation of growth-limiting substrate (magnesium) was less efficient in the cultures of low population density. Thus, although the method used for determining extracellular magnesium was insufficiently sensitive to detect small changes ($< 0{\cdot}05$ μg. Mg^{2+}/ml.) in the steady-state growth-limiting substrate concentration, the mixed culture experiments indicated clearly that for *Bacillus subtilis* and *B. megaterium*, at least, the relationship between growth-limiting substrate concentration (s) and growth rate (μ) varied with population density when the availability of magnesium limited growth.

Although the Michaelis–Menten type of equation (1) may describe accurately the relationship between μ and s in many real chemostat cultures, clearly it is inadequate for cultures in which growth is affected by bacterial products which accumulate in the environment. In such cases a 'population factor' (or, more accurately, a 'population-product factor') must be incorporated in the growth equation. But how equation (1) should be modified to accommodate such a factor is a matter for conjecture, since it is not certain whether its effect is primarily on the saturation constant (K_s) or on the maximum growth rate value (μ_{max}) or on both. Recent work by Dr E. O. Powell (personal communication), using a turbidostat to determine values of μ_{max}, showed that magnesium-limited *Bacillus subtilis* organisms secreted a substance (or substances) which increased the maximum growth-rate of *B. subtilis* growing in a low magnesium medium; whether the saturation constant (K_s) was also affected could not be determined. If the growth-promoting substance affected μ_{max} only,

then the simplest relationship that can be derived to accommodate its effect on growth is as follows:

$$\mu = \mu_{\max}\left(\frac{1+\lambda p}{1+p}\right)\left(\frac{s}{K_s+s}\right), \tag{2}$$

where p represents the concentration of extracellular growth-promoting metabolite and λ is a constant; $\mu_{\max}$ is defined as the maximum growth rate when $p = 0$. Thus, plotting the relationship between s and μ for magnesium-limited chemostat cultures of *B. subtilis* that differed only in the concentration of magnesium in the input medium (and hence in culture population density and concentration of extracellular growth-promoting substance) would produce a family of curves, similar to those shown in Fig. 1, each describing the relationship between s and μ at a particular culture population density. At low population densities the concentration of growth-promoting substance would be negligible, and then the relationship between growth rate and growth-limiting substrate concentration given by equation (2) would reduce to that given by equation (1). At high population densities, on the other hand, the concentration of growth-promoting substance would reach a 'saturation' value at which it would exert its maximum effect; thus $(1+\lambda p)/(1+p)$ would approximate to λ and the maximum growth rate approach a value equal to $\lambda\,\mu_{\max}$.

Powell (1958) and Jannasch (1967) concluded that the fate of a contaminant organism in a chemostat culture depended on many factors including the nature of the growth-limiting component of the medium, the dilution rate, temperature and culture pH value. Our results show that under some conditions at least, the culture population density is a further factor.

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