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Carotenoid-glycoprotein of *Sarcina flava* Membrane

By D. THIRKELL AND M. I. S. HUNTER
Department of Biochemistry, Bute Medical Buildings, St Andrews, Fife

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SUMMARY

The carotenoid-glycoprotein isolated from the membranes of *Sarcina flava* by using synthetic detergents was not resolved into subfractions. It was resistant to extremes of heat and pH value. The peptide moiety carried little net charge and the carotenoid was not easily released.

INTRODUCTION

All of the carotenoid in *Sarcina flava* is localized in the membranes (Strang, 1968). Two general methods are available for membrane solubilization: the use of synthetic detergents (Salton & Netschey, 1965; Salton, 1967*a*, *b*) and prolonged ultrasonic treatment (Salton, 1967*a*). The use of synthetic detergents has been studied and non-ionic detergents found to be the most effective. Most carotenoproteins studied have been of invertebrate origin (Fox, 1955; Cheesman, 1958; Cheesman, Lee & Zagalsky, 1967). The link between carotenoid and protein, in these instances, is easily broken and a total recovery of carotenoid made. Since the carotenoid-glycoprotein from the membranes of *S. flava* is associated with carbohydrate and protein (Thirkell, Hunter, Crawford & Fracassini, 1969), the carotenoid may be glycosidically bound to carbohydrate. Such a situation exists in *Mycobacterium phlei* (Hertzberg & Jensen, 1967). The present work was undertaken to study some of the properties, particularly the nature of the bonding of carotenoid, of a detergent-solubilized membrane fraction from *S. flava*.

METHODS

Growth and harvesting of bacteria, preparation and solubilization of the membrane: *Sarcina flava* (NCTC 7503) membranes were prepared as previously reported (Thirkell *et al.* 1969). Solubilization was achieved by repeated extractions with either 1 % (v/v) Lubrol L (I.C.I. Ltd.) or Crillet 1 (Croda Ltd.), both non-ionic polyoxyethylene derivatives. The solubilized material was exhaustively dialysed against tap water and then distilled water, and the carotenoid-glycoprotein purified by repeated ammonium sulphate precipitation.

Attempted resolution of Crillet 1 solubilized carotenoid-glycoprotein. Three techniques were used: (*a*) Electrophoresis in free buffer film. Separation was attempted on a vertical flow machine with buffers of different ionic strengths and with pH values ranging from 3·9 to 8·6. The material was exhaustively dialysed against the buffers for at least 48 hr before being pumped into the machine; (*b*) Sephadex gel filtration. Concentrated material was applied to columns (26 × 1·5 cm.) of Sephadex G-100 and of

G-200 and the columns were developed with 0·1% (w/v) NaCl. Fractions were collected on a Central Ignition fraction collector; (*c*) ultracentrifugation. The sedimentation patterns of the material in distilled water were studied on the ultracentrifuge for up to 2 hr after it had reached top speed (59,780 rev./min.).

Examination of Lubrol L solubilized carotenoid-glycoprotein. (*a*) Effect of heat and pH value on the absorption spectrum. The absorption spectrum was determined over a range of temperatures (15 to 100°) and pH values (4·0, 7·0, 10·0); (*b*) effect of heat on optical rotation. Optical rotation was studied over a temperature range of 18 to 78°; (*c*) amino acid analysis. A sample was freeze-dried for 36 hr and hydrolysed in a sealed tube with 6N-HCl at 120° for 24 hr. The hydrolysate was made acid free and applied to a Technicon automatic amino acid analyser; (*d*) attempts to release free carotenoid. (1) Partition with an equal volume of ether, (2) dilution with an equal volume of acetone and ether extracted (Cheesman, 1958), (3) made 4 M with respect to urea, stirred (12 hr) and ether extracted, (4) made 4 M with respect to urea and 1 % (v/v) with respect to thioglycollic acid, stirred (12 hr) and ether extracted, (5) carotenoid-glycoprotein titrated with either 0·1N-HCl or 0·1N-NaOH to give samples at pH 4·3, 5·3, 7·0, 8·9, 11·8 and ether extracted, (6) boiled for 15 min. and ether extracted, (7) material saponified with 10 % (w/v) methanolic KOH up to 7 days and ether extracted, (8) digestion with papain. The solution was made 0·1 M with respect to phosphate buffer (pH 7·6), 5 mM with respect to EDTA (chelation of metal ions), 1·0 mM with respect to cysteine (enzyme -SH groups maintained in the reduced form) and 1 % (w/v) with respect to papain. Digestion was at 40° in the dark in an atmosphere of nitrogen. Two recharges of EDTA + cysteine + papain were made at 5 hr intervals and the digestion then left overnight. The digest was extracted with ether until the ether phase was colourless. The total etherial extract was dried over anhydrous sodium sulphate, taken to dryness at 25° and tested for the presence of carotenoid (spectrum), carbohydrate (Whistler & Wolfrom, 1962) and peptide (amino acid analysis).

RESULTS

Resolution. With electrophoresis in free buffer film, no resolution was achieved in any system. The carotenoid-glycoprotein did not appear to be charged at any pH value. No resolution was achieved with Sephadex and on the ultracentrifuge a single symmetrical peak was seen after 1 hr. These results suggest that a single molecular species had been solubilized.

Table 1. *Carotenoid-glycoprotein of Sarcina flava membrane: relative concentrations of amino acids present*

Amino acid	(%)*	Amino acid	(%)
Alanine	9·66	Lysine	trace
Aspartic acid	8·73	Ornithine	trace
Glutamic acid	7·98	Phenylalanine	6·73
Glycine	9·78	Serine	10·35
Histidine	5·30	Threonine	6·04
Isoleucine	8·19	Tyrosine	4·00
Leucine	14·20	Valine	9·04

* Amino acid analysis could not be calculated on a dry weight basis since only drying in a vacuum oven at 85° for 4 days will remove all the water associated with the detergent (checked by infrared spectroscopy). Such treatment partially degrades the samples.

Spectra. The $\lambda_{max.}$ were 226, 276, 282, 297, 416, 444 and 474 mμ. The peaks in the visible region are similar to those given by the free pigments of *Sarcina flava* in organic solvents (Thirkell, Strang & Chapman, 1967). This suggests that any binding of carotenoid to other molecules does not alter the main chromophore (unlike the situation with carotenoproteins of invertebrates). The peaks at 276 and 282 mμ are due to detergent and aromatic amino acids and that at 226 mμ to peptide bonding. No change in $\lambda_{max.}$ or peak heights was detected over the range of temperature or pH value studied, and prolonged boiling did not cause precipitation (mutual stabilization of components, Cheesman *et al.* 1967).

Optical rotation. No change was detected suggesting that no alteration in the secondary or tertiary structure of the peptide had occurred.

Amino acid composition. The results are shown in Table 1.

Attempted release of free carotenoid. The findings are summarized in Table 2. When the effects of urea or urea + thioglycollic acid were examined on the ultracentrifuge, equilibrium patterns were produced. Thus, no conclusions could be drawn as to whether a split into smaller subunits had occurred.

Table 2. *Carotenoid-glycoprotein of Sarcina flava membrane: attempts to release free carotenoid from the complex*

Method	Result	Comment
1	No release	Association through a definite type of bonding rather than a loose non-specific association.
2	No release	Carotenoid bound in a manner different to that found in invertebrate carotenoproteins which are split by this method, after which 100% of the carotenoid is extractable into ether (Cheesman *et al.* 1967). The effect of acetone is to denature the protein/peptide and thus the integrity of the tertiary structure of this is not necessary for the bond.
3	No release	Hydrogen bonding not involved.
4	No release	Hydrogen bonding not involved; split of any S–S bridges in the protein/peptide moiety does not aid pigment release.
5	No release	Electrostatic binding not involved.
6	No release	(i) Hydrogen bonding or π–π electron interactions not involved since the thermal energy supplied should be in excess of that required to break such bonds. (ii) No precipitation occurred which suggests that the components of the complex mutually stabilize each other.
7	Some carotenoid released after 6 days.	Suggests that the type of bond ruptured is very weakly alkali labile.
8	Pigment release	A substantial amount of pigment was released into the ether phase; analytical tests showed that it was not free carotenoid but that it was still associated with carbohydrate, a short peptide and detergent. The effect of papain was that it decreased the peptide to a length which made some of the material preferentially ether-soluble rather than water-soluble.

DISCUSSION

The solubilized membrane fraction of *Sarcina flava* appears to be a single molecular species. The amino acid analysis revealed little or no basic amino acids and a very high ammonia peak suggests that most, if not all, of the dicarboxylic amino acids may be

in the amide form. This would explain why no charge was noticeable during electrophoresis in free buffer film, why the authors could not obtain any reasonable titration curve for the material and why it did not migrate from the origin on disc-gel electrophoresis. The fraction is extremely stable to extremes of temperature (up to 100°) and pH value (pH 4 to 10). Release of free carotenoid is difficult to achieve indicating that the bonding is covalent (glycosidic?). Papain digestion produced smaller units which became ether-soluble, but further degration of these subunits would be required to identify the molecules involved in the links between the components of the complex. The carotenoid-glycoprotein studied was rich in detergent. To what extent the presence of detergent influenced these results cannot be determined since it is impossible to remove all traces of the detergent from the material.

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The Polar Carotenoid Fraction from *Sarcina flava*

By D. THIRKELL AND M. I. S. HUNTER
Department of Biochemistry, Bute Medical Buildings, St. Andrews, Fife

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SUMMARY

The polar carotenoid fraction from *Sarcina flava* does not not appear to be a free carotenoid, but a carotenoid associated with glucose and a peptide. The glucose seems to be glycosidically bound to the carotenoid through its reducing group. Presumably the peptide is linked to the glucose through either the primary or one of the secondary hydroxyl groups on the carbohydrate molecule. A model of the *in vivo* state of the carotenoid complex in the bacterial membrane has been proposed.

INTRODUCTION

Thirkell, Strang & Chapman (1967) examined the carotenoids which could be isolated from *Sarcina flava*; two of these were shown to be C_{50} compounds. However, a polar fraction (designated fraction 4) was purified and little positive information was obtained about its constitution. As some of the carotenoids in the membranes of this bacterium are associated with carbohydrate and peptide (Thirkell, Hunter, Crawford & Fracassini, 1969), the present work was undertaken in an attempt to reveal the nature of this polar material.

METHODS

Organism. Approximately 3 kg. of deep frozen *Sarcina flava* (NCTC, 7503) were obtained from Imperial College, London. The organisms had been grown at 37° in nutrient broth (Oxoid Ltd.) with 1 % (w/v) glucose added.

Chemicals. All solvents used were Analar grade and were dried and redistilled before use.

Isolation of the polar carotenoid fraction. This was done by the method previously reported (Thirkell et al. 1967).

Resolution of the polar carotenoid fraction. The total fraction was resolved into four subfractions by thin-layer chromatography on 0·5 mm. layers of silica gel G (Merck) with chloroform + methanol (90 + 10, v/v) as solvent. The subfractions were again chromatographed to establish their purity and labelled 1 to 4 in order of decreasing polarity. Since subfractions 1 and 3 were in approximately equal concentration and constituted about 80 % of the total fraction 4, all subsequent investigations were done only with these two subfractions.

Tests used for the characterization of the subfractions

Acetylation. (Kuhn & Sørensen, 1938; Jensen, 1962). A sample of chromatographically pure material was taken to dryness on a rotary evaporator and then freeze-dried

for 24 hr. The fraction was dissolved in 1 ml. pyridine, 0·1 to 0·2 ml. acetic anhydride added and the reaction allowed to proceed at room temperature in the dark under an atmosphere of nitrogen. Samples were withdrawn every 30 min. for the first 5 hr. and then at regular intervals up to 36 hr, by which time the reaction was complete. Each sample was examined by thin-layer chromatography with 20 % (v/v) acetone in light petroleum (b.p. 60 to 80°) as solvent. The intermediates were identified visually and after spraying with a saturated solution of antimony trichloride in chloroform (Morton, 1942). Thus the initial compound, the formation of intermediates and the formation of the final ester could be monitored. Spectroscopic examination was used before and after the reaction to test that no degradation had occurred.

Test for tertiary hydroxyl groups after acetylation. (S. L. Jensen, private communication). The esters from above were dried and dissolved in 0·5 ml. dry pyridine, 0·2 ml. hexamethyldisilane and 0·1 ml. trimethylchlorosilane were added and the reaction allowed to proceed at room temperature in the dark and under an atmosphere of nitrogen. After 1 hr, carbon tetrachloride was added and the solutions taken to dryness. The products in methanol were examined by thin-layer chromatography as before.

Methylation of carboxyl group. (Metcalfe & Schmitz, 1961). A sample of the subfractions was dried as for acetylation and dissolved in dry methanol, 0·5 ml. methanolic boron trifluoride added and the mixtures refluxed for 3 min. The products were removed into ether and examined chromatographically.

Partition ratio. (Petracek & Zechmeister, 1956). 95 % (v/v) methanol in water and hexane were equilibrated with each other and the samples dissolved in a small volume of equilibrated methanol. An equal volume of equilibrated hexane was added, and after shaking the two layers were collected. The concentration of pigment in each layer was determined spectrophotometrically.

Spectroscopy. The absorption spectra of the subfractions and their acetates in methanol were determined with a Unicam SP 800 spectrophotometer.

Infra-red spectroscopy. This was done with a Unicam SP 200 G instrument with liquid cells containing solutions in carbon tetrachloride and in carbon disulphide (spectro-quality reagents).

Carbohydrate estimation. The sensitive but general phenol–sulphuric acid method of Whistler & Wolfrom (1962) was used with solutions of glucose (25 to 100 μg./ml.) as standard.

Identification of carbohydrate. Salton & Freer (1965) reported that the carbohydrates present in the membranes of the related micro-organisms *Sarcina lutea* and *Micrococcus lysodeikticus* were glucose, galactose, mannose and ribose. These sugars were used as standards throughout this part of the work. The subfractions were hydrolysed with N-HCl in a sealed tube for 18 hr at 120° after which the hydrolysates were made acid-free in the normal way. Chromatography was done in a single dimension on Whatman No. 1 chromatography papers with the following descending solvents: (i) *n*-butanol + pyridine + water (6 + 4 + 3; by vol.); (ii) *n*-butanol + acetic acid + water (6 + 1 + 2; by vol.); (iii) *n*-butanol + acetone + water (4 + 5 + 1; by vol.) The standard carbohydrates were run alone and as a mixture, and the hydrolysates were run alone and co-chromatographed with each standard. Since carotenoid in the membranes of *Sarcina flava* had been shown in some cases to be associated with both carbohydrate and peptide (Thirkell *et al.* 1969), in all instances,

duplicate chromatograms were developed, one of which was stained with 0·1 M-anisidine phthalate in ethanol (Pridham, 1956) and the other with 2 % (w/v) ninhydrin in acetone. The former chromatograms were viewed in daylight and under ultraviolet illumination.

Separation of the carbohydrate and peptide by two-dimensional paper chromatography. An attempt was made to separate any free sugar from peptides (or glycopeptides) in the N-HCl hydrolysates on Whatman No 1 chromatography papers with *n*-butanol + acetic acid + water (6 + 1 + 2; by vol.) as first solvent and phenol (400 g. phenol/100 ml. water) + water as the second. In each instance, duplicate chromatograms were developed, after removal of the phenol with ether, one was stained with ninhydrin and the other with the anisidine phthalate reagent.

Peptide resolution. Since ninhydrin-positive spots were detected on the above chromatograms and since hydrolysis with N-HCl would be more likely to produce small peptides rather than free amino acids, the hydrolysates were resolved by two-dimensional thin-layer chromatography on 0·25 mm. layers of silica gel G (Merck) with *n*-butanol + acetic acid + water (6 + 2 + 2: by vol.) as first solvent and *n*-propanol + water (64 + 36, v/v) as second solvent (Randerath, 1964). The plates were stained with ninhydrin.

Amino acid analysis. A sample of each subfraction was taken to dryness and freeze-dried for 24 hr in a weighed vessel. The weights were determined and the fractions hydrolysed with 6N-HCl in a sealed tube for 18 hr at 120°. The hydrolysates were made acid-free, and then in distilled water they were applied to a Technicon auto-analyser, with norleucine as internal standard.

Estimation of carotenoid. This was estimated spectrophotometrically using a theoretical extinction coefficient of 3000 based on the similarity of the chromophore to that of neurosporene or ε-carotene. Such an estimation assumes that the absorption characteristics of the free and bound carotenoid are similar.

RESULTS

The results for part of the chemical analysis of the subfractions are shown in Table 1. For the tests shown in the table, the results from the two subfractions were identical.

Chromatography

Identification of carbohydrate. The N-HCl hydrolysates were resolved into four spots which stained with anisidine phthalate reagent. That with the highest R_F value co-chromatographed with glucose, the others did not co-chromatograph with any standard used. However, duplicate chromatograms stained with ninhydrin showed eight spots, four of which had the same R_F values as the carbohydrate spots when the chromatograms were run in a single dimension.

Separation of carbohydrate from peptide (glycopeptide). Two-dimensional paper chromatography showed that the carbohydrate spot which co-chromatographed with glucose was a free sugar. The other spots which stained with anisidine phthalate were associated with peptide. Ninhydrin-staining material was also detected which did not appear to be associated with sugar.

Resolution of peptides. Two-dimensional thin-layer chromatography showed that the peptides present in the two hydrolysates were not identical. Calculation of R_F

values showed that most, if not all, of the spots were peptides rather than free amino acids.

Link of carotenoid to glucose. The anisidine phthalate reagent stains with reducing groups only, and the subfractions before hydrolysis did not take up the stain.

Table 1. *Sarcina flava. Some analytical results obtained from the pigment subfractions 4 (i) and 4 (iii)*

Test	Result	Comment
Acetylation	Positive	At least 6 hydroxyl groups (primary or secondary) were acetylated. After acetylation, both subfractions were still polar, suggesting the presence of polar groups other than hydroxyl in the subfractions
Tertiary hydroxyl group	Negative	—
Methylation of carboxyl group	Positive	Methylation of carboxyl groups on dicarboxylic amino acids
Partition ratio	100% in methanol	Very polar compound
Spectra	max.$^{-415,437,467}$ mμ with original and final acetate	Chromophore not unduly altered from that shown by free pigments (Thirkell *et al.* 1967). Association of other molecules with carotenoid does not involve any electrostatic linkages with the electron-dense double bonds of the central conjugated chain.
Infrared spectroscopy	No *cis* peak	Carotenoid in *all-trans* form. The infrared spectrum showed many peaks and proved almost impossible to interpret. This finding might be explained by the presence of carotenoid, carbohydrate and amino acids in the subfractions

Table 2. *Sarcina flava. Relative amino acid composition of pigment subfractions 4 (i) and 4 (iii) expressed in μM*

Amino acid	Fraction 4 (i)	Fraction 4 (iii)
Aspartic acid	1·3	2·0
Threonine	1·2	1·2
Serine	3·0	3·0
Glutamic acid	3·2	5·0
Glycine	6·1	7·1
Alanine	2·1	3·0
Valine	1·0	2·0
Isoleucine	0·8	1·8
Leucine	1·0	2·1
Tyrosine	0·8	1·0
Phenylalanine	0·8	1·1
Ornithine	1·4	2·8
Lysine	1·0	2·0
Histidine	0·9	1·1

Since the hydrolysates in each case did stain, and since the presence of glycopeptides was demonstrated in them, we suggest that the carotenoid is linked glycosidically to the reducing group of glucose. One would expect such a linkage to be split on hydrolysis as reported.

Amino acid analysis

The amino acid composition of the two subfractions is shown in Table 2 where the relative amounts of each amino acid are expressed in μM.

Constitution of the subfractions by chemical analysis. The relative percentages of carotenoid, glucose and amino acids found were:

	Carotenoid	Glucose	Amino acid
Fraction 4 (i)	42	23	24
Fraction 4 (iii)	40	24*	27

* This value was checked by using the anthrone method (Scott & Melvin, 1953).

DISCUSSION

Both subfractions examined contain carotenoid, glucose and amino acids. The carotenoid is presumed to be the C_{50} dihydroxy compound (the most polar free carotenoid) isolated by Thirkell *et al.* (1967) which has become bound to form an integral part of the membrane structure where all of the carotenoid is localized in *Sarcina flava* (Strang, 1968). The carotenoid seems to be bound to glucose through a glycosidic linkage involving the reducing group of the hexose. The amino acids (peptides) associated with the subfractions must be linked to the hexose in some way involving at least one of the remaining primary or secondary hydroxyl groups of the glucose. If

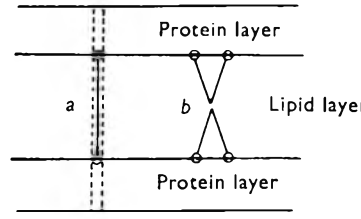

Fig. 1.

one considers how this type of complex could be incorporated into the bacterial membrane, let us represent the dihydroxy carotenoid as: O————O, where O = hydrophilic hydroxyls and ———— = hydrophobic central carbon chain. This could fit into the lipid layer of a bimolecular leaflet membrane model as shown in Fig. 1. Formation (*b*) can be excluded since it would require the central double bond to be *cis* and it is known to be *trans*. Thus, formation (*a*) is more likely and the dotted lines would represent the type of material of which these subfractions are constituted. The length of the carotenoid molecule is of the right order to straddle the lipid layer in this way. Figure 2 would represent a more detailed explanation of Fig. 1.

If the results presented here are considered in relation to the model, the following observations can be made. (1) The hydroxyl groups acetylated are probably unsubstituted primary or secondary hydroxyls on the glucose molecules. Thus, according to the model, six hydroxyl groups should be available for acetylation and this was demonstrated; (2) the results from amino acid analysis show an amino acid content which in relation to glucose suggests very few amino acid residues attached to each

glucose molecule. However, during the isolation and purification of these fractions, saponification was used. In the study of glycoprotein structures, β-elimination is frequently used to verify the existence of O-glycosyl linkages with serine or threonine (Neuberger, Gottschalk & Marshall, 1966). Although stronger alkali is used for β-elimination, the conditions used in saponification could well result in the complete loss of amino acids (peptides) from certain glucose molecules if this type of linkage was present. The amino acids with the exception of ornithine (some or all of which may be derived from arginine during saponification) are present in a definite molar ratio, suggestive of a repeating unit of peptide in the protein layer of the membrane. The component amino acids are the same 14 as those found in carotenoid-glycoprotein isolated by using synthetic detergents (Thirkell & Hunter, 1969). Their relative concentrations differ, suggesting that the lengths of the peptides are not the same. One would expect a longer peptide to be more soluble in an aqueous detergent solvent than in the system used in this work.

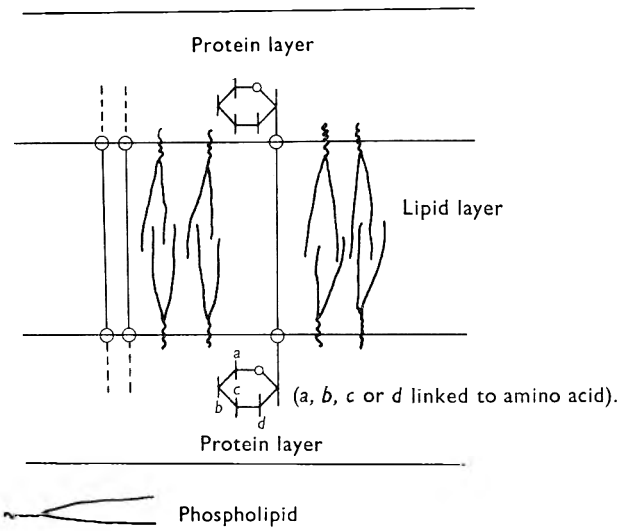

Fig. 2. Proposed model to show incorporation of carotenoid in *Sarcina flava* membrane.

If there is a repeating unit in the protein layer of the membrane, and if the distance between the carotenoid molecules is less than the length of this repeating unit, then the latter must be fragmented during the isolation of the subfractions. This would release glycocarotenoid to which only part of the unit is bound. Thus, only when a mixture of these fragments is studied should a repeating unit be apparent and be shown by a definite molar ratio of the amino acids present. Such a situation is reported in this work.

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Resistance of *Escherichia coli* and *Salmonella typhimurium* to Carbenicillin

By H. C. NEU AND H. SWARZ

Department of Medicine, College of Physicians and Surgeons, Columbia University, New York, New York 10032, U.S.A.

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SUMMARY

Carbenicillin, a 9-alpha carboxybenzyl penicillin, is bactericidal only for *Escherichia coli* strains which are resistant to less than 125 μg./ml. of ampicillin. All *E. coli* and *Salmonella typhimurium* strains in which penicillinase is a surface enzyme are resistant to carbenicillin. *E. coli* and *S. typhimurium* strains in which production of beta-lactamase is episomally mediated are resistant to both ampicillin and carbenicillin. A non-hydrolyzable penicillin (methicillin or dicloxacillin) does not allow carbenicillin to exert its antibacterial effect against resistant strains of *E. coli*, *Pseudomonas*, *Klebsiella* or *Enterobacter*. Carbenicillin shows no synergy with penicillinase-resistant penicillins.

INTRODUCTION

Carbenicillin is an alpha carboxybenzyl penicillin synthetised from the 6-aminopenicillanic acid nucleus. It differs from ampicillin in that a carboxyl group is substituted for an amino group at the alpha position. The drug is hydrolyzed by β-lactamases to produce alpha carboxybenzyl penicilloic acid. Since its introduction for the treatment of *Pseudomonas* and *Proteus* infections (Brumfitt, Percival & Leigh, 1967), strains of ampicillin-resistant *Escherichia coli* which are also sensitive to this drug have been reported (Acred *et al.* 1967, Bodey & Terrell, 1968). Previous work from this laboratory on the location of β-lactamases in *E. coli* (Neu, 1968) suggested that carbenicillin sensitivity was related to the low level of penicillinase production of certain *E. coli* strains.

In the present investigation we have studied carbenicillin resistance in *Escherichia coli* and *Salmonella typhimurium* and related it to ampicillin resistance and surface location of penicillinase, whether that enzyme is episomally mediated or not.

Synergism between combinations of penicillins has been reported for ampicillin and penicillinase resistant penicillins such as methicillin and cloxacillin (Sutherland & Batchelor, 1964). We have therefore sought synergism between carbenicillin and the β-lactamase-insensitive penicillins in a variety of Gram-negative rods.

MATERIALS AND METHODS

Materials. Carbenicillin was obtained from Beecham Inc. Dicloxacillin and methicillin were obtained from Bristol Laboratories.

Organisms. Escherichia coli, *Pseudomonas*, *Proteus*, *Klebsiella* and *Enterobacter* strains were obtained from the diagnostic laboratories of the Presbyterian Hospital.

Salmonellae were a generous gift of Dr Winter of the Beth Israel Salmonella Centre. *E. coli* isolates had been selected on the basis of resistance to 10 μg. ampicillin discs.

Tube dilution sensitivity tests. A dilution, containing 10^4 organisms, of an overnight trypticase soy broth (BBL) culture of the test organisms was added to tubes containing dilutions of carbenicillin or ampicillin (final vol. 1·0 ml.) and incubated at 37° for 18 hr. and then observed for growth. Bactericidal levels were obtained by plating clear tubes.

Broth dilution tests for synergy. A checkerboard arrangement of test tubes was used to test the effect of pair antibiotics. Tubes were arranged in five rows of 12 tubes each. Each row contained the same concentration of penicillinase inhibitor (methicillin or dicloxacillin; 0, 100, 500, 1000 or 2000 μg./ml.) and successive tubes in an alternate row contained carbenicillin 0, 2, 4, 8, 16, 32, 64, 125, 250, 500, 1000 and 2000 μg./ml. Antibiotics were prepared fresh for each test; inoculation and incubation were as above. The first clear tube was taken as the minimum inhibitory concentration (MIC) for that organism; clear tubes were plated to obtain the bactericidal level.

Carbenicillin assay. A 12 × 12 in. glass plate was poured with 450 ml. of trypticase soy agar (BBL) seeded with 5 ml. of an overnight culture of the Elsworth pseudomonas strain. Wells of 6 mm. were punched in the agar, and 0·06 ml. of the test solution placed in the wells. The plate was incubated at 35° for 18 hr. Concentration of carbenicillin was determined using 5, 10, 20 and 40 μg. carbenicillin standards and measuring diameters in mm. of the zone of growth inhibition.

Procedure for osmotic shock. Stationary-phase organisms were harvested at 16 hr after a 1% inoculation into Penassay broth (Difco) and washed twice at 21° with 0·03 M-NaCl + 0·01 M Tris-HCl (pH 7·3). They were suspended in 0·5 M-sucrose + M Tris-HCl (pH 7.5) at 21° at 10^{10} cells/ml. EDTA was added to 1 mM and mixed by shaking for 5 min. The organisms were removed by centrifugation at 0°, and resuspended in cold distilled water with mixing for 5 min. The organisms were removed by centrifugation and the supernatant fluid assayed for β-lactamase activity.

Bacterial extracts were prepared with a Branson Sonifer Model 75 using 2 min. sonic disintegration at 0° in 15 sec. bursts. R-factors were detected by the method of Watanabe (1964). Penicillinase activity was determined using a modification of Novick's (1962) iodometric assay with benzylpenicillin G as substrate.

RESULTS

Fourteen strains of *Escherichia coli* in which the β-lactamase was released by osmotic shock, and was thus a surface enzyme (Neu & Chou, 1967), were all resistant to carbenicillin. Of 21 strains of *E. coli* in which the penicillinase was not released by osmotic shock 20 or 95% were sensitive to 25 μg./ml. or less of carbenicillin.

Escherichia coli strains which were sensitive to carbenicillin (Table 1) were resistant to less than 125 μg./ml. of ampicillin.

Table 2 is a comparison of the carbenicillin and ampicillin sensitivity of some *Escherichia coli* and *Salmonella typhimurium* strains in which the β-lactamase is either a surface or internally located enzyme. There do not appear to be significant differences in MIC levels of carbenicillin and ampicillin for either high level or low level penicillinase-producing strains. Strains of either organism in which the penicillinase is surface located showed high level resistance. When the penicillinase was an internal enzyme resistance did not exceed 250 μg./ml.

None of 15 *Escherichia coli* and 15 *Salmonella typhimurium* strains in which production of the β-lactamase is episomally mediated were sensitive to either ampicillin or carbenicillin. Several strains of *E. coli* has surface penicillinases but transfer of resistance to another strain was not possible.

Table 1. *Sensitivity of ampicillin resistant Escherichia coli to carbenicillin*

Ampicillin resistance μg./ml.	Carbenicillin resistance		Sensitive (%)
	No. tested	No. sensitive*	
10–50	18	16	90
50–250	21	19	90
> 250	14	0	0

* Sensitive to 25 μg./ml. or less of carbenicillin.

Table 2. *Comparison of carbenicillin-ampicillin sensitivity and location of penicillinase*

Escherichia coli strain	Penicillinase location	Ampicillin μg./ml.	Carbenicillin μg./ml.
DB 131	Surface	> 4000	> 4000
DB 130	Internal	3·2	3·2
DB 111	Internal	64	6·4
DB 100	Internal	250	100
DB 112	Internal	8	4
FW/R 126	Surface	> 2000	> 2000
DB 103	Surface	> 2000	> 2000
Salmonella typhimurium			
BH 19	Surface	> 8000	> 8000
BH 16	Internal	16	32
A 100	Surface	4000	4000

Table 3. *Correlation of growth rates of two strains of Escherichia coli with the destruction of carbenicillin**

ime hr)	*E. coli* DB 131 (Surface penicillinase)		*E. coli* DB 307 (Internal penicillinase)	
	o.d.	Carbenicillain % left	o.d.	Carbenicillin % left
0	0·025	100	0·025	100
1	0·130	75	0·085	85
2	0·408	33	0·200	60
3	0·748	5	0·451	50
4	1·020	0	0·785	6

* Overnight cultures were inoculated to 10^4 cells/ml. After 1 hr at 35° on a rotary shaker, 100 μg. per ml. carbenicillin was added. Growth was followed by turbidimetric change at 600 mμ. Carbenicillin was assayed (see 'Methods') on aliquots removed at noted times. DB 131 was resistant to 2000 μg. carbenicillin/ml. and DB 307 was resistant to 125 μg. carbenicillin/ml.

Variation in the size of the *Escherichia coli* inoculum from 10^3 to 10^7 organisms/ml. in the carbenicillin tube dilution did not lower the level of resistance of the high level resistant strains. Strains of *E. coli* resistant to less than 30 μg./ml. rarely showed an effect of inoculum size. Strains such as DB 108, in which the level of resistance was 63 μg./ml. at an inoculum of 10^3 organisms, rose to 200 μg/ml. with an inoculum of 10^7

organisms. Highly resistant strains of *E. coli.* or *Salmonella typhimurium* in which the penicillinase was surface located probably destroyed the carbenicillin so rapidly that the effect of inoculum size was not seen. Table 3 illustrates that the rapid growth rate of high level carbenicillin-resistant, surface penicillinase strains in the presence of carbenicillin, in contrast to these strains with lower resistance and an internal enzyme, is related to destruction of carbenicillin.

Table 4. *Test for synergy of carbenicillin and methicillin*

MIC with: Strain	Carbenicillin (μg./ml.)	Methicillin (μg./ml.)	Carbenicillin + Methicillin (μg./ml.)		
Pseudomonas a. 2	250	1000	4	+	1000
Escherichia coli 132	6	500	2	+	500
E. coli 18	4	500	0·5	+	500
Enterobacter 10	64	1000	4	+	500
Klebsiella 273	250	1000	32	+	1000
Klebsiella 272	125	500	32	+	100
E. coli 131	4000	>4000	4000	+	>4000
S. typhimurium A 100	1000	2000	1000	+	1000
Pseudomonas a. 101	8000	16000	4000	+	8000
Proteus m.	4000	>4000	4000	+	4000

Since carbenicillin is hydrolyzed by β-lactamases of most Enterobacteriaceae we attempted to lower the level of resistance of various organisms by performing the tube dilution in the presence of a penicillinase-resistant penicillin such as methicillin. Using a checkerboard method of broth dilution it is possible to determine whether combinations of antibiotics are synergistic, antagonistic or additive in effect. Using either methicillin or dicloxacillin, only 1 strain of 32 tested showed synergy. Eleven *Pseudomonas aeruginosa*, 8 *Escherichia coli*, 4 *Enterobacter*, 7 *Klebsiella* and 2 *Proteus* were tested. Table 4 demonstrates that only klebsiella 272 showed true synergy. In the few cases where the MIC of carbenicillin was lowered the MIC of methicillin in the combination was equal to the MIC of methicillin alone. Fifty per cent of these strains showed a synergistic effect of ampicillin and methicillin.

DISCUSSION

Carbenicillin, which is hydrolyzed by β-lactamases of both Gram-positive and Gram-negative bacteria, had been thought to be active against some ampicillin-resistant *Escherichia coli.* These investigations show that *E. coli* which possess surface penicillinases are resistant to carbenicillin. Not all of these strains with surface penicillinases could transfer resistance to ampicillin and carbenicillin to another strain of *E. coli.* In all of these episome strains the penicillinase was a surface enzyme. Carbenicillin is effective only against ampicillin-resistant *E. coli* in which resistance is at a low level.

It is not readily apparent why carbenicillin shows less synergist action than ampicillin when used with penicillinase-resistant penicillins such as methicillin. It is possible that the entry into the cell of carbenicillin is more rapid or that its affinity for β-lactamases is greater than that of ampicillin. There is no practical value to combinations of carbenicillin with other penicillins since concentrations from 3000 to 20,000 μg./ml. can be obtained in urine (Neu & Swarz, 1969).

Although carbenicillin is unlike ampicillin in that it is active against pseudomonas strains, its activity against members of the Enterobacteriaceae is similar to ampicillin, particularly in regard to β-lactamase producing strains.

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Components of Conidial and Hyphal Walls of *Penicillium chrysogenum*

By V. RIZZA* AND J. M. KORNFELD
Microbiology Section, Biological Sciences Group, University of Connecticut, Storrs, Connecticut 06268, U.S.A.

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SUMMARY

Cell walls were prepared mechanically from both hyphae and conidia of *Penicillium chrysogenum*. The major carbohydrate components of cell wall hydrolysates were glucose, galactose and glucosamine, with lesser amounts of mannose and rhamnose. The conidia were richer in galactose and the hyphae contained more glucosamine. The distribution of the monomers in acid-soluble and alkali-soluble fractions was different in the two kinds of cell wall; conidial walls contained higher concentrations and a greater variety of amino acids than hyphal walls.

INTRODUCTION

In contrast to the intensive investigations of the polymers constituting the cell walls of bacteria, the study of the structural macromolecules of the cell walls of filamentous fungi has been very limited. Many of these studies, dating from the last century, were restricted to histological observations (van Wisselingh, 1898; Melzer, 1924). More recent reports have been concerned with direct chemical analyses. In most cases (Hamilton & Knight, 1962; Crook & Johnston, 1962; Manocha & Colvin, 1967; Applegarth, 1967), however, analyses have been restricted to only the hyphal stage in the developmental cycle, although the acetyl content of hyphal and spore walls of *Asperigillus phoenicis* has been compared (Bloomfield & Alexander, 1967).

Penicillium chrysogenum, a filamentous fungus, possesses at least two morphologically distinct stages representing a seemingly primitive morphogenetic system. The conidia are oblate spheroids, green in colour, and possessing rigid cell walls, whereas the hyphae which develop from them are filamentous, flexible and white. These morphological differences suggest differences in chemistry. Mahadevan & Tatum (1965) have detected a number of quantitative differences in the cell walls of a variety of mutants of *Neurospora crassa*. They concluded that the morphology is a direct consequence of the cell wall composition and that changes from wild-type growth to other forms may be caused by alterations in the relative concentrations of the structural polymers of the cell wall. It seems reasonable to expect that functionally comparable structures which differ morphologically at different stages in the life cycle of an organism would also differ chemically. Because of the paucity of comparative data and to uncover basic molecular changes in the conversion of conidium to hyphal cell, studies were undertaken to determine whether the cell walls of the two stages are, in fact, biochemically distinct.

* Present address: Department of Biochemistry, University of Kentucky School of Medicine, Lexington, Kentucky, U.S.A.

METHODS

Organism and growth conditions. Penicillium chrysogenum, strain NRRL 1951-B25, was maintained on Sabouraud glucose (SG) slopes: 4% glucose, 1% 'neopeptone', 1·8% agar. After sporulation, the cultures were refrigerated.

Roux culture bottles containing SG agar were inoculated with conidia from sporulated slopes and incubated at ambient temperature (about 25°) until sporulation occurred, which was usually 4 days. Sterile water was added, and the conidia removed by scraping the surface with a sterile aluminium spatula. The conidial suspension was filtered through sterile Pyrex glass wool so that the spore harvest would be free from mycelial fragments, as verified by microscopic examination. After at least five cycles of washing and centrifugation, the conidia were suspended and maintained in cold M/15 potassium phosphate buffer (pH 7·4).

Hyphae were grown by inoculating a spore suspension into 6 l. of SG broth in a Microferm Laboratory Fermentor (New Brunswick Scientific Co., Inc., New Brunswick, New Jersey, U.S.A.). Incubation continued for 48 hr at ambient temperature under constant aeration and agitation. The hyphae were collected on filter paper and washed four times with cold distilled water.

Preparation of spore walls. Washed spores (10 g. wet weight) were suspended in 10 ml. cold phosphate buffer (pH 7·4) to which 50 g. of glass beads (0·5 mm. diameter) were added. Spores were disrupted in a Bronwill Mechanical Cell Homogenizer maintained at 5°. When disruption was essentially complete (as determined by the release of protein into the supernatant fluid and verified microscopically), homogenization was discontinued. The mixture was centrifuged at 500 *g* to separate the spore walls from the glass beads. The crude wall fraction was collected by centrifugation at 4000 *g*. The pellet was suspended in 100 volumes of distilled water and agitated on a wrist action shaker overnight at 4°. The suspension was centrifuged and washed successively with distilled water and phosphate buffer (pH 7·4) until no extinction was detectable in the supernatant at 260 mμ and 280 mμ, indicating the absence of cytoplasmic contaminants. After collection, the pellet was extracted for 1 hr in each of the following solvents: 95% (v/v) ethanol in water at 5°, three times with boiling ethanol + ether (1 + 3, v/v), and finally boiling ether. After extraction the walls were lyophilized.

Preparation of hyphal walls. Hyphae were disrupted in a Sorvall Omnimixer (Ivan Sorvall, Inc., Norwalk, Connecticut, U.S.A.) with glass beads. Purification procedures were the same as described for the preparation of spore walls.

Isolation of carbohydrates. The procedure described by Mahadevan & Tatum (1965) was adopted: 49 mg. samples of wall material were treated with 2 N-NaOH for 16 hr at room temperature, centrifuged, and the supernatant liquid treated with 2 vol. 95% (v/v) ethanol in water. The resulting precipitate was suspended in water, dialysed against water, and lyophilized; this fraction was designated F 1. The residue remaining after the treatment with NaOH was resuspended in N-sulphuric acid, incubated at 90° for 16 hr and centrifuged. The pellet was washed and the washings were added to the supernatant fluid. The supernatant fluid was adjusted to pH 7 with $Ba(OH)_2$, and the resulting precipitate was removed by centrifugation and discarded; the material in the supernatant fluid was designated F 2. The residue remaining from the sulphuric acid treatment was resuspended in 2 N-NaOH for 30 min. at room temperature and

centrifuged and 2 vol. ethanol added to the supernatant liquid. The precipitate was centrifuged down, suspended in water, dialysed against water, and lyophilized; this fraction was designated F3. The residue from the second NaOH treatment was suspended in water, dialysed, lyophilized, and designated F4. Before electrophoresis, the fractions were hydrolysed according to the procedure of Mahadevan & Tatum (1965).

Extraction of amino acids. Samples (50 mg.) of either conidial or hyphal walls were heated in 6N-HCl in evacuated tubes at 105° for 18 hr. The hydrolysates were filtered through 'Millipore' filters. The filtrates were taken to dryness by heating *in vacuo* in a hot water bath. Each hydrolysate was dissolved in 1 ml. 10% (v/v) isopropanol in water and stored at 0°.

Separation of components. Separation of amino acids was accomplished by electrophoresis with the high voltage electrophorator, Model D (Gilson Medical Electronics, Middletown, Wisconsin, U.S.A.). Hydrolysate samples or standards were applied at 5 cm. intervals parallel to the short side along an origin line drawn 12·7 cm. from the end of a 46·4 × 57·2 cm. sheet of Whatman 3 MM chromatography paper. The paper was then sprayed with a mixture of formic acid + acetic acid + water (6 + 24 + 170 by vol.) at pH 2 until wet and transferred to the electrophoresis tank with the origin line proximal to the anode. The samples were run in the same buffer at 30 V/cm., current 80 to 85 mA, for 90 min. at 10 to 12°. After electrophoresis, the paper was dried at 90° for 20 min. Neutral sugars were run in a boric acid buffer adjusted with NaOH to pH 9·4 at 240 V/cm., current 4 to 5 mA, for 150 min. at 10 to 15°. After removal from the electrophoresis chamber, the paper was dried as described above. Because of trailing, the resolution obtained with amino sugars upon direct electrophoresis was insufficient for identification or quantitation, so the samples were oxidized with ninhydrin before electrophoresis; this converted any hexosamines present to their corresponding pentoses (Stoffyn & Jeanloz, 1954). The oxidized samples were run as described for neutral sugars.

Amino acids were made visible by dipping the electrophoretograms in 1·5% ninhydrin in 95% (v/v) ethanol in water, drying at room temperature and counterstaining with a cupric nitrate solution (Mabry & Todd, 1963). Sugars were made visible with the diphenylamine-*p*-anisidine reagent (Mabry, Gryboski & Karam, 1965).

In situations where components could not be separated by electrophoresis, the electrophoretograms were cut into strips parallel to the direction of migration. The strips were sewn on to Whatman No. 1 paper and chromatographed in the second dimension with 2-butanone + propionic acid + water (75 + 25 + 30, by vol.). By this procedure, amino acids which had similar electrophoretic mobilities were separated and amino acids whose mobility properties left them within the glucosamine streak were resolved.

Identification and estimation of components. The amino acids and sugars were identified by three properties: (1) electrophoretic mobility compared with standard samples run simultaneously; (2) characteristic tints after colour development; (3) rates of colour development. Electrophoretic mobility values were also compared with published values (Mabry & Todd, 1963; Himes & Metcalfe, 1959).

Quantitative estimates were made by cutting the developed sheets into strips 3·2 cm. wide and scanning them in a Model RB Analytrol densitometer-integrator (Spinco Division, Beckman Instruments, Inc., Palo Alto, California, U.S.A.). For the amino

acids, 500 mμ interference filters were used, and for the sugars, 450 mμ interference filters. Proportionality between integration units and concentration was obtained in the range 0 to 400 μg. for the sugars and 0 to 100 μg. for the amino acids.

Nitrogen analysis. Nitrogen was determined as ammonia by the Kjeldahl-Nessler method described by Wilson & Knight (1952).

RESULTS

The distribution of monosaccharides in hydrolysates of the series of extracts from conidial and hyphal cell walls is shown in Table 1. The first alkali-soluble fraction (F 1) of conidial walls contained glucose, galactose and mannose. Hydrolysis of hyphal F 1 also gave glucose and galactose, but no mannose. Quantitatively, the two fractions differed markedly in the relative amounts of glucose and galactose. The glucose : galactose ratio of conidial F 1 was 0·32, whereas in hyphal F 1, the ratio was 7·5. The total weight of the two fractions were similar, conidial F 1 being slightly greater.

Table 1. *Distribution of monosaccharides in the fractions obtained by sequential extraction of conidial and hyphal walls of Penicillium chrysogenum*

	Conidial walls						Hyphal walls						
	F1	F2	F3	F4	Total	Mole ratio	F1	F2	F3	F4	Total	Mole ratio	Mole* ratio
	(mg./100 mg. wall)						(mg./100 mg. wall)						
Glucose	6·12	5·92	14·30	—	26·34	10·23	15·31	12·24	6·33	—	33·88	12·77	8·8
Galactose	18·80	0·61	—	—	19·41	7·54	2·04	5·10	—	—	7·14	2·70	2·7
Mannose	2·25	0·33	—	—	2·58	1·00	—	2·65	—	—	2·65	1·00	1·0
Rhamnose	—	0·31	—	—	0·31	0·13	—	0·88	—	—	0·88	0·37	0·5
Glucosamine	—	4·08	—	7·35	11·43	4·49	—	9·39	—	8·57	17·96	6·86	4·4
Residue	6·95	—	—	11·02	17·97	—	10·20	—	5·72	6·12	22·04	—	—
Total	34·12	11·25	14·30	18·37	78·04	—	27·55	30·26	12·05	14·69	84·55	—	—

* Data of Hamilton & Knight, 1962.

Both F 1 fractions were non-dialysable and could be precipitated from NaOH with ethanol or ammonium sulphate. However, the conidial F 1 was dark green, which was not the case with hyphal F 1. Despite the intense colour, no extinction bands were detectable in a scan of the material in the visible spectrum. The extinction curve obtained was suggestive of light scattering of sufficient intensity to mask any specific absorption bands. On standing at room temperature for extended periods of time, the colour passed through a dull brown colour to a mustard yellow. This colour change was accelerated by reducing agents, e.g. sodium dithionite, and reversed by hydrogen peroxide. The hyphal F 1, in contrast, did not exhibit these oxidation-reduction characteristics. The nature and function of the green pigment is unknown.

The F 2 fractions of conidial and hyphal walls contained all five detectable monosaccharides. However, they differed quantitatively, each monosaccharide being present in higher concentration in the hyphal walls. Consequently, hyphal F 2 constituted a significantly greater fraction (30%) than conidial F 2 (11%).

The two fractions F 3 and F 4 appeared to be the only homopolymeric fractions. On hydrolysis, F 3 gave only glucose, and F 4 only glucosamine. There were about the same amounts of glucosamine in the F 4 fractions of either wall. The conidial F 3 contained twice as much glucose as in hyphal F 3.

Comparison of the total amounts of each monosaccharide indicates that only galactose and glucosamine differed markedly in the two kinds of wall. The galactose

content of the conidial walls was 2·7 times that found in the hyphal walls. The hyphal walls, on the other hand, were richer in glucosamine, containing 1·6 times that found in conidial walls. Glucose was present at slightly greater concentration in the hyphal walls. Although the rhamnose content of hyphal walls was greater than in conidial walls, the concentrations in both kinds of wall was sufficiently low so that rhamnose may be described as a minor component.

Other differences in the composition of the cell walls became apparent from the distribution of the monosaccharides in the several fractions. All of the mannose of hyphal walls was in the F 2 fraction, whereas virtually all appeared in fraction F 1 of conidial walls. Although glucose was found in fractions F 1, F 2 and F 3 of both types of walls, it was concentrated in F 3 in conidial walls, whereas it was present in largest amounts in F 1 and F 2 of hyphal walls. Essentially all of the galactose of conidial walls was recovered in F 1, but in hyphal walls it was found in F 1 and in F 2, with 70% of the total galactose in F 2. Glucosamine was found at about the same concentration in F 4 of both hyphal and conidial walls. However, hyphal F 2 contained significantly more glucosamine than did conidial F 2.

Included in Table 1 are mole ratios for monosaccharide components of *Penicillium chrysogenum* hyphal walls reported by Hamilton & Knight (1962). The present results appear to be in good agreement with those data for galactose, mannose and rhamnose, but are high with respect to glucose and glucosamine. In the paper by Hamilton & Knight (1962), xylose was reported as a component of the hyphal walls; this pentose has also been reported as a trace component in *P. digitatum* and *P. italicum* (Grisaro, Sharon, & Barkai-Golan, 1968). Although the presence of xylose had been expected, we found no xylose in the hydrolysates of any fraction. Neither Crook & Johnston (1962) nor Applegarth (1967) detected xylose in hydrolysates of the closely related *P. notatum*. Since the organism used in the present work is the strain Hamilton & Knight (1962) used, it seems likely that the absence of xylose had resulted from differences in growth conditions.

Table 2. *Amino acid components of conidial and hyphal walls of Penicillium chrysogenum*

	Conidial walls		Hyphal walls	
Amino acid	(mg./100 mg. wall)	Mole ratio	(mg./100 mg. wall)	Mole ratio
Aspartic acid	3·92	1·73	0·61	3·40
Glutamic acid	2·78	1·10	0·29	1·46
Threonine	0·25	0·12	—	—
Leucine	0·83	0·38	0·22	1·17
Isoleucine	0·83	0·38	0·20	1·13
Valine	1·22	0·61	0·20	1·27
Serine	0·67	0·38	0·13	0·92
Alanine	1·51	1·00	0·12	1·00
Glycine	1·47	1·15	—	—
Arginine	1·88	0·64	—	—
Lysine	1·58	0·64	—	—
Total	16·94	—	1·77	—

The amino acid composition of the two types of wall differed not only in total amount and relative proportions, but qualitatively as well (Table 2). No tyrosine, phenylalanine, tryptophan, cysteine, methionine or histidine was detected in the conidial or hyphal walls. Furthermore, hyphal walls did not contain any glycine,

threonine, lysine or arginine, all of which were found in conidial walls. The absence of these amino acids raised the question of their possible destruction during hydrolysis. There have been reports of the degradation of aromatic and sulphur-bearing amino acids during acid hydrolysis in the presence of carbohydrate (Lugg, 1933, 1938). However, all of these absent amino acids have been recovered in acid hydrolysates of *Penicillium notatum* hyphal walls, with the exception of methionine (Crook & Johnston, 1962) and tryptophan (Applegarth, 1967). Since the carbohydrate content of *P. notatum* does not differ significantly from *P. chrysogenum*, and the conditions of hydrolysis in those studies were essentially the same as used here, it appears unlikely that the amino acids absent from the hydrolysates, with the possible exception of methionine and tryptophan, were initially present in either complex. With respect to the four amino acids found in conidial wall hydrolysates (arginine, lysine, threonine, glycine) but not found in hyphal wall hydrolysates, there is little doubt that they were not part of the hyphal wall complex. Had they been present initially in the hyphal walls, one would expect that their destruction would have occurred during hydrolysis of both conidial and hyphal walls, since both kinds of walls contain approximately the same amount of carbohydrate and were hydrolysed under identical conditions.

The amino acid data for hyphal walls are not in agreement with the values reported for hyphal walls of *Penicillium notatum* by Applegarth (1967) who found threonine, glycine, arginine and lysine as components of hyphal wall hydrolysates, the latter two as major constituents (all absent from hyphal walls in *P. chrysogenum*).

Quantitatively, the most significant difference appears in the total amino acid content of the two types of wall of *Penicillium chrysogenum*. Conidial walls appeared to contain 10 times as much amino acid as hyphal walls. The value of 1·77% for total amino acid is in good agreement with the figure of 2% suggested by Hamilton & Knight (1962) for hyphal walls of the same organism.

Table 3. *Recovery of nitrogen in hydrolysate components and in Kjeldahl digests of conidial and hyphal walls of Penicillium chrysogenum*

Calculated from components	Spore wall (μg. N/mg. wall)	Hyphal wall (μg. N/mg. wall)
Amino Acid N	24·60	1·97
Glucosamine N	8·99	14·11
Total N	33·59	16·08
Found in Kjeldahl digests of walls	36·57	17·00
Calculated/found × 100	91·8%	94·6%

When the total contributions of each of the nitrogen-containing components are summed, excellent agreement is obtained with the direct nitrogen analysis of walls (Table 3), 92% of conidial wall nitrogen and 95% of hyphal wall nitrogen can be accounted for as glucosamine and amino acids; the data support the validity of the quantitative method used, and the high recovery of nitrogen supports the contention that certain amino acids were not present in the cell walls.

DISCUSSION

The significance of any analyses of fungal cell walls, particularly in regard to amino acid composition, depends upon the purity of the preparation. The 'cleanliness' of wall preparations has been judged by a variety of criteria. Crook & Johnston (1962) considered the isolation of the walls to be complete when all materials which stained intensely with methylene blue had been removed, or, as in the cases of *Sporobolomyces roseus* and *Penicillium notatum*, when cytoplasmic particles could not be detected by examination with the electron microscope. Applegarth (1967) washed the disrupted hyphae of *P. notatum* several times, but still detected some protein contamination of the preparation by electron microscopy. He then treated the material with 0·1% trypsin for 24 hr, after which electron microscopic examination showed a 'good' wall preparation. Mahadevan & Tatum (1965) washed the disrupted hyphae of *Neurospora crassa* with water and increasing concentrations of ethanol and determined the cleanliness of the material by microscopic examination. In the present work, the cell walls were judged pure when ultraviolet absorbing material was no longer released during the series of washings described. None of these criteria, however, is wholly reliable for judging the purity of walls, since adsorbed materials would not be detected. Even if it could be demonstrated unequivocally that the wall preparations were uncontaminated by cytoplasmic material, the preparations might differ from the native complexes. In preparations treated with lytic enzymes after disruption but before hydrolysis, it is difficult to determine whether the enzyme treatment is removing contaminating substances or removing material native to the complex as well or if the enzyme has become adsorbed. An indication that the walls used in the present work were not contaminated by cytoplasmic material is that the preparations lacked several amino acids normally found in proteins. The absence of these amino acids also argues for the absence of cytoplasmic membranes if the assumption is made that fungal membranes are similar to bacterial membranes, which contain all the known amino acids (Weibull & Bergstrom, 1958; Brown, 1961; Yudkin, 1966). The alternative assumption, that fungal membranes are radically different from bacterial membranes and do not contain certain amino acids allows for the possibility of membrane contamination. This appears to be unlikely, since the data would require not only that hyphal membrane be different from conidial membrane, containing even fewer amino acids, but in addition, the amino acids present in the two types of membrane would have to be present in different proportions.

Some insight into the nature of the polymers is provided by the data. In both types of wall, the F 3 fraction is probably a glucan. A similar fraction has been isolated from hyphal walls of *Neurospora crassa* which was susceptible to the β-1,3-glucanase complex derived from Streptomyces C-3 (Mahadevan & Tatum, 1965). The finding of only glucosamine in the F 4 fractions suggests a chitinous material, as was reported for the F 4 fraction of *N. crassa*. Such components have also been reported for other fungi (Applegarth, 1967; Chattaway, Holmes & Barlow, 1968; Grisaro *et al.* 1968). The kinds of polymers in our F 1 and F 2 fractions are considerably in doubt, since these fractions are so complex. No information is available whether these multicomponent fractions represent sets of homopolymers or heteropolymers. Possibly, there might be micelles of one polymer embedded within a matrix of another polymer. Some of the polymers of *Penicillium chrysogenum* hyphal wall have been degraded enzymically

(Horikoshi, Koffler & Garner, 1961; Troy & Koffler, 1966), but enzymic digestion of conidial wall fractions has not been attempted. Intact conidia, however, have been treated in this laboratory with a variety of enzymes in an attempt to produce conidial protoplasts, but unsuccessfully. The intact conidia were resistant to the action of 'Pronase' chitinase, keratinase and the complex of enzymes derived from snail digestive juice. That conidia are refractory to snail enzyme has been corroborated by C. C. Remsen (personal communication). Hyphal walls of *Aspergillus phoenicis* are extensively digested by a mixture of purified chitinase and *B*-1, 3-glucanase, but the conidia of this organism are resistant to these enzymes, although they contain both glucose and N-acetylhexosamine in their walls (Bloomfield & Alexander, 1967).

The data in Table 1 suggest the possibility of a lamellar arrangement of wall polymers. Most of the glucose in the conidial walls was released by the second NaOH treatment, rather than the first, and thus required the acid extraction between the NaOH treatments in order to be solubilized. It seems likely, therefore, that the F 3 fraction, in which most of the glucose is located, is covered by a base-resistant material which prevents F 3 from being solubilized simultaneously with F 1. Similar arguments could be advanced for the fact that glucosamine appears in both F 2 and F 4, rather than F 2 only. Preliminary examination of thin sections of conidia by electron microscopy appear to substantiate the presence of a lamellar array. The inability to convert conidia to protoplasts, therefore, may have resulted from the absence of one or more of the enzymes necessary for the sequential hydrolysis of the several major structural polymers of the cell wall.

The differences in distribution and concentration of the sugars and amino acids clearly indicates that conidial and hyphal walls are biochemically distinct.

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The Effects of Oxygen on the Possible Repair of Dehydration Damage by *Escherichia coli*

By S. J. WEBB

Department of Bacteriology, University of Saskatchewan, Saskatoon, Saskatchewan, Canada

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SUMMARY

Proflavine in the post-aerosolization plating medium lowered by over 80% the number of viable *Escherichia coli* B/R organisms recovered from aerosols of 55% relative humidity (RH) and above, but had no effect on the recovery of viable *E. coli* $WP2_S$ organisms. Oxygen enhanced the lethal action of semi-dehydration on *E. coli* strain B/R, held at 50% RH and below, but had less effect on the survival of *E. coli* $WP2_S$. In addition, *E. coli* B/R organisms displayed an increased rate of respiration, and discharged 260 mμ absorbing material, when held at intermediate values of RH. These phenomena were not observed with *E. coli* $WP2_S$ organisms. Thus *E. coli* B/R seems to possess an energy consuming mechanism by which it is able to survive semi-dehydration above 55% RH by a discharge of damaged cell components. This mechanism is very slowly destroyed when the organisms are semi-dehydrated in nitrogen but, below 50% RH, it is rapidly inactivated by oxygen. It is suggested that the mechanism is a membrane-bound system, possibly involving the cytochrome chain.

INTRODUCTION

When bacteria are held in an aerosolized state for prolonged periods, the rate at which they lose viability depends on the quantity of water vapour and oxygen present in their gaseous environment and on the environmental temperature. Much of the relevant work done between 1920 and 1953 has been summarized by Wells (1955) and by Webb (1965). From these investigations it was concluded that one of the main factors which determines the rate of loss of viability is the partial pressure of water vapour. Between 10 and 40° the partial vapour pressure and temperature, usually expressed as a relative humidity (RH), controls the rate at which organisms die, rather than the absolute water content of the air (Wells, 1955), apparently because the RH determines the quantity of water bound to macromolecules within the cell (Webb, 1960). Most investigators have found organisms held at 25° and at relative humidities above 75% to be stable. In contrast, however, Cox (1967, 1968) found that *Escherichia coli* died rapidly when held in an atmosphere of nitrogen at relative humidities between 85 and 75%.

Oxygen appears to enhance the rate at which organisms die only when they are held at RH values below 40% (Webb, 1965, 1967; Hess, 1965), suggesting that the presence of water prevents the oxidation of cell constituents. The mechanism by which they are killed at relative humidities between 80 and 40%, therefore, appears to differ from that responsible for their death at 40% RH and below. Results are described here

which indicate that the respiratory mechanisms of bacteria are damaged by oxygen when they are held below 40% RH but that, at higher RH values, death appears to be due, in part, to the destruction of a mechanism by which some strains are able to repair the dehydration damage which otherwise would impair their ability to manufacture protein and nucleic acid.

METHODS

Organisms. (*a*) *Escherichia coli* B/R (NCIB 9485), a strain resistant to ultraviolet radiation (u.v.) due to its ability to dark repair DNA damage, and (*b*) *Escherichia coli* $WP2_s$, a tryphtophan requiring mutant of *E. coli* B/R, which is unable to perform host cell reactivation of bacteriophage, is highly sensitive to u.v. and has a reduced ability to dark repair u.v. damage (Hill, 1965; Ashwood-Smith & Bridges, 1966).

Preparation of aerosols in air. Escherichia coli, strains B/R and $WP2_s$, were grown for 24 hr at 37° in Bacto Nutrient Broth (NB), harvested and washed twice in de-ionized distilled water and resuspended in either (*a*) distilled water or (*b*) 0·3 M-raffinose or 0·3 M-myo-inositol to a concentration of 1×10^{10} bacteria/ml. These organisms were then aerosolized for 5 min. into a 800 l. stainless steel rotating drum by using a Collison spray operating at 25 p.s.i. The air was supplied from a carbon ring compressor and, before use, was passed through two bacterial filters (to remove fine particles) and two columns each of activated charcoal and silica gel. The RH value was pre-set by spraying distilled water into the drum until a given RH value had been obtained. Samples were collected from the aerosol by using a liquid impinger containing 9 ml. 0·5M-glucose in 0·85% (w/v) NaCl. Serial ten-fold dilutions then were made in nutrient broth and 0.2 ml. of each dilution plated onto nutrient agar and nutrient agar containing 5 μg. proflavine/ml. To the remainder of the initial sample 1 ml. 0·1% (w/v) casamino acids in potassium phosphate buffer (pH 6·9) was added and immediately a 2 ml. sample withdrawn. The rest was placed in an incubator at 37° and allowed to incubate for 45 min. The 2 ml. portion originally removed was passed through a Millipore filter to remove bacteria, and the same treatment was given to the incubated bacteria following the 45 min. incubation period. The difference between extinction at 260 mμ. of filtrates from the initial and the incubated samples was determined with a Beckman DB spectrophotometer.

One of the difficulties in the estimation of the respiration rate of bacteria after periods of storage in air is the rapid loss of viability encountered at low RH values. To overcome this problem the storage time in aerosols was decreased to 5 min., samples were taken as above and the organisms in each sample concentrated in 0·1 M-phosphate buffer, to 5×10^8 bacteria/ml., by centrifugation. The bacterial concentration was standardized with a Coleman nephelometer and, therefore, the bacterial count at the time the O_2 uptake measurements were made was a total count, not a viable one. Viable counts were made by serial dilution, plating on nutrient agar and counting the colonies after 24 hr of incubation. After the bacteria had been concentrated, 2 ml. portions were placed in the main chamber of a Bronwell Warburg flask and 0·5 ml. of a solution of 0·1 M-glucose + 500 μg. casamino acids/ml. placed in the side arm. Oxygen uptake was measured at 37° with double capillary manometers.

Preparation of aerosols in nitrogen. The bacterial suspensions were prepared as above, but using deaerated (boiled) distilled water. The suspension was then placed into a Collison spray and nitrogen gently bubbled through it for about 60 min. For

use in the aerosol studies the nitrogen was washed successively with (*a*) alkaline pyrogallol, (*b*) water, (*c*) N-H_2SO_4, (*d*) N-NaOH, (*e*) water, and then passed through the silica driers and charcoal columns to the aerosol drum. The drum was purged with the cleaned nitrogen for 2 hr and then a Collison spray, containing water free from dissolved oxygen, was introduced into the system. The spray was operated with nitrogen until a given RH value was reached and then replaced by the spray containing the bacterial suspension which was 'atomized' into the drum for 5 min. All other procedures were as above. The reasons for the extensive washing of the nitrogen will be evident from the text.

Measurement of release of thymidine-2-^{14}C. Bacteria of both strains were grown at 37° for 24 hr in a medium consisting of: 0·1 M-glucose, 500 μg. casamino acids/ml. and 0·8 μc. [^{14}C]thymidine (34 mc./m-mole)/ml. all in 0·1 M-phosphate buffer (pH 6·9). The [^{14}C]-labelled thymidine was purchased from New England Nuclear Corp., Boston, Mass., U.S.A. The bacteria were harvested, washed, aerosolized and collected as above. After 15 min. of storage, triplicate 10 ml. samples were taken and casamino acids (to 500 μg./ml.) and, in some cases, proflavine (to 5 μg./ml.) added to each. The samples were incubated for 45 min. at 37° and the bacteria removed by passage through Millipore filters of 0·45 μ porosity. Each filtrate was evaporated to dryness *in vacuo*, resuspended in 0·5 ml. of distilled water and placed into a 1 in. (2·54 cm.) stainless steel planchette containing a 0·75 in. (1·9 cm.) diameter filter paper disc previously glued to the bottom of the planchette. The samples were then dried under an infrared lamp and the radioactivity present determined with a Phillips Gas Flow Counter.

RESULTS

Effects of oxygen and relative humidity

The two strains of *Escherichia coli* responded differently to changes in the RH value of the atmosphere in which the organisms were held. *E. coli* B/R, both in nitrogen and in air, was considerably more stable than the WP2$_s$ strain, especially at relative humidities above 40% (Fig. 1). In addition, the ratio between the percentage recoveries of strains B/R and WP2$_s$ after 15 min. in air or nitrogen showed that the difference in stability of the two strains was greatest when the bacteria were held at intermediate humidity values (Fig. 1 *c*). Oxygen affected the survival of strain B/R to a greater extent than it did that of strain WP2$_s$. However, oxygen enhanced the death rate only at relative humidities below 50% (Fig. 1).

Effect of proflavine

Webb & Tai (1968) showed that the ability of airborne organisms to repair u.v.-induced damage was gradually destroyed when they were held at RH values below 55%. Since the two strains of *Escherichia coli* used vary in their ability to repair damage induced by u.v. radiation, it seemed possible that the added resistance of strain B/R to dehydration at RH values above 50% might reside in an ability to repair comparable damage induced by semi-dehydration. Proflavine in the plating medium, as well as related acridine dyes and caffeine, apparently inhibit the repair system (Witkin, 1963; Lieb, 1964). Following aerosolization between 55 and 75% RH proflavine in the growth medium greatly diminished the recoverable number of colony-forming organisms of *E. coli* B/R; the recovery of *E. coli* WP2$_s$ was not affected (Fig. 2).

This decrease in the number of surviving bacteria of strain B/R, due to proflavine, occurred both when the bacteria were held in air or in nitrogen at RH values between 75 to 55%. Holding them in air at RH values below 55%, however, resulted in a rapid decrease in the action of proflavine (Fig. 2 *c*, *d*).

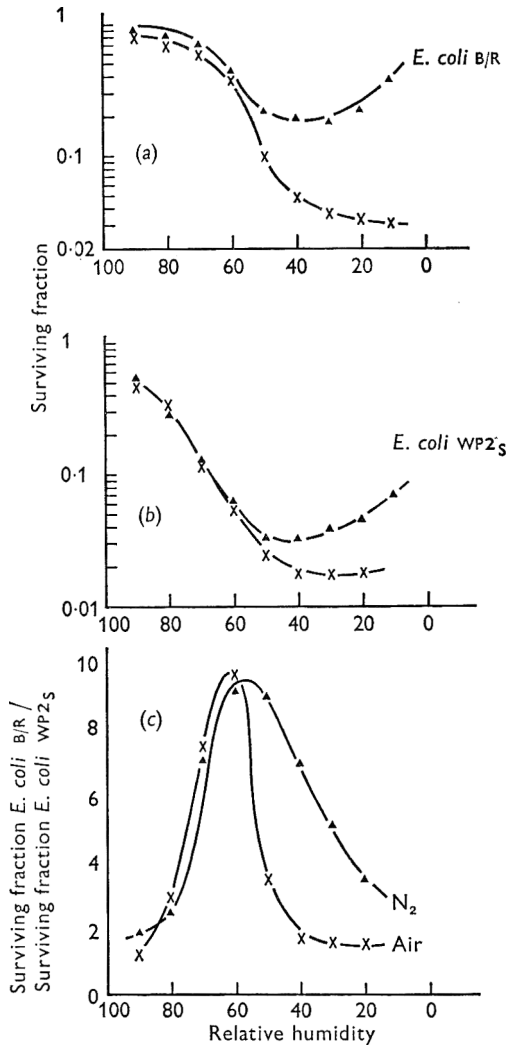

Fig. 1. The effect of relative humidity and oxygen on the survival of airborne *Escherichia coli* strains B/R and WP2s after storage for 15 min. (*a*) *E. coli* B/R (▲) bacteria stored in N_2, (×) bacteria stored in air. (*b*) *E. coli* WP2s (▲) bacteria stored in N_2, (×) bacteria stored in air.

Release of 260 mμ.-absorbing material and [^{14}C]thymidine

Since it was possible that the proflavine-sensitive function was similar to that by which the bacteria were able to repair u.v. damage, the presence of 260 mμ.-absorbing material in the filtrate of bacteria incubated after periods of storage was examined. *Escherichia coli* WP2s held at RH values between 90 and 10% and incubated for 45

min. following aerosolization showed no significant increase in 260 mμ.-absorbing material in supernatant or filtrate fluids. However, an increase in u.v.-absorbing material in the filtrate fluids of strain B/R was observed. This reached its maximum quantity after organisms had been stored at about 60% RH (Fig. 3), coinciding with both the relative sensitivities of the two strains used and the effect of proflavine in the plating medium; maximum differences between the two strains occurred when the bacteria were held at 55 to 60% RH.

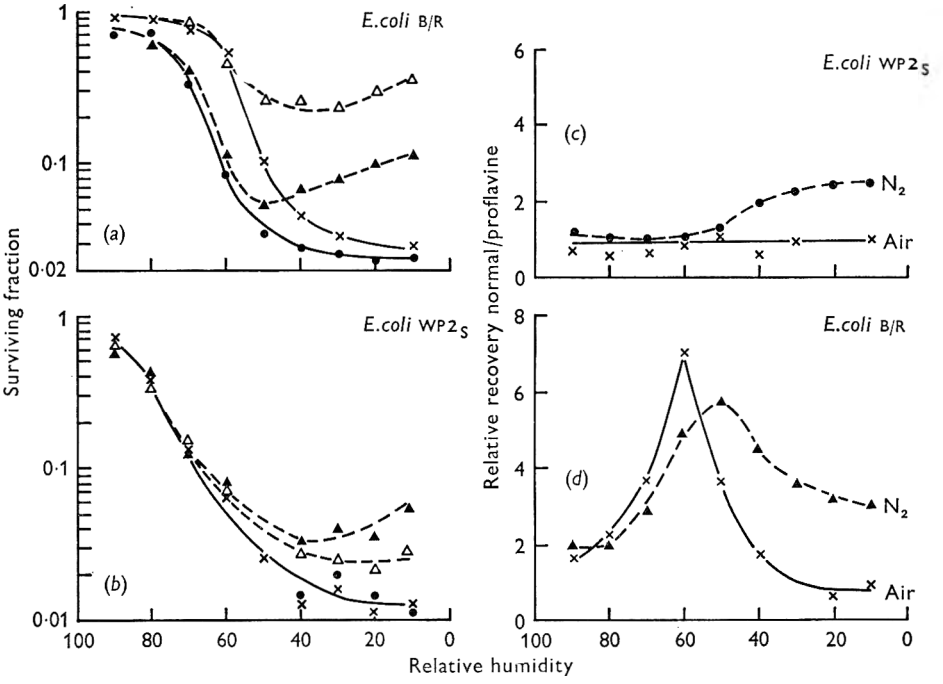

Fig. 2. The effect of relative humidity and proflavine on the recovery of *Escherichia coli* STRAINS B/R and WP2S from aerosols in N_2 and air. (*a*) *E. coli* B/R (△) bacteria held in N_2 plated on nutrient agar; (▲) bacteria held in N_2 plated on nutrient agar containing 5 μg. proflavine/ml.; (×) bacteria held in air plated on nutrient agar; (●) bacteria held in air plated on nutrient agar plus 5 μg. proflavine/ml.; (*b*) *E. coli* WP2S (▲) bacteria held in N_2 plated on nutrient agar; (△) bacteria held in N_2 plated on nutrient agar + proflavine; (●), (×) bacteria held in air and plated on nutrient agar and nutrient agar + proflavine, respectively; (*c*) *E. coli* WP2S; relative recovery = fraction surviving on nutrient agar/fraction surviving on nutrient agar plus proflavine; (*d*) *E. coli* B/R relative recovery same as for (*c*) above. N_2 and air = gas in which bacteria were held. Storage time, 15 min.

In an attempt to ascertain whether the released u.v.-absorbing material was DNA, the radioactive counts/min. from a standard number of [^{14}C]thymidine-labelled bacteria, before and after 45 min. of incubation, was determined. This procedure failed to reveal DNA loss because the quantity of radioactive material released represented considerably less than 1% of the total cell DNA and the experimental error in radioactive counting was greater than 1%. The radioactivity found in the filtrates, however, simulated their 260 mμ. extinction value; the amount present in filtrates from *Escherichia coli* B/R suspensions was greatest after storage at 60% RH and decreased as the RH was decreased, especially when the organisms were held in air.

A similar trend was found with strain WP2s, but the quantity of released material was small. Proflavine decreased the radioactivity in filtrates of *Escherichia coli* B/R, but not that in *E. coli* WP2s filtrates (Table 1). It appeared, therefore, that the survival of *E. coli* B/R at RH values above 60% depended on its ability to reject damaged material.

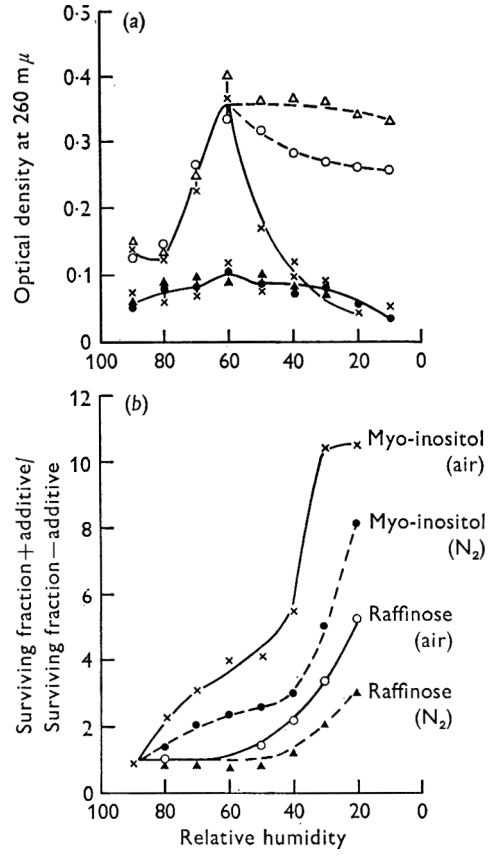

Fig. 3. The survival and release of 260 mμ.-absorbing material from *Escherichia coli* strains B/R and WP2s after being held for 15 min. in aerosols of various relative humidities with protective additives. (*a*) The release of 260 mμ.-absorbing material from bacteria held in air for 15 min. *E. coli* B/R aerosolized from a suspension in 0·3 M-myo-inositol (△), 0·3 M-raffinose (○), H_2O (×); *E. coli* WP2s aerosolized from a suspension in 0·3 M-myo-inositol (▲), 0·3 M-raffinose (●), H_2O (×); (*b*) ratio of the surviving fractions of *E. coli* B/R obtained from aerosols in air and N_2 in the presence of myo-inositol and raffinose.

Effect of protective compounds

To examine the above possibility, the ability of two compounds to preserve the mechanism by which damaged material was discharged from the organism was examined. Myo-inositol, a compound able to enter the cell, and raffinose, a compound unable to permeate through the cell membrane, were both able to preserve this mechanism at all relative humidities studied (Fig. 3). There was, however, a difference in the ability of these two compounds to preserve the viability of the organisms. Myo-inositol afforded protection to bacteria held either in nitrogen or in air at all the RH values studied. Raffinose, on the other hand, offered little protection to organisms held

at high RH value, but displayed a high protective ability at the low RH values (Fig. 3 *b*). This particular finding with raffinose agrees with the observations of Cox (1968). Raffinose afforded *Escherichia coli* strain WP2$_S$ organisms only a small measure of protection at low relative humidities when they were held in either air or nitrogen and the protection afforded them by myo-inositol was not as great as that afforded to *E. coli* strain B/R organisms.

Table 1. *The influence of relative humidity on the release of* [^{14}C]*thymidine from Escherichia coli after storage in aerosols for 15 min.*

E. coli strain B/R and WP2$_S$ were held for 15 min. in N_2 or air at set values of relative humidity (RH), resuspended in glucose + casamino acids with (†) and without (*) 5 μg. proflavine/ml., incubated at 37° for 45 min. and the bacteria removed by Millipore filtration. The filtrates were evaporated *in vacuo*, the solids dissolved in 0·5 ml. water, deposited into stainless steel planchettes, dried under an infrared lamp and radioactive counts determined with a gas flow counter. The numbers presented are the radioactive counts/min. from 10 ml. of culture filtrate and are the averages of six independent determinations.

Strain	Holding gas	Non-dried control	Holding aerosol relative humidity					
			30	40	50	60	70	80
			radioactivity (counts/min.)					
B/R	Air	*21	26	101	214	621	533	241
	N_2	*14	246	543	510	640	430	284
	N_2	†16	44	61	66	88	43	51
	Air	*16	22	19	31	21	33	69
WP2$_S$	N_2	*14	42	53	71	92	66	48
	N_2	†21	48	62	59	74	76	52

Effects on respiration

The rate at which *Escherichia coli* B/R consumed oxygen increased after they had been stored in air or nitrogen at relative humidities between 80 and 55%. At RH values below this, however, storing the organisms in air substantially decreased the respiration rate, but storage in nitrogen did not. *E. coli* WP2$_S$, on the other hand, displayed no increase in respiration rate but a decrease, which seemed to parallel the loss in viability (Fig. 4 & Table 2).

Influence of N_2 purity

Throughout these investigations differences seemed to exist between the behaviour of the two strains of *Escherichia coli* used and that of *E. coli* K12 as reported by Cox (1968). Both of the strains used here were stable at relative humidities above 80%, which contrasted with the behaviour of the *E. coli* K12 strain used by Cox, which apparently is unstable in aerosols at high RH values. The reasons for this discrepancy are not understood, but such effects may result from traces of gaseous toxic materials in the local air emanating from newly painted laboratories, the use of laboratory insecticides, and from the presence of toxic materials in crude nitrogen. Often traces of these toxic materials cause a tendency to high death rates at high relative humidities (Webb, 1965). The effects of using the nitrogen directly from the supply tank and of washing through alkaline pyrogallol only are shown in Fig. 5. When the bacteria were held in aerosols generated with the crude unwashed nitrogen they tended to die more

rapidly in atmospheres of 70 to 80% RH. This particular effect was greatly enhanced when the nitrogen was washed through alkaline pyrogallol only; presumably small amounts of pyrogallol in the nitrogen led to the high death rates.

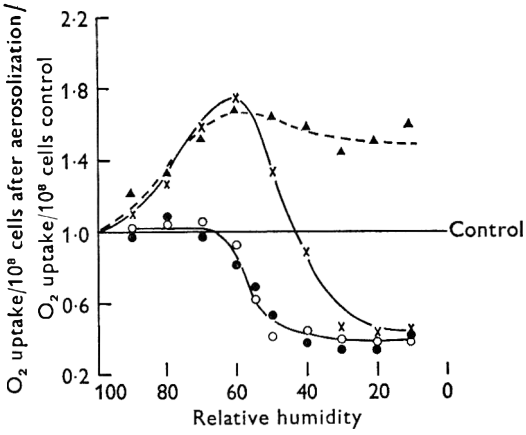

Fig. 4

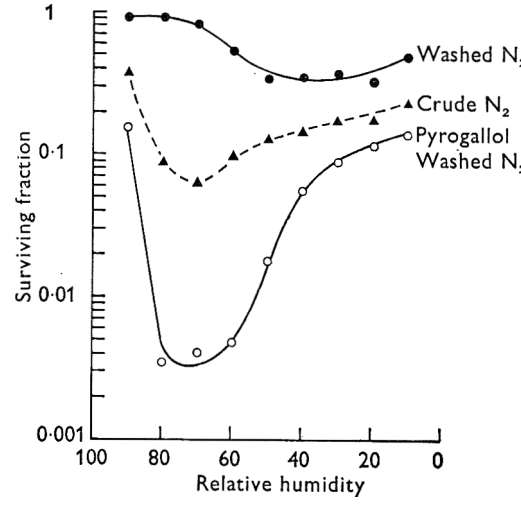

Fig. 5

Fig. 4. The relative rate of glucose oxidation by *Escherichia coli* strains B/R and WP2$_S$ after storage in air or N_2 for 15 min. *E. coli* B/R in N_2 (▲), in air (×); *El coli* WP2$_S$ in N_2 (●), in air (○).

Fig. 5. The effect of the N_2 washing procedure on the deaths of *E. coli* B/R held for 15 min. in aerosols at various relative humidities.

Table 2. *The influence of relative humidity and storage gas on the uptake of O_2 by Escherichia coli after 5 min. in aerosols*

After aerosolization the bacteria were resuspended in 0·1 M-potassium phosphate buffer to a total bacterial concentration of 5 × 10^8/ml. The number was determined by nephelometry. Triplicate samples of 2 ml. were used to measure O_2 uptake in a glucose + casamino acids medium (A) and 1 ml. portions for viable counts on nutrient agar (B). A, O_2 uptake μl./hr; B, % recovery.

		Non-dried control		Holding aerosol relative humidity											
				30		40		50		60		70		80	
Strain	Storage gas	A	B	A	B	A	B	A	B	A	B	A	B	A	B
B/R	Air	58	100	18	21	44	38	76	61	104	81	87	88	73	96
	N_2	53	100	74	63	81	76	88	79	93	88	86	89	61	94
WP2$_S$	Air	56	100	17	22	18	24	16	29	31	48	62	74	61	86
	N_2	54	100	16	30	17	35	24	40	36	59	59	68	58	81

DISCUSSION

It seems clear that the survival of these two strains of *Escherichia coli* in aerosols of above 50% RH is independent of the presence of oxygen. The greater sensitivity of *E. coli* WP2$_3$ over *E. coli* B/R to semi-dehydration at RH values above 60% seems to be due, in part, to an inability of the latter strain to carry out some kind of repair involving the elimination of 260 mμ.-absorbing material from the cell. *E. coli* B/R, on the

other hand, seems capable of overcoming damage produced at high relative humidities by some mechanism which is inhibited by the presence of proflavine in the plating medium. The increased respiration of *E. coli* B/R organisms after a period of storage above 60% RH, a phenomenon which does not occur with *E. coli* $WP2_s$, suggests that the mechanism requires energy. Moreover, part of the protective action of both raffinose and myoinositol appears to be to prevent the destruction of this particular mechanism in *E. coli* B/R cells. The fact that both the increased respiration and the release of u.v.-absorbing and [^{14}C]thymidine labelled material from the organisms rapidly decreases when they are held in air below 50% RH and, at this particular RH value, oxygen begins to have a lethal action, all suggests that at low RH values the repairing mechanism is inactivated by oxygen. The ability of raffinose to preserve the integrity of this system when the bacteria are held at low RH values, plus the apparently necessary enhanced respiration, suggests that the mechanism of dehydration repair is controlled by some function of the cytoplasmic membrane.

Bacteria do not possess mitochondria and the mitochondrial function may be exercised by the cytoplasmic membrane. In addition, portions of the bacterial chromosome are known to be in contact with the cytoplasmic membrane at various places. It is possible, therefore, that a compound unable to enter the cell, but able to penetrate partially into the cytoplasmic membrane, would be able to preserve the integrity of that portion of the membrane system involving energy production and the possible repair of those segments of the bacterial chromosome associated with the membrane. The release of u.v.-absorbing materials from organisms held in aerosols at high RH values and those protected by myo-inositol at low RH values has been reported earlier (Webb, 1965). The liberation of this material from the bacteria has been found to be associated with survival rather than with death. To the author's knowledge, however, this release of material has not been suggested to be characteristic of particular strains. The nature of this repair system remains to be elucidated, but it is possibly the same mechanism as that responsible for the 'dark' repair by u.v.-induced damage. Because oxygen seems to be required to inactivate the system, some easily oxidizable cellular constituent appears to be involved in the repair mechanism. These types of compound are involved also in the respiratory systems of the organism which seem to be stimulated during the process of repair. Such considerations, along with the diminished respiration of the organisms after storage in air of low relative humidities, points to death resulting from the inactivation at low RH values of some part of the mitochondrial or cytochrome system which, in some strains, is able to repair semi-dehydration damage. The apparent action of oxygen on this repair mechanism may also explain why oxygen seems to play no role in the inactivation of airborne viruses (deJong & Winkler, 1968). Certain experimental procedures, such as the exposure of bacteria to 365 mμ. light (Kelner, 1949), holding them in water or at elevated temperatures (Stein & Meutzner, 1950) will induce some cells to repair u.v. damage of DNA. Whether or not these procedures stimulate the operation of parts of a single metabolic process is uncertain; however, all of them, including the 'dark' repair of u.v. or semi-dehydration damage, seem to rely on an enhanced activity of the cytochrome chain produced either by an increase in respiration rate or the absorption of 365 mμ. light by the quinones or flavenoid pigments of this chain. The finding that some bacteria possess a system by which they are able to overcome semi-dehydration damage presents the interesting possibility that those repair systems able to cope with damage due to 254 mμ. u.v.

radiation, a wavelength not present in sunlight, may have evolved in response to other physical stresses such as semi-dehydration and extremes of temperature. Indeed, in some cases, these systems now may have evolved to a point where the continuity of biological life cycles, especially the germination of spores or seeds, depends on the activation of the system through exposure to periods of semi-dehydration, extremes of temperature and even sunlight itself (Borthwick *et al.* 1952).

The apparent difference between the behaviour at 80% RH of the two strains of *Escherichia coli* used here and that used by Cox (1968) may be due to the condition of the air existing in respective laboratories or the purity of the nitrogen used. Alternatively, the strain used by Cox may lack a repair system or its prophage may be a defective one. The latter is suggested since semi-dehydration at RH values above 70% will induce some prophages and, should the prophage be a defective one or made defective by the dehydration process, simple plaque counts would not detect induction (Webb & Dumasia, 1967*a*, *b*).

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Incorporation of Cholesterol by Membranes of Bacterial L-phase Variants

WITH AN APPENDIX

On the Determination of the L-phase Parentage by the Electrophoretic Patterns of Cell Proteins

By S. RAZIN AND ZEHAVAH SHAFER

Department of Clinical Microbiology, The Hebrew University-Hadassah Medical School, Jerusalem, Israel

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SUMMARY

L-phase variants of *Streptobacillus moniliformis* and *Proteus mirabilis* resembled mycoplasmas in their ability to incorporate considerable amounts of exogenous cholesterol into their cell membranes. Much smaller quantities were incorporated by L-phase variants of *Streptococcus pyogenes* and *Staphylococcus aureus*, by the parent bacteria of all L-phase variants except *S. moniliformis*, and by a series of wall-covered bacteria, including their protoplasts and spheroplasts. Cholesterol-binding ability corresponded roughly to sensitivity to growth inhibition and lysis by digitonin. Like mycoplasma, the *S. moniliformis* L-phase had a non-enzymic cholesterol uptake mechanism, but its growth in a cholesterol-free medium was not improved by adding cholesterol. It is concluded that a bacterium, on transforming to its stable L-phase, does not necessarily develop the ability to bind cholesterol; if it does, the cholesterol bound is not essential for growth of the L-phase, unlike cholesterol-requiring mycoplasmas.

INTRODUCTION

Cholesterol, though an important constituent of membranes of eucaryotic organisms (Rothfield & Finkelstein, 1968), is not usually found in microbial membranes (Fiertel & Klein, 1959). Most mycoplasma species form an exception: cholesterol, supplied by the growth medium, is an essential component of their cell membrane (Razin, 1969). They also differ from other bacteria in that, like the stable L-phase variants of bacteria, they have no cell wall. As early as 1941 Partridge & Klieneberger reported the presence of large quantities of cholesterol in *Streptobacillus moniliformis* L-phase (L-1) cultures. Later, cholesterol originating from serum in the growth medium was detected in the L-phase of *Proteus mirabilis* (Rebel, Bader-Hirsch & Mandel, 1963) and in the L-phase of *Agrobacterium tumefaciens* (Santaolalla, 1966). Cholesterol was located in the cell membrane of the *S. moniliformis* L-phase by Razin & Boschwitz (1968).

The large quantities of cholesterol in L-phase membranes raise the question: is the transformation of a bacterium to its stable L-phase directly connected with the

microbial plasma membrane developing ability to incorporate cholesterol? Cholesterol has a stabilizing effect on artificial phospholipid+cholesterol membranes (Willmer, 1961; De Gier, Mandersloot & Van Deenen, 1969) and also on mycoplasma membranes (Razin, 1967). A fair hypothesis was that a capacity to integrate cholesterol into the microbial plasma membrane made for better adaptation to growth without a protective cell wall. To establish whether there is a connexion between cholesterol incorporation and L-phase transformation, cholesterol uptake was tested in a series of bacteria, protoplasts, spheroplasts, L-phase variants and their presumed parent bacteria and experiments were made to find whether the cholesterol, if in fact incorporated, was an integral part of the cell membrane and essential to the growth of the organism after it had lost its cell wall. The findings of Edward (1953), Smith & Rothblat (1960, 1962) and Rebel *et al.* (1963), which furnish considerable information on the matter, have thus been extended and re-evaluated.

METHODS

Organisms. Streptobacillus moniliformis ATCC 14647, *S. moniliformis* L-phase (L-1) ATCC 14075, *Proteus mirabilis* (strain 9) ATCC 14273, *P. mirabilis* L-phase (strain 'L 9') ATCC 14168, *Staphylococcus aureus* (Smith) ATCC 19636, and *S. aureus* L-phase (Smith L form) ATCC 19640 were obtained from the American Type Culture Collection (Rockville, Md. U.S.A.) through the courtesy of Dr R. G. Wittler. *Streptococcus pyogenes* and its derived stable L-phase variant were obtained from Dr C. Panos (Albert Einstein Medical Centre, Philadelphia, Penn., U.S.A.). *Mycoplasma mycoides* var. *capri* (PG 3) was obtained from Dr D. G. ff. Edward (The Wellcome Research Laboratories, Beckenham, Kent). *M. laidlawii* (oral strain) was isolated in our laboratory and *M. gallisepticum* (A 5969) was provided by Dr M. E. Tourtellotte (Department of Animal Diseases, The University of Connecticut, Storrs, Conn. U.S.A.). *Bacillus megaterium* (KM) and *Sarcina lutea* were the gift of Dr C. Weibull (Lund University, Sweden). All other bacteria were taken from our departmental collection.

Growth conditions. The organisms were grown in a modified Edward medium (Razin, 1963) containing 3% (v/v) horse serum instead of Difco-PPLO serum fraction. For growth of the L-phase variants of the Gram-positive bacteria and their presumed parents NaCl was added to the medium to 3% (w/v). For satisfactory growth of *Streptobacillus moniliformis* and the L-phase variant of *Streptococcus pyogenes* the concentration of the serum in the medium had to be increased to 10% (v/v). The organisms were grown at 37° in 0·5 l. volumes dispensed in 1 to 2 l. flasks. The mycoplasma and L-phase cultures were incubated statically, while all other bacterial cultures were shaken. The organisms were harvested after 24 to 48 hr of incubation by centrifugation at 23,000 *g* for 20 min. Since growth of the L-phase of *Staphylococcus aureus* was slow it was usually harvested after 72 hr of incubation. The sedimented organisms were washed twice in 0·5M-NaCl at 4°. Cell protein was determined according to Lowry, Rosebrough, Farr & Randall (1951) with crystalline bovine plasma albumin as standard.

Preparation of protoplasts and spheroplasts and isolation of cell membranes. Protoplasts of *Micrococcus lysodeikticus* and *Sarcina lutea* were prepared by using lysozyme (Gilby & Few, 1960) and protoplasts of *Bacillus megaterium* were obtained by the

method of Miller (1963). Spheroplasts of *Escherichia coli* were prepared by the combined action of lysozyme and EDTA (Birdsell & Cota-Robles, 1967). The mycoplasma and L-phase membranes were isolated by osmotic lysis of the organisms as described previously (Razin, Morowitz & Terry, 1965; Razin & Boschwitz, 1968). The membranes were washed twice in 0·01M-phosphate buffer (pH 7·5) containing 0·05M-NaCl.

Cholesterol uptake by growing organisms. The organisms were grown in 500 ml. volumes of Edward medium supplemented with 0·2 μc of either [4-^{14}C]cholesterol (specific activity 55·8 mc/m-mole) or cholesteryl-[1-^{14}C]oleate (specific activity 10·7 mc/m-mole) (purchased from The Radiochemical Centre, Amersham, England). The organisms were harvested and washed as described above and their lipid extracted twice with chloroform + methanol (2 + 1, by vol.) first for 1 hr and then overnight at 4°. Radioactivity measurements showed that this extraction removed all the radioactive cholesterol or cholesteryl oleate from the organisms. Cholesterol and cholesteryl esters in the lipid extracts were separated by thin-layer chromatography and estimated by the $FeCl_3$ reaction and by radioactivity measurements (Argaman & Razin, 1965). Correlation between the two methods was good; in most experiments the radioactivity technique was used. Radioactivity was determined in a Packard Tri-Carb liquid scintillation spectrometer using 0·3% (w/v) 2,5-diphenyloxazole and 0·01% (w/v) 1,4-bis-2-(5-phenyl-oxazolyl)-benzene in toluene as the scintillation liquid. The efficiency of the ^{14}C counting was 80%.

Cholesterol uptake by washed organisms and cell membranes. A mixture of 2 ml. cell or membrane suspension (containing 2 mg. protein), 0·5 ml. horse serum containing 0·05 μc of labelled cholesterol or cholesteryl oleate, and 17·5 ml. of 0·5 M-NaCl in 0·067M-phosphate buffer (pH 7·5) was shaken in a 37° water bath; 2 ml. samples were withdrawn at various times and passed through membrane filters (type HA, 0·45 μ pore size, Millipore Corp, Bedford, Mass., U.S.A.). Filtration was by suction, through application of a negative pressure of 60 mm. of mercury. The filters were washed with 30 ml. of a cold 0·5M-NaCl solution containing 0·067M-phosphate buffer (pH 7·5) and 3% (v/v) horse serum. The filters were air-dried and their radioactivity determined. Mixtures without cells or membranes, which served as controls for the non-specific adsorption of radioactive cholesterol or cholesteryl oleate to the filters, showed very low radioactivity values.

Cholesterol exchange. The method of Rothblat *et al.* (1966) was used in a modified form. The organisms were grown in Edward medium containing [4-^{14}C]cholesterol, washed twice and suspended in the uptake mixture described above which contained [1α-^{3}H]cholesterol (specific activity 500 mc/m-mole, The Radiochemical Centre, Amersham, England) instead of [4-^{14}C]cholesterol. The suspension was shaken in a 37° water bath and 2 ml. samples withdrawn at various times and filtered. The filters were assayed for both ^{14}C and ^{3}H by liquid scintillation spectrometry. The efficiency of ^{14}C counting was 54% and that of ^{3}H was 20%.

Inhibition of growth and lysis of micro-organisms by digitonin. Inhibition of growth was tested in Edward medium containing 3% (v/v) horse serum. An ethanolic solution (0·5 ml.) of digitonin (40 mg./m .) was added to 9·7 ml. of Edward medium without serum. The ethanol was then e aporated by boiling the medium, and 0·3 ml. horse serum was added after cooling Serial two-fold dilutions of this medium were made in 5 ml. quantities of the serum-containing Edward medium. Each test-tube received 0·1 ml. of a young culture of the test organism and the tubes were incubated at 37°. Lysis

by digitonin was examined in test-tubes containing different concentrations of digitonin in 3 ml. of 0·5M-NaCl. Each test-tube received 0·1 ml. of a washed suspension of organism and the extinction was read at 500 mμ after 30 min. incubation at 37°.

Table 1. *Uptake of cholesterol by growing or washed organisms and their sensitivity to digitonin**

Organism	[4–^{14}C]cholesterol uptake (counts/min./mg. cell protein)		Minimal concentration of digitonin inhibiting growth§ (μg./ml.)
	Growing organisms†	Washed organisms‡	
Streptobacillus moniliformis			
L-phase	3515	19,820	15
Bacterial phase	1674	17,000	60
Proteus mirabilis			
L-phase	2032	3200	120
Bacterial phase	152	250	> 2000
Streptococcus pyogenes			
L-phase	148	1115	1000
Bacterial phase	161	1100	> 2000
Staphylococcus aureus			
L-phase	442	195	120
Bacterial phase	541	540	> 2000
Mycoplasma gallisepticum	4372	3180	30
M. mycoides var. *capri*	2154	5530	30
M. laidlawii	1224	2050	500
Diplococcus pneumoniae	627	ND	500
Mycobacterium phlei	90	125	> 2000
Corynebacterium diphtheriae	612	1725	> 2000
Pseudomonas aeruginosa	37	1500	2000
Escherichia coli K 12			
Bacterial phase	102	1430	> 2000
Spheroplasts	62	1500	ND
Bacillus megaterium			
Bacterial phase	9	1360	> 2000
Protoplasts	11	1400	ND
Micrococcus lysodeikticus			
Bacterial phase	102	1745	> 2000
Protoplasts	74	1520	ND
Sarcina lutea			
Bacterial phase	15	1260	> 2000
Protoplasts	8	1630	ND

* Each result represents the average of 3–5 experiments.

† Grown in Edward medium containing 0·2 μc of [4–^{14}C]cholesterol/500 ml. of medium.

‡ Uptake determined after 3 hr incubation at 37° in a reaction mixture containing [4–^{14}C]cholesterol as described in Methods.

§ Tested in Edward medium containing 3% (v/v) horse serum. Results read after 48 hr incubation at 37°. *S. moniliformis* and *S. pyogenes* bacterial and L-phase organisms were tested in Edward medium containing 10% (v/v) horse serum.

ND, not done.

RESULTS

Incorporation of cholesterol by growing organisms. The L-phase variants of the Gram-negative bacteria resembled mycoplasmas in their marked ability to incorporate cholesterol from the growth medium (Table 1). The L-phase of *Proteus mirabilis*

incorporated far more cholesterol than its parent bacterium, while in *Streptobacillus moniliformis* the difference was less pronounced. Phase-contrast microscopy of *S. moniliformis* cultures showed both rods and spherical forms resembling those of the L-phase variant. The percentage of the spherical forms was very variable. The ability of the L-phase variants of the Gram-positive bacteria to incorporate cholesterol was low and resembled that of their presumed parents and other wall-covered bacteria investigated. Cholesterol uptake by wall-covered bacteria was generally low, but somewhat higher in *Diplococcus pneumoniae*, *Corynebacterium diphtheriae* and *Staphylococcus aureus* (Table 1). When the cell wall of bacteria grown with [4-^{14}C]-cholesterol was completely or partly removed at least half of the radioactivity remained in the resulting protoplasts or spheroplasts (Table 1). Chromatographic analysis of the extracted cell lipids showed that in most organisms grown with [4-^{14}C]cholesterol, it was incorporated unchanged, except in *Mycobacterium phlei* and *Escherichia coli*, where 23 to 50% was esterified.

Table 2. *Uptake of cholesterol by L-phase and mycoplasma membranes*

Membranes	Incubation time (min.)	[4-^{14}C]cholesterol uptake (counts/min./mg. membrane protein)
L-phase of *S. moniliformis*	0	700
	60	13,100
	180	19,400
	240	23,200
L-phase of *P. mirabilis*	0	30
	60	1050
	180	2000
	240	3400
L-phase of *S. pyogenes*	0	300
	60	800
	180	1600
	240	1600
M. laidlawii	0	80
	60	700
	180	2020
	240	3300

Incorporation of cholesterol by washed organisms and cell membranes. Generally, cholesterol uptake by washed organisms resembled that of growing organisms, but washed organisms of *Streptobacillus moniliformis* L-phase incorporated cholesterol much better than all the other organisms tested, including the mycoplasmas (Table 1). The wall-covered bacteria and their protoplasts and spheroplasts bound only small amounts of cholesterol, and the uptake usually was instantaneous. Cholesterol uptake of the L-phase variants of *S. moniliformis*, *Proteus mirabilis* and the mycoplasmas depended on time (Fig. 1). *S. moniliformis* L-phase incorporated cholesterol faster than any of the mycoplasmas, and usually reached saturation after 1 hr (Fig. 1). Table 2 shows that isolated L-phase and mycoplasma membranes incorporated cholesterol; the membranes of the *S. moniliformis* L-phase showed the highest values. Growing L-phase variants of *S. moniliformis*, *P. mirabilis*, *M. laidlawii* and *M. mycoides* var. *capri* incorporated much less cholesteryl oleate than free cholesterol (Table 3).

Growth inhibition and lysis by digitonin. The sensitivity of organisms to growth inhibition by digitonin roughly paralleled their ability to incorporate cholesterol. Thus,

Streptobacillus moniliformis L-phase and the parasitic mycoplasmas were the most sensitive, while the other L-phase variants and *Mycoplasma laidlawii* were less sensitive (Table 1). All the wall-covered bacteria except *Diplococcus pneumoniae* were essentially resistant to digitonin. The organisms whose growth was inhibited by digitonin were also lysed by it (Fig. 2).

Factors influencing cholesterol uptake. Streptobacillus moniliformis L-phase, having the highest cholesterol uptake, was chosen for this part of the study. Cholesterol

Table 3. *Uptake of cholesteryl oleate by growing organisms compared to free cholesterol uptake*

Organism*	Radioactivity (counts/min./mg. cell protein)	
	Cholesteryl-[1-^{14}C]oleate uptake	[4-^{14}C]cholesterol uptake
L-phase of *S. moniliformis*	234	3095
L-phase of *P. mirabilis*	98	1170
S. pyogenes		
L-phase	53	97
Bacterial phase	75	97
M. mycoides var. *capri*	192	1987
M. laidlawii	14	1607
M. lysodeikticus	10	77
M. phlei	4	92

* The organisms were grown in Edward broth containing 0·2 μc of either cholesteryl-[1-^{14}C]-oleate or [4-^{14}C]cholesterol/500 ml. medium.

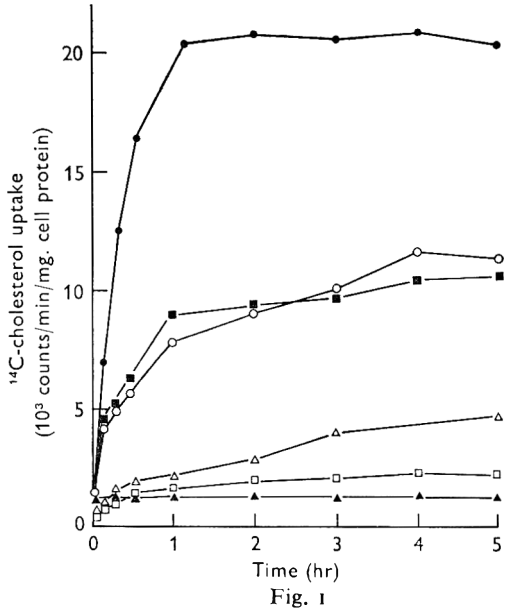

Fig. 1

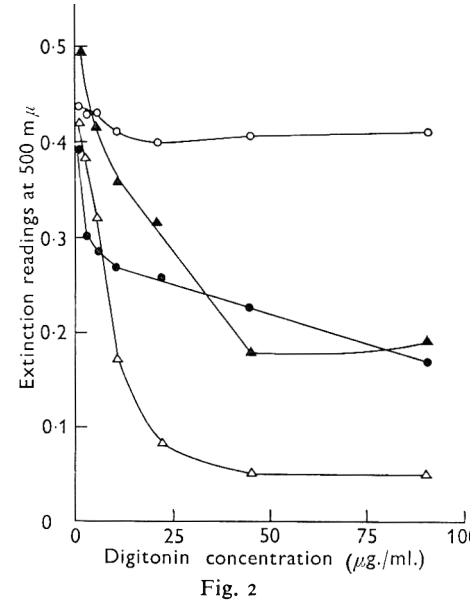

Fig. 2

Fig. 1. Cholesterol uptake by washed organisms of the L-phase of *Streptobacillus moniliformis* (●); the L-phase of *Proteus mirabilis* (■); *Mycoplasma mycoides* var. *capri* (○); *Mycoplasma gallisepticum* (△); *Mycoplasma laidlawii* (□); *Escherichia coli* (▲).

Fig. 2. Lysis of washed organisms by digitonin. L-phase of *Streptococcus pyogenes* (○); L-phase of *Proteus mirabilis* (▲); L-phase of *Streptobacillus moniliformis* (●); *Mycoplasma laidlawii* (△).

uptake of washed organisms depended on temperature (Fig. 3), but after heating at 60° for 30 min. they still incorporated about two-thirds of the amount incorporated when unheated. The decrease was apparently due to the organisms aggregating when heated so that the cell surface available for cholesterol binding was smaller. Within the range of pH 5·0 to 9·0 the pH value had little effect on the cholesterol uptake. At more acid pH values the serum proteins carrying the cholesterol precipitated and the organisms agglutinated, decreasing the amount incorporated. Cholesterol uptake by

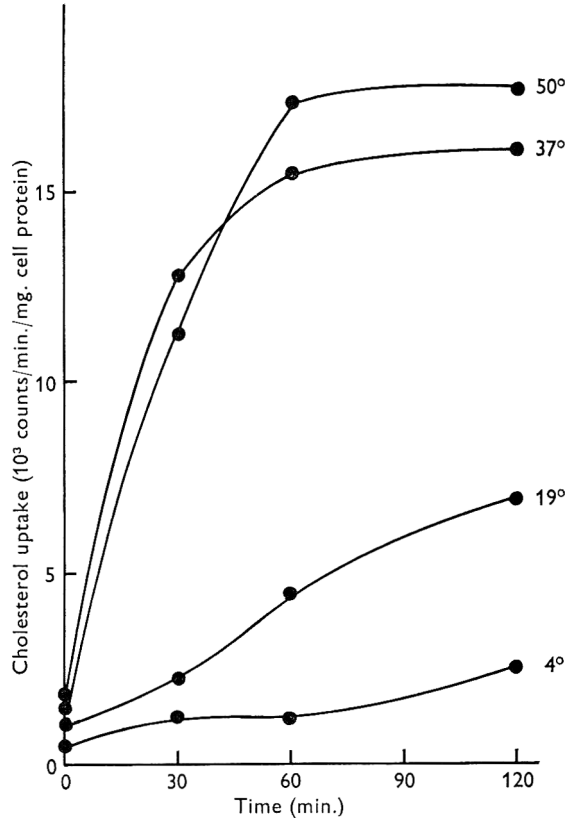

Fig. 3. Temperature dependence of the cholesterol uptake by washed organisms of the *Streptobacillus moniliformis* L-phase.

washed organisms of the *S. moniliformis* L-phase, *Mycoplasma gallisepticum* and *Streptococcus pyogenes* was unchanged when the NaCl concentration in the mixture used for measuring uptake was changed from 0 to 0·5 M, but was lower when Tween 80 was used as emulsifier and carrier of cholesterol instead of serum.

The cholesterol incorporated was firmly bound to the cell components. During incubation of the *Streptobacillus moniliformis*-phase, prelabelled with [4–^{14}C]cholesterol, in a fresh medium containing [1α–^{3}H]labelled cholesterol very little ^{14}C was lost but ^{3}H was incorporated (Fig. 4). The results with *Mycoplasma gallisepticum* were essentially the same.

The effect of cholesterol on the growth of the Streptobacillus moniliformis L-phase. Since the *Streptobacillus moniliformis* L-phase resembles the parasitic mycoplasmas

in its ability to incorporate appreciable amounts of cholesterol into its membrane (see also Razin & Boschwitz, 1968) we re-examined the role of cholesterol in the growth of this L-phase variant. The *S. moniliformis* L-phase could not be grown in the partially defined medium of Razin & Cohen (1963) suitable for *Mycoplasma laidlawii* growth, even when this was supplemented with yeast extract. It could, however, be grown in Edward broth in which the serum component had been replaced by 0·01% (w/v) Tween 80 + 1% (w/v) bovine serum albumin fraction V. No cholesterol could be detected by chromatographic analysis in the lipid (98 mg.) extracted from the constituents of this serum-free medium (sufficient to make up 200 ml.). Figure 5 shows that

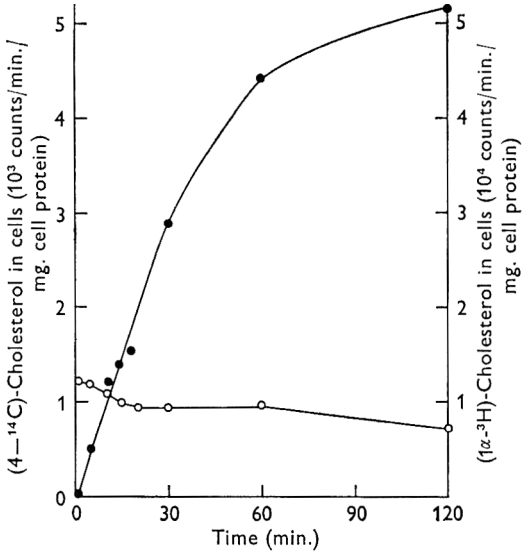

Fig. 4

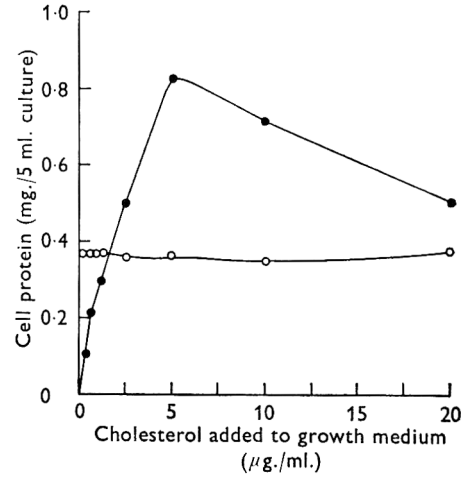

Fig. 5

Fig. 4. Uptake and loss of cholesterol by washed organisms of the *Streptobacillus moniliformis* L-phase. The organisms were grown in Edward medium containing [4–^{14}C]cholesterol, harvested, washed and tested for additional [1α –^{3}H]cholesterol uptake (●) and [4–^{14}C]-cholesterol loss (○) as described under Methods.

Fig. 5. The effect of cholesterol on growth of the *Streptobacillus moniliformis* L-phase (○) and *Mycoplasma gallisepticum* (●). The organisms were grown in Edward broth devoid of serum, but with 0·01% (w/v) Tween 80, 1% (w/v) bovine serum albumin fraction V, 12 μg/ml. palmitic acid and various concentrations of cholesterol solubilized in Tween 80. The organisms were harvested after 120 hr incubation at 37°, washed twice in 0·5 M-NaCl and their cell protein was determined.

cholesterol added to this medium did not improve the growth of *S. moniliformis* L-phase, but had a most pronounced effect on the growth of *M. gallisepticum*. The *S. moniliformis* L-phase could be subcultured several times in serum-free medium with no apparent decrease in growth, and after their second subculture no cholesterol could be detected in the lipid fraction (3·1 mg.) of the L-phase cells. The minimal amount of cholesterol detected by the thin-layer chromatographic method used was 1 μg. included in a spot of 4 mm. diameter.

DISCUSSION

Our results show that the transformation of a bacterium to its L-phase is correlated with a cholesterol-binding capacity in *Proteus mirabilis* but not in the Gram-positive cocci. Bacterial-phase cultures of *Streptobacillus moniliformis* incorporated slightly less cholesterol than did the L-phase. However, here the transformation to the L-phase occurs spontaneously (Dienes, 1939), so that bacterial-phase cultures always contained a high percentage of L-phase forms, which may account for their high cholesterol incorporation.

Chemical analyses of isolated cell membranes (Razin, 1967; Razin & Boschwitz, 1968) have suggested that the cholesterol taken up by mycoplasmas and L-phase variants is located in the cell membrane and we have now shown marked cholesterol uptake by isolated L-phase and mycoplasma membranes. The sensitivity of mycoplasmas to lysis by digitonin (Smith & Rothblat, 1960, 1962; Razin & Argaman, 1963) suggests that the cholesterol becomes an integral part of their membrane. Saponin, which resembles digitonin in action and is known to form holes in biological and artificial cholesterol-containing membranes, has recently been shown to produce holes in *Mycoplasma mycoides* var. *mycoides* membranes (Chu & Horne, 1967). Our study extends these previous observations by showing that digitonin inhibits mycoplasma and L-phase growth. Since the sensitivity of the various organisms to growth inhibition by digitonin roughly paralleled their cholesterol-binding capacity, this method may be useful in comparing the cholesterol content of different microbial membranes.

Smith & Rothblat (1962) reported that digitonin did not lyse the *Streptobacillus moniliformis* L-phase but caused agglutination of the organisms. Since we were able to show distinct lysis of this L-phase by digitonin, it seems probable that Smith & Rothblat were using organisms which had already been lysed during washing before being tested for digitonin sensitivity. They used physiological saline (0·15 M-NaCl) and not 0·5M-NaCl which in our experience protects the fragile L-phase organisms much better against lysis during washing. The sensitivity of *Diplococcus pneumoniae* to growth inhibition and lysis by digitonin distinguishes it from the other wall-covered bacteria. Digitonin sensitivity depends on the pneumococcus being grown in the presence of cholesterol (Klein & Stone, 1931; Smith & Rothblat, 1960). Probably the mechanism of lysis involves activation of autolytic enzymes which solubilize the rigid cell wall, as postulated to explain the lysis of this bacterium by surface-active agents (MacLeod, 1965).

Smith & Rothblat (1960) with mycoplasmas and Rothblat *et al.* (1966) with tissue-culture cells, noted that the adsorbed cholesterol was firmly attached to the cells and that there was no exchange with exogenous cholesterol. Graham & Green (1967), on the other hand, claimed that cholesterol in a variety of animal cell membranes was exchanged with plasma cholesterol. However, the rate of exchange as shown by Bruckdorfer, Graham & Green (1968) was rather slow. Our experiments also showed a slow rate of exchange of the cholesterol bound to the *Streptobacillus moniliformis* L-phase and *Mycoplasma gallisepticum* with exogenous cholesterol, but so slowly that serum could be used in the washing fluid to remove radioactive cholesterol weakly adsorbed to the cells and to the membrane filters.

The present findings corroborate the conclusion of Smith & Rothblat (1960) and of Rothblat *et al.* (1966) that the mycoplasma and L-phase cells take up cholesterol by a

physical adsorptive non-enzymic mechanism. Both the L-phase variants and the mycoplasmas investigated here incorporated much less cholesteryl oleate than free cholesterol as had been found with mycoplasmas (Argaman & Razin, 1965), the L-phase of *Proteus mirabilis* P 18 (Rebel *et al.* 1963), and tissue-culture cells (Rothblat *et al.* 1966). The cholesterol in biological membranes is found almost exclusively in the free state (Ashworth & Green, 1966; Nelson, 1967; Pfleger, Anderson & Snyder, 1968). The rather tight packing of lipids in cellular membranes, due to the presence of protein septa might, according to Bruckdorfer *et al.* (1968), explain the rejection by the membranes of sterols with bulky side-chains and the absence of cholesteryl esters in them.

Our findings support the suggestion of Edward (1953) that the requirement of cholesterol for growth is a valid criterion for differentiating between bacterial L-phase variants and mycoplasmas. The ability of the L-phase variants of *Streptococcus pyogenes* (van Boven, Kastelein & Hijmans, 1967), *Listeria monocytogenes* (Edman, Pollock & Hall, 1968), *Bacillus subtilis* (Burmeister & Hesseltine, 1968), *Proteus mirabilis* (Rebel *et al.* 1963) and *Agrobacterium tumefaciens* (Santaolalla, 1966) to grow in cholesterol-free media proves their independence of cholesterol, while most mycoplasmas are dependent on it for growth. One of our main objectives was to show that the *Streptobacillus moniliformis* L-phase also conforms to this rule, although it incorporates large quantities of cholesterol into its cell membrane. Edward (1953) could not show a requirement for cholesterol by the *S. moniliformis* L-phase but found that the serum had to be replaced by acetone-insoluble lipid from egg yolk and bovine serum albumin. Smith & Rothblat (1962) argued that the complete absence of sterol from Edward's experiments was not proved. Our study supports and strengthens Edward's observations by demonstrating the absence of cholesterol from the serum-free medium, and in the L-phase organisms grown in it. The L-phase of *S. moniliformis* thus resembles all the other L-phase variants investigated in so far as it does not depend on cholesterol for growth, although it has a marked ability to incorporate it. It may be concluded that in none of the L-phase membranes tested does cholesterol play the essential role which it does in the mycoplasma membrane.

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APPENDIX

On the Determination of the L-phase Parentage by the Electrophoretic Patterns of Cell Proteins

The electrophoretic technique devised by us for the identification of mycoplasmas (Razin & Rottem, 1967; Razin, 1968) and Gram-negative bacteria (Sacks, Haas & Razin, 1969) was used to determine whether the L-phase variants are the descendants of the bacteria presumed to be their parents. Washed organisms were dissolved in phenol + acetic acid + water (2 + 1 + 0·5, by vol.) and the dissolved material was run in polyacrylamide gels containing 35% acetic acid and 5M-urea. The gels were stained with 1% Amido Black 10B (Razin & Rottem, 1967) and densitometer tracings of the stained gels were made in a Gilford Model 2410 Scanner. Figure 1 (A to C) shows that the electrophoretic patterns of the *Streptobacillus moniliformis*, *Proteus mirabilis* and *Streptococcus pyogenes* L-phase variants were very close to those of their presumed parents. The differences were quantitative rather than qualitative, for almost all protein bands appeared both in the L-phase and bacteria patterns. The electrophoretic pattern of the L-phase of *Staphylococcus aureus*, however, differed fundamentally from that of its presumed parent (Fig. 1D). This strongly indicates that *S. aureus* (Smith) ATCC 19636 cannot be the parent of the *S. aureus* (Smith) L-phase ATCC 19640, since the genetic identity of micro-organisms seems to be reflected in the electrophoretic patterns of their cell proteins (Razin & Rottem, 1967) and the L-phase variant should be genetically indistinguishable from its parent bacterium (McGee, Rogul & Wittler, 1967).

The electrophoretic technique accordingly seems to be useful in determining the parentage of L-phase variants, and might well replace the far more complex nucleic acid hybridization techniques. Its use for the identification of L-phase variants is also advocated in the recent report of Theodore, King & Cole (1969).

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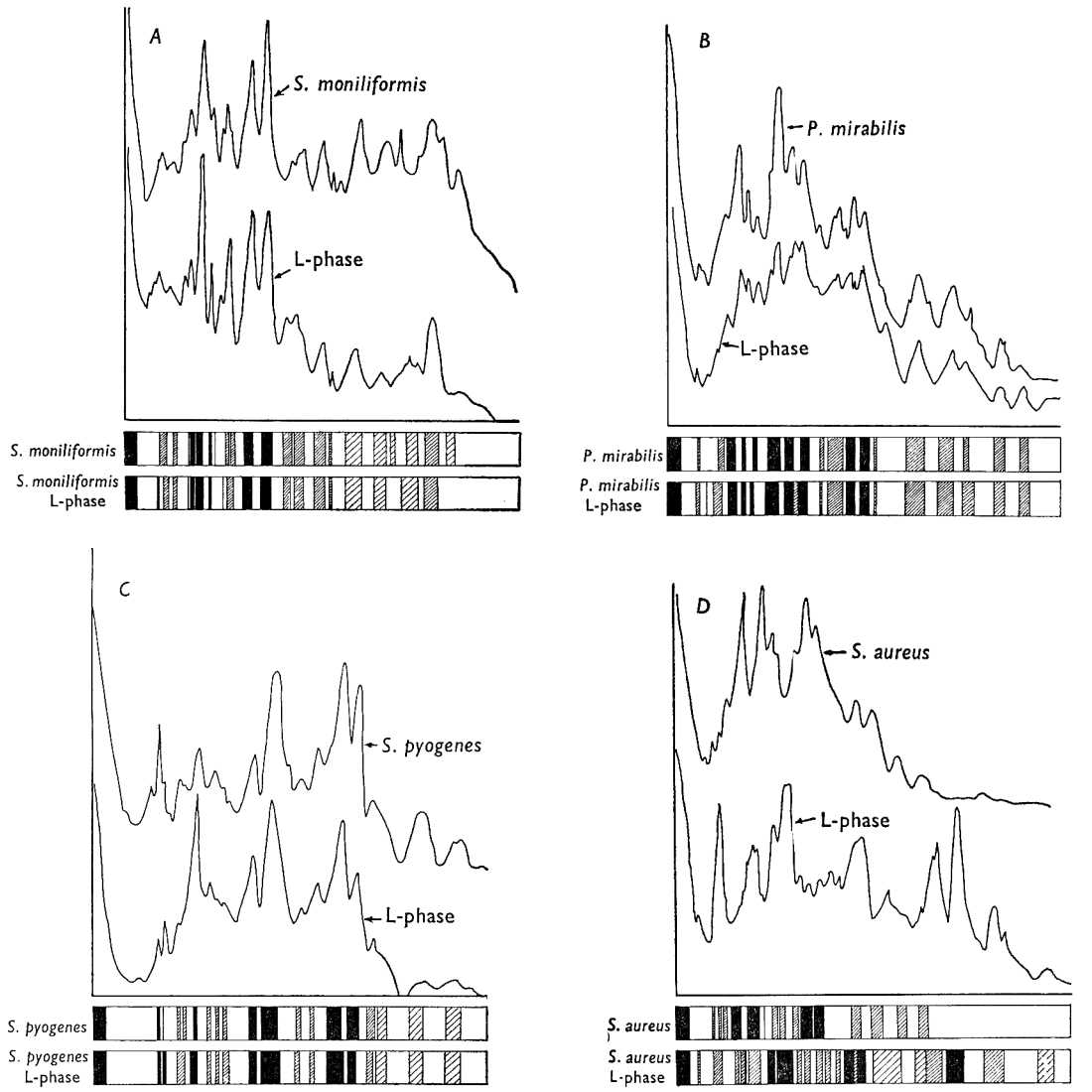

Fig. 1. Densitometer tracings and schematic representation of the electrophoretic patterns of cell proteins of bacterial L-phase variants and their presumed parents. (A) *Streptobacillus moniliformis* ATCC 14647 (upper diagram and curve) and the L-phase of *S. moniliformis* (L-1) ATCC 14075 (lower diagram and curve). (B) *Proteus mirabilis* 9, ATCC 14273 (upper diagram and curve) and the L-phase of *P. mirabilis* (L–9) ATCC 14168 (lower diagram and curve). (C) *Streptococcus pyogenes* (upper diagram and curve) and the L-phase of *S. pyogenes* (lower diagram and curve). (D) *Staphylococcus aureus* Smith ATCC 19366 (upper diagram and curve) and the L-phase of *S. aureus* (Smith-L) ATCC 19640 (lower diagram and curve).

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A Study of Vegetative Reproduction in *Endomycopsis platypodis* by Electron Microscopy

By N. J. W. KREGER-VAN RIJ
Laboratory of Bacteriology and Serology, State University, Groningen, Netherlands
AND M. VEENHUIS
Laboratory of Ultrastructural Biology, State University, Haren, Netherlands

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SUMMARY

The hyphal cross-walls in *Endomycopsis platypodis* had dolipores and, depending on the conditions of growth, dolipores were sometimes present in the walls between budding cells also. The formation of walls, both in hyphae and between budding cells, was initiated by an electron-light primary wall. This was later thickened at both sides with electron-dense material, and the dolipore exhibited an extra swelling around the pore channel. Buds separated from their parents at the primary wall, which remained attached to the mother cell.

INTRODUCTION

The yeast species *Endomycopsis platypodis* Baker & Kreger-van Rij (1964) reproduces vegetatively by the formation of round to oval cells and of branched hyphae with cross-walls. The round or oval cells may remain attached to each other forming pseudomycelium. They are also formed laterally or terminally on the hyphae, and hyphae may arise from round or oval cells. Kreger-van Rij & Veenhuis (1969) observed plugged dolipores in the cross-walls of the hyphae. The wall between budding cells was generally of normal thickness, but occasionally swollen in the middle.

Bud formation and the formation of hyphae with cross-walls are characteristics used in yeast taxonomy. However, insufficient is known to make an absolute distinction between both forms of reproduction. *Endomycopsis platypodis* is suitable material for the closer examination of these forms. The present study is concerned with the formation of cross-walls and with the development of buds in this species as observed in ultrathin sections under the electron microscope.

METHODS

The type strain of *Endomycopsis platypodis* (CBS 4111) was studied. In this strain the formation of a cross-wall took only about 8 min., while the swelling of the dolipore was visible within one hr. To observe different stages of the formation of cross-walls and of dolipores, a preparation containing many growing tips of hyphae was required. A 24 hr culture in a Petri dish with a thin layer of malt extract inoculated heavily with round cells fulfilled this requirement since, under these conditions, many cells formed hyphae. On the other hand, in a 24 hr shaken culture in malt extract, budding cells exclusively were formed. Both cultures were prepared and incubated at 27°. The

harvested cells were washed with water and, after fixation with 1·5% aqueous $KMnO_4$ solution for 20 min. at room temperature, suspended in agar. The agar blocks were dehydrated in an acetone series and poststained with a saturated solution of uranyl acetate in 100% acetone for one hr. After washing with acetone to remove excess uranyl acetate, the specimens were embedded in Vestopal W at 60°, and were cut with a diamond knife on an LKB Ultrotome. The sections were poststained with lead citrate (Reynolds, 1963). They were examined with a Philips EM 100 electron microscope.

RESULTS

Formation of cross-walls in hyphae in standing culture

The beginning of cross-wall formation was observed in sections as a narrow electron-light outgrowth of the lateral wall (Pl. 1, fig. 1). This primary wall had a wedge-like edge in the lateral wall, which surrounded the primary wall at its base (Pl. 1, fig. 2). Nothing on the outside of the lateral wall served to indicate the presence of a cross-wall. The primary wall grew centripetally leaving a pore in the centre (Pl. 1, fig. 3). In the next stage, the wall thickened at both sides with secondary electron-dense layers with an extra swelling around the pore. This pore, surrounded by the swelling, was the dolipore which at first formed an open connexion between the two adjacent hyphal cells (Pl. 1, fig. 4), but later became plugged (Pl. 1, fig. 5). Generally, from the beginning of its formation, the cross-wall tended to bulge in the direction of the hyphal tip, probably owing to a strong protoplasmic stream; at the concave side of the cross-wall dense protoplasm often occurred, and at the convex side a vacuole was sometimes present.

Formation of buds in shaken culture

The first stage of bud formation observed is shown in Pl. 2, fig. 6. The bud was still small; its wall arose from under the wall of the mother cell. The electron-micrographs suggest that later between the walls of mother cell and bud, an electron-light collar was formed which appeared in sections as a wedge (Pl. 2, fig. 7, 8). In these first stages, mitochondria and endoplasmic reticulum moved into the bud, followed by the nucleus which elongated and split on arriving at the isthmus between mother cell and bud. Thereupon, a primary electron-light wall was formed centripetally across the isthmus (Pl. 2, fig. 9). Like the primary wall of the cross-walls of hyphae, this wall in a budding cell had a wedge-like edge. This edge touched the electron-light collar and thus split the original wall of the bud. The primary wall was thickened with electron-dense material at each side (Pl. 2, fig. 10, 11). The bud was finally separated from the mother cell by splitting along the primary wall in such a way that this wall remained attached to the mother cell (Pl. 3, fig. 12, 13.) The rim of the bud scar consisted of the edge of the mother cell and that of the white collar. On the young bud a wide scar was visible with a vague narrow ridge. The latter constituted the breaking point between mother cell and bud. The primary wall between mother cell and bud in sections often had an undulating appearance. When the secondary wall was formed it was straight. After separation of the bud from the mother cell, both bud- and birth scars bulged outwards.

Formation of buds in standing culture

In a standing culture, apart from hyphae, buds were formed on the round cells. The buds arose as described in the previous paragraph; at the junction between mother cell and bud they were surrounded by a two-layered collar on the mother cell. Some of the buds developed with a thin wall between mother cell and bud. Others, however, were separated from the mother cell by a wall with a dolipore and plugs (Pl. 3, fig. 14). The actual pore was sometimes a very narrow channel. These cells remained attached to each other as pseudomycelium, but sometimes there were also loose cells with a thickened scar wall, presumably resulting from the swelling of the dolipore. No trace of the channel was found.

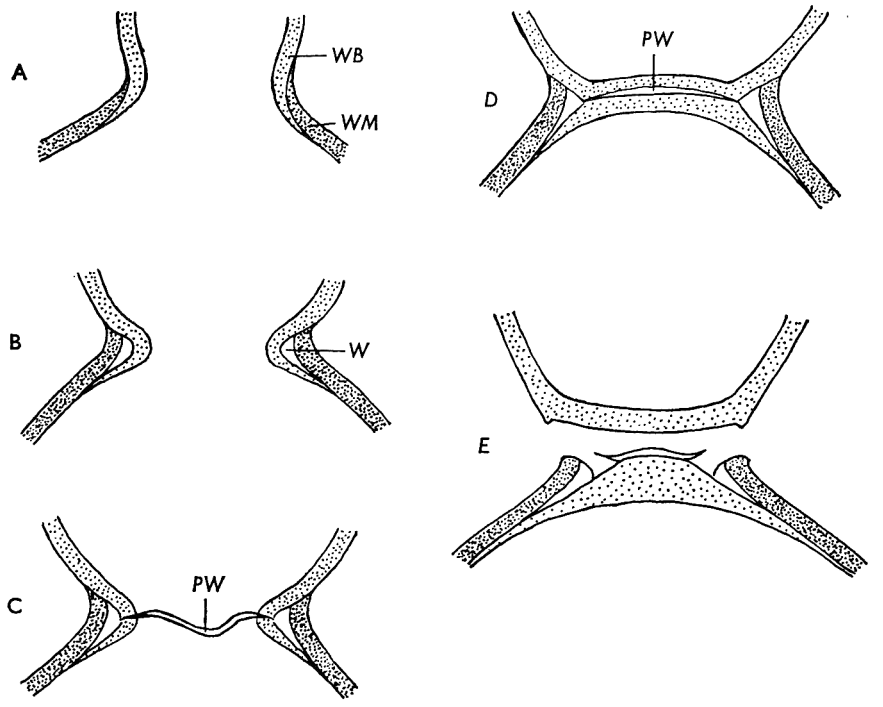

Fig. 1. Diagram of bud formation in *E. platypodis*. *WM* = cell wall of mother cell, *WB* = cell wall of bud, *W* = electron-light collar, *PW* = primary wall.

Hyphae arose on round cells from a narrow neck with a collar on the mother cell and were separated from the latter by a cross-wall with a dolipore in the narrow part (Pl. 3, fig. 15). A short-oval cell sometimes grew out on a wider base to form a hypha. No distinct collar was visible in this case, and the cross-wall in the hypha was formed at some distance from the mother cell (Pl. 3, fig. 16).

DISCUSSION

The development of a cross-wall with a dolipore in *Endomycopsis platypodis* proceeded via the formation of a primary electron-light layer. This layer was thickened with an extra swelling around the pore. In contrast with the dolipore of the Basidio-

mycetes, no pore cap was formed, but at both sides of the pore plugs were present which were probably dilations of the endoplasmic reticulum. The plugs were formed at an early stage.

In the first stage of bud formation observed, the bud was already distinct, its wall different from that of the mother cell. No earlier stage was recognized as such, so it is not known how the wall of the mother cell was penetrated by the bud. The double-layered elevated edge of the scar was present as a collar when the bud was still in open connexion with the mother cell. This collar was also distinct when a hypha was formed on a narrow base on a round cell. With hyphae that arose on a broad base from oval cells, the collar was indistinct or not visible at all.

Dolipores were always present in the cross-walls of the hyphae, whether from a standing malt extract culture or from the surface of an agar medium. However, in the cell walls between round or oval cells the formation of dolipores was observed in standing cultures only; in shaken cultures they did not appear. Round or oval cells often remained together as pseudomycelium and then the walls between them showed dolipores; but loose cells with thickened bud- or birth scar plugs, probably resulting from dolipores, were also observed.

Attention has been drawn above to three particular features. (1) The join between two adjacent cells was narrow or wide. It was narrow in budding, the separating wall forming in the narrow part with a collar on the mother cell at the base of the bud. The collar occurred between round or oval cells, and between hyphae and round or oval cells. A wide join between cells, without any narrowing at the junction of the lateral wall and cross-wall, was typical of hyphae. (2) A dolipore was present in the separating wall, both of hyphae and of some budding cells. (3) Complete separation of the cells at the intervening wall was only observed for round and oval cells.

These features indicate some of the differences and resemblances between hyphae and budding cells. Other aspects, such as zones of growth of the wall and the site of formation of hyphal cross-walls, have yet to be studied.

Since bud formation in ascogenous yeasts has been mainly studied in *Saccharomyces cerevisiae*, budding in *Endomycopsis platypodis* has been compared with that in *Saccharomyces cerevisiae* as described by others and as observed by the present authors. A similar development was found in both species which differs, however, in some respects from the recent description of *S. cerevisiae* by Marchant & Smith (1968). In the first place, in *S. cerevisiae* the wall of the bud did not appear to be continuous with that of the mother cell, the wall of the bud protruding from the inside of the mother cell as is clearly shown in Pl. 3, fig. 17. However, the part of the cell wall of the bud within the mother cell was not always distinctly visible. The observation that the walls of mother cell and bud were not continuous agrees with the description of bud formation on a regenerating protoplast by Nečas & Svoboda (1967). Secondly, in both *E. platypodis* and *S. cerevisiae* the electron-light layer of the wall between mother cell and bud was found to be the primary wall which later extended to both sides with electron-dense material (Pl. 3, fig. 18). Sentandreu & Northcote (1969) also described the formation of a primary wall which later thickened in *S. cerevisiae*. In the electron-micrographs published by Marchant & Smith (1968) the electron-light layer was distinct, but was not described as a primary wall. Splitting of the bud from the mother cell occurred along this wall, and the electron-light layer, which was rather thin in *S. cerevisiae*, remained attached to the mother cell (Pl. 3, fig. 19).

Houwink & Kreger (1953) found chitin on the plug of the bud scars of baker's yeast and of *Candida tropicalis*; Bacon, Davidson, Jones & Taylor (1966), after chemical and enzymatic treatment of the cells of baker's yeast, retained a residue consisting mainly of chitin and formed entirely of bud scars. These observations in connection with our findings suggest that the electron-light parts in sections of the scars may consist of chitin.

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EXPLANATION OF PLATES

Symbols: *LW* = lateral wall, *PW* = primary wall, *WM* = cell wall of mother cell, *WB* = cell wall of bud, *S* = swelling, *P* = plug, *N* = nucleus, *ER* = endoplasmic reticulum, *M* = mitochondria. The marker represents 0·5 μ. All figures are made of material fixed with $KMnO_4$ and poststained with lead.

PLATE 1

Longitudinal sections through hyphae of *Endomycopsis platypodis*.

Fig. 1. Initial stage of the formation of a cross-wall. A nucleus is visible.

Fig. 2. Section through a primary wall beside the pore.

Fig. 3. shows a partly thickened primary wall with pore.

Fig. 4. Section through a cross-wall with swelling, but still without plugs.

Fig. 5 shows a completed cross-wall with dolipore and plugs.

PLATE 2

Sections through yeast cells of *E. platypodis*.

Fig. 6. Early stage of bud formation. The wall of the bud arises from under the wall of the mother cell. *ER* lies along the wall of the bud.

Figs. 7 and 8. Later stages in bud formation. Between the walls of bud and mother cell an electron-light wedge is visible. In fig. 7 mitochondria and *ER* are present in the bud. In fig. 8 the nuclei of mother cell and bud are observed separately.

Fig. 9, 10 and 11 show three stages of wall formation between mother cell and bud. In fig. 9 the electron-light primary wall is visible. In fig. 10 and 11 the wall has thickened at both sides with more electron-dense material.

PLATE 3

Fig. 12 to 16 are cells of *E. platypodis*, figs. 17 to 19 of *S. cerevisiae*.

Fig. 12 and 13. Sections of partly and wholly detached buds. The primary wall remains attached to the mother cell. In fig. 13 the two layers of the rim of the scar on the mother cell, namely the edge of the mother cell and that of the electron-light collar, are distinctly visible. On the bud a vague rim of the birth scar is present (arrowed). Both bud and birth scars bulge outwards.

Fig. 14. Budding cells with a dolipore in the wall.

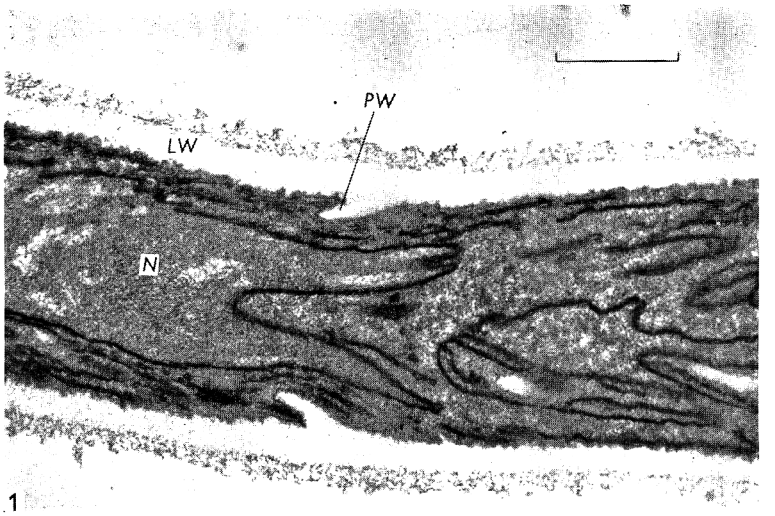
Fig. 15. Section through a round cell with a hypha arising from it.

Fig. 16. Section through an oval cell growing out with a hypha.

Fig. 17. Section through a budding cell of *S. cerevisiae*. The wall of the bud arises from under the wall of the mother cell.

Fig. 18 shows the electron-light primary wall thickened with darker material in *S. cerevisiae*.

Fig. 19. Section through a bud scar in *S. cerevisiae* showing the electron-light primary wall left on the mother cell.



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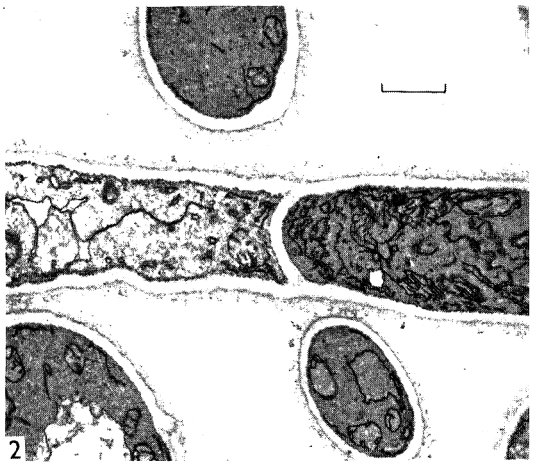

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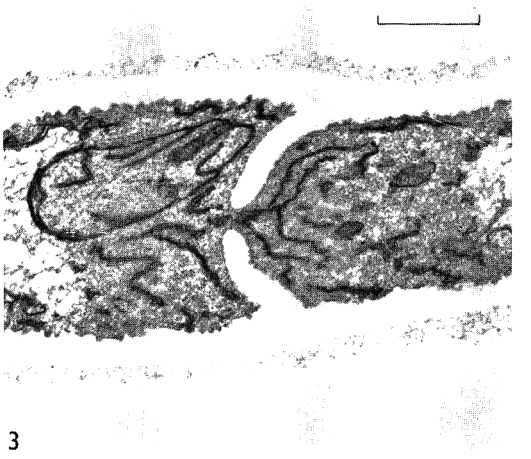

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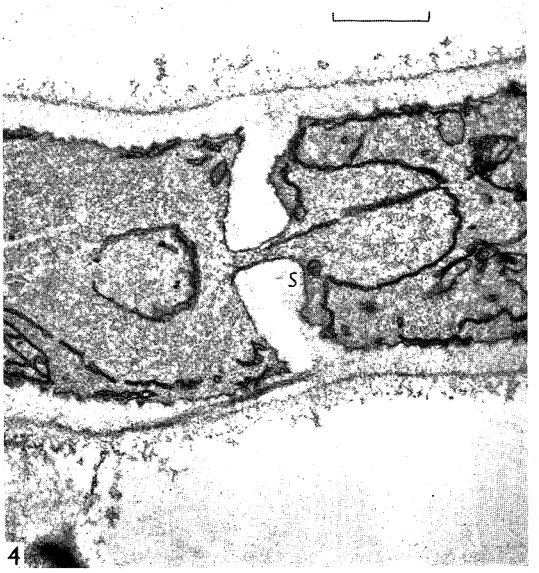

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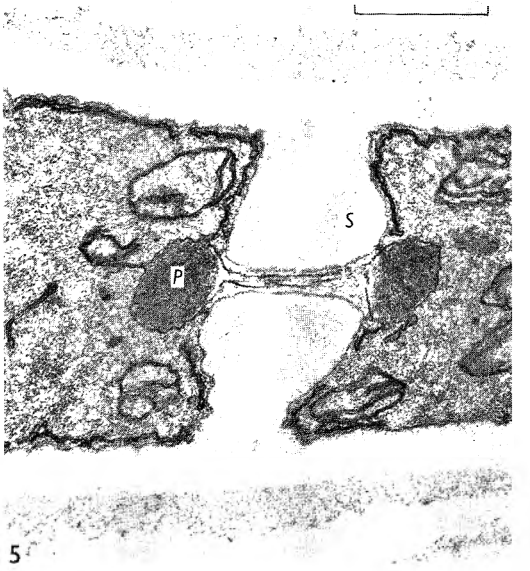

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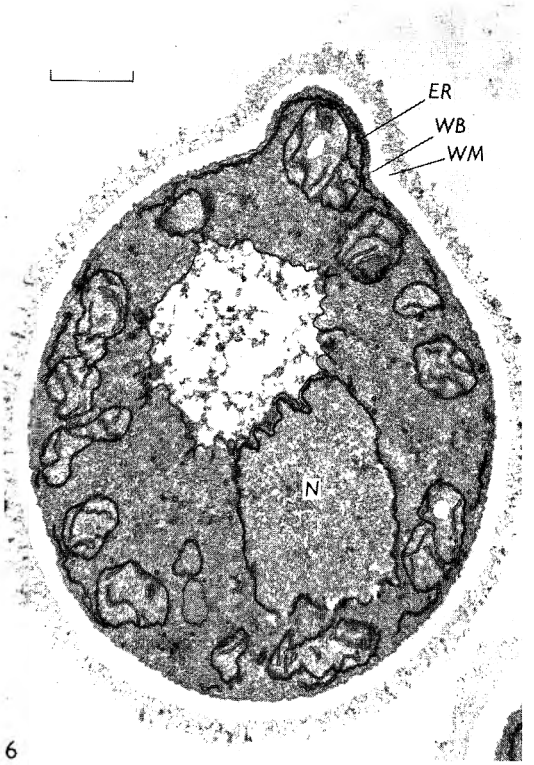

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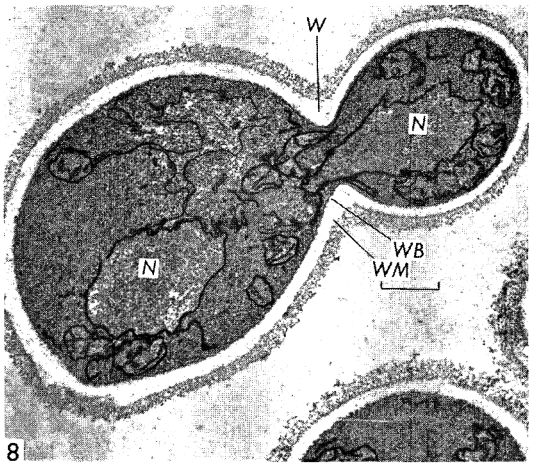

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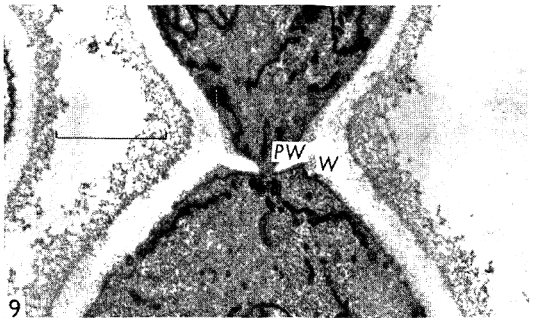

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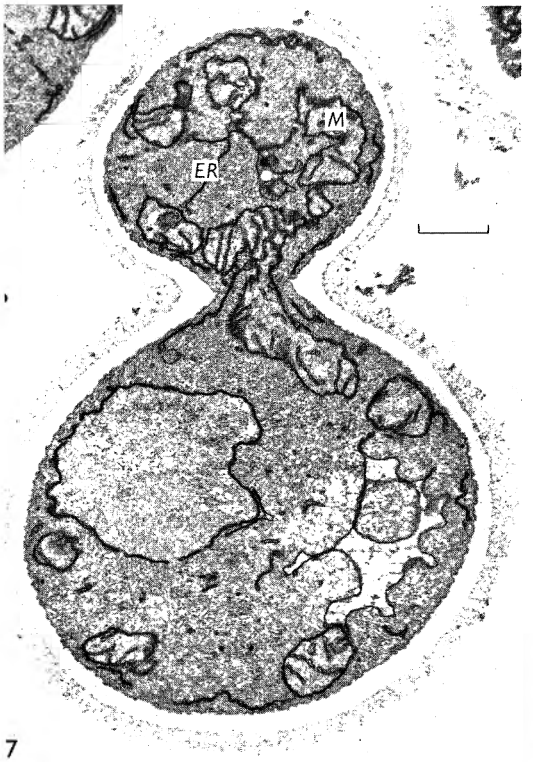

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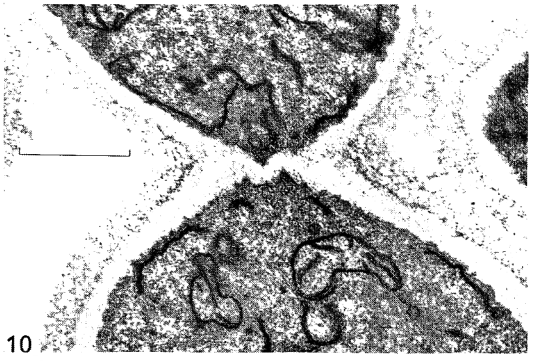

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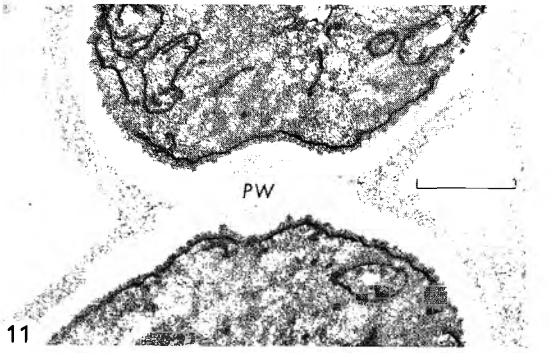

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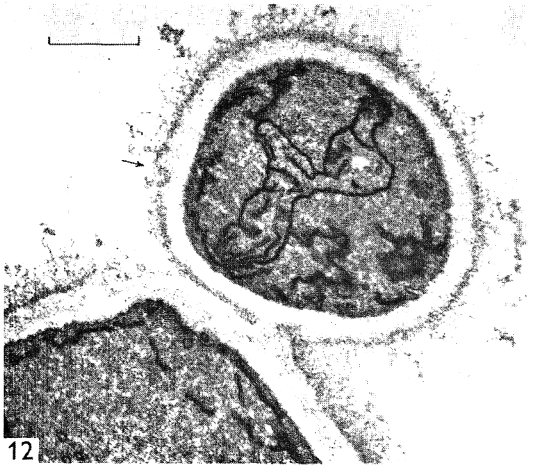

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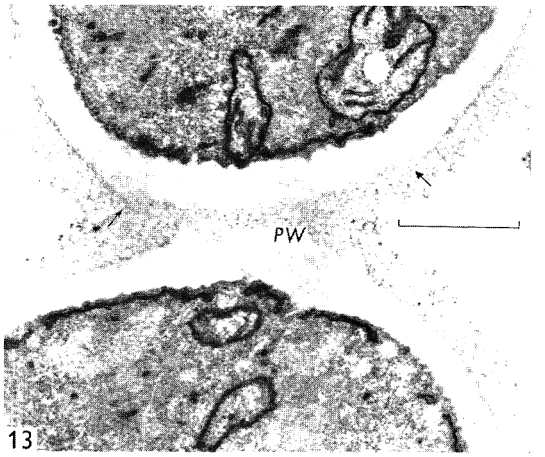

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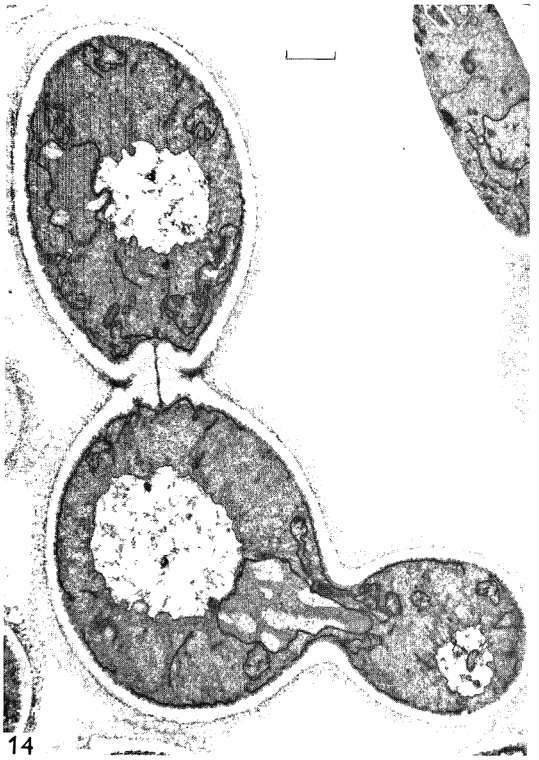

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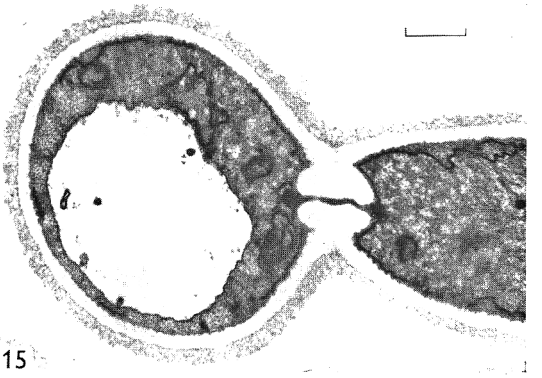

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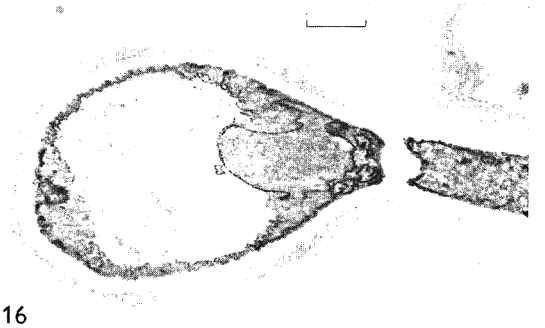

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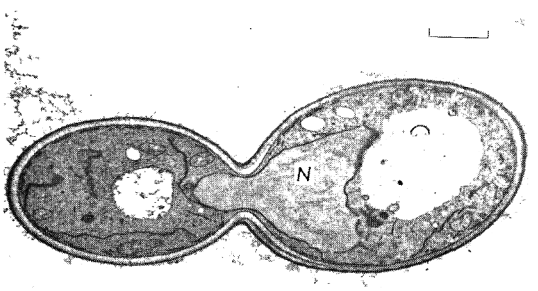

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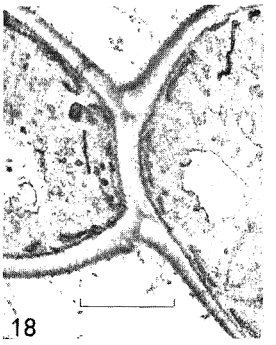

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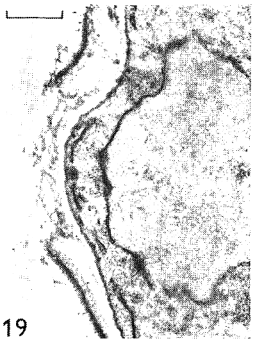

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Degradation of Cell Constituents by Starved *Streptococcus lactis* in Relation to Survival

By T. D. THOMAS* AND R. D. BATT

New Zealand Dairy Research Institute, Palmerston North, New Zealand and Chemistry and Biochemistry Department, Massey University, Palmerston North, New Zealand

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SUMMARY

Streptococcus lactis organisms were grown in lactose-limited batch culture and transferred, after washing, to phosphate buffer at the growth temperature. Soluble protein was released from viable organisms into the suspending buffer and the intracellular free amino acid pool declined steadily with the components appearing in the suspending buffer; a net increase in the total amount of free amino acid indicated some protein hydrolysis. RNA was hydrolysed, resulting in the release of u.v.-absorbing bases and ribose from the organisms. Conditions which promoted rapid RNA breakdown also produced rapid death rates and long cell division lags in surviving organisms. After 28 hr starvation in buffer containing Mg^{2+}, the bacterial dry wt decreased by 26 %; loss of RNA, protein and free amino acids accounted for 10·3 %, 7·3 % and 2·7 % of the total bacterial mass loss. The products of polymer hydrolysis appeared to be released in an undegraded form into the external buffer and there was no appreciable formation of lactate, ammonia or volatile fatty acids, possibly indicating the absence of any important endogenous energy sources. There was no appreciable degradation of DNA or carbohydrate but phospholipid was broken down on prolonged starvation. No polyglucose or poly-β-hydroxybutyrate was detected in the organisms.

INTRODUCTION

The progressive loss of cell constituents which generally occurs when bacteria are starved in buffer at the growth temperature is normally a result of an imbalance in the total anabolic and catabolic reactions. It may also result from other processes such as the leakage or secretion of cell components and intracellular pools. Limited resynthesis or turnover of cell polymers may occur but the net metabolism is catabolic and will eventually result in the death of the organism. The over-all metabolic activities of starved bacteria are normally referred to as their 'endogenous' metabolism which, according to Powell (1967), is effectively a measure of the rate at which bacteria break down their own mass. This field has been extensively investigated with aerobic organisms and published work has been reviewed in a symposium (Lamanna, 1963) and by Dawes & Ribbons (1962, 1964). The functions of endogenous metabolism, as envisaged by Dawes & Ribbons (1962), include the provision of energy for turnover of protein and nucleic acids, osmotic regulation, pH control and the supply of suitable

* Present address: Sub-Department of Chemical Microbiology, Department of Biochemistry, University of Cambridge, Cambridge, Great Britain.

substrates for the resynthesis of essential bacterial constituents. However, the control and economy of these energy-requiring processes are ill-defined (Strange, 1967) and, while starved bacteria may exhibit high initial O_2-uptakes, the O_2-uptake may fall to a negligible level though significant decline in viability may not occur for a considerable period (Burleigh & Dawes, 1967), which suggests that the amount of energy required for survival may be very small.

Components reported to be degraded in starved organisms include carbohydrate, RNA, protein, free amino acids, peptides, lipids and certain specialized 'reserve' materials. The rates and orders of substrate degradation and the general pattern of endogenous metabolism vary (1) in different organisms, (2) with the growth conditions and hence the chemical and physiological state of the organism and (3) with the physico-chemical conditions of the starvation environment (Strange, 1967). Profound changes in the composition of starved bacteria without concurrent viability loss are well documented. Up to 25 % of the total cell protein was catabolized before a significant decrease in the viability of *Aerobacter aerogenes* occurred (Strange, Dark & Ness, 1961) and it appeared that RNA was to some extent expendable, since up to 50 % of the ribosomal RNA of some organisms was metabolized without death taking place. Within a single species, organisms grown at rapid rates contain much more RNA (Schaechter, Maaløe & Kjeldgaard, 1958) and die more slowly when starved than organisms grown at slow rates (Postgate & Hunter, 1962). Although many conditions which delay RNA degradation, such as the presence of Mg^{2+} (Strange & Hunter, 1967) or D_2O (Lovett, 1964), also delay death, no absolute correlation between RNA degradation and death rate has been established (see Postgate, 1967). Burleigh & Dawes (1967) were unable to correlate loss in viability of starved *Sarcina lutea* with the degradation of any single cellular constituent.

An important function of research laboratories serving the dairy industry is the maintenance of lactic acid cultures with high acid producing activities for use as cheese 'starters'. An understanding of the factors affecting the survival and activity of these organisms could therefore be valuable. Only a small number of reports on the metabolism of starved lactic acid bacteria have been published and no correlations between viability and endogenous metabolism have been reported. Most of the reported studies on the metabolism of viable, starved bacteria have involved Gram-negative organisms which can accumulate 'reserves' such as glycogen or poly-β-hydroxybutyrate. Since lactic streptococci have no reported 'reserves' and very limited catabolic activities, it was considered that extension of endogenous metabolism studies to *Streptococcus lactis* could be informative. The report that exogenous Mg^{2+} suppressed RNA degradation but had no effect on the survival of starved *Sarcina lutea* (Burleigh & Dawes, 1967), suggested that some of the important survival characteristics of this Gram-positive organism may be markedly different from those for Gram-negative organisms. Similar findings were made by Dawes with two other Gram-positive organisms (see Discussion, Strange & Hunter, 1967). Further investigations with Gram-positive organisms have been indicated and might more clearly define the general parameters for bacterial survival.

Studies on the metabolism of viable, starved organisms are likely to be significant only if factors affecting survival are investigated concurrently. The results of such an investigation with *Streptococcus lactis* have been previously reported (Thomas & Batt, 1968).

METHODS

Organism and culture methods. Streptococcus lactis ML_3 was maintained and subcultured using methods and media described previously (Thomas & Batt, 1968).

Preparation of starved suspensions. Organisms were harvested at the end of the growth phase by centrifuging (30,000 *g* for 1 min.) and the deposited bacteria were then rinsed thoroughly, dispersed in phosphate buffer (see below), centrifuged (30,000 *g* for 1 min.), rinsed and finally resuspended in buffer. The temperature was maintained at about 30° and the procedures, which were performed with aseptic precautions, were completed in 15 to 20 min., thus minimizing changes in temperature and the chemical environment. The phosphate buffer used was 0·075 M-$Na_2HPO_4 + KH_2PO_4$ (pH 7·0) containing 10^{-5} M-EDTA (plus substrates, etc., where specified).

Bacterial mass and viability (by slide culture) were determined by methods described previously (Thomas & Batt, 1968). Where some settling occurred after prolonged starvation, the organisms were gently agitated by means of a magnetic stirrer. Correction was made for evaporation loss by adding appropriate amounts of sterile water. Samples of supernatant buffer solutions were obtained for chemical analyses by centrifugation and filtration through a membrane filter (0·45 μ; Millipore Filter Corp., Bedford, U.S.A.). When analyses on the bacteria were required, the packed organisms were washed in phosphate buffer and normally resuspended in deionized water. Bacterial mass determinations were carried out at the sampling time, while suspension samples and cell-free supernatant solutions were stored at $-20°$ when not analysed immediately.

Analytical procedures. Colorimetric and spectrophotometric measurements in the visible and ultraviolet (u.v.) region were made with either a Beckman DB or a Zeiss PMQII spectrophotometer using 1 cm. glass or silica cells. Polyglucose and poly-β-hydroxybutyrate were assayed by the methods of Strange *et al.* (1961) and Williamson & Wilkinson (1958) respectively.

Protein was determined by the biuret method (Stickland, 1951). Suspensions of *Streptococcus lactis* required heating at 100° for 20 min. in 0·75 N-NaOH for maximum colour development. Dried bovine serum albumin (Sigma, A grade), containing 13·6 % N, was used as the standard. The alternative method of Lowry, Rosebrough, Farr & Randall (1951) gave similar results to the biuret method with samples of both supernatants and alkali-hydrolysed cell suspensions. Total cell N and supernatant N were determined by the micro-Kjeldahl method described by Humphries (1956) using a Se/K_2SO_4 catalyst and methyl red-methylene blue indicator.

Amino acids in bacterial extracts and supernatant samples were measured colorimetrically by the ninhydrin method of Yemm & Cocking (1955). Glycine standards were used and $(NH_4)_2SO_4$ standards were included to correct for NH_3, which was determined separately by the micro-diffusion method of Conway (1947). Buffer samples were adjusted to pH 5·0 before amino acid analysis by addition of HCl or dilution in citrate buffer (pH 5·0). Quantitative measurement of individual amino acids was obtained using a Beckman model 120C automatic amino acid analyser with Beckman custom spherical ion exchange resin. The short column for basic amino acids contained resin type PA 35 to a height of 8 cm. The elution buffer pH was 5·28 and the flow rate 68 ml./hr. The long column for acidic and neutral amino acids contained resin type PA 28 to a height of 58 cm. Buffer, pH 3·28, was used as eluant, being

replaced after 90 min. by a second buffer at pH 4·25. The ninhydrin flow rate was 34 ml./hr and analyses took place over a 4 hr period. Peaks were identified from standard elution times and integrated by the height–width method. The total cellular amino acid composition was determined on organisms harvested at the end of the growth phase and washed twice in distilled water. Washed organisms were freeze-dried, hydrolysed (6N-HCl for 24 hr at 110° in a vacuum sealed tube) and the hydrolysate analysed. The minimum time in boiling water required for complete release of the amino acid pool of *Streptococcus lactis* was 12 ± 3 min. For routine extracts, bacteria were washed and resuspended in deionized water and the suspensions heated for 20 min. at 100° in stoppered tubes. Bacterial debris was removed by centrifugation and the clear extract was pipetted from the tube. A second extraction yielded only a further 1 to 3 % of ninhydrin-positive material and was therefore not carried out routinely.

Bacterial RNA was determined by the method described by Munro & Fleck (1966). Perchloric acid-washed bacteria (10 min. at 0° in 0·5N-$HClO_4$) were subjected to alkaline hydrolysis in 0·5N-KOH at 37° for 60 min.; this was shown to give a complete extraction of ribonucleotides. (For convenience, samples and standards were often stored at −20° in alkaline suspension before hydrolysis; storage for up to 24 hr at −20° did not affect analyses.) The hydrolysate was chilled to 0° and ice-cold $HClO_4$ was added to a final concentration of 0·5N. After 10 min. at 0° the insoluble fraction was sedimented and washed twice in ice-cold $HClO_4$ (0·5N). Centrifugation was carried out in a Sorvall refrigerated centrifuge (30,900 *g* for 1 min.). The alkali extract and washings were combined and made up to 25 ml. Samples were filtered through a sintered glass filter (porosity 5/3) before extinction measurement at 260 mμ. Soluble yeast RNA (Sigma, type III) was used as a standard and was treated in the same way as samples. The standard RNA contained 7·85 % phosphorus and was assumed to be 83 % RNA. Bacterial DNA was extracted and estimated by the methods of Burton (1956). Deoxyribose was used as the standard. Before measuring the extinction of supernatant samples at 257 mμ, the solutions were deproteinized with 5 % trichloroacetic acid, and the appropriate blanks were included.

Free and purine-bound ribose was estimated in cell-free supernatant or alkali-soluble nucleotide extracts using the orcinol method (Mejbaum, 1939). Total cellular hexose was estimated as glucose by the anthrone procedure of Trevelyan & Harrison (1952) or by the reducing sugar method of Nelson (1944). Lactic acid was measured in deproteinized supernatant samples by the method of Barker & Summerson (1941). Volatile fatty acids were separated by exhaustive steam distillation in a Markham still, after adjusting the supernatant and standard samples to pH 1·0 with H_2SO_4 and distillates were titrated with 0·01N-NaOH in a stream of N_2, using phenolphthalein as the indicator.

Lipids were extracted directly from washed organisms by the methanol-chloroform procedure of Vorbeck & Marinetti (1965*a*). A second extraction recovered a further 6 to 9 % of the total lipid, and acid hydrolysis (2·5N-HCl, 16 hr at 100°) of the residue yielded a further 3 to 4 % of the total lipid. Two methanol-chloroform extractions were normally carried out. Extracts were washed with aqueous $CaCl_2$ (Folch, Lees & Stanley, 1957) to remove non-lipid material and the solvents were removed using a rotary evaporator at 40°. The tared flasks were finally dried to constant weight in a vacuum desiccator over P_2O_5 and the lipids were estimated gravimetrically. Silicic

acid column chromatography was ued to separate lipids into neutral and polar fraction (Hirsch & Ahrens, 1958). Lipid (200 mg.) was applied to the top of the column (250 × 17 mm.) in the minimum amount of chloroform-methanol (4:1) and the neutral lipid was completely eluted with ethanol-free chloroform containing 1 % methanol (500 ml.). Polar lipid was then eluted completely using chloroform-methanol (2:1, v/v, 300 ml.). Column separations were monitored routinely by both TLC and phosphorus analyses and the fractions obtained were dried and weighed as described previously.

Glycerides were hydrolysed by refluxing with aqueous 5N-KOH:CH_3OH (1:1, v/v) for 5 hr; non-saponifiable material was removed by ether extraction. After acidification, free fatty acids were extracted into diethyl ether, dried over anhydrous Na_2SO_4 and esterified with diazomethane (Schlenk & Gellerman, 1960). Analysis of the methyl esters was carried out on an Aerograph 600 gas chromatograph (Wilkins Instrument & Research, U.S.A.) using a hydrogen flame ionization detector. Apiezon L on 80-100 mesh celite (10 %, w/w) at 196° and polyethylene glycol adipate at 180° were used as stationary phases and the columns were prepared as described by James (1960). The detector and columns were checked periodically with fatty acid standards and the procedures recommended by Horning *et al.* (1964). Peaks were identified by comparison with retention data of standards or with published values; the % composition of the fatty acids was estimated by the height–width method (Horning *et al.* 1964).

Thin-layer chromatography was carried out on glass plates with a layer of silicic acid (silica gel G, Merck) following the procedures of Mangold (1961). Chromatograms, which had been developed in either hexane:diethyl ether:acetic acid (70:30:1, v/v/v) or di-isobutyl ketone:acetic acid:water (80:50:8, v/v/v), were dried before spraying with ninhydrin and the spots giving a positive reaction were marked. Phosphorus-containing components were detected by using the molybdenum spray reagent of Dittmer & Lester (1964). The plate was finally sprayed with 10 % H_2SO_4 and charred at 100° to show all components. Phosphorus was estimated by the method of Burton & Petersen (1960).

Materials. Solid reagents for analytical procedures were recrystallized and solvents redistilled if analytical reagent grades were not available. All water was distilled and then deionized by passage through a mixed bed ion-exchange resin (Permutit 'Biodeminrolit').

RESULTS

'Reserve' polymers. Organisms (1·2 g. dry wt) were harvested from the end of the growth phase in the routine medium and extracted for polyglucose and poly-β-hydroxybutyrate (PHB). Analysis of the extracts revealed no trace of either polymer. However, the routine medium was lactose-limiting, so that optimum conditions may not have existed for polymer accumulation. When organisms were harvested at the end of the growth phase and resuspended in the routine medium minus the casamino acids and peptone components, no detectable synthesis of either polyglucose or PHB occurred. Conditions similar to these had been found to be optimal for intracellular polyglucose synthesis in *Streptococcus salivarius* (Hamilton, 1968). Therefore it seemed unlikely that *Streptococcus lactis* was capable of synthesizing these polymers which had been reported as present in many bacterial species. Electron micrographs of thin sections showed no evidence of polyphosphate granules (Thomas & Batt, 1969).

Changes in bacterial protein and total N. Little, if any, net protein breakdown occurred in starved suspensions (Fig. 1). Protein was estimated by the biuret method, which measures peptide bonds and by the Folin–Ciocalteu procedure which measures tyrosine (see Methods). Only traces of free tyrosine were released from starved organisms (Table 2). Protein accounted for 48 % of the initial bacterial dry wt and starvation for the 28 hr period resulted in a 26 % bacterial mass loss (Fig. 1).

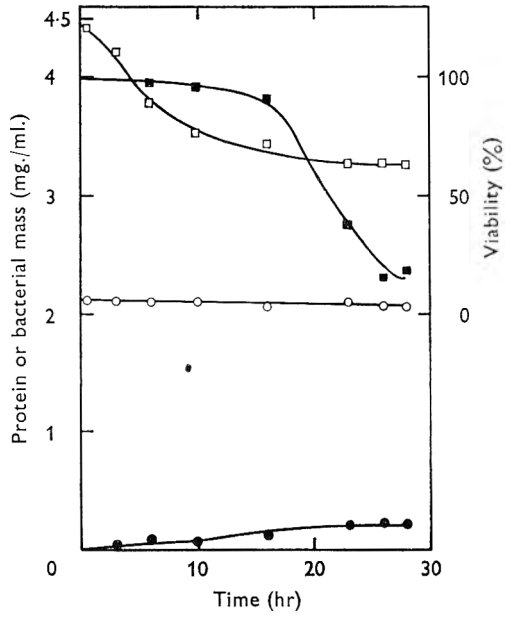

Fig. 1. Changes in protein in starved *Streptococcus lactis*. Bacteria were harvested from the end of the growth phase, washed and resuspended at 30° in phosphate buffer (0·075 M, pH 7·0 containing 1 mM-$MgSO_4$ + 10 μM-EDTA). At the times indicated, samples of the suspension were removed and a portion immediately deep-frozen together with supernatant samples which were obtained after centrifugation and filtration. Protein analyses were performed on whole suspensions (○) and supernatant samples (●). At each sample time, bacterial density (□) was determined by both turbidity and dry wt measurements and viability (■) was estimated by the slide-culture procedure.

Table 1. *Release of protein from starved Streptococcus lactis*

Suspensions were prepared as for Fig. 1 at initially 4·5 mg. dry wt/ml. but with organisms resuspended in (1) 0·075 M-$MgSO_4$ + 10 μM-EDTA and (2) as for (1) + casamino acids (0·5 %, Difco) + arginine (0·1 %). Supernatant protein was precipitated with 5 % TCA, washed and estimated by the method of Lowry *et al.* (1951); results are expressed as mean mg./ml. of four determinations.

	Suspension (1)		Suspension (2)	
Starvation period (hr)	Viability (%)	Supernatant protein	Viability (%)	Supernatant protein
2·5	99	0·03	—	0·02
6	98	0·06	99	0·06
18·5	93	0·17	99	0·17
23	71	0·20	97	0·21
42	0	0·29	48	0·28

Soluble protein (0·22 mg./ml.), amounting to 10 % of the total bacterial protein, was released from starved organisms into the external medium (Fig. 1). The total N in this culture was constant at 0·49 ± 0·018 mg./ml. and the amount of N released into the suspending buffer after 28 hr starvation (0·091 ± 0·016 mg./ml.) suggested that a considerable amount of non-protein N was involved (protein release accounted for approx. 0·02 mg. N/ml.). The initial turbidity of the whole suspension was 23·1 and the turbidity of supernatant samples was always less than 0·005. The rate of protein release appeared to decrease at about the same time as the death rate increased.

Table 2. *Release of amino acids from starved Streptococcus lactis organisms*

Bacteria were harvested, washed twice and resuspended at 15 mg. dry wt/ml. in buffer (Fig. 1). The intracellular amino acid pool was extracted with deionized water at zero time (see Methods). After 28 hr starvation at 30° the organisms in the buffer suspension were washed and extracted in deionized water. The supernatant buffer was deproteinized by addition of TCA (final concentration 5 %). Samples (0·5 ml.) of the extracts, supernatant and hydrolysed bacteria were analysed with an amino acid analyser (see Methods). Results are expressed as μg. amino acid or NH_3/mg. dry wt bacteria at 0 hr.

	Intracellular pool		Supernatant	Total pool	Hydrolysed bacteria
Amino acid	0 hr	28 hr	(28 hr)	(28 hr)	(0 hr)
Lysine	0·95	0·38	8·9	9·3	48·5
Histidine	0·24	0·03	0·71	0·74	8·0
Arginine	—	—	—	—	18·6
Aspartic acid	4·25	0·15	0·70	0·85	67·1
Threonine	1·32	0·10	2·24	2·34	24·9
Serine	0·20	0·03	0·32	0·35	22·5
Glutamic acid	16·3	3·44	11·6	15·0	88·2
Proline	—	0·11	1·27	1·38	19·0
Glycine	1·05	0·22	5·2	5·5	28·8
Alanine	7·3	1·24	15·1	16·4	56·6
Cystine	0·19	—	0·21	0·21	—
Valine	0·33	0·05	1·37	1·42	23·2
Methionine	0·05	—	0·25	0·25	9·7
Isoleucine	0·09	0·03	0·81	0·84	18·1
Leucine	0·35	0·05	1·40	1·45	32·3
Tyrosine	0·17	—	0·57	0·57	13·3
Phenylalanine	0·35	—	0 71	0·71	16·4
Ammonia	0·17	0·25	2·18	2·43	11·5
Total amino acid	33·14*	5·83	51·36†	57·31	495·2‡

* Plus six unidentified peaks which comprised 3·5% total pool.
† Plus four unidentified peaks which comprised < 1% total pool.
‡ Plus one peak which comprised 3% total pool (probably hydroxylysine).

However, the rate and amount of protein release was not influenced by the presence of exogenous amino acids although these reduced the death rate (Table 1).

Release of amino acids and ammonia. The intracellular amino acid pool of *Streptococcus lactis* accounted for more than 3 % of the dry wt of freshly suspended organisms and this pool was rapidly depleted on starvation (Fig. 2). Ninhydrin-reactive material appeared concurrently in the external medium and there was a net increase in the total free amino acids on starvation.

The 73 % net increase in the total free amino acids after 28 hr starvation (Table 2) suggested that, in addition to leakage of intracellular amino acids, some protein degradation occurred. This increase in amino acid concentrations would correspond

to a 5 % breakdown of the total bacterial protein. Colorimetric methods of protein estimation were presumably too insensitive to estimate these small changes accurately (see Fig. 1). No low molecular weight peptides were observed on the amino acid traces but it is possible that if they were present they may have been removed when the samples were deproteinized. The total amount of amino acids obtained from hydrolysed organisms (Table 2) was consistent with earlier analyses for bacterial protein.

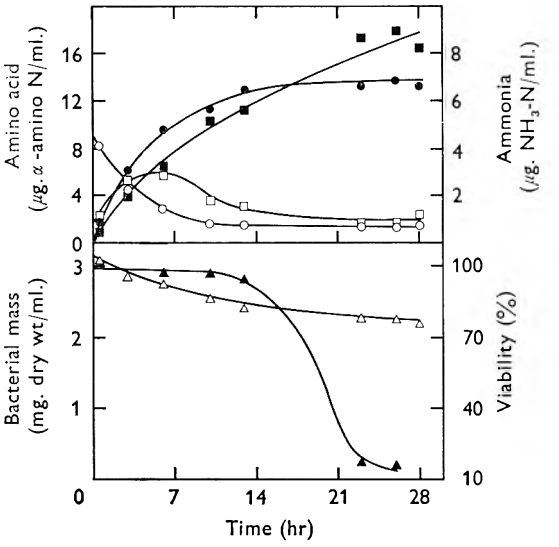

Fig. 2. Release of ninhydrin-reactive material and ammonia from starved *Streptococcus lactis* organisms. Bacteria were harvested, resuspended and sampled at intervals, as described for Fig. 1. Bacterial extracts were prepared and all samples were deep-frozen and later analysed (see Methods). Intracellular free amino acid and ammonia are shown as ○, □; supernatant amino acid and ammonia are shown as ●, ■. Bacterial mass (△) and viabilities (▲) were determined as described in Methods.

Glutamic acid, alanine and aspartic acid made up 49·2 %, 22·0 % and 12·8 % respectively of the initial amino acid pool. The total amount of free aspartic acid was substantially reduced on starvation while the glutamic acid level showed a slight decrease (Table 2). There have been no reports of the catabolism of these amino acids by *Streptococcus lactis*. The total amount of free lysine increased considerably on starvation while other amino acids increased by varying amounts. The levels of amino acids in the intracellular pool reflected the total amino acid composition of the organisms (Table 2). Neither free arginine nor ornithine were detected in any samples and only a small amount of NH_3 was produced (Table 2). These results suggested that starved *Streptococcus lactis* was unlikely to obtain substantial energy from the amino acid pool.

Changes in nucleic acids. A substantial amount of material, with an absorption maximum of 257 mμ, was released from starved organisms (Fig. 3). This could be correlated with nucleic acid breakdown and the release of ultraviolet (u.v.)-absorbing purine and pyrimidine fragments.

In the absence of added Mg^{2+}, bacterial RNA was broken down at a rapid rate

from the onset of starvation, the degradation products being released into the suspending buffer. Organisms initially contained 20·8 % RNA, which was reduced after 28 hr starvation to 5·5 % RNA (calculated on the initial bacterial dry wt). With added Mg^{2+}, the death rate was reduced and RNA was broken down only after a considerable lag, the initial RNA level of 20·7 % dry wt being reduced to 10·4 % after 28 hr (Fig. 3).

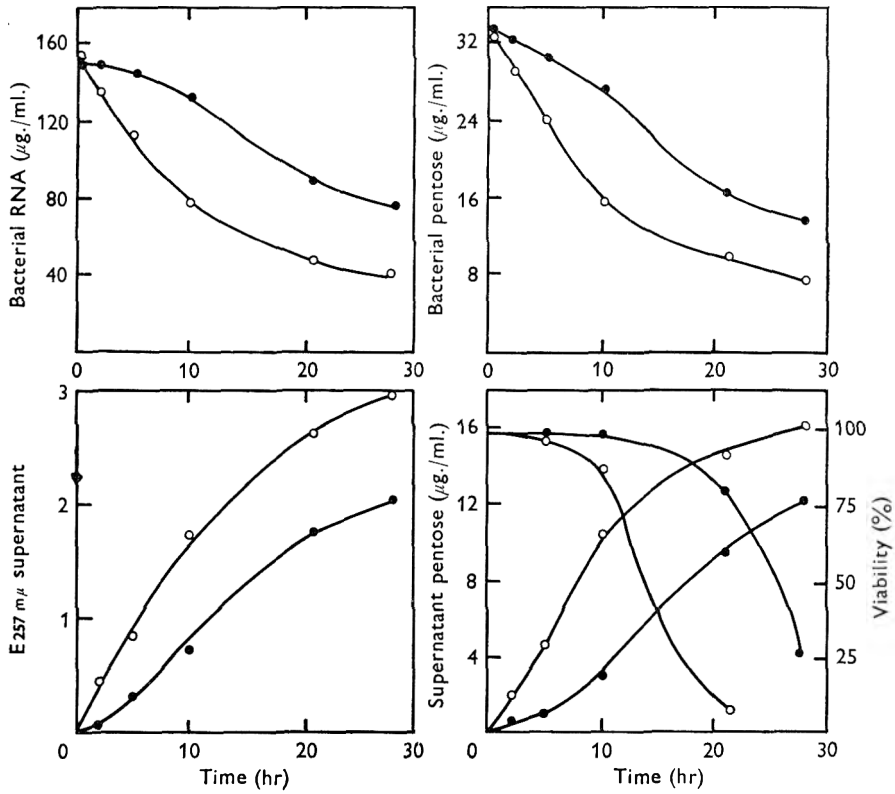

Fig. 3. Breakdown of RNA in starved *Streptococcus lactis* organisms. Bacteria were harvested from routine growth medium at the end of the log-phase, washed once and resuspended at 30° in 0·075 M-phosphate buffer (pH 7·0, + 10 μM-EDTA) containing: ○, no addition; ●, 1 mM-$MgSO_4$. Bacterial masses were initially 0·74 and 0·73 mg. dry wt/ml. respectively. Samples were removed at the times indicated and immediately centrifuged. Supernatant and cellular extracts were prepared as described in Methods, frozen and later analysed.

Without added Mg^{2+}, most organisms were still viable when 50 % of the cellular RNA had been lost, although with added Mg^{2+} most organisms were non-viable at this point (Fig. 3).

In both suspensions loss of cellular orcinol-reactive material was similar to RNA loss but was not balanced by a corresponding increase in supernatant orcinol-reactive material (Fig. 3), suggesting that some ribose may have been catabolized. However, lactic acid was not produced from exogenous ribose (Thomas & Batt, 1968), and since the orcinol reaction does not estimate pyrimidine bound ribose it may not be possible to balance ribose concentrations. In later experiments measurements of the release of u.v.-absorbing material into the suspending buffer were used as a measure of RNA

breakdown after samples had been treated with TCA to remove u.v.-absorbing protein (see Methods).

The rates of RNA breakdown and loss of viability were measured in the presence of substrates which produced the maximum range of death rate and cell division lag time (Fig. 4). Glucose-accelerated death was accompanied by extremely rapid RNA breakdown. Organisms starved in phosphate buffer also showed rapid rates of death and RNA breakdown. Addition of Mg^{2+} suppressed RNA degradation and the death rate but the presence of exogenous amino acids had only a slight effect on RNA breakdown although they markedly reduced the death rate.

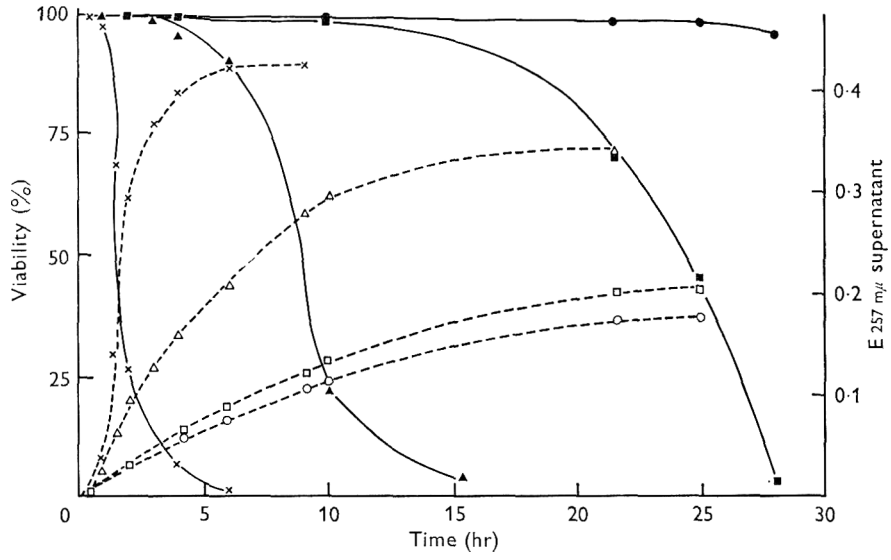

Fig. 4. RNA breakdown and death rate of starved *Streptococcus lactis* organisms. Washed organisms were resuspended at 0·09 mg. dry wt/ml. in phosphate buffer containing 10 μM-EDTA. Supernatant $E_{257m\mu}$ values for the suspensions: ○, +0·5 % casamino acids (Difco) +0·1 % arginine+ 1 mM-Mg^{2+}; □, + 1 mM-Mg^{2+}; △, no addition; ×, + 10 mM-glucose; are indicated by dashed lines. Viability curves of organisms in the same systems have solid lines.

No detectable change in bacterial DNA occurred in two experiments where organisms were starved for 16 hr at 0·24 mg. dry wt/ml. in phosphate buffer. The total DNA content of the suspensions remained at 8·84 ± 0·17 μg./ml. (DNA accounted for 3·7 % of the bacterial dry wt).

Changes in carbohydrates. Analyses for cellular anthrone-positive material and reducing sugars indicated that there was little, if any, carbohydrate breakdown in starved organisms (Table 3). Carbohydrate fermentation would be expected to produce predominantly lactic acid. The small amount of lactate produced (Table 3) suggested that about 2 % of the total cellular carbohydrate may have been fermented. Most of the hexose in *Streptococcus lactis* is likely to have a structural role. Other investigators working with other organisms (Strange *et al.* 1961; Postgate & Hunter, 1962; Ribbons & Dawes, 1963; Dawes & Ribbons, 1965; Burleigh & Dawes, 1967) have shown that structural carbohydrate is only slightly degraded, if at all, in starved bacteria.

Changes in lipids. Streptococcus lactis ML_3, grown in the routine medium, contained

3·8 % lipid on a dry wt basis (Table 4). A net loss of 10 % of this lipid occurred during a 55 hr starvation period. During this time the polar lipid fraction decreased from 85 % to 70 % of the total lipid and the neutral lipid fraction increased from 12 % to 30 % of the total lipid (Table 4). Spot intensities on H_2SO_4-charred thin-layer chromatograms indicated a marked increase in the free fatty acid component of the neutral

Table 3. *Cellular carbohydrate in starved Streptococcus lactis organisms*

Bacteria from the end of the growth phase were washed and resuspended in 0·075 M-phosphate buffer (pH 7·0 + 10 μM-EDTA) at 30° and 1·0 mg. dry wt bacteria/ml. Supernatant samples were removed and frozen at intervals. Anthrone-reactive carbohydrate was determined directly on washed organisms. Reducing sugar was measured in washed organisms after storage at −20° in 2·5 N-H_2SO_4 before hydrolysis. Carbohydrate is expressed as the mean μg. glucose equivalents/ml. of four determinations.

		Cellular carbohydrate		Supernatant	
Starvation period (hr)	Viability (%)	Anthrone + ve material	Reducing sugar	Anthrone + ve material	Lactate (μg./ml.)
0·2	98	77	107	0·2	0·6
1·2	97	77	102	0·6	0·7
2	95	74	105	0·5	0·9
3	95	75	109	0·7	1·2
4	96	74	106	1·0	1·0
5	93	72	106	0·9	1·6
10	68	73	103	0·9	1·8
22	12	74	102	0·8	1·9

Table 4. *Changes in lipid components of starved Streptococcus lactis*

Bacteria were grown at 30° in 3 × 10 l. flasks containing 10 l. routine medium, agitation being provided by a magnetic stirrer. Growth was followed turbidimetrically, and at the times indicated from the end of growth bacteria were harvested using a Sorvall continuous flow centrifuge. With a flow rate of 6 l./hr at 31,000 *g*, turbidity measurements on the supernatant indicated recovery of > 99 % bacterial dry wt. The packed bacteria were washed twice in distilled water and the lipids extracted, washed and analysed as described in Methods.

Time from end of growth (hr)	0	24	55
Bacterial yield* (g. dry wt)	6·72	5·73	4·97
Total lipid (g.)*	0·256	0·244	0·228
(% bacterial dry wt)	(3·81)	(4·25)	(4·59)
Neutral lipid (g.)*	0·032	0·036	0·070
(% total lipid)	(12·5)	(14·6)	(30·6)
Polar lipid (g.)*	0·218	0·201	0·159
(% total lipid)	(85·2)	(82·5)	(69·7)
Lipid recovery (%)	97·7	97·1	100·3
Polar lipid P (%)	2·37	2·28	1·86
Polar lipid N (%)	0·72	0·67	0·95
Viability (%)	99	96	32

* Mean of two or more batches.

lipid on starvation. Hydrolysis of polar lipid may explain these changes. The polar lipid phosphorus and nitrogen values observed (Table 4) were similar to those reported by Ikawa (1963) for a range of lactic acid bacteria.

Analysis of the fatty acids in the neutral and polar lipid fractions from *Streptococcus lactis* revealed a composition very similar to that previously reported for this organism

by MacLeod, Jensen, Gander & Sampugna (1962). Starvation brought about changes in the fatty acid composition of the two fractions (Table 5). The relative amount of hexadecanoic acid decreased in the neutral lipid fraction while tetradecanoic acid increased. The cyclopropane acid, lactobacillic acid, increased in both neutral and polar lipid fractions with a corresponding decrease of its immediate precursor, *cis*-vaccenic acid. Knivett & Cullen (1967) reported that *Escherichia coli*, in the post-

Table 5. *Changes in fatty acids in starved Streptococcus lactis.*

The neutral and polar lipid fractions of the lipid extracts in Table 4 were hydrolysed, the fatty acids isolated, esterified and finally identified and quantitatively measured by gas-liquid chromatography (see Methods). Results are the means of four analyses and are expressed as % of the total fatty acid ester in each sample.

Fatty acid (probable identity)	Neutral lipid			Polar lipid		
	0 hr	24 hr	55 hr	0 hr	24 hr	55 hr
^{c}12:0	1·1	1·3	0·9	0·1	0·0	0·2
^{c}14:1	0·5	0·6	0·8	0·4	1·1	1·3
^{c}14:0	8·7	10·3	13·9	13·7	17·4	14·9
^{c}16:1	3·9	4·1	4·2	4·1	3·7	4·2
^{c}16:0	34·7	26·8	25·9	20·8	20·7	21·4
^{c}18:1	24·6	26·8	21·0	30·2	20·3	18·1
^{c}18:0	2·7	2·4	1·4	0·1	1·1	0·8
^{c}19:0$^{\nabla}$	24·8	27·6	31·7	30·8	35·7	39·2

^{c}18:1 represents *cis*-vaccenic acid. ^{c}19:0$^{\nabla}$ represents lactobacillic acid.

Table 6. *Summary of changes in components of Streptococcus lactis organisms starved at 30° in 0·075 M-phosphate buffer (pH 7·0, 10 μM-EDTA, 1 mM-$MgSO_4$)*

(1) Depletion of polymers and amino acid pool (results are expressed as % initial bacterial dry wt)

Starvation period (hr)	Protein*	Amino acid† pool	RNA‡	DNA	Carbohydrate§	Lipid‖	Total	Bacterial dry wt*
0	48	3·3	20·7	3·7	7·7	3·8	87·2	100
28	40·7¶	0·6	10·4	3·7	7·2	3·6	66·2	74
Loss	7·3	2·7	10·3	0·0	0·5	0·2	21·0	26

(2) Formation of products (results of analyses on the suspending buffer expressed as % initial bacterial dry wt)

Starvation period (hr)	Lactate§	NH_3†	Volatile fatty acid (as acetic acid)	Amino acid†
28	0·21	0·22	0·36	5·73

* Fig. 1.
† Table 2. ‡ Fig. 3. § Table 3 (carbohydrate = anthrone+ve).
‖ Table 4 (24 hr).
¶ Estimated from protein released* and increase in total free amino acids†.

exponential growth phase, showed increases in cyclopropane fatty acids with decreases in the corresponding monoenoic acids and similar changes have been reported with many bacterial species after growth has stopped (see Kates, 1964).

A small amount of volatile fatty acid (0·6 μeq./mg. dry wt bacteria/24 hr) was released into the suspending buffer (0·075M-phosphate + 1 mM-$MgSO_4$) by starved *Streptococcus lactis* organisms. The source of this material has not been defined.

A summary of the chemical changes which occurred when *Streptococcus lactis* was starved at the growth temperature is presented in Table 6.

DISCUSSION

Streptococcus lactis did not accumulate detectable amounts of polyglucose or poly-β-hydroxybutyrate in conditions which might be expected to be favourable for their synthesis. No reports have appeared in the literature indicating the presence of these or other storage polymers in lactic streptococci. Only one report has been published on the metabolism of *S. lactis* starved at growth temperatures and this claimed that (*a*) 'the organism had a substantial endogenous respiration' and (*b*) 'endogenous lactate or succinate was oxidized after a lag' (Spendlove, Weiser & Harper, 1957). These results were not confirmed by Thomas (1968) and it seems unlikely that lactate or succinate could function as endogenous substrates for an organism which is a homolactic fermenter, possessing no terminal respiratory system.

Whether bacteria possess polymeric storage reserves or not, the constitutive material of starved organisms is ultimately degraded and death ensues. Most of the decrease in cell mass of starved *Streptococcus lactis* organisms could be accounted for by RNA and protein degradation. The rates of RNA breakdown and death were reduced by the addition of Mg^{2+}, in contrast to the findings of Burleigh & Dawes (1967) with *Sarcina lutea*. However, these authors examined the effect of added Mg^{2+} with suspensions of high bacterial density (8·8 mg. dry wt/ml.) and it is possible that if Mg^{2+} was liberated by the organisms as their RNA was degraded, the effect of added Mg^{2+} on survival may have been masked (see Thomas & Batt, 1968) since RNA appeared to be to some extent expendable. The protective effect of Mg^{2+} on Gram-negative organisms under conditions of stress has been demonstrated with bacterial concentrations of 20 μg. dry wt/ml. or less (see Postgate & Hunter, 1962, 1964; Strange & Postgate, 1964; Strange & Dark, 1965). These stresses showed population density effects so that the response of *S. lutea* to added Mg^{2+} may not be basically different from that defined for Gram-negative organisms. *S. lactis* has both a high RNA content, typical of an organism grown at a rapid rate, and a high Mg content, which is consistent with the probable interdependence of these constituents (Tempest & Strange, 1966). The molar ratio of RNA/Mg was approximately 50, which is of the same order as that observed for *Aerobacter aerogenes* (Tempest & Strange, 1966).

Conditions which accelerated RNA breakdown—such as buffer systems which either contained glucose alone or did not contain Mg^{2+}—also produced increased death rates. However, in the presence of Mg^{2+}, arginine only slightly suppressed RNA degradation although it extended survival. Hence no general correlation exists between RNA degradation and loss of viability. Although considerable RNA may be degraded without affecting viability, in agreement with results for many other starved bacteria (see Burleigh & Dawes, 1967), it seems likely that a degree of ribosome stability is important for survival of *Streptococcus lactis*. Conditions which accelerated RNA breakdown in other bacteria were generally more lethal (e.g. see Strange & Shon, 1964; Strange & Dark, 1965) and Postgate (1967) has concluded that RNA degrada-

tion is a critical process in the survival of *Aerobacter aerogenes*. However, no absolute correlation between the rates of death and RNA breakdown could be demonstrated.

No evidence was obtained for the catabolism of ribose or bases from degraded RNA, in contrast to findings with most other starved organisms (see Dawes & Ribbons, 1964). It has been suggested that RNA breakdown may continue in viable bacteria as long as mechanisms for RNA resynthesis from precursors remain intact (Burleigh & Dawes, 1967). The present results show that conditions producing maximum rates of RNA degradation and death also produce increased cell division lag times of surviving organisms (Thomas & Batt, 1968). These lags are probably directly influenced by the amount of polymer degradation which has taken place, particularly that of RNA, since the RNA content of bacteria increases with the growth rate (Tempest & Strange, 1966) while the rate of protein synthesis per ribosome particle is constant in growing bacteria (Tempest, Herbert & Phipps, 1967). Most of the protein lost from starved organisms appeared in the external medium as biuret-positive material although the net increase in total free amino acids indicated that some protein had been hydrolysed. Protein hydrolysis occurs in many starved bacteria with subsequent metabolism of the released amino acids (see Dawes & Ribbons, 1964). Although the over-all level of free aspartate (and to a lesser extent glutamate) was reduced, no evidence was obtained for appreciable catabolism of components of the free amino acid pool in *Streptococcus lactis*.

The loss of RNA and protein from starved *Streptococcus lactis* appeared to involve only hydrolytic reactions with the release of undegraded products into the external medium. Viability was not immediately affected and there was no evidence for appreciable energy-yielding catabolism of endogenous substrates. This was consistent with the formation of only trace amounts of the normal end products of fermentation, namely lactate, NH_3 and volatile fatty acids. The cellular anthrone-reacting material and reducing sugar of *S. lactis* (which probably occurs mainly in structural polymers), together with cellular DNA, were not appreciably degraded in starvation conditions. These results are in agreement with literature reports for other starved organisms. However, Postgate (1967) and Burleigh & Dawes (1967) pointed out that chemical analyses would not detect structural changes in DNA that could cause loss of viability.

Polar lipid and in particular phospholipid, constitutes the main lipid fraction in most lactic acid bacteria (Kates, 1964). In *Streptococcus faecalis*, 94% of the total lipid was found in the membrane fraction (Vorbeck & Marinetti, 1965*b*). In the present investigation, a substantial amount of phospholipid breakdown occurred on prolonged starvation of *Streptococcus lactis*. Since these compounds are known to have important structural and physiological roles in bacterial membranes (Brown, 1964; Salton, 1967) any breakdown of phospholipids may be expected to impair permeability barriers. The possible significance of these observations in the survival of starved *S. lactis* is discussed elsewhere (Thomas & Batt, 1969).

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Synthesis of Protein and Ribonucleic Acid by Starved *Streptococcus lactis* in Relation to Survival

By T. D. THOMAS* AND R. D. BATT

New Zealand Dairy Research Institute, Palmerston North, New Zealand, and Chemistry and Biochemistry Department, Massey University, Palmerston North, New Zealand

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SUMMARY

Protein synthesis, as estimated from incorporation studies with [^{14}C]valine, was barely detectable when organisms were starved in phosphate buffer containing Mg^{2+}. The addition of an energy source promoted limited protein synthesis. In this respect glucose produced a much higher rate of [^{14}C]valine uptake and incorporation than arginine. Although arginine prolonged survival, experiments with inhibitors showed that this was unlikely to be due to the protein synthesis which had been promoted. The ability of starved organisms to assimilate [^{14}C]valine and to incorporate the isotope into protein in the presence of glucose appeared to be correlated with survival.

Synthesis of RNA in starved organisms, as determined from [^{14}C]uracil incorporation experiments, depended on exogenous glucose. During brief exposures to [^{14}C]uracil plus glucose at intervals during starvation, organisms incorporated decreasing amounts of isotope into RNA although incorporation by non-viable organisms occurred at approximately half the initial rate. Although resynthesis of both protein and RNA was demonstrated with added energy sources, no evidence was obtained to suggest that this favoured survival.

INTRODUCTION

When bacteria are starved of a nitrogen source, net protein and RNA synthesis stops but protein and RNA turnover may continue for several hours. Degradation of pre-existing protein and RNA provides amino acids and bases for resynthesis (Mandelstam, 1960, 1963). Although the rates of protein degradation may be similar in both nitrogen- and carbon-limited media (Willetts, 1967), the rate of protein synthesis depends on an energy source (Schlessinger & Ben-Hamida, 1966). These authors reported declining, although significant protein turnover in nitrogen-starved *Escherichia coli* for at least 20 hr. Ben-Hamida & Schlessinger (1966) recorded a much lower rate for RNA turnover in nitrogen-starved *E. coli* and concluded that ribosomes were not resynthesized and that the net effect of the turnover process was to transfer amino acids and nucleotides from a surplus of ribosomes to the soluble proteins, energy supply and reserves required for subsequent adaptations.

Macromolecule resynthesis may represent a mechanism whereby cell constituents, whose loss is particularly likely to result in death, may be selectively reformed from dispensible material (Burleigh & Dawes, 1967). The present paper describes experiments undertaken to determine the role of polymer resynthesis in the survival of starved *Streptococcus lactis*.

* Present address: Sub-Department of Chemical Microbiology, Department of Biochemistry, University of Cambridge, Cambridge, Great Britain.

METHODS

General procedures. The organism (*Streptococcus lactis* ML_3), the conditions of growth and starvation and the methods used for determining bacterial mass and viability were described in preceding papers (Thomas & Batt, 1968, 1969*a*).

Measurement of protein synthesis. Protein synthesis in starved organisms was estimated by following the incorporation of [^{14}C]valine into cell protein (Willetts, 1967). Culture samples (2 ml.) were added to an equal volume of trichloroacetic acid (TCA, 10 %, w/v) and, after heating 30 min. at 90° (see Marchesi & Kennell, 1967), the precipitates were filtered off on membrane filters (25 mm., pore size 0·45 μ; Millipore Filter Corp., U.S.A.). Each filter was washed successively with three 1 ml. volumes of TCA (5 %, w/v) containing unlabelled DL-valine (150 μg./ml.), TCA (5 %, w/v) alone and acetic acid (1 %). Material isolated by this procedure may include cell wall substance as well as 'true' protein. However, lactic acid bacteria are reported to contain little valine in the cell walls (Ikawa & Snell, 1960).

For the measurement of [^{14}C]valine uptake by whole cells, culture samples (2 ml.) were filtered on membrane filters, washed with ten 2 ml. volumes of 0·075 M-phosphate buffer containing unlabelled L-valine (200 μg./ml.) and dried. The washing procedures used for incorporation and uptake measurements removed all radioactivity from the membrane filters in control systems to which cells had not been added. [^{14}C]Valine incorporation into protein was estimated with a standard deviation of ± 4 % (16 determinations); for [^{14}C]valine uptake by cells it was ± 6 % (8 determinations).

Measurement of RNA synthesis. Estimates of RNA synthesis were made by analogous procedures with [^{14}C]uracil. Trichloroacetic acid at 0° was added to culture samples giving a final concentration of 5 %. After 30 to 60 min. at 0°, the acid-insoluble material was collected on a membrane filter, washed at 0° with 5 % TCA (10 × 2 ml.) and finally with 0·1 N-HCl (2 × 2 ml.); the filters were then dried. For the measurement of [^{14}C]uracil uptake by whole cells, the organisms were collected on a membrane filter and washed with ten 2 ml. volumes of 0·075 M-phosphate buffer containing uracil (200 μg./ml.). [^{14}C]Uracil incorporation into RNA was estimated with a standard deviation of ± 2 % (16 determinations); for [^{14}C]uracil uptake by cells it was ± 4 % (8 determinations).

The radioactivity on the dried membrane filter discs was measured with a Packard Tri-Carb 2000 series liquid scintillation spectrometer (gain settings 10 %, window settings 50–1000) with an efficiency of 70·5 % as determined by channels ratio measurements. The scintillation solution consisted of 2,5-diphenyloxazole (5·0 g.) and 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene (0·3 g.) per litre of toluene. [^{14}C]-DL-valine and [^{14}C]uracil were obtained from the Radiochemical Centre, Amersham.

RESULTS

Protein synthesis. Protein synthesis, determined by the measurement of [^{14}C]valine incorporation into hot TCA-insoluble material, required an exogenous energy source (Table 1); there appeared to be no endogenous energy source capable of supporting protein synthesis. Exogenous glucose produced approximately four times as much protein synthesis as arginine.

Uptake of [^{14}C]valine by starved organisms occurred without an exogenous energy

source but to a much lesser extent (Table 1). This initial uptake of a small amount of [^{14}C]valine and the subsequent loss of activity (Table 1) may indicate the presence of a small ATP pool at the onset of starvation. Retention of the accumulated TCA-soluble [^{14}C]valine by starved organisms required an exogenous energy source. At the bacterial density used in Table 1, 10 mM-glucose would be completely fermented in approx. 2 hr while 10 mM-arginine would require about 7 hr for complete metabolism (Thomas & Batt, 1969*b*).

Table 1. [^{14}C]*Valine uptake and incorporation by starved Streptococcus lactis*

Washed organisms, 0·64 mg. dry wt/ml., were starved at 30° in 0·075 M-phosphate buffer (pH 7·0) containing 10 μM-EDTA, 1 mM-$MgSO_4$, [1-^{14}C]-DL-valine (25 μc./ml., 6 μg./ml.) and L-valine (200 μg./ml.). Additions or omissions from this buffer are given in the first column. Samples (2 ml.) were removed at intervals and treated as described in Methods. Counts of total [^{14}C]valine uptake are given in parentheses.

	Time (min.)							
	7	17	30	45	60	90	120	190
Additions to above buffer	[^{14}C]valine incorporation and uptake (c.p.m./mg. dry wt bacteria*).							
None	9 (619)	11 (659)	11 (446)	— —	14 (318)	— —	20 (185)	11 (154)
+Glucose (10 mM)	247 (1513)	471 (1981)	626 (2227)	— —	801 (3144)	— —	1080 (3090)	1137 (3180)†
+L-arginine (10 mM)	44 (705)	91 (898)	167 (934)	— —	243 (1364)	— —	242 (1381)	265 (1378)
+L-arginine (10 mM)+casamino acids (2·78 mg./ml.)+L-valine	61 (499)	115 (703)	228 (845)	— —	319 (904)	— —	331 (912)	340 (856)
+Glucose (10 mM)+chloramphenicol (200 μg./ml.)	—	—	—	152 (1934)	—	214 (2224)	—	—
L-arginine (10 mM) +chloramphenicol (200 μg./ml.)	— —	— —	— —	28 (981)	— —	34 (1209)	— —	— —

* Based on initial bacterial mass. † Equivalent to approx. 0·5 % total [^{14}C]valine.

Net protein synthesis appeared to stop when the glucose was exhausted. By contrast, net synthesis stopped in the presence of excess arginine, even with casamino acids present (Table 1). *Streptococcus lactis* ML_3 organisms contained 23 μg. valine/mg. dry wt (Thomas & Batt, 1969*a*). Assuming that all the valine is present in cell protein and that the protein synthesized in starved organisms contained the above proportion of valine, then the amount of protein synthesis in starved organisms could be calculated from the rates of [^{14}C]valine incorporation. On this basis, with exogenous glucose and arginine, protein synthesis would amount to about 2 % and 0·5 % of the total cell protein in the first hour of starvation. The apparent cessation of protein synthesis after a period of starvation in the presence of arginine may have resulted from a balance being reached between protein synthesis and degradation. However, no relevant data were available to estimate the rate of protein degradation in the presence of exogenous arginine. Any TCA-insoluble protein released from starved organisms into the external medium during these experiments would be measured as cell protein. The concentration of free valine in *S. lactis* accounted for only 1 % of the total amino

acid pool (Thomas & Batt, 1969*a*). On incubation with glucose and radioactive valine, the concentration of free intracellular valine was calculated to increase by about three-fold so that the over-all change in the free amino acid pool during incorporation experiments was probably small and the differences in the rates of protein synthesis are not likely to result from differences in the levels of free amino acids.

Chloramphenicol caused 78 % and 86 % inhibitions of protein synthesis in the presence of glucose and arginine respectively (Table 1) while little or no inhibition of [^{14}C]valine uptake into the TCA-soluble pool was detected. (For these calculations, 45 and 90 min. values were obtained by expressing the data graphically.) This finding is consistent with the reports that chloramphenicol does not inhibit the transport or accumulation of other amino acids by micro-organisms (Gale & Paine, 1951; Holden, 1965).

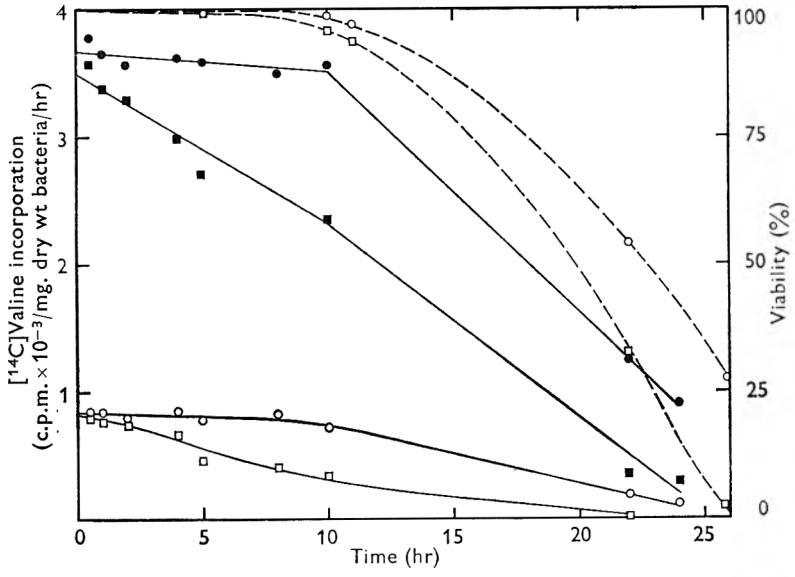

Fig. 1. Ability of starved *Streptococcus lactis* to assimilate and incorporate [^{14}C]valine. Washed organisms were resuspended at a density of 0·93 mg. dry wt/ml. in 0·075 M-phosphate buffer (pH 7·0, + 10 μM-EDTA + 1 mM-$MgSO_4$) containing: L-arginine (10 mM), (○, ●); no addition (□, ■). The cultures were starved at 30°, samples (2 ml.) were withdrawn at intervals and added to 2 ml. 0·075 M-phosphate buffer containing $MgSO_4$ (1 mM), D-glucose (20 mM), [1-^{14}C]-DL-valine (0·5 μc./ml., 12 μg./ml.) and L-valine (400 μg./ml.). After incubation for 1 hr at 30°, the incorporation of [^{14}C]valine into cells (closed symbols) and protein (open symbols) was determined (see Methods). Results are based on the initial bacterial mass. The dashed lines indicate slide-culture viabilities.

The ability of starved organisms to assimilate [^{14}C]valine and to synthesize protein appeared to be correlated with survival (Fig. 1). With the bacterial concentration used in this experiment, 10 mM-arginine would be metabolized in approx. 4 hr (Thomas & Batt, 1969*b*) and consequently survival was not enhanced to the same extent as at lower bacterial densities. Although chloramphenicol inhibited protein synthesis, it did not increase the death rate and, in fact, there appeared to be a reduction in the death rate in some systems (Table 2).

RNA synthesis. RNA synthesis in starved organisms, as determined by the incorporation of [^{14}C]uracil into cold TCA-insoluble material, required exogenous

glucose (Table 3). Exogenous arginine gave only trace [^{14}C]uracil incorporation in contrast to its effect on [^{14}C]valine incorporation (Table 1). Net RNA synthesis stopped in the presence of excess glucose and uracil after a short starvation period (Table 3). This may have been due to either a shortage of other intermediates or to a balance being reached between synthesis and breakdown.

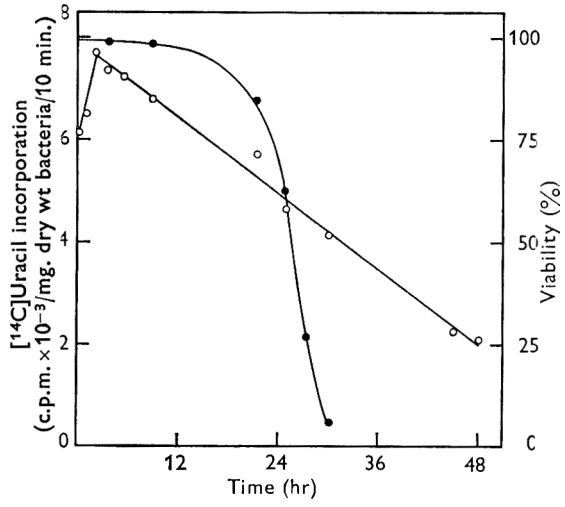

Fig. 2. Ability of starved *Streptococcus lactis* to incorporate [^{14}C]uracil. Washed organisms were resuspended at a density of 0·96 mg. dry wt/ml. in 0·075 M-phosphate buffer (pH 7·0, 30°) containing 10 μM-EDTA and 1 mM-$MgSO_4$. Samples (2 ml.) were removed at intervals and added to 20 μl. buffer containing [^{14}C]uracil (0·5 μc., 60 μg.) and glucose (20 μmoles). After incubation for 10 min. at 30°, the incorporation of [^{14}C]uracil into RNA was determined (○) (see Methods). Results are based on the initial bacterial mass and viability (●) was determined by slide-culture.

Table 2. *Effect of chloramphenicol on the survival of Streptococcus lactis*

Washed organisms, 30 μg. dry wt/ml., were starved at 30° in phosphate buffer (0·075 M, pH 7·0, + 10 μM-EDTA + 1 mM-$MgSO_4$) containing the additions given in the first column. Samples were removed at intervals and the organisms washed (except where indicated). Viability was determined by slide-culture.

Additions to phosphate buffer	Time (hr) Viability (%)							
	0	0*	16	20	24	28	40	45
None	99	99	96	81	79	37	1	0
+ Chloramphenicol (200 μg./ml.)	99	0	98	91	92	80	6	1
+ Glucose (10 mM)	99	99	69	43	26	14	15	3
+ Glucose (10 mM) + chloramphenicol (200 μg./ml.)	99	0	97	87	78	82	26	1
+ Arginine (10 mM)	99	99	99	96	98	94	85	52
+ Arginine (10 mM) + chloramphenicol (200 μg./ml.)	99	0	98	99	99	87	76	31

* Slide-cultures prepared with unwashed organisms.

The rate of RNA synthesis rose to a reproducible maximum soon after the onset of starvation and then fell steadily (Fig. 2). Non-viable organisms still incorporated [^{14}C]uracil into RNA at an appreciable rate.

When a brief incubation of organisms with [^{14}C]valine plus glucose or with [^{14}C]uracil plus glucose was followed by a 100-fold excess of unlabelled valine or uracil, most of the isotope incorporated was retained in the starved organisms. Therefore exchange of the incorporated isotope does not occur readily with the external medium. Since *Streptococcus lactis* does not metabolize either valine or uracil, conversion of the label would not have affected the interpretation of the present results.

Table 3. [^{14}C]*Uracil uptake and incorporation by starved Streptococcus lactis*

Washed organisms, 0·36 mg. dry wt/ml., were starved at 30° in 0·075 M-phosphate buffer (pH 7·0) containing 10 μM-EDTA, 1 mM-$MgSO_4$, [^{14}C]uracil (0·25 μc./ml., 200 μg./ml.) and the substrates given below. Samples (2 ml.) were removed at intervals and treated as described in Methods. Results are given as c.p.m./mg. initial dry wt bacteria.

Starvation period (min.)	Additions to buffer: None		Glucose (10 mM)		Arginine (10 mM)	
	Uptake	Incorp.	Uptake	Incorp.	Uptake	Incorp.
7	16	0	2242	875	0	0
18	174	11	4322	1230	168	9
30	568	3	5639	1277	364	13
60	477	14	7002	1273	547	31
120	116	0	7650	1112	502	14
180	160	6	7201*	1003	566	29

* Equivalent to approx. 1 % total [^{14}C]uracil.

DISCUSSION

Since macromolecule synthesis is an energy-consuming process, protein and RNA synthesis in starved *Streptococcus lactis* was not expected as no substantial endogenous energy source has been detected (Thomas & Batt, 1969*a*). The present investigation demonstrated that the accumulation and incorporation of [^{14}C]valine and [^{14}C]uracil required an exogenous energy source. Exogenous glucose promoted greater isotope accumulation and subsequent incorporation than arginine, perhaps because glucose metabolism produced a higher rate of ATP production (Thomas & Batt, 1969*b*). The acceleration of [^{14}C]valine uptake in the presence of an added energy source and the energy requirement for the retention of accumulated amino acid was consistent with mechanisms for the accumulation of amino acids in other bacteria (see Holden, 1962).

The survival capacity of starved organisms could be correlated with the ability to synthesize protein which may in turn be influenced by RNA stability. In contrast, non-viable organisms retained the ability to synthesize RNA at a substantial rate. Schlessinger & Ben-Hamida (1966) and Ben-Hamida & Schlessinger (1966) reported rapidly declining protein and RNA synthesis in nitrogen-starved *Escherichia coli* from the onset of starvation although it is not clear whether or not the energy source was exhausted after a period giving rise to the reduced isotope incorporation.

No evidence has been obtained in the present investigation to suggest that resynthesis of protein or RNA is important for survival. In fact, exogenous glucose, which gave maximum rates of resynthesis, increased the death rate and the rate of RNA hydrolysis (Thomas & Batt, 1969*a*). Experiments with chloramphenicol indicated that the pro-

longed survival with added arginine is unlikely to be a result of the limited protein synthesis which took place. In some starvation environments chloramphenicol was shown to reduce the death rate. As well as inhibiting protein synthesis, chloramphenicol may indirectly inhibit protein degradation (Schlessinger & Ben-Hamida, 1966; Willetts, 1967) and hence may exert some sparing action on specific cellular proteins which are essential for survival of *Streptococcus lactis*. All reported studies involving the starvation of vegetative bacteria indicate that, while macromolecule degradation may be balanced by synthesis during the initial starvation period if an adequate energy source is present, diminishing synthesis and a net increase in catabolism eventually results.

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Metabolism of Exogenous Arginine and Glucose by Starved *Streptococcus lactis* in Relation to Survival

By T. D. THOMAS* AND R. D. BATT

New Zealand Dairy Research Institute, Palmerston North, New Zealand, and Chemistry and Biochemistry Department, Massey University, Palmerston North, New Zealand

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SUMMARY

Arginine or glucose accelerated death of *Streptococcus lactis* starved in phosphate buffer. Both substrates were metabolized at linear but different rates and the rate of ATP production from glucose metabolism was about 7·5 times that for arginine metabolism. Mg^{2+} reduced glucose-accelerated death and abolished arginine-accelerated death to prolong survival. When glucose was fed continuously to organisms starved in phosphate buffer containing Mg^{2+}, survival was related to the rate of glucose addition and hence to the rate of metabolism. By feeding low concentrations of glucose, the viability of most of the population was extended from about 1 to 4 days. The maintenance energy requirement appeared to be in the region of 0·045 to 0·09 mg. glucose/mg. dry wt bacteria/hr. The glycolytic activity of starved organisms declined at varying rates depending on the starvation environment but could not be correlated directly with loss of viability. Exogenous arginine substantially reduced the lethal effect of adverse pH values.

INTRODUCTION

Streptococcus lactis ferments carbohydrate mainly by means of the Embden-Meyerhof pathway, and in an earlier investigation (Thomas & Batt, 1969*a*) the lactic acid produced was found to account for approximately 93 % of the glucose fermented. Apart from carbohydrate, arginine is the only substrate reported from which *S. lactis* can obtain energy. Catabolism of arginine by *S. lactis* produced ornithine, carbamyl phosphate and ammonia and the enzymes catalysing these reactions were characterized by Korzenovsky & Werkman (1953, 1954). When *S. lactis* was starved in the presence of a fermentable carbohydrate, accelerated death occurred (Thomas & Batt, 1968). Addition of Mg^{2+} reduced this effect while incubation of starved organisms with arginine plus Mg^{2+} gave extended survival indicating that the nature of the energy source was critical for survival.

Postgate & Hunter (1962) reported that glycerol-limited *Aerobacter aerogenes* died more rapidly when washed and starved in phosphate buffer containing glycerol than in phosphate buffer alone. This phenomenon was termed 'substrate-accelerated death' and has since been investigated intensively for *A. aerogenes* (see review by Postgate, 1967). Strange & Dark (1965) reported that the death rate of *A. aerogenes* increased with the rate of substrate metabolism while exogenous Mg^{2+} decreased the rate of

* Present address: Sub-Department of Chemical Microbiology, Department of Biochemistry, University of Cambridge, Cambridge, Great Britain.

degradation of intracellular RNA without significantly affecting substrate metabolism. The mechanism of substrate-accelerated death is not fully understood. However, Strange & Dark (1965) attributed glycerol-accelerated death, at least in part, to the formation of an unidentified toxic product of glycerol metabolism. They suggested that added Mg^{2+} prevented the accumulation or formation of this product. During glucose-accelerated death of *A. aerogenes* the bacterial ATP pool increased significantly whereas in the presence of glucose plus Mg^{2+} there was little change (see Discussion, Strange & Hunter, 1967). In this connexion, Postgate & Hunter (1964) showed that glycerol-accelerated death of *A. aerogenes* was largely abolished by uncoupling agents. Any proposed mechanism should be consistent with the fact that while exogenous glucose accelerates death the death rate may decrease in parallel with increasing intracellular polyglucose levels (Strange, Dark & Ness, 1961).

In contrast to the effect of carbon substrates on starving organisms discussed above however, there are several recent reports of decreased death rates resulting from the intermittent addition of small amounts of glucose to *Escherichia coli* starved in buffer containing Mg^{2+} (Clifton, 1966; McGrew & Mallette, 1962, 1965; Mallette, 1963). However, the evaluation of some of these results is difficult since the experimental procedure did not preclude regrowth (see Dawes & Ribbons, 1964; Postgate, 1967).

The present investigation was undertaken in an attempt to define the role of exogenous, energy-yielding substrates in relation to bacterial survival.

METHODS

General procedures. The organism (*Streptococcus lactis* ML_3), the conditions of growth and starvation and the methods used for determining bacterial mass and viability were described in preceding papers (Thomas & Batt, 1968, 1969*b*). With experiments which involved the continuous feeding of glucose, washed organisms suspended in phosphate buffer were placed in a triple-necked quickfit flask (1400 ml. capacity) held in an incubator at 30°. The culture was agitated by a magnetic stirrer and phosphate buffer containing glucose was added at a constant flow rate using a D.C.L. Micropump (F. A. Hughes and Co., England) attached to a reservoir flask fitted with a constant head device.

Analytical methods. The products of arginine metabolism were estimated using the basic column of a Beckman model 120C automatic amino acid analyser. The glycolytic activity of starved organisms was determined by the method of Thomas & Batt (1969*a*).

RESULTS

The effect of energy sources on the survival of starved *Streptococcus lactis* is shown in Fig. 1. Both arginine and glucose accelerated the death rate. Mg^{2+} reduced glucose-accelerated death while arginine + Mg^{2+} gave extended survival times.

Arginine was metabolized at a linear rate of 2·35 μmole/mg. dry wt bacteria/hr (Fig. 2). This rate theoretically corresponds to 2·35 μmole ATP/mg. dry wt bacteria/hr (based on the stoichiometric reaction:

$$\text{arginine} + H_2O + P_i + ADP \rightarrow \text{ornithine} + 2NH_3 + CO_2 + ATP).$$

The glycolytic activity of *Streptococcus lactis* ML_3 was 0·29 μmole lactate/mg. dry wt bacteria/min. (Thomas & Batt, 1969*a*) and, since anaerobic fermentation of

glucose produces 1 mole of ATP/mole lactate, this glycolytic activity represents the formation of 17·7 μmole ATP/mg. dry wt bacteria/hr. Therefore the theoretical rate of ATP generation by *S. lactis* is about 7·5 times greater with 10 mM-glucose than with 10 mM-arginine. Arginine metabolism yielded the theoretical amount of ornithine and slightly less than the theoretical amount of ammonia (Fig. 2), confirming the findings of Korzenovsky & Werkman (1954). In earlier survival experiments (Thomas & Batt, 1968) washed suspensions with 20 μg. dry wt bacteria/ml. were incubated with 10 mM-arginine. Assuming the above linear rate, these organisms would require more than 200 hr for complete arginine metabolism, so that arginine was probably not limiting in these experiments.

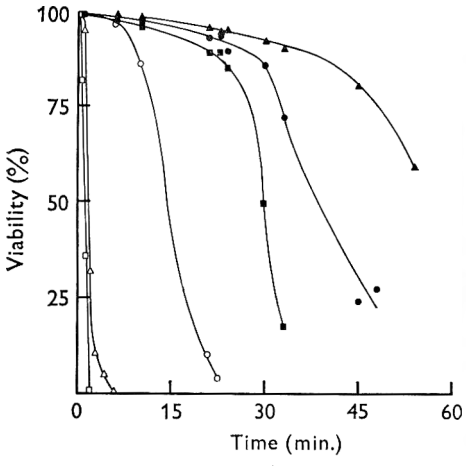

Fig. 1

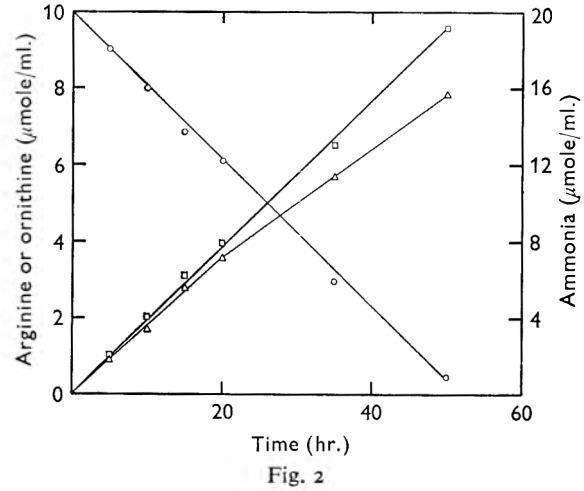

Fig. 2

Fig. 1. Effect of glucose and arginine on survival of *Streptococcus lactis*. Washed organisms, harvested from the end of the growth phase in routine medium, were resuspended at 20 μg. dry wt/ml. in 0·075 M-phosphate buffer (pH 7·0, 10 μM-EDTA) containing: ○, no addition; ●, 1 mM-$MgSO_4$; □, 10 mM-glucose; ■, 10 mM-glucose + 1 mM-$MgSO_4$; △, 10 mM-arginine; ▲, 10 mM-arginine + 1 mM-$MgSO_4$. Cultures were incubated at 30° and viability was determined by slide-culture.

Fig. 2. Arginine metabolism by *Streptococcus lactis*. Bacteria were harvested from the routine medium at the end of the growth phase, washed twice and resuspended in 0·075 M-phosphate buffer (pH 7·0; containing 10 μM-EDTA, 1 mM-$MgSO_4$ and 10 mM-L-arginine). The organisms were incubated at 30° at 4·9 mg. dry wt/ml. At intervals, samples were withdrawn, immediately cooled to − 10°, centrifuged and the supernatants filtered. Supernatant samples were frozen (− 20°) and later analysed for arginine (○), ornithine (□) and ammonia (△), using the basic column of an amino acid analyser (see Methods).

Certain published findings with other bacteria have suggested that the extended survival of starved *Streptococcus lactis* with added arginine, as compared with glucose, may have been due to a lower rate of substrate metabolism (see Discussion). In order to test this possibility, experimental conditions were established so that glucose could be fed continuously to starved organisms. Buffered glucose solutions were added to cultures at constant flow rates with different glucose concentrations being used in each experiment. Some typical results of viability measurements on the starved organisms are shown in Fig. 3. Starved *S. lactis* is capable of fermenting glucose at a maximum rate of about 8·8 μmole/mg. dry wt bacteria/hr (Thomas & Batt, 1969*a*).

When glucose was fed continuously to starved organisms in excess of this rate the death rate increased as expected (Fig. 3). By adding 1·5 μmole glucose/mg. dry wt bacteria/hr, a level which would be expected to produce ATP at about the same rate as added arginine (see above), the survival curve resembled that of organisms starved in the presence of arginine + Mg^{2+} (Fig. 1). Feeding lower levels of glucose further enhanced survival until an optimum level was reached at 0·25 to 0·5 μmole glucose/mg. dry wt bacteria/hr (Fig. 3). After 120 hr starvation at a feed rate of 0·25 μmole glucose/mg. dry wt bacteria/hr (viability 40 %), organisms were harvested for examination by electron microscopy (Thomas & Batt, 1969*d*).

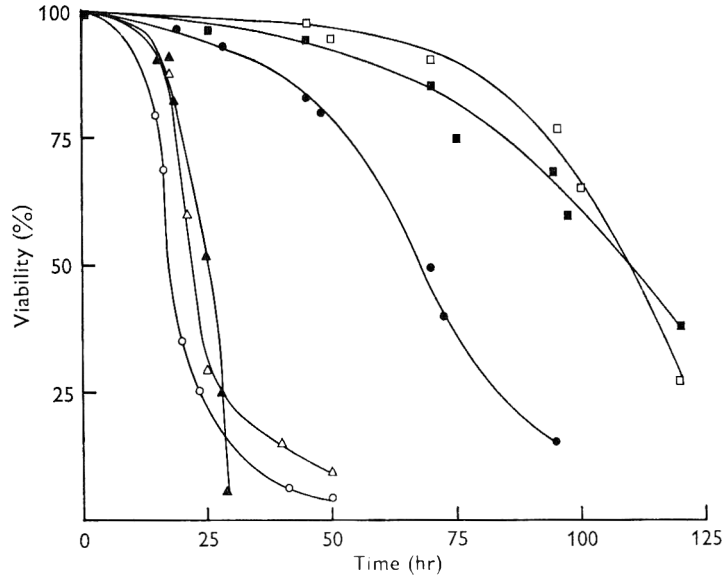

Fig. 3. Effect of feeding starved *Streptococcus lactis* continuously with different concentrations of glucose. For each experiment, washed organisms harvested from 35 ml. routine medium were resuspended at 30° in 500 ml. 0·075 M-phosphate buffer (pH 7·0) containing 1 mM-$MgSO_4$ and 10 μM-EDTA. The initial bacterial mass was 45–50 μg. dry wt/ml. Phosphate buffer, containing different glucose concentrations in each experiment, was added continuously (13 additions/min.) to the stirred culture at a flow rate of 7·0 ml./hr. Samples (0·2 ml.) were removed periodically for determination of viability, pH and turbidity. Glucose feed rates (μmole glucose/mg. dry wt bacteria/hr) were: ○, 15·0; ●, 1·5; □, 0·5; ■, 0·25; △, 0·05; ▲, 0·00.

Starved cultures were progressively diluted but since survival was independent of bacterial density in the presence of added Mg^{2+} (Thomas & Batt, 1968), this should not have influenced the present results. The pH drop was always less than 0·15 units. At low feed rates the turbidity of starved cultures, after correction for dilution, showed a slow decline while at higher feed rates the decline was more rapid. At low glucose feed rates, viable organisms after 50 to 100 hr starvation had short division lag times similar to those at the onset of starvation. By contrast, rapid glucose metabolism gave rise to long division lags (Thomas & Batt, 1968) and more rapid RNA breakdown (Thomas & Batt, 1969*b*).

Organisms which were starved under conditions giving the maximum range of

death rate were examined for comparative glycolytic activities. No general correlation between glycolytic activity and survival was found (Table 1). Incubation of starved organisms with glucose caused a more rapid loss of glycolytic activity (Table 1). With the bacterial concentration used, 10 mM-glucose was metabolized in about 5 hr and 10 mM-arginine in about 20 hr. The survival curve for the high glucose feed rate flattened off unexpectedly (Fig. 3), possibly as a result of decreased glycolytic activity. Similarly the survival curves at low feed rates flattened off at low viabilities but in these experiments the lower glucose feed rates may have been capable of supporting only part of the total population.

Table 1. *Glycolytic activity of starved Streptococcus lactis*

Organisms were washed and resuspended at 30° in 100 ml. 0·075 M-phosphate buffer (pH 7·0, 10 μM-EDTA) containing the additions given in the first column. The cell mass was 0·21 mg. dry wt/ml. At intervals, samples (10 ml.) were removed and centrifuged, the organisms were washed and resuspended in phosphate buffer (2 ml.) and the glycolytic activity determined as described in Methods. Glycolytic activity is given in terms of μmoles lactate/mg. dry wt bacteria/min., % viabilities are given in parentheses.

	Time (hr)							
	0·5	1·5	3	5	8	10	22	27
Additions to above buffer	Glycolytic activity							
None	0·274 (99)	0·247	0·230	0·194 (96)	0·175 (57)	0·174 (15)	0·155 (1)	0·133
+ $MgSO_4$ (1 mM)	0·279 (99)	0·264	0·234	0·206 (99)	0·185 (96)	0·164 (98)	0·158 (72)	0·155 (36)
+ $MgSO_4$ (1 mM) + L-arginine (10 mM)	0·285 (99)	0·269	0·262	0·218	0·189	0·193 (97)	0·148 (98)	0·144 (91)
+ $MgSO_4$ (1 mM) + L-arginine (10 mM) + casamino acids (0·5 %)	0·286 (99)	0·272	0·241	0·231	0·178	0·170 (98)	0·157 (95)	0·140 (87)
+ Glucose (10 mM)	0·269 (99)	0·243 (72)	0·181 (19)	0·162 (11)	0·137 (1)	0·118	0·052	0·045
+ $MgSO_4$ (1 mM) + glucose (10 mM)	0·277 (99)	0·260	0·205	0·171 (99)	0·121 (84)	0·091 (61)	0·016 (23)	0·011 (4)

Starved organisms were previously shown to be very sensitive to pH changes, the optimum pH for survival being near 7·0 (Thomas & Batt, 1968). Since Dawes & Ribbons (1962, 1964) have suggested that an energy source is required for intracellular pH control in starved bacteria it seemed appropriate to re-examine the pH effect on *Streptococcus lactis* in the presence of an added energy source. As addition of either carbohydrate or arginine alone produced accelerated death of starved *S. lactis* (Fig. 1) it was decided to re-examine the pH effect with added Mg^{2+}, which abolished arginine-accelerated death (Fig. 1), in addition to incubations with arginine + Mg^{2+}. Addition of Mg^{2+} decreased the death rate at all pH values (Fig. 4*b*), while arginine + Mg^{2+} produced a further marked decrease in the death rates (Fig. 4*c*). The initial pH values in Fig. 4 (*a*), (*b*) did not change significantly over the starvation period. After 48 hr starvation with 10 mM-arginine (Fig. 4*c*), initial pH values had risen by only about 0·2 pH units. This rise was presumably due to ammonia production.

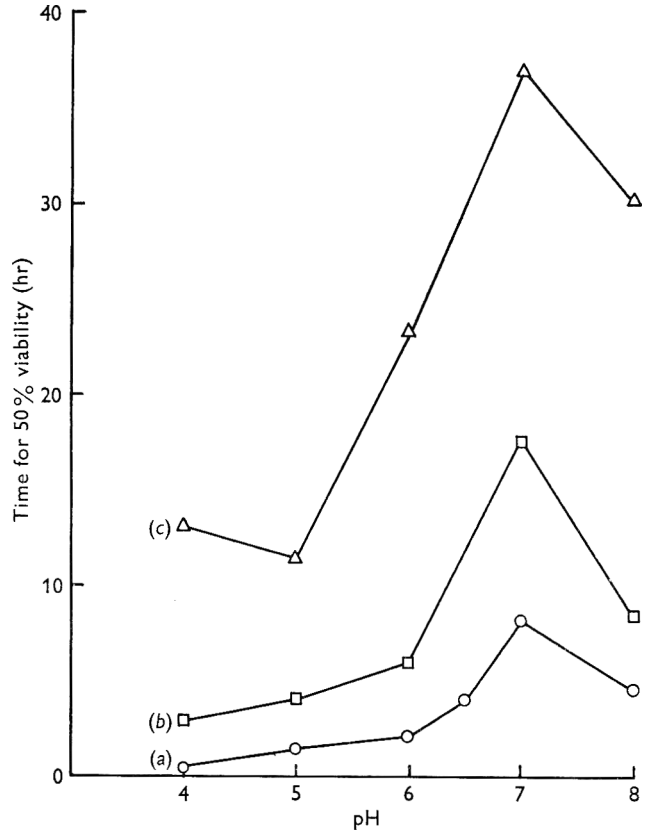

Fig. 4. Effect of pH on survival of *Streptococcus lactis* ML_3. Buffers were prepared from 0·1 M-Na_2HPO_4 and 0·05 M-citric acid containing 10 μM-EDTA. Curve (*a*). Bacteria were harvested from 20 ml. culture at the end of the growth phase, washed and resuspended in 5 ml. phosphate-citrate buffer (pH 7·0), 0·5 ml. of this suspension was added to 10 ml. buffer at 30° to give 190 μg./ml. and final pH values indicated. Curve (*b*). Suspensions prepared as for (*a*) at similar pH values; each suspension contained 1 mM-Mg^{2+}. Curve (*c*). Suspensions prepared as for (*a*) but at 80 μg. dry wt bacteria/ml. The buffer contained 10 mM-arginine + 1 mM-$MgSO_4$ and pH values were adjusted to those above by addition of HCl. Results were graphed and a summary is presented. Before viability determination samples were diluted in phosphate-citrate buffer containing 1 mM-Mg^{2+} (pH 7·0).

DISCUSSION

The addition of either glucose or arginine to *Streptococcus lactis* starved in phosphate buffer resulted in accelerated death while the presence of Mg^{2+} either reduced or eliminated this effect. The only metabolic product likely to be common to both glucose and arginine metabolism is ATP. Both arginine and glucose were metabolized at constant rates by starved organisms and the rate of ATP generation was calculated to be about 7·5 times greater with 10 mM-glucose than with 10 mM-arginine, in agreement with the results of Forrest (1965) for starved *Streptococcus faecalis*. Assuming 93 % conversion, a glycolytic activity of 0·29 μmole lactate/mg. dry wt bacteria/min. corresponds to fermentation of about 1·7 mg. glucose/mg. dry wt bacteria/hr. Even allowing for energy yield differences from glucose metabolism, this rate was much greater than

the maintenance energy requirement of approximately 0·07–0·09 mg. glucose/mg. dry wt bacteria/hr for aerobic metabolism of *Escherichia coli*, *Aerobacter aerogenes* and *Aerobacter cloacae* (see Pirt, 1965). Assuming a similar maintenance energy requirement for *S. lactis*, then clearly there is no economy of metabolic energy for maintenance of starved *S. lactis* and the linear rate of glucose fermentation (Thomas & Batt, 1969*a*) indicates that the metabolism of starved organisms continues at rapid rates. From data obtained for the growth of *S. lactis* (Thomas, 1968) it can be calculated that growing organisms should produce approximately 1·2 μmoles lactate/mg. dry wt bacteria/min. while glycolytic activity measurements (Thomas & Batt, 1969*a*), showed that non-growing organisms produced 0·29 μmole lactate/mg. dry wt bacteria/min. A simple explanation for the observed linear rates of glycolysis and arginine fermentation in starved *S. lactis* would be that these processes were not subject to feedback control and hence ATP could be generated in amounts which were far in excess of requirements. A similar conclusion was drawn by Forrest & Walker (1965*a*) using *S. faecalis*.

It seemed possible that the extended survival of starved *Streptococcus lactis* organisms with added arginine, compared with glucose, was due to the lower rate of ATP production since McGrew & Mallette (1962) reported an increase in the survival times of starved *Escherichia coli* on the intermittent addition of very small amounts of glucose. In contrast, most other workers have used relatively high carbohydrate/cell mass ratios and have not observed extended survival. Glycerol- and succinate-accelerated death decreased on reducing the substrate concentration and was undetectable at low concentrations (Postgate & Hunter, 1964). It could be informative to re-examine the effect of low glycerol concentrations on survival in the presence of added Mg^{2+}. Strange & Dark (1965) have observed that increased rates of glycerol oxidation produced increased death rates adding to a considerable amount of evidence which suggested that the rate of substrate metabolism, and the rate of ATP production, may be critical for survival. In the present investigation experiments involving the continuous addition of glucose to starved *S. lactis* indicated that survival was profoundly affected by the rate of glucose metabolism. An optimum feed rate of 0·25–0·5 μmole glucose/mg. dry wt bacteria/hr extended survival from about 1 to 4 days. This corresponds to a maintenance energy requirement for starved *S. lactis* of approximately 0·045–0·09 mg. glucose/mg. dry wt bacteria/hr which is of the same order as that reported for aerobic bacteria (see Pirt, 1965). Although glucose fermentation by *S. lactis* yields less energy than in aerobic organisms, Marr, Nilson & Clark (1963) calculated that for *E. coli* approximately half the maintenance energy requirement may be used for resynthesis so that the actual maintenance requirement for *S. lactis* may be lower than that for *E. coli* since resynthesis of protein and RNA by starved *S. lactis* occurs to a much more limited extent (Thomas & Batt, 1969*c*).

In the absence of Mg^{2+}, the death rate of *S. lactis* when harvested from the lactose-limited growth medium and starved in phosphate buffer was accelerated to similar degrees by the addition of either glucose, galactose, fructose or lactose (Thomas & Batt, 1968), all of which are considered to be metabolized via the glycolytic pathway. By contrast, the death rate of glycerol-limited *Aerobacter aerogenes* was not accelerated by added glucose or ribose (Postgate & Hunter, 1964). Although these substrates were shown to be metabolized, there was no indication that the rate of their metabolism was similar to that in organisms grown with glucose or ribose limitations where these

substrates accelerated the death rate of starved organisms. Since the activity of constitutive enzymes may vary according to growth conditions (Pardee, 1961), it seems possible that substrates not included in the growth medium may be metabolized at slower rates by starved organisms. However, the strain of *A. aerogenes* used by Strange & Dark (1965) showed carbon-accelerated death with carbon sources besides the one which had been used as the growth-limiting substrate.

Several reports have indicated that rapid metabolism of energy-yielding substrates by starved bacteria is accompanied by an increased rate of polymer hydrolysis (e.g. Strange & Dark, 1965; Thomas & Batt, 1969*b*) while other investigations have demonstrated that the breakdown of protein and nucleic acid in starved bacteria is suppressed by agents inhibiting energy-yielding reactions (see Halvorson, 1962). Slow substrate metabolism may supply the maintenance energy requirement without deleterious effects.

The metabolism of exogenous substrates does not always appear to be coupled to the energy requirements of starved organisms and it seems possible that the rapid depletion of reserve polymers in some bacteria may present a similar situation with respect to endogenous substrates. Burleigh & Dawes (1967) have suggested that the rapid metabolism of polyglucose may cause substrate-accelerated death. Reserve polymers may exert a sparing action on essential cell constituents but they seem to enhance survival only if they are broken down at comparatively slow rates (e.g. see Strange *et al.* 1961; Sierra & Gibbons, 1962; Sobek, Charba & Foust, 1966; Zevenhuizen, 1966). However, Strange & Hunter (1967) also pointed out that the enhanced survival of nitrogen-limited *Aerobacter aerogenes* may be due to the relatively high Mg^{2+} content rather than the glycogen reserves.

In a number of starvation environments, the glycolytic activity of *Streptococcus lactis* fell steadily from the onset of starvation and was not correlated with viability. Other workers have, however, found a correlation between the activity of the catabolic enzymes and survival in support of an early hypothesis associating bacterial death with enzyme inactivation (Rahn & Schroeder, 1941). For example, Postgate & Hunter (1962) found that the glycerol dehydrogenase and glycerol oxidase activities of starved *Aerobacter aerogenes* declined in parallel with viability, while the endogenous respiration rate of the glycerol-limited organisms was not directly related and, in fact, declined more rapidly. Similarly, Burleigh & Dawes (1967) reported a correlation between the survival of aerobically starved *Sarcina lutea* and their ability to oxidize exogenous glutamate and glucose. In the present study the glycolytic activity of *S. lactis* was not maintained with exogenous carbohydrates or amino acids including arginine, in contrast to the findings of Walker & Forrest (1964) and Forrest & Walker (1965*b*) with *Streptococcus faecalis*. Indeed, added glucose produced a more rapid decline in glycolytic activity.

Although it has been proposed that energy is required for intracellular pH control in starved bacteria (Dawes & Ribbons, 1962, 1964), this has not been experimentally demonstrated. *Streptococcus lactis* had a sharp pH optimum for survival which is perhaps consistent with the absence of an endogenous energy source. In this connexion, the addition of arginine was shown to produce a marked decrease in death rates at all pH values. At acid pH values this effect could have been due to a neutralizing action of the NH_3 produced from arginine metabolism. However, arginine also prolonged survival at alkaline pH and it seemed likely that the energy produced from

arginine metabolism was directly responsible for reducing the lethal effect of adverse pH values.

The growth of lactic acid bacteria in milk normally ceases when the lactic acid formed produces inhibitory acid conditions. Although growth ceases, the organisms remain viable and continue to ferment the large excess of lactose present but at a much slower rate. It is possible that the energy produced by the continuing fermentation could be an important factor for the survival of these organisms in sour milk.

We thank Dr R. C. Lawrence for many helpful discussions.

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Changes in Permeability and Ultrastructure of Starved *Streptococcus lactis* in Relation to Survival

By T. D. THOMAS,* PAMELA LYTTLETON, K. I. WILLIAMSON AND R. D. BATT

New Zealand Dairy Research Institute, Palmerston North, Department of Chemistry and Biochemistry, Massey University, and Plant Chemistry Division, D.S.I.R., Palmerston North, New Zealand

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SUMMARY

When *Streptococcus lactis* was starved in phosphate buffer the intracellular amino acid pool was rapidly released into the external medium from the onset of starvation whereas lactic dehydrogenase and DNA appeared in the suspending buffer only as organisms began to lose viability. Addition of spermine enhanced survival and suppressed the release of ultraviolet-absorbing material.

Thin sections of *Streptococcus lactis* were examined by electron microscopy at intervals during starvation in a number of environments. Ribosome particles rapidly disappeared from organisms starved in the absence of Mg^{2+} and non-viable organisms remained structurally intact; in the presence of Mg^{2+} ribosomes were retained but, after prolonged starvation, some organisms autolysed soon after the death rate increased. Addition of a suitable energy source maintained cell structures intact for a much longer period but again lysis occurred as the death rate increased. Starvation led to unfolding or extrusion of the mesosomes and dislocation of the nuclear material. The death rate of starved *S. lactis* organisms in phosphate buffer partly depended on the presence of Mg^{2+}, which probably acted by promoting polymer stability, particularly RNA. In this environment, a suitable exogenous energy source further enhanced survival which may ultimately be a function of cell wall and/or membrane stability.

INTRODUCTION

Vegetative bacteria starved in buffer at their optimum growth temperature may survive for several days but death ultimately occurs. The reasons for bacterial death in starvation environments may be impossible to define but some authors have claimed that certain possibilities can be excluded. For instance, starved *Aerobacter aerogenes* were considered to maintain functional membranes after death (Postgate & Hunter, 1962). An earlier investigation (Thomas & Batt, 1969*a*) showed that a substantial amount of phospholipid breakdown occurred on prolonged starvation of *Streptococcus lactis*. This process would be expected to impair permeability barriers since phospholipids are important membrane components (Salton, 1967). The present paper describes a study of the changes in permeability and ultrastructure of starved *S. lactis* in relation to survival.

* Present Address: Sub-Department of Chemical Microbiology, Department of Biochemistry, University of Cambridge, Cambridge, Great Britain.

METHODS

General procedures. The organism (*Streptococcus lactis* ML_3), the conditions of growth and starvation and the methods used for determining bacterial mass and viability were described in preceding papers (Thomas & Batt, 1968, 1969*a*).

Thin-layer chromatography. Amino acids were separated by thin-layer chromatography (TLC) on mixed cellulose-silica gel layers and detected with ninhydrin (Turner & Redgwell, 1966). Plates were developed with phenol-water (80:20, w/v) in the first direction (5 hr) and dried overnight at 40 to 50°. Butanol:acetic acid:water (5:1:4, v/v/v, upper phase) was used in the second direction (4 hr) and the spots were identified by comparison with standard amino acids. Supernatant buffer samples were desalted (Dreze, Moore & Bigwood, 1954) before analysis.

Enzyme assays. Lactic dehydrogenase was assayed using the method described by Kornberg (1955). Supernatant samples (2·8 ml.) were adjusted to pH 7·4, 10 mM-Na pyruvate (0·1 ml.) and 4 mM-nicotinamide-adenine-dinucleotide (NADH, 0·1 ml.) were added and the rate of oxidation of NADH at 30° was determined using a Zeiss PMQII recording spectrophotometer at 340 mμ. The unit of activity was taken as the amount of enzyme which caused an initial rate of oxidation of 1 μmole NADH per minute. β-Galactosidase activity of supernatant samples was assayed by the *o*-nitrophenyl-β-D-galactopyranoside hydrolysis method as described by Citti, Sandine & Elliker (1965).

Deoxyribonucleic acid. Cell-free DNA was estimated by the method of Burton (1956).

Electron microscopy. The organisms were examined in the electron microscope as thin sections, prepared by the following methods.

Fixation. Method 1. The organisms were fixed with osmium tetroxide and post-fixed in uranyl acetate according to the method of Kellenberger, Ryter & Sechaud (1958). This method is subsequently referred to as R-K fixation.

Method 2. The organisms underwent a primary fixation in a glutaraldehyde preparation followed by a secondary fixation in osmium tetroxide according to the following schedules: (*a*) 2 ml. fixative (1 % glutaraldehyde in 0·1 M-phosphate buffer, pH 6·1, containing 6 mM-$MgCl_2$) was added to 20 ml. of culture. After mixing, the culture was centrifuged (1000 *g* for 5 min.) and the organisms resuspended infixative (2 ml.) and left for 3 hr at 4°. After further centrifugation, the organisms were washed twice in 0·1 M-phosphate buffer (pH 6·1) containing 0·2 M-sucrose. The pellet was then resuspended in 0·1 M-phosphate buffer (pH 6·1) containing 1 % osmium tetroxide and left for 2·5 hr at 4°. After two further washes in phosphate buffer the organisms were set in 2 % agar (aq.) at 45°; the agar blocks were cut into small pieces and immersed in uranyl acetate (0·5 %, aq.) for 2 hr at room temperature (Kellenberger *et al.* 1958). (*b*) 2 ml. fixative (1 % glutaraldehyde in 0·05 M-cacodylate buffer, pH 6·3, containing 0·01 M-$MgCl_2$) was added to 20 ml. of culture. After mixing, the culture was centrifuged (1000 *g* for 5 min.) and the organisms were resuspended in fixative (2 ml.) and left for 3 hr at 4°. After further centrifugation, the organisms were washed twice in 0·05 M-cacodylate buffer, pH 6·3, containing 0·5 % NaCl. The pellet was resuspended in 0·05 M-cacodylate buffer (pH 6·3) containing 1 % osmium tetroxide and 0·5 % NaCl, and left for 2·5 hr at 4°. The suspensions were diluted with cacodylate buffer and centrifuged. The organisms were then set in agar and small pieces of the agar blocks were immersed in uranyl acetate according to the procedure of Kellenberger *et al.*

(1958). (*c*) Using the fixative (1 % glutaraldehyde in 0·05 M-cacodylate buffer (pH 6·3) containing 0·5 % NaCl) the same procedure as (*b*) was followed.

These double fixation methods are subsequently referred to as G-Os (*a*) (*b*) and (*c*).

Dehydration and embedding. The agar blocks obtained from each fixation method were dehydrated in graded aqueous ethanol solutions according to the following schedule: 25 %—15 min., 50 %—15 min., 75 %—overnight at 4°, 95 %—30 min., 100 %—30 min. (twice). The blocks were then washed with propylene oxide and embedded in Araldite resin using the method of Luft (1961).

Sections were cut with either a glass or diamond knife, using an L.K.B. ultramicrotome and picked up on copper grids having either a collodion-carbon supporting film or no supporting film. Sections were stained by floating the grids on lead salt solutions (Millonig, 1961; Reynolds, 1963) for 1 min. followed by washing with distilled water and they were subsequently examined in a Philips EM 200 electron microscope.

RESULTS

Measurement of bacterial lysis. Chromatographic analysis indicated a progressive loss of intracellular amino acids with a corresponding increase in the concentrations of most of the supernatant amino acids, suggesting that leakage of the intracellular pool occurred on starvation.

The release of an intracellular enzyme, such as β-galactosidase, into the external medium has been used as an index of cell lysis (Pollock, 1961). In the present study however, no detectable β-galactosidase activity was found in cell-free samples of *Streptococcus lactis* suspensions starved at a bacterial density of 4·8 mg. dry wt/ml.

Table 1. *Release of lactic dehydrogenase (LDH) and deoxyribonucleic acid (DNA) from starved Streptococcus lactis*

Washed organisms were resuspended at 4·6 mg. dry wt/ml. in phosphate buffer containing 10 μM-EDTA + 1 mM-$MgSO_4$. Samples were removed at the times indicated, centrifuged and the supernatant buffers assayed as described in Methods.

Starvation period (hr)	Supernatant		Viability (%)
	LDH (Units/ml.)	DNA (μg./ml.)	
1	0·000	0·0	—
3	0·002	0·0	99
5	0·006	0·2	—
8	0·011	0·2	99
10	0·069	0·7	—
12	0·138	1·3	94
21	0·319	4·0	32
24	0·505	6·8	17
29	0·547	11·2*	3

* Equivalent to 6·6 % total cell DNA.

Citti *et al.* (1965) reported that the β-galactosidase released from five out of six strains of *S. lactis* by shock treatment was very unstable, so that if this enzyme had been released in the present study its activity may have been rapidly lost. Assays for lactic dehydrogenase (LDH) indicated that this enzyme was released slowly from viable organisms (Table 1) but release of LDH appeared to increase markedly at the onset

of cell death. Control experiments showed that the LDH in cell-free samples was unstable under the incubation conditions used. Assays of 24 hr samples (Table 1), after 5 hr and 22 hr incubation, indicated a loss of 11 % and 53 % of the original activity. Therefore, the results for LDH release in Table 1 are only approximate.

Release of diphenylamine reactive material (expressed as DNA—Table 1) from starved organisms increased markedly as the death rate accelerated.

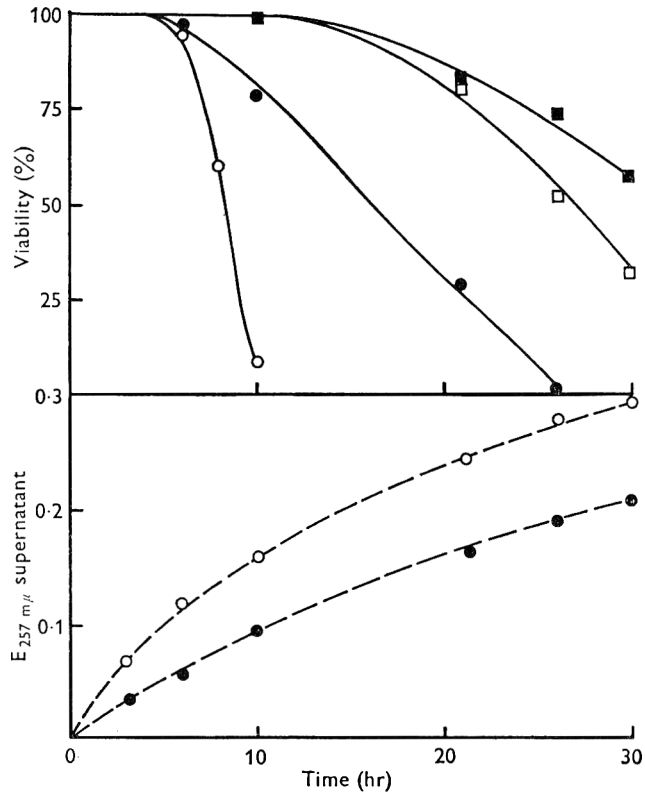

Fig. 1. Effect of spermine on starved *Streptococcus lactis*. Organisms were washed and resuspended at 20 μg. dry wt/ml. in 0·075 M-phosphate buffer (pH 7·0, 10 μM-EDTA). Viabilities of suspensions containing: no addition, ○; spermine (1 mM), ●; $MgSO_4$ (1 mM), □; $MgSO_4$ (1 mM)+spermine (1 mM), ■. Release of u.v.-absorbing material is indicated by dashed lines; symbols as above.

Effect of spermine. Certain aliphatic diamines, especially spermine, have a marked stabilizing effect on osmotically sensitive bacteria and protoplasts (Mager, 1959; Tabor, 1962), suppressing the leakage of ultraviolet (u.v.)-absorbing material and cell lysis. Incubation of starved *Streptococcus lactis* organisms with spermine reduced the death rate and the release of u.v.-absorbing material (Fig. 1). Spermine did not however greatly enhance the normal stabilizing effect of Mg^{2+}.

General ultrastructural features of Streptococcus lactis. The morphology of organisms from the growth phase (Plate 1) reflects various stages in the division cycle. This sequence illustrates separation of the nuclear material followed by septum formation.

Prominent mesosomes (Fitz-James, 1960) are associated with the ingrowing cell wall and membrane and also with the nuclear material. The cytoplasmic membrane is 70 to 80 Å thick and consists of two prominent electron-dense layers separated by a light layer (Plate 1). The cell wall occurs as a diffuse band approximately 200 to 250 Å thick. Cole (1965) investigated cell wall growth in *Streptococcus pyogenes* using an immunofluorescence technique and considered that cell wall replication was initiated along an equatorial ring. Transverse septa grew centripetally with the peripheral wall replicating simultaneously so that the new hemispheres of the daughter cocci were initiated back-to-back. New equatorial sites of wall and transverse septa formation were initiated before completion of the previous transverse septum. In contrast, Chung, Hawirko & Isaac (1964) found that *Streptococcus faecalis* had only one site of cell wall synthesis per coccus. This latter mode of cell wall replication appears to operate in *Streptococcus lactis* since only one site of transverse septum formation per coccus was seen.

The fixation method of Kellenberger *et al.* (1958) (Method 1) preserved the nuclear material with a typical fibrillar structure (Plate 1). Glutaraldehyde-osmium tetroxide fixation (Method 2) procedures clearly demonstrated electron-dense, granular particles 100 to 200 Å in diameter (Pl. 2, fig. 4). Most investigators consider that these particles are ribosomes (see Kellenberger & Ryter, 1964).

Ultrastructural changes in starved cells. Although the appearance of ribosomes in thin sections from any system showed some variation, examination of a large number of sections prepared by different techniques suggested a rapid depletion of ribosomes when organisms were starved in buffer without Mg^{2+} (Pl. 2, fig. 5). In only a few of these organisms were ribosomes seen. Magnesium starvation of *Escherichia coli* (Morgan, Rosenkranz, Chan & Rose, 1966) and *Aerobacter aerogenes* (Kennell & Kotoulas, 1967) resulted in similar ribosome depletion. Starvation of *Streptococcus lactis* in the presence of Mg^{2+} and amino acids substantially reduced the depletion (Pl. 2, fig. 6).

The death of *Streptococcus lactis* in buffer without Mg^{2+} occurred while peripheral cell structures appeared to remain intact (Pl. 2, fig. 5). However, R-K fixation procedures showed disappearance of mesosomes and a pronounced dislocation of nuclear material. After 17 hr starvation intrusions of the membrane and cell wall were occasionally observed and the membrane appeared to be in direct contact with the nuclear material (Pl. 3, fig. 7). The appearance of these organisms is similar to that of organisms which have extruded their mesosomes (Ryter & Landman, 1964; Ryter, 1968). In this connexion it has been reported that mesosome extrusion may occur without loss of viability (Ryter, 1968).

After 30 hr starvation in buffer containing Mg^{2+}, viability had fallen to 42 % and examination of micrographs indicated that at least 12 % of the population had lysed. Intact organisms (G-Os fixation [a]) retained a dense ribosome pattern and organisms fixed by the R-K method showed fragmentary mesosomes. By 40 hr most of the organisms had lysed and the viability was < 1 % (Pl. 3, fig. 8–11). Lysis appeared to result from rupture of both the membrane and cell wall, and after lysis membranes tended to remain relatively intact while cell walls fragmented (Pl. 3, fig. 11). It is not clear whether the extrusion of cytoplasmic material occurred at specific sites but the ruptures did not appear to be randomly distributed over the cell surface. The micrographs of lysing cocci (Pl. 3, fig. 8–11) appear similar to those of lipase-treated bacteria

(Ghosh & Murray, 1967) where breakdown of the cytoplasmic membrane and cell wall eventually resulted in the liberation of cytoplasmic material.

It seems that autolytic enzymes are present in most bacteria, and appear to be activated whenever normal growth of the organism is disrupted (Stolp & Starr, 1965; Shockman, 1965). For example, Shockman (1965) has observed that cell wall preparations from *Streptococcus faecalis* in the growth phase were slowly autolysed when incubated in phosphate buffer. It is generally considered that the bacterial cell wall has a predominantly mechanical role, conferring cell shape, rigidity and resistance to the high osmotic pressure created by the selectivity of the cytoplasmic membrane. As *Streptococcus lactis* lysed, the cytoplasmic membrane pulled away from the rigid wall and eventually collapsed (Pl. 3, fig. 8–11), presumably due to the decreasing osmotic pressure. The membranes of Gram-positive bacteria do not appear to be bound to the cell walls (Salton, 1967).

In this study, exogenous amino acids have been shown to maintain organisms intact and viable for a longer period (Pl. 3, fig. 12). A dense ribosome pattern was still visible after prolonged starvation (Pl. 2, fig. 6) and lysed organisms appeared as the death rate began to increase (Pl. 3, fig. 12). Sections prepared by the R-K fixation procedure showed some organisms with normal mesosome structures consisting of concentric whorls of membrane but most mesosomes appeared to have unfolded (Pl. 4, fig. 13).

Maximum survival of starved *Streptococcus lactis* was achieved by the continuous feeding of low concentrations of glucose (Thomas & Batt, 1969*b*). After 120 hr starvation the population was 40 % viable and thin sections of these organisms showed marked changes in the mesosomes and nuclear material (Pl. 4, fig. 14, 15; Pl. 5, fig. 16, 17). Unfolding of mesosomes often resulted in what appeared to be a multiple cytoplasmic membrane system (Pl. 4, fig. 14, 15). In some Gram-positive bacteria a close morphological and functional relationship has been shown to exist between the mesosomes and the nuclear material (Ryter & Landman, 1968). When mesosome extrusion or unfolding occurred in the present study, the nuclear material become dislocated from its central position contiguous with the mesosome and often appeared in direct contact with the cytoplasmic membrane (Pl. 5, fig. 17). Changes similar to these seem to occur in all bacteria which have lost their mesosomes, e.g. protoplasts (Ryter & Landman, 1968). Although some of the changes observed in starved *Streptococcus lactis* are typical of Gram-positive bacteria undergoing plasmolysis, extruded mesosomes were only rarely observed in pockets between the cell wall and cytoplasmic membrane as described by Fitz-James (1964). This finding, together with the fact that the cell walls of lysed organisms were broken down into small fragments, may indicate that cell wall autolysis is responsible for the loss of viability of *S. lactis* under these conditions. Lysed organisms appeared in viable cultures containing amino acids (Pl. 3, fig. 12) and also in glucose-fed populations (Pl. 5). In the latter system, some organisms appeared to have undergone considerable growth without division; ribosomes were still present (Pl. 5, fig. 16) and surviving organisms had short division lags similar to those at the onset of starvation (Thomas & Batt, 1968, 1969*a*).

DISCUSSION

The steady leakage of intracellular amino acids from starved *Streptococcus lactis* into the external medium is consistent with the absence of an endogenous energy source (Thomas & Batt, 1969*a*) since bacteria having such energy sources appear to retain their free amino acid pools when starved (Dawes & Holms, 1958; Postgate & Hunter, 1962; Dawes & Ribbons, 1965). Conditions promoting release of components from metabolic pools may initiate polymer breakdown and increase the death rate and this would be consistent with the observation that spermine enhanced survival and suppressed the release of u.v.-absorbing material from starved *S. lactis*. As well as promoting ribosome stability, added Mg^{2+} could have an important function in maintaining permeability barriers. The finding that high EDTA concentrations were lethal (Thomas & Batt, 1968) supports this view, since EDTA appears to remove cell wall and membrane Mg^{2+} by chelation. After approximately 12 hr starvation of *S. lactis* in phosphate buffer containing Mg^{2+}, the death rate began to increase and was accompanied by the release of lactic dehydrogenase and DNA. The release of intracellular macromolecules, before lysis was detectable by electron microscopy, may have resulted from a partial rupture of the cell wall and membrane.

Without exogenous Mg^{2+} ribosome particles rapidly disappeared from the cytoplasm in agreement with earlier chemical evidence (Thomas & Batt, 1969*a*) and loss of viability occurred while organisms retained intact cell wall and cytoplasmic membrane structures. In the presence of exogenous Mg^{2+} some lysis was evident as the death rate increased while intact organisms still retained dense ribosome patterns. When organisms were starved with Mg^{2+} plus a suitable energy source, cell structures and ribosomes were maintained for a longer period. However, lysis eventually occurred when the death rate increased and this could have led to some high viability estimates, since lysed organisms may not always be counted on slide-cultures.

Conclusions. The existence of minimum growth rates for bacteria (Tempest, Herbert & Phipps, 1967) implies a state of minimum subsistence which must be maintained for survival. In this state, essential polymers are present at the lowest level capable of initiating regrowth and any breakdown of these components results in loss of viability. It is now well established for some species of bacteria that organisms produced at maximum growth rates may contain material in excess of these minimum levels so that considerable degradation may take place before death occurs. The products of polymer hydrolysis in most bacteria are metabolized producing energy but with *Streptococcus lactis* these products generally appear to be released into the external medium in an undegraded form.

Although it has been suggested that maintenance of bacteria in a viable state requires energy, the amount of energy is ill-defined. *Streptococcus lactis* maintained complete viability for approximately 15 hr when starved in buffer containing Mg^{2+}, yet no absolute requirement for an energy source could be determined. At the onset of starvation an ATP pool was probably present and small amounts of energy may have been produced by endogenous metabolism, expecially if *S. lactis* contains constitutive kinases for nucleotide metabolism (see Gronlund & Campbell, 1965). However, leakage of the intracellular free amino acid pool occurred as soon as the organisms were starved, there was no appreciable protein or RNA synthesis and, in addition, starved organisms were extremely pH sensitive. This suggested that an

appreciable endogenous energy source was not involved in maintaining the viability of organisms starved in phosphate buffer. It would appear that these organisms remained viable as long as the degradation of polymers, perhaps RNA, had not proceeded beyond some irreversible point and the present evidence suggests that the death rate was dependent on the presence of compounds promoting polymer stability. When Mg^{2+} was added to buffered suspensions, a suitable exogenous energy source further enhanced survival. This energy source appeared to suppress the release of the free amino acid pool and allow limited polymer synthesis and pH control. In this environment survival may then be a function of cell wall and/or membrane stability. *S. lactis* does not appear to have any survival mechanism based on an endogenous metabolism. The ability to withstand low pH values may be the most important factor for the survival of this organism in milk.

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EXPLANATION OF PLATES

PLATE 1

Division sequence of *Streptococcus lactis* ML_3 from the logarithmic growth phase (R-K fixation, Reynolds stain) Scale lines = 0·2 μ

Fig. 1. Initial stage of cell division showing the undivided nuclear material (*N*) and small invaginations where the mesosome (*M*) is continuous with the cytoplasmic membrane (*CM*).

Fig. 2. An intermediate stage showing the divided nuclear material (*N*) in close contact with the mesosome (*M*).

Fig. 3. Transverse wall formation is complete with a clearly defined membrane. The new mesosome (*M*), formed at the next division site, is continuous with the cytoplasmic membrane.

PLATE 2

Ribosome changes

Fig. 4. Organisms from the logarithmic growth phase, showing an even distribution of ribosomes (G-Os fixation (*b*), Reynolds stain).

Fig. 5. Organisms after 17 hr starvation in 0·075 M-phosphate buffer (pH 7·0, 10 μM-EDTA), viability 16 %, initial bacterial mass 100 μg. dry wt/ml. Note the absence of ribosomes in most cells (G-Os fixation (*c*), Reynolds stain).

Fig. 6. Organisms after 48 hr starvation in phosphate buffer containing 10 μM-EDTA, 1 mM-$MgSO_4$, 10 mM-arginine and casamino acids (1 %, Difco), viability 92 %, initial bacterial mass 100 μg dry wt/ml. Ribosomes are still present and appear to be concentrated near the cytoplasmic membrane (G-Os fixation (*a*), Millonig stain).

PLATE 3

Lysis of *Streptococcus lactis*

Fig. 7. Organisms after 17 hr starvation in phosphate buffer (10 μM-EDTA), viability 2 %. Before any visible lysis, some organisms showed intrusions of the membrane (arrowed) in direct contact with the low density nuclear material (*N*) (G-Os fixation (*a*), Reynolds stain).

Fig. 8–11. Organisms after 40 hr starvation in phosphate buffer containing 1 mM-$MgSO_4$, viability < 1 %, 2 mg. dry wt/bacteria/ml. Bacterial lysis has occurred leaving the remains of cytoplasmic membranes (*CM*) and cell walls (*CW*). Membranes tend to remain intact while cell walls fragment. Lysed organisms show the extrusion of cytoplasmic material into the external medium through ruptures (arrowed) in the membrane and cell wall (G-Os fixation (*a*); Fig. 8, 10, Reynolds stain; Fig. 9, 11, Millonig stain).

Fig. 12. Organisms after 48 hr starvation in phosphate buffer containing Mg^{2+} and amino acids (see Pl. 2, fig. 6), viability 92 %. Lysed organisms are visible at a high viability (G-Os fixation (*a*), Millonig stain).

PLATE 4

Changes in membrane structures (R-K fixation, Reynolds stain)

Fig. 13. Organisms after 48 hr starvation in phosphate buffer containing Mg^{2+} and amino acids (see Pl. 2, fig. 6), viability 92 %. The mesosome (*M*) appears to have unfolded and is still in close contact with the separated nuclear material.

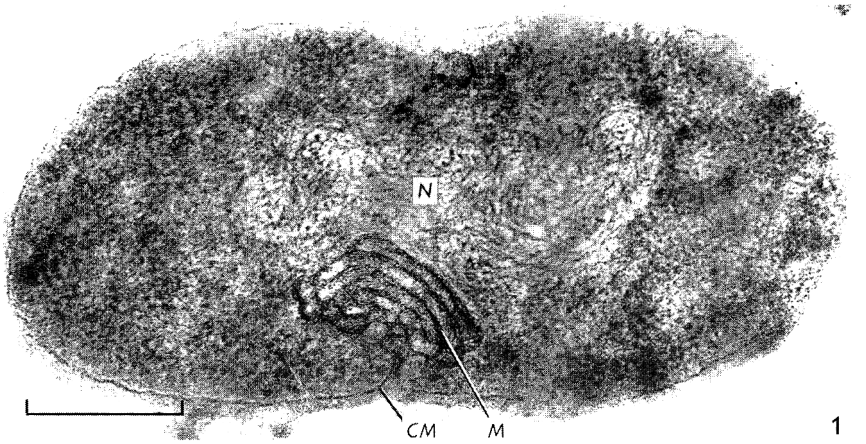
Fig. 14, 15. Organisms after 120 hr starvation in phosphate buffer supplied with a continuous glucose feed (0·25 μmole glucose/mg. dry wt bacteria/hr—see Thomas & Batt, 1969*b*), viability 40 %. In Fig. 14 three membrane layers (*M*) are visible which may result from unfolding of the mesosome (see Fig. 15).

PLATE 5

Changes in membrane structures and nuclear material.

Organisms after 120 hr starvation in phosphate buffer supplied with a continuous glucose feed (see Pl. 4, fig. 14, 15), viability 40 % (R-K fixation, Reynolds stain).

Fig. 16, 17. Typical changes in mesosome structures (*M*) and nuclear material (*N*). Ribosomes (*R*) were clearly visible and some organisms had lysed (*L*).



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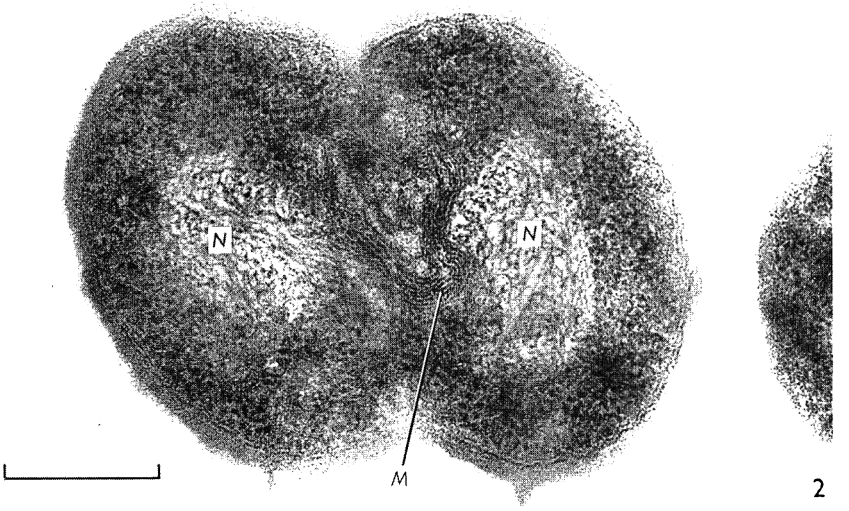

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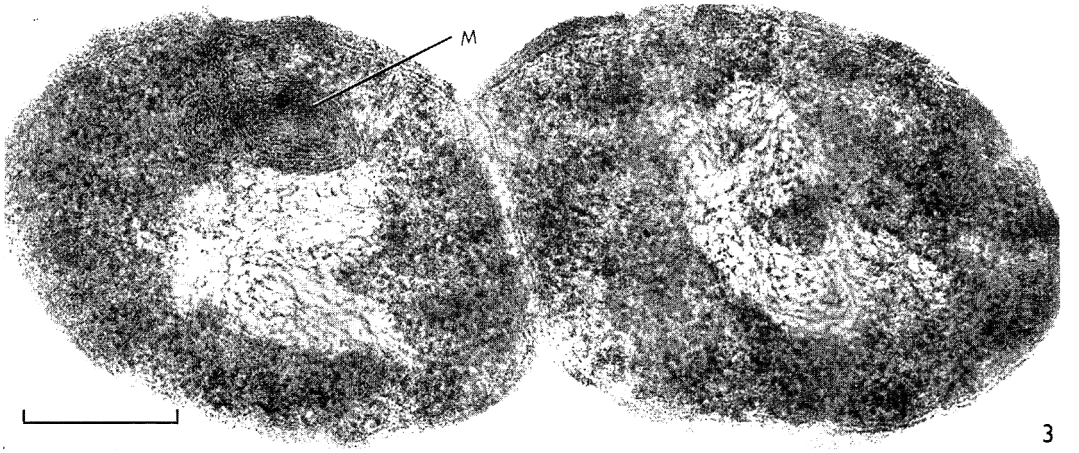

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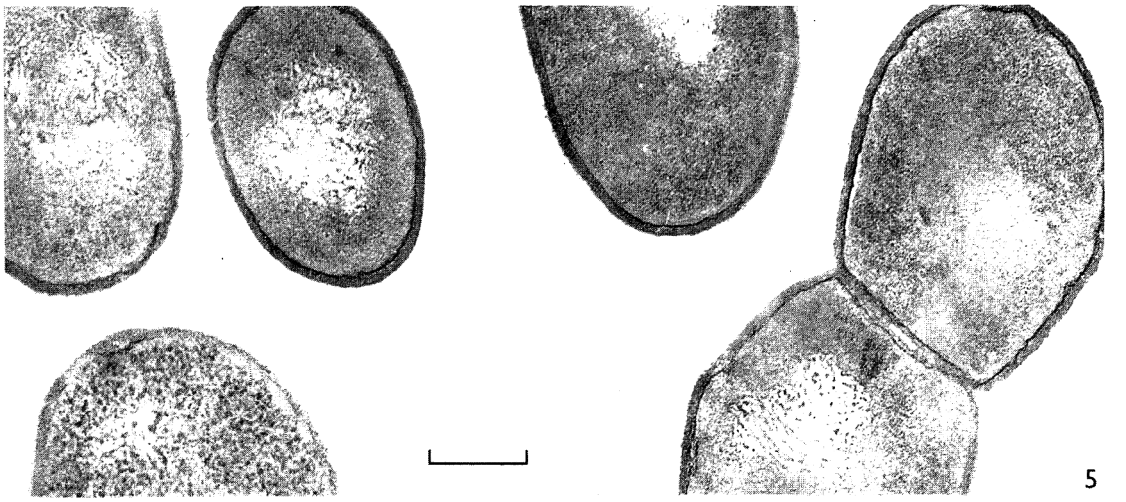

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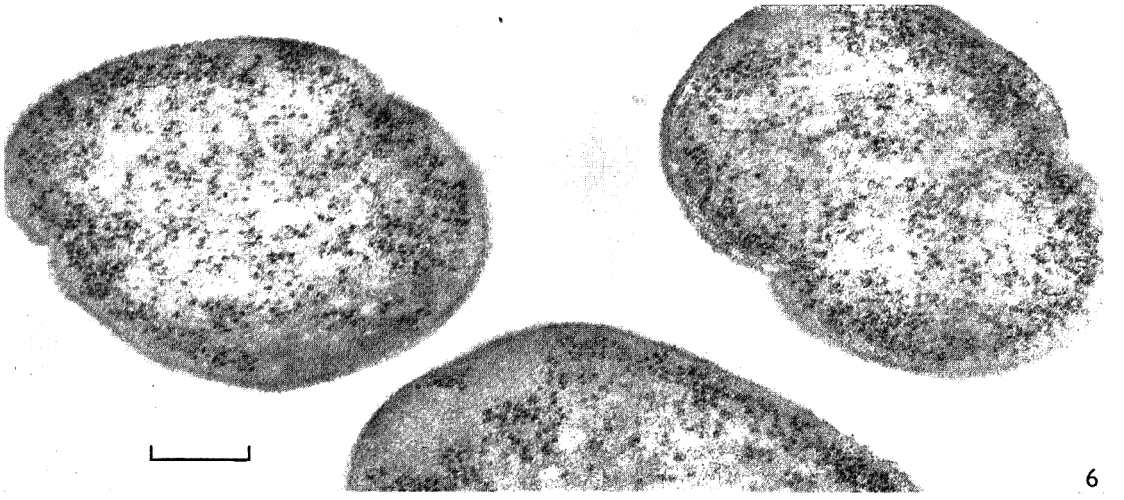

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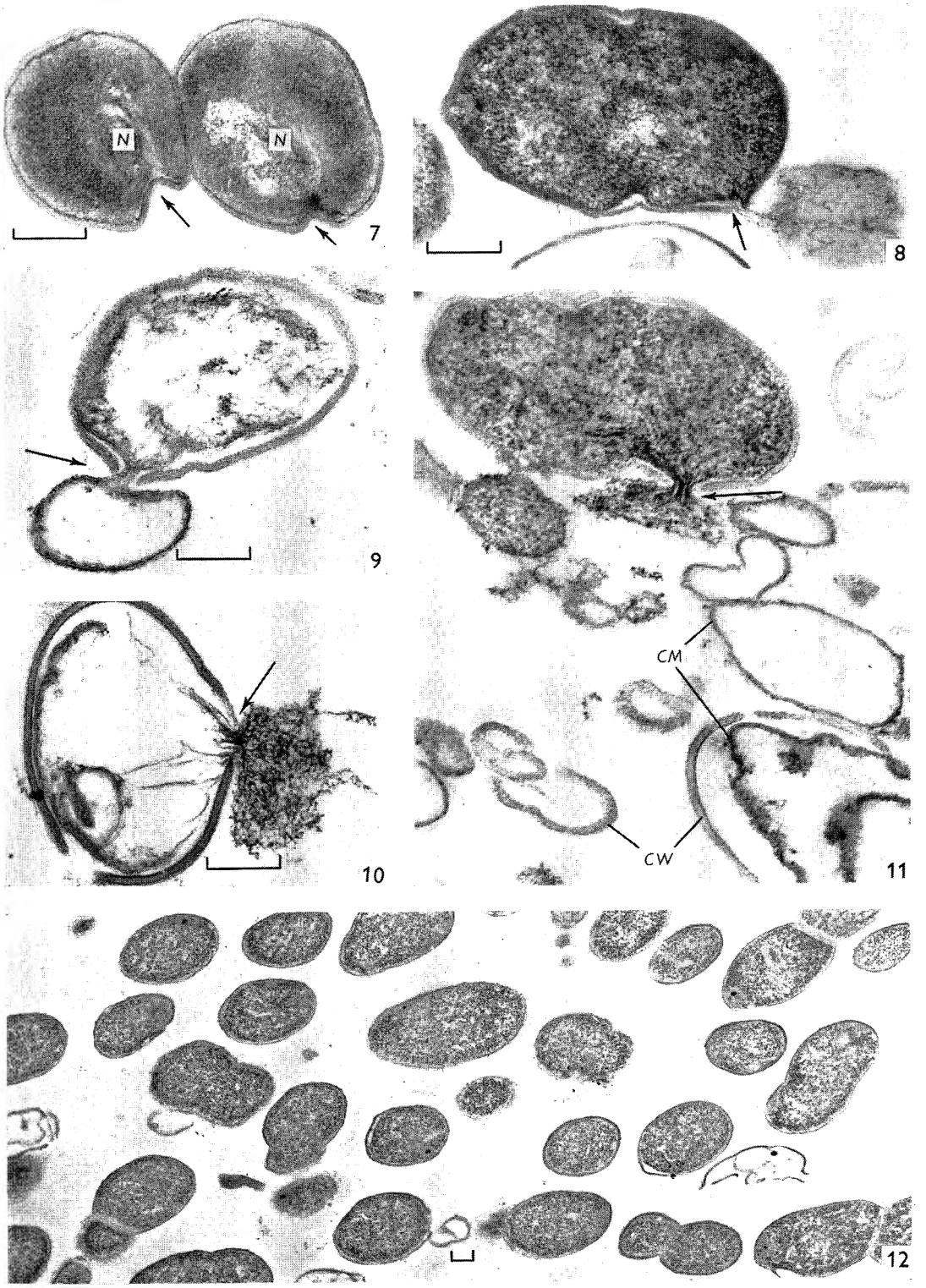
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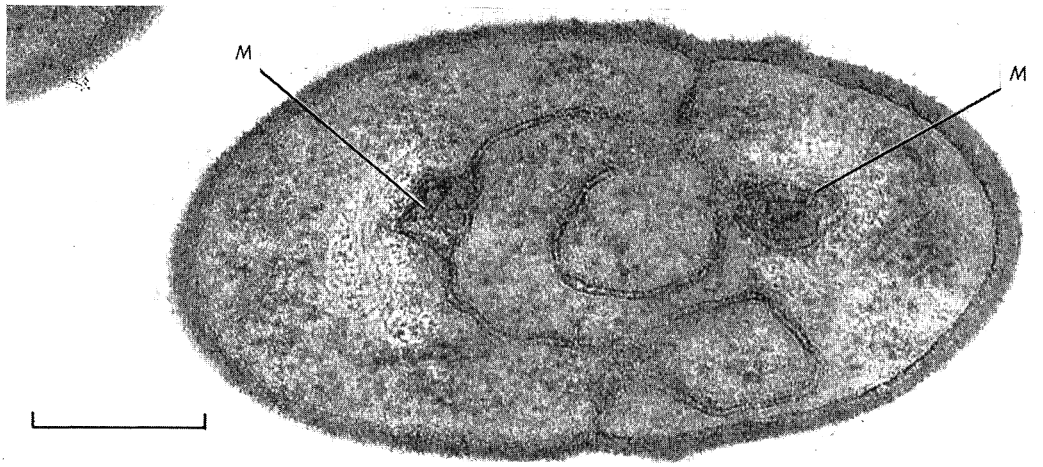

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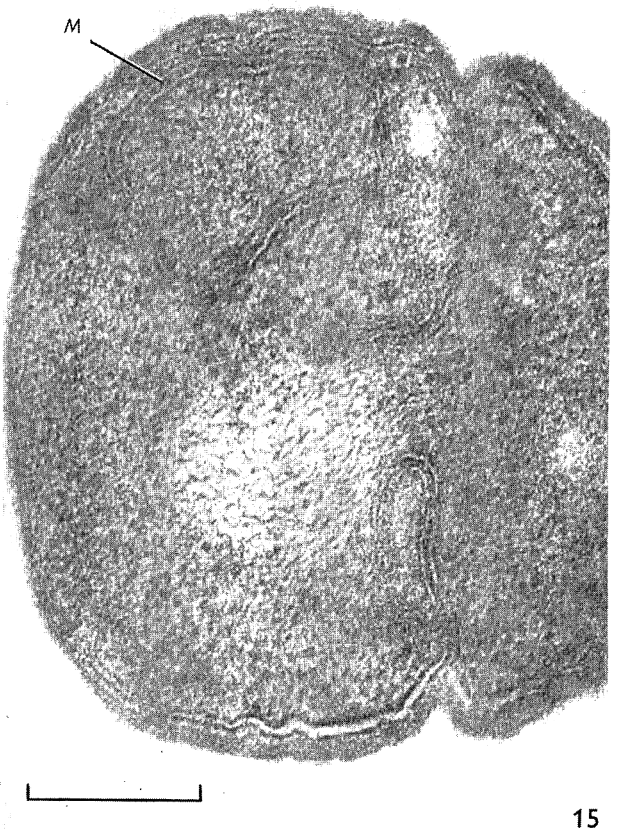

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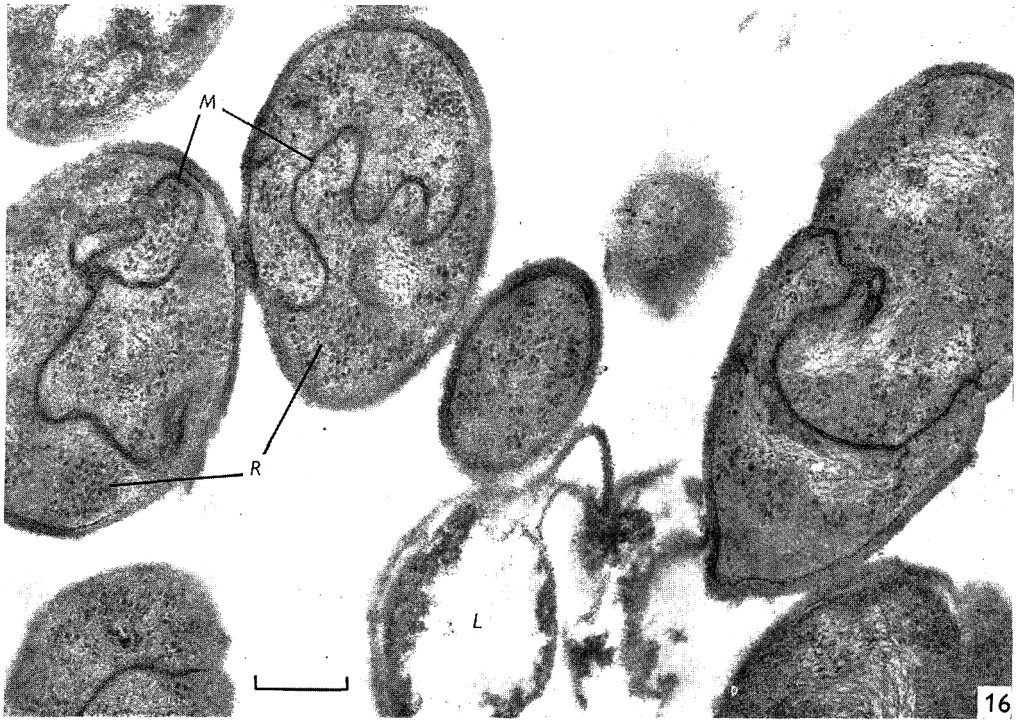
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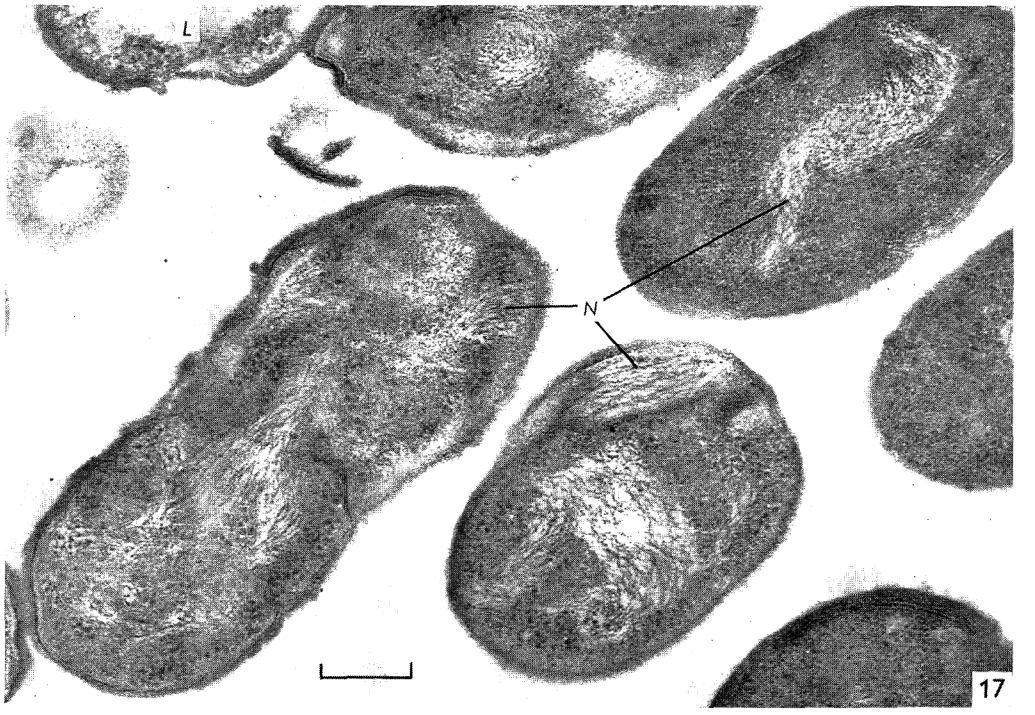
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The Susceptibility of Brassica Callus to Infection by *Peronospora parasitica*

By D. S. INGRAM
Botany School, Downing Street, Cambridge, CB2 3EA

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SUMMARY

Axenic combinations of *Peronospora parasitica* and callus of *Brassica oleracea* var. *capitata*, Drumhead cabbage, and other *Brassica* species were established. The fungus grew well in callus culture and sometimes had many of the characteristics of an aggressive parasite, in contrast with its growth habit in intact Brassica cotyledons. The susceptibility of different Brassica callus clones and root organ cultures to *P. parasitica* was not constant, and results obtained with callus were frequently at variance with results obtained using intact Brassica plant parts.

INTRODUCTION

Combinations of obligate parasites and host callus cultures suitable for experimental study have been achieved only by Morel (1944, 1948) with *Plasmopara viticola* and vine; by Hotson & Cutter (1951) and Cutter (1959) with *Gymnosporangium juniperi-virginianae* and juniper; by Cutter (1960) with *Uromyces ari-triphylli* and *Arisaema triphyllum;* by Strandberg, Williams & Yukawa (1966) and Ingram (1969*a*, *b*) with *Plasmodiophora brassicae* and Brassica. Most other combinations, including the downy mildew *Peronospora tabacina* and *Nicotiana tabacum* combination of Izard, Lacharpagne & Schiltz (1964) have been short lived (Brian, 1967). Nakamura (1965) reported briefly on the dual culture of *Peronospora parasitica* and turnip. Cultures of *Pseudoperonospora humuli* and hop callus (Griffin & Coley-Smith, 1968) grew very slowly, and their use as experimental tools was thus precluded.

The expression of resistance to fungi in tissue culture has been investigated by Ingram & Robertson (1965), Ingram (1966, 1967) and Robertson *et al.* (1968) using the *Phytophthora infestans* + *Solanum tuberosum* combination. In addition, Maheshwari, Hildebrandt & Allen (1968) have studied abnormal resistance of Antirrhinum callus to infection by *Puccinia antirrhini* spores.

Methods for the successful growth of *Peronospora parasitica* in Brassica callus are reported here, together with some observations on the susceptibility and resistance of various callus clones of three *Brassica* species to invasion by *P. parasitica.*

METHODS

Organisms. Peronospora parasitica (Pers. ex Fr.) Fr. was isolated from leaves of *Brassica oleracea* var. *gemmifera*, Sutton's Fillbasket Brussels sprout, plants collected in Cambridge. Conidia were produced abundantly on the surface of detached leaves incubated for 72 hr in a moist chamber in a cold frame, and a suspension containing about 3×10^5 conidia/ml. water was prepared. Drops (0·1 ml.) of suspension were

applied to the upper surfaces of cotyledons from 7- to 9-day-old *B. oleracea* var. *capitata*, Early Drumhead or Primo cabbage, seedlings. These cotyledons had previously been removed in pairs, together with a 1·5 cm. length of hypocotyl, from seedlings which had been soil-grown in a greenhouse at 20° to 25°, having supplementary lighting for 12 hr each day from six 125 W daylight fluorescent tubes. The cotyledon pairs were laid out in rows on moist filter paper (crinkled grey) contained in 17 cm. × 11 cm. × 4 cm. clear plastic boxes, and were incubated, after inoculation, in an incubator at 15° lit for 12 hr each day by six 8 W warm white fluorescent tubes. This procedure was a modification of a method reported by Channon & Hampson (1968).

Table 1. *Details of Brassica callus tissue cultures used in infection studies with Peronospora parasitica*

Parent species	Origin of the tissue culture	Clone no.	Date of establishment	Characteristics of callus
B. oleracea var. *capitata* (Early Drumhead cabbage)	Surface sterilized mature leaf	CL	Sept. 1966	Slow growing firm white callus
	Root from a 7-day sterile seedling	CR	Sept. 1966	Slow growing, friable, mucilagenous callus of discreet meristematic centres
B. rapa (Golden Ball turnip)	Surface sterilized expanded root/hypocotyl from a 9-week plant	GBC1	Nov. 1966	Fast growing, firm orange coloured callus
	Surface sterilized *Plasmodiophora brassicae* clubbed root of a 9-week plant	GBB5	Nov. 1966	Fast growing, dry, friable callus
	Surface sterilized expanded root/hypocotyl from a 5-week plant	GB(H)	July 1967	Fast growing, firm, orange coloured callus
	Surface sterilized *Plasmodiophora brassicae* clubbed root of a 5-week plant	GB(i)	July 1967	Fast growing, firm, orange coloured callus
B. napus (Wilhelmsburger swede)	Surface sterilized expanded root/hypocotyl from a 9-week plant	DG	Sept. 1966	Fast growing, dry, friable orange coloured callus
	Hypocotyl from a 7-day sterile seedling	DH	Nov. 1966	Fast growing, firm, orange coloured callus

Sporulation of *Peronospora parasitica* occurred after 5 to 8 days of incubation and conidia were used to inoculate a new batch of cotyledons. Non-sterile stock cultures of the fungus were maintained by transfer to fresh cotyledons every 7 days.

Callus culture clones were established from organs of *Brassica oleracea* var. *capitata*, Early Drumhead cabbage, *B. rapa*, Golden Ball turnip, and *B. napus*, Wilhelmsburger swede, as shown in Table 1 and according to the methods of Ingram (1969*a*). The callus culture medium was supplemented with 2,4-dichlorophenoxyacetic acid 6·0 mg./l., α-naphthylacetic acid 0·1 mg./l. and coconut milk 150 ml./l. (Ingram 1969*a*).

Stock callus cultures were incubated at 25° in the dark, but callus cultures infected with *Peronospora parasitica* were incubated either at 22° in the dark or in the 15° illuminated incubator described above. All callus experiments were made with 20 ml. of medium solidified with 0·6% Davis agar and contained in 25 mm. × 150 mm. Pyrex glass test tubes.

Root organ cultures were established from 7-day sterile seedlings of *Brassica oleracea* var. *capitata*, Drumhead and Primo, and *B. rapa*, Golden Ball, in a liquid medium (Street & Henshaw, 1966) supplemented with *meso*-inositol 50·0 mg./l., kinetin 0·00125 mg./l. and α-naphthylacetic acid 0·00005 mg./l. (Ingram 1969*a*).

Establishment of Peronospora parasitica in callus culture. Cotyledon pairs from 7-day *Brassica oleracea* var. *capitata*, Drumhead, seedlings were inoculated with conidia of *Peronospora parasitica* and incubated at 15° as described above. After 72 hr the cotyledons were detached from the hypocotyl segments, washed in 95% (v/v) ethanol in water and surface sterilized for 5 min. in a 5% (w/v) filtered calcium hypochlorite solution containing a drop of detergent. After three washes in sterile distilled water each cotyledon was cut in half and the halves placed upper-side downwards on solidified callus culture medium in test tubes and incubated at 22° in the dark. Cotyledon halves which remained sterile and gave rise to aerial mycelium of *P. parasitica* were placed on the surface of growing *B. oleracea* var. *capitata*, Drumhead, leaf calluses in culture tubes and were further incubated at 22°.

Histological procedures. Calluses and detached cotyledons infected with *Peronospora parasitica* were fixed in glutaraldehyde and embedded in wax (Ingram, 1969*a*). Sections (16 μ) were stained with Delafield's haematoxylin (Ingram, 1967); or with resorcin blue (Griffin & Coley-Smith, 1968); or with safranin-O (ethanolic safranin-O 1·0 g. + Cellosolve (2-ethoxyethanol) 50·0 ml. + 95% ethanol in water 25·0 ml. + sodium acetate 1·0 g. + formalin 2·0 ml. + distilled water 25·0 ml.) for 30 min. followed by a wash in running water, counterstaining in fast green (0·5% in equal volumes clove oil, absolute ethanol and Cellosolve) for 10 sec., a wash in clove oil and 5 min. in a clearing mixture (50 vol. clove oil + 25 vol. absolute ethanol + 25 vol. xylene). Small pieces of fresh infected callus were also examined whole or as freehand sections or squashes.

Infection test with Brassica plant parts. The detached cotyledon technique described above was used to test the susceptibility of *Brassica rapa*, Golden Ball, and *B. napus* Wilhelmsburger, cotyledons to *Peronospora parasitica*. Conidia obtained from detached cotyledons of *Brassica oleracea* var. *capitata*, Drumhead, infected with *P. parasitica* were used as inoculum. When the susceptibility of seedling roots or hypocotyls was to be tested the technique was modified: washed 7-day seedlings were laid out in the plastic boxes instead of detached cotyledon pairs. Tests were replicated 20 times and were repeated. In one test the susceptibility of root/hypocotyl slices from mature plant of *Brassica rapa*, Golden Ball, and *B. napus*, Wilhelmsburger, was investigated. Plants of these two species were raised in soil in the greenhouse and were harvested at 9 weeks. The swollen root/hypocotyls were washed thoroughly in water, surface sterilized by immersion in a 5% filtered calcium hypochlorite solution for 10 min. and washed twice in sterile distilled water. Slices, 0·5 cm. thick, were laid on moist sterile filter paper in Petri plates. The slices were each inoculated at the centre with 0·1 ml. of a suspension containing 3×10^4 conidia of *P. parasitica* and were incubated in the 15° illuminated incubator.

The pathogenicity of callus grown *Peronospora parasitica* to cotyledons of *Brassica oleracea* var. *capitata*, Drumhead, was tested using the detached cotyledon techniques where a conidial suspension obtained from an infected callus culture of *B. rapa*, clone GBC 1, was substituted for the cotyledon-produced conidia, or where explants (3·0 mm.^{3}) of infected *B. rapa*, clone GBC 1, callus and *B. oleracea* var. *capitata*, Drumhead, leaf callus were applied in 0·05 ml. drops of water to the upper surfaces of cotyledons. The callus fragments were removed from the cotyledons after 24 hr.

Infection tests with tissue cultures to assess the ability of *Peronospora parasitica* to colonize callus of the Brassica clones listed in Table 1 were carried out by placing an explant (3·0 mm.^{3}) of *Brassica rapa*, clone GBC 1, callus, permeated with mycelium of *P. parasitica*, on the surface of the culture medium and in contact with a freshly transferred callus of the clone to be tested. Incubation was then at 22° in the dark. Alternatively, calluses were each inoculated with 0·1 ml. of a sterile suspension containing 10^3 conidia/ml. distilled water, obtained from infected cultures of *B. rapa*, clone GBC 1, and were incubated at 15° in the dark for 48 hr and then at 22° in the dark.

Root organ culture infection tests were made by using 4·0 cm. root tip segments, from liquid culture, placed on the surface of the organ culture medium solidified with 0·6% Davis agar and contained in Petri plates. Each root segment was inoculated with 0·1 ml. of a sterile suspension containing 10^3 conidia/ml. sterile water, obtained from infected calluses of *Brassica rapa*, clone GBC 1. Incubation was at 15° in the dark for 48 hr and then at 22° in the dark.

All infection tests with calluses and organ cultures were replicated at least five times and were repeated twice.

RESULTS AND DISCUSSION

Establishment of Peronospora parasitica in callus culture

Aerial mycelium of *Peronospora parasitica* developed abundantly on seven out of 16 infected surface-sterilized cotyledons of *Brassica oleracea* var. *capitata*, Drumhead, incubated on callus medium at 22° in the dark for 5 days. The cotyledons became yellow during this time, but remained turgid. Aerial mycelium did not develop on eight of the cotyledons and one became contaminated with bacteria. When half cotyledons covered with aerial mycelium were placed in contact with six root calluses and seven leaf calluses of *B. oleracea* var. *capitata*, Drumhead, contaminant bacteria developed on one root callus and on four leaf calluses. *P. parasitica* from the cotyledon halves did not invade the root calluses, but did invade and became established in the three uncontaminated leaf calluses. After 14 days of incubation at 22° in the dark the latter were brown and moribund and were covered with aerial mycelium of the fungus; the cotyledon inoculum had withered completely.

Leaf callus infected with *Peronospora parasitica* could not be subcultured to fresh medium since the infected tissues aged rapidly. In contrast, healthy leaf callus could be subcultured readily and proliferated well on fresh medium. It is regrettable that the infected calluses could not be transferred directly, since this would have facilitated their use as experimental tools. Sterile fungus + callus combinations could be perpetuated, however, by inoculation of fresh, uninfected calluses with a 3·0 mm.^{3} explant of infected tissue from a moribund culture, and incubation at 22° in the dark. Fungus

hyphae usually grew from the inoculum and invaded the new tissue after about seven days. Cultures of *P. parasitica* on *Brassica oleracea* var. *capitata* leaf callus were maintained by inoculation of fresh tissues ever 14 to 21 days. It was later found more convenient to substitute the faster growing *B. rapa*, clone GBC 1, callus for the *B. oleracea* var. *capitata* tissue. Fungus + clone GBC 1 callus combinations were usually maintained by inoculation of fresh calluses with explants from moribund cultures as described above. However, infected clone GBC 1 calluses could also be transferred directly: about 4 weeks after infection of GBC 1 calluses with *P. parasitica*, by which time cultures were brown and apparently senescent, small nodules of new tissue began to arise on the callus surface and frequently became infected by the fungus. These new infected tissues could be removed to fresh medium where they proliferated, or the whole callus could be transferred and the nodules allowed to grow to form larger masses of tissue. The nodules, which sections showed were formed from deep-seated uninfected layers of cells, sometimes did not become infected by *P. parasitica*. The phenomenon of re-growth of the infected clone GBC 1 calluses might have useful applications.

Conidia of *Peronospora parasitica* were not produced readily on callus cultures incubated at 22° in the dark, but were produced abundantly on infected calluses transferred to the 15° illuminated incubator, particularly when 0·5 ml. of sterile distilled water was applied to the surface of the culture medium. Davison (1968) commented on the effects of light on sporulation of *P. parasitica*. However, it is not possible to draw definite conclusions from the observations reported here, except to comment that conditions in the 15° illuminated incubator probably resembled more closely the natural conditions for growth and sporulation of *P. parasitica* than did conditions in the 22° dark incubator. The conidia formed on callus were typical of *P. parasitica* (Pl. 1, fig. 1, 2) and were pathogenic to detached cotyledons of *Brassica oleracea* var. *capitata*, Drumhead, or leaf callus of this species or callus of *B. rapa*, clone GBC 1. On inoculated calluses and on cotyledons, typical *P. parasitica* conidial heads were produced after 5 to 8 days of incubation at 15° in the illuminated incubator. Moreover, explants of moribund infected callus were capable of infecting detached Drumhead cotyledons, leading to formation of conidia. At no time were spores similar to the spores of any fungus other than *P. parasitica* observed on infected calluses.

Peronospora parasitica maintained in callus culture was always restricted to the callus tissue, and hyphae did not make any growth on to the surrounding culture medium. This is in contrast to the findings of Griffin & Coley-Smith (1968), who showed that *Pseudoperonospora humuli* frequently grew for short distances away from hop calluses on the culture medium.

Histological investigation of infected calluses

The growth habit of *Peronospora parasitica* was compared in detached cotyledons of *Brassica oleracea* var. *capitata*, Drumhead, and in callus of *B. oleracea* var. *capitata*, Drumhead leaf and of *B. rapa*, clone GBC 1. At the time of fixation, cotyledons infected with *P. parasitica* had a dense cover of conidiophores bearing conidia. Infected calluses were covered with a layer of sterile aerial hyphae.

Detached cotyledons of Brassica oleracea var. capitata, Drumhead were fixed 7 days after infection, and sections cut in a near-paradermal plane. Mycelium in the cotyle-

dons was diffuse and predominantly intercellular, with hyphae present in all tissues except the xylem. Individual hyphae were between 10·0 μ and 16·0 μ diameter. Haustoria were present and were normally simple, flask shaped and about 11·0 μ wide by 22·0 μ long (Pl. 1, fig. 3, 4); bilobed haustoria were occasionally noted. A callose haustorial sheath, which stained blue with resorcin blue, was often present. Between one and four haustoria were present in each penetrated host cell. These findings were in agreement with the findings of Fraymouth (1956). Conidiophores usually emerged in groups of three to eight through stomata on both surfaces of the cotyledons; oospores were not noted. There was no evidence that *P. parasitica* had killed or macerated host cells.

Leaf callus of Brassica oleracea var. capitata, Drumhead, was fixed 7 days after infection. The distribution of mycelium in callus tissues was quite different from that in detached cotyledons. The outer layer (three or four cells deep) of each callus had been heavily invaded by coenocytic hyphae which were both inter- and intra-cellular. Cells in this layer were frequently packed with hyphae or with enlarged and swollen haustoria (Pl. 2, fig. 5, 6), while individual haustoria were often compound, having two to four branches (Pl. 2, fig. 7). In many cases it was impossible to determine whether cells were filled with enlarged haustoria or with intracellular hyphae. Butler & Jones (1961) figured a similar aggressive growth habit of *Peronospora parasitica* in stems of *Capsella bursa-pastoris*. Below the outer layer of heavily invaded Drumhead cells occurred another layer, also three to four cells thick, where growth of *P. parasitica* was more diffuse, hyphae tending towards an intercellular growth habit with cells penetrated by smaller, simple or bilobed haustoria. The central regions of infected calluses were completely free of mycelium. This could have been due to a decreased availability of oxygen in the deeper seated tissues or to the fact that the central regions of calluses usually consisted of dead or old cells.

Calluses of Drumhead leaf fixed 21 days after infection with *Peronospora parasitica* were also examined. These calluses were brown in colour and were completely senescent. Fungus distribution was the same as in 7-day infected calluses. However, all heavily invaded tissues were quite dead and individual cell walls were thickened and golden brown in colour. These thickened walls did not take up Delafield's stain.

Calluses of Brassica rapa, clone GBC1 were fixed 7 days after infection. The distribution of hyphae was similar to that in Drumhead leaf calluses, infection being restricted to the outer layers of cells, with the central region free from the parasite. An important difference, however, was that in the clone GBC1 calluses hyphae were predominantly intercellular, and only occasionally intracellular. Moreover, haustoria resembled the haustoria found in Drumhead cotyledons, being restricted in size and only occurring in groups of one to three per cell. A sheath was frequently present and both simple and bi- and trilobed haustoria were found (Pl. 2, fig. 8). Oospores were not detected in callus cultures, and there was no evidence for tissue maceration.

Clearly, *Peronospora parasitica* had a more aggressive growth habit in the *Brassica cleracea* var. *capitata* leaf calluses than in the *B. rapa*, clone GBC1, calluses and the *B. oleracea* var. *capitata* cotyledons. It is likely that the vigour of fungal growth in callus is related both to the physiological state of the calluses at the time of inoculation and to the sources from which the fungal cultures and the callus cultures are derived.

Infection tests

Tests with detached plant parts. The results of these tests are summarized in Table 2. The isolate of *Peronospora parasitica* used in the experiments was pathogenic to, and became systemic in, detached cotyledons or whole seedlings of *Brassica oleracea* var.

Table 2. *Growth of Peronospora parasitica on callus tissues and detached plant parts of three Brassica species*

Inoculum consisted of explants of *B. rapa*, clone GBC 1, callus infected with *P. parasitica* in the callus tests, and conidia from cotyledons of *B. oleracea* var. *capitata*, Drumhead, in the detached plant part tests.

Growth of *P. parasitica* on test calluses: + + + = aerial mycelium covering the whole callus in 7 to 14 days; + + = aerial mycelium covering the whole callus in 14 to 21 days; + = invasion of callus not occurring until 4 weeks after infection, and then being restricted; — = callus not invaded. Susceptibility of detached organs: S = susceptible; H = hypersensitive reaction; R = resistant. N.T. = not tested.

		Callus tissues			Plant parts
Brassica species	Plant part	Callus clone no.	Growth of *P. parasitica*	Time to onset of senescence of callus (days)	Resistance or susceptibility
B. oleracea var. *capitata*, Early Drumhead cabbage	Mature leaf	CL	+ + +	14<	N.T.
	Seedling cotyledon	N.T.	N.T.	N.T.	S*
	Seedling hypocotyl	N.T.	N.T.	N.T.	S*
	Seedling root	CR	—	56<	S*
B. rapa, Golden Ball turnip	Mature root/ hypocotyl	GBC 1	+ + +	14–21<	S
	Mature root/ hypocotyl	GB(H)	+ + + / + + / ±	14<	
	Plasmodiophora brassicae-clubbed root	GBB 5	—	56<	N.T.
	Plasmodiophora brassicae-clubbed root	GB(i)	+ + +	28<	N.T.
	Seedling cotyledon	N.T.	N.T.	N.T.	H
	Seedling hypocotyl	N.T.	N.T.	N.T.	H
	Seedling root	N.T.	N.T.	N.T.	R
B. napus, Wilhelmsburger swede	Mature root/ hypocotyl	DG	+	21–28<	S
	Seedling cotyledon	N.T.	N.T.	N.T.	H
	Seedling hypocotyl	DH	+ + + / + + / ±	14<	H
	Seedling root	N.T.	N.T.	N.T.	R

* Infection of these tissues also occurred systemically.

capitata, Drumhead and Primo, irrespective of whether the conidial inoculum was applied to the roots, the hypocotyls or the cotyledons. Normally all seedlings in these tests were susceptible. It was not possible to infect roots, hypocotyls or cotyledons of *B. rapa*, Golden Ball, or *B. napus*, Wilhelmsburger, seedlings successfully with a conidial inoculum of *P. parasitica*. Attempted infection of Golden Ball and Wilhelmsburger cotyledons and hypocotyls led to necrotic flecking characteristic of a hypersensitive reaction. Occasionally, when Wilhelmsburger cotyledons under test were becoming senescent a few sparse conidiophores of *P. parasitica* were produced, but this was not a normal occurrence. In contrast, when a conidial suspension was applied to mature root/hypocotyl slices of Golden Ball and Wilhelmsburger, the fungus rapidly became established in the tissues and sporulation occurred within 7 days. Infection always took place, irrespective of whether tissue slices were taken from the apical or the basal ends of the roots.

Host specificity did appear to be expressed by the *Peronospora parasitica*, a finding which is in keeping with the work of Natti, Dickson & Atkin (1967), who identified two physiological races of the fungus and demonstrated the existence of two dominant resistance genes in *Brassica oleracea*. The anomalous results obtained with the Golden Ball and Wilhelmsburger root slices may have been due to the fact that conidia were applied to cut rather than intact tissue surfaces.

Tests with callus tissues. The result of tests to assess the ability of *Peronospora parasitica* to invade various *Brassica* callus tissues are summarized in Table 2. (Fragments of tissue from an infected *Brassica rapa* clone GBC1 callus were used as inoculum.) Invasion of calluses was assessed on the basis of aerial mycelium production and examination of hand sections.

Peronospora parasitica did not invade callus of *Brassica rapa*, Golden Ball, clone GBB5 root/hypocotyl (already infected with *Plasmodiophora brassica* plasmodia) or of *B. oleracea* var. *capitata*, Drumhead, root. There was no evidence that a diffusible inhibitor was produced by the resistant calluses, for aerial mycelium was always produced on the inoculum explants in contact with the tissues under test. Calluses of *B. napus*, Wilhelmsburger, clone DG, root were only invaded when the tissues were becoming less vigorous, about 3 to 4 weeks after inoculation. Such invasions were restricted to an area about 5·0 mm. diameter around the point of contact between the inoculum and the test callus: this area enlarged as the calluses senesced. Infection was accompanied by darkening of the tissues.

Calluses of Wilhelmsburger seedling hypocotyl, clone DH, did not give consistent results in infection tests: some cultures were susceptible and were invaded by *Peronospora parasitica* in 7 to 14 days, although invasion was accompanied by browning of the infected tissues and senescence of the calluses by 14 days. Other clone DH calluses were less susceptible and were frequently only partially invaded by the fungus. The uninfected remainders of such calluses usually continued to grow and did not senesce until 6 to 8 weeks old. Yet other clone DH calluses were totally resistant and never became invaded by *P. parasitica*, although the fungus proliferated on the inoculum explants. *Brassica rapa*, Golden Ball, clone GB(H), also gave inconsistent results, some calluses being susceptible to invasion by *P. parasitica*, and others being only partly susceptible or completely resistant.

Calluses of *Brassica oleracea* var. *capitata*, Drumhead, leaf and *B. rapa*, Golden Ball, root/hypocotyl clones GB(i) (already infected with plasmodia of *Plasmodiophora*

brassicae) and GBC 1 were consistently susceptible to *Peronospora parasitica*, and were completely covered with mycelium 7 days after infection. Senescence occurred most readily in the slow-growing Drumhead leaf calluses, beginning at about 14 days, when calluses became brown and growth stopped. Senescence of clone GBC 1 calluses infected with *P. parasitica* was slower to begin, while senescence of infected clone GB(i) calluses did not begin until 28 days after infection. Senescence of uninfected calluses did not usually occur until they were 6 to 8 weeks old. Re-growth of Golden Ball clones GBC 1, GB(H) and GB(i) calluses, in the form of small tissue nodules which proliferated and became infected with *P. parasitica*, occurred at 4 weeks after infection.

When *Brassica oleracea* var. *capitata*, Drumhead, leaf and root calluses and *B. rapa* clone GBC 1 calluses were inoculated with 0·1 ml. of a suspension containing 10^3 *Peronospora parasitica* conidia/ml., the fungus became established in the Drumhead leaf calluses and in the GBC 1 calluses, but infection of Drumhead root calluses did not occur.

Tests with root organ cultures. It was thought that root organ cultures might resemble intact plants more closely than callus in their response to infection by *Peronospora parasitica.* This was not so, however. Direct microscopic examination of Petri plate cultures 14 days after inoculation showed that *P. parasitica* conidia had germinated on, and in contact with, root hairs and roots of *Brassica oleracea* var. *capitata*, Drumhead and Primo, and of *B. rapa*, Golden Ball. However, infection of the roots did not normally occur. Limited invasion of two of the Primo root cultures took place, and hyphae could be seen growing for short distances on the root surfaces. These infections did not develop and the fungus did not sporulate. In those cultures of Drumhead, Primo and Golden Ball where penetration had not taken place there was no evidence that spore germination had been inhibited by the root organ cultures or that germ tubes were orientated either away from or towards the roots.

It is quite clear that the expression of resistance to *Peronospora parasitica* in callus culture and in root organ culture is variable and cannot be related directly to findings with intact plants. This is in contrast to the work of Ingram and Robertson (1965) and Ingram (1966, 1967), who found good correlation between intact plants and tissue cultures of *Solanum tuberosum* in the expression of R-gene resistance to *Phytophthora infestans.* Maheshwari *et al.* (1968) demonstrated production of an abnormal metabolite by Antirrhinum callus which was toxic to *Puccinia antirrhini.* Abnormal antimicrobial metabolite production by callus of lettuce and cauliflower (Campbell, Chan & Barker, 1965) and by callus of *Populus tremula* (Mathes, 1963) has also been demonstrated. It is possible that a similar phenomenon may have been operative in the abnormally resistant *Brassica oleracea* var. *capitata* root calluses. The unexpected susceptibility of many of the *B. rapa*, Golden Ball, and *B. napus*, Wilhelmsburger, calluses to *P. parasitica* is difficult to explain, although it is significant that root slices of these two species were susceptible to the fungus, and like calluses were inoculated on surfaces not possessing an epidermis. It may also be relevant that Ingram (1966) and Robertson *et al.* (1968) observed a decrease in the resistance of *S. tuberosum* calluses to *P. infestans* which could be correlated with the length of time that the tissues had been in culture: all of the Brassica callus cultures used in the tests described here had been in culture for more than one year. It is possible that freshly isolated calluses would behave in a similar way to intact plants in response to infection with *P. parasitica.*

The results of the infection tests serve to emphasize the caution necessary in relating results obtained on the growth of fungi in tissue culture to their growth in intact plants.

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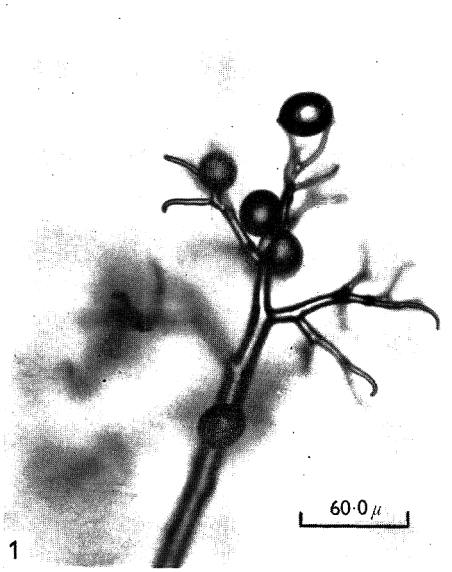

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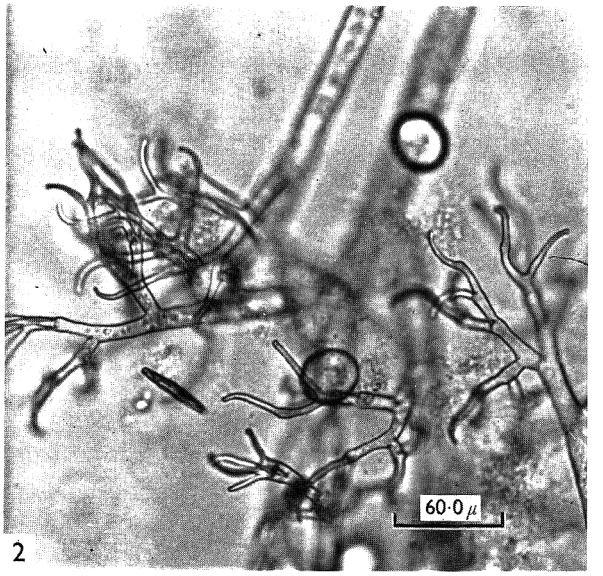

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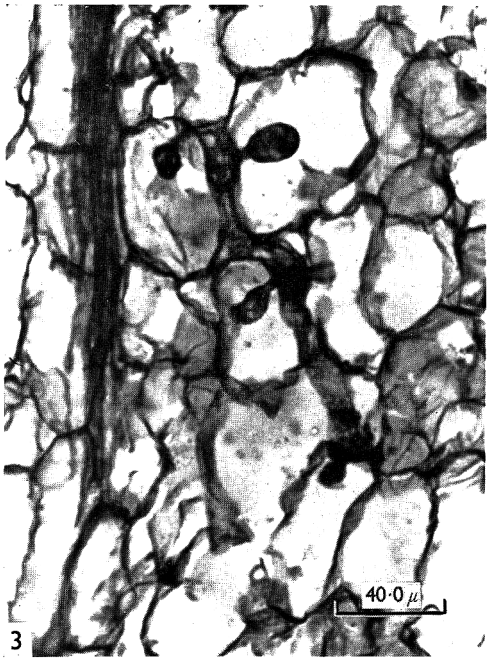

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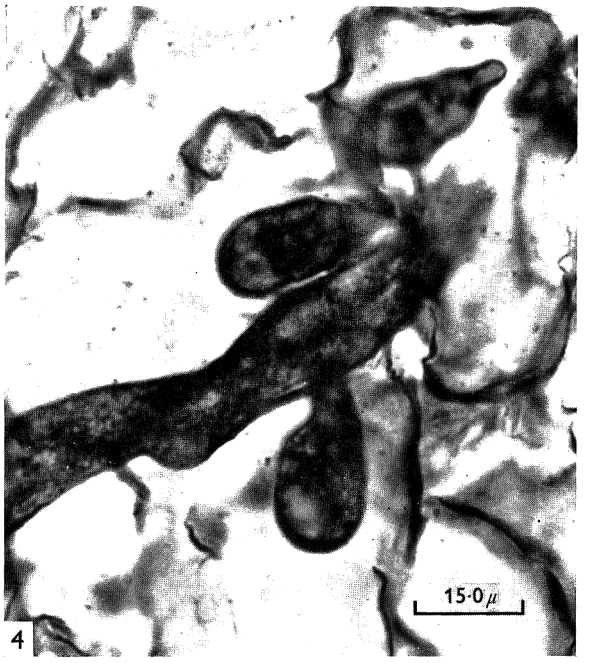

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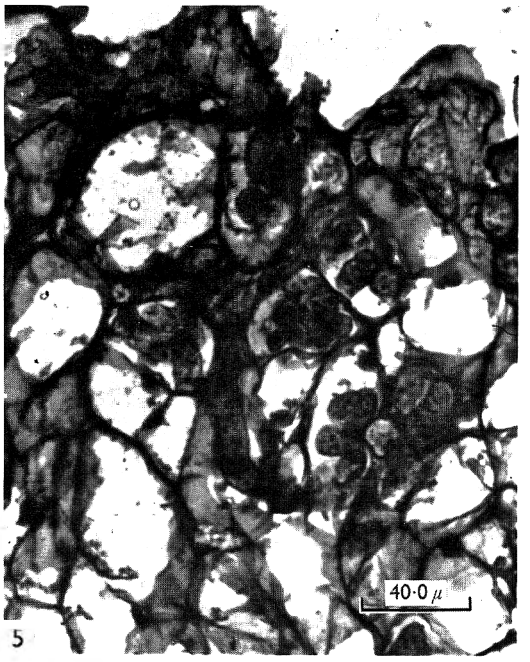
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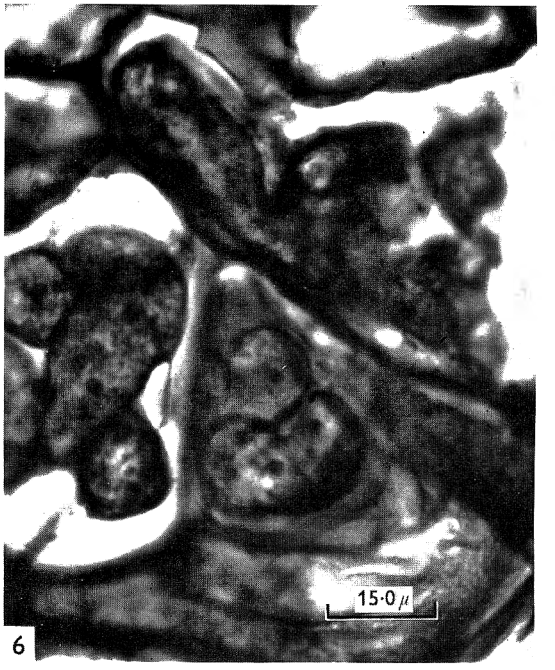
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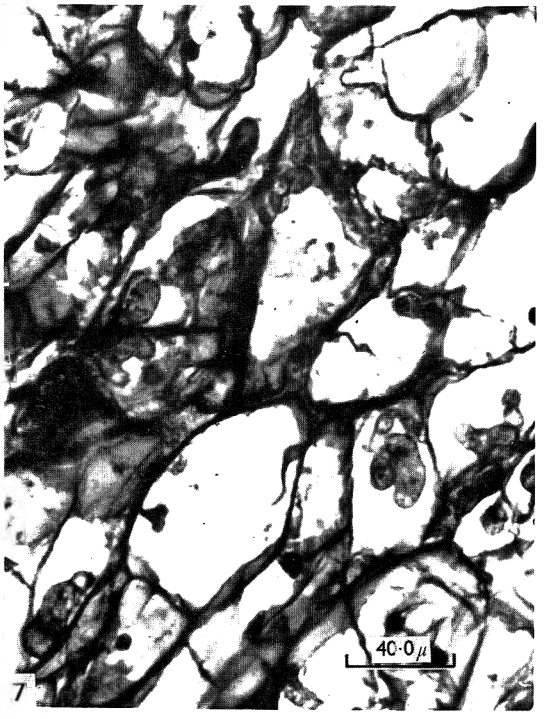
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EXPLANATION OF PLATES

PLATE 1

Fig. 1 and 2. Typical conidia and conidiophores of *Peronospora parasitica* grown on *Brassica rapa*, clone GBC 1, callus. Unstained (fig. 1) and a squash stained with cotton blue (fig. 2).

Fig. 3 and 4. Intercellular hyphae and haustoria growing in a detached cotyledon of *Brassica oleracea* var. *capitata*, Drumhead cabbage. Near paradermal sections stained with Delafield's haematoxylin.

PLATE 2

Fig. 5, 6 and 7. Inter- and intracellular hyphae and haustoria of *Peronospora parasitica* in *Brassica oleracea* var. *capitata*, Early Drumhead cabbage, leaf callus. Stained with Delafield's haematoxylin.

Fig. 8. A three-lobed haustorium of *Peronospora parasitica* in an enlarged cell of *Brassica rapa*, clone GBC 1, callus. Stained with Delafield's haematoxylin.

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Growth and Toxin Synthesis in Batch and Chemostat Cultures of *Corynebacterium diphtheriae*

R. C. RIGHELATO* AND P. A. VAN HEMERT
Rijks Instituut voor de Volksgezondheid, Bilthoven, The Netherlands

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SUMMARY

Toxin synthesis in restricted-iron batch cultures of *Corynebacterium diphtheriae* CN2000 (a $PW8_s$ PD strain) was proportional to the bacterial mass above a minimum bacterial concentration. During glucose-limited growth 0·30 g. toxin was released/g. bacterial protein synthesized. In excess of glucose 0·06 g. toxin was released/g. bacterial protein synthesized. Toxin release in glucose-limited continuous cultures followed similar kinetics to the batch cultures.

Iron in the restricted-iron medium, 6 μg. atom/l., limited the quantities of iron taken up by the bacteria and of three iron-containing enzymes, but did not restrict bacterial concentration over the range 2·0–4·5 mg. bacterial protein/ml. The addition of 90 μg. atom Fe/l. to a restricted-iron culture rapidly inhibited toxin release.

INTRODUCTION

Diphtheria toxin is a synthesized by *Corynebacterium diphtheriae* when grown in medium whose iron content is very low (Pappenheimer & Hendee, 1947). It is released from intact organisms (Pappenheimer, Miller & Yoneda, 1962) and the very low intracellular concentrations of the toxin (Raynaud *et al.* 1954) indicate that it is released as soon as it is synthesized. The relationship between the formation of toxin and the growth of the bacterium is of interest for the light it may shed on the role of the toxin in the physiology of *C. diphtheriae*; a critical evaluation of this relationship is a prerequisite for the study of toxinogenesis.

Diphtherial toxin has been reported to be produced by the organisms under a variety of growth conditions. Hirai, Uchida, Shinmen & Yoneda (1966) described a system in which toxin synthesis occurred for short periods of time (3 to 4 hr) without concomitant increase in cell mass. To obtain high titres of toxin, however, growing bacteria are used. Raynaud *et al.* (1954) showed an approximately constant relationship between increase in toxin concentration and increase in bacterial nitrogen over the greater part of the toxin production phase in batch cultures. Toxin release begins during the exponential phase of growth after a short lag and continues for variable lengths of time after exponential growth has ceased (Raynaud, 1966; van Hemert & van Wezel, 1966).

The shape of the growth curves on both the complex and the defined media used for diphtheria toxin production show that the cultures pass through at least two exponential stages, followed by a phase of decreasing growth rate (Raynaud, 1966). More-

* Present Address: Glaxo Laboratories Limited, Ulverston, Lancashire

over, as growth progresses the bacteria are exposed to simultaneous changes in the concentrations of substrates utilized for growth, as well as of the products of growth. Steady-state chemostat cultivation (Monod, 1950; Novick & Szilard, 1950) eliminates the problem of sequential changes in the environment of the bacteria and allows the independent investigation of the effects of growth rate and cell concentration on toxinogenesis. The present paper shows that toxinogenic *Corynebacterium diphtheriae* can be maintained in steady-state continuous culture for indefinite lengths of time; this allowed the method to be used to study the inhibition of toxin synthesis by iron over long periods, whilst maintaining steady-state growth at the same rate.

METHODS

Organism. Corynebacterium diphtheriae CN2000, capable of yielding up to 0·30 μg. toxin protein/g. cell protein, was used. This strain originated from the PW8 strain (Park & Williams, 1896). Professor W. L. Barksdale kindly examined this strain and has identified it as the $PW8_S$ (PD) strain (Barksdale, Garmise & Rivera, 1961).

Medium. A papain digest of beef supplemented with salts and vitamins (Linggood & Fenton, 1947), containing Kjeldahl-N 5 g./l. was used. The medium was ultra-filtered and analytical grade glucose added in the concentrations stated below. The medium contained 5 to 7 μg. atom Fe/l. unless otherwise stated.

Apparatus. Batch and continuous cultures were done in the glass-walled Bilthoven Unit of 10 l. total capacity (van Hemert, 1964). A vaned disc impellor operating at 650 rev./min. and producing a considerable vortex provided agitation and aeration. The partial pressure of dissolved oxygen (pO_2) was measured with a steam-sterilizable galvanic electrode (van Hemert, Kilburn, Righelato & van Wezel, 1969). The pO_2 was controlled at 25 mm. Hg, by adding oxygen or nitrogen to a carrier stream of air in response to the signal of the electrode, while the total gas flow was held constant by a differential pressure regulator (Fischer and Porter) and adjusted to give an effluent carbon dioxide concentration of 5 to 7 % (v/v). The medium was controlled at pH 7·3 by adding 2 N-HCl or 4 N-NaOH. An antifoam concentration of 1·0 ml./l. medium was maintained by adding 3 μl. antifoam (polypropylene glycol P2000, Dow Chemical Co.) at intervals of 0·5 to 2·0 min., depending on the dilution rate used. Temperature was controlled at 35°.

Assays. Diphtheria toxin concentration was measured by Ramon flocculation against the Dutch National Standard Antitoxin.

Extinctions of the bacterial suspensions were measured with a Vitatron photo-electric Colorimeter.

Cellular and extracellular protein was measured by the biuret method of Stickland (1951) with crystalline bovine serum albumin as standard.

For all assays on cell material bacteria were washed twice in the cold with 0·05 M-phosphate buffer (pH 7·3) and disrupted at 0 to 5° for 30 min. in an MSE 20 Kc/s ultrasonic disintegrator.

The enzyme assays and the methods for the measurement of iron in cells or medium are those described by Righelato (1969).

Glucose concentration was measured by a glucose oxidase method (blood sugar kit; Boehringer and Soehne GmbH, Mannheim, Germany).

RESULTS

Toxin synthesis in batch cultures

On Linggood & Fenton (1947) medium containing 25 g. maltose/l. after the initial exponential growth phase during which toxin synthesis began, the growth rate was probably limited by the rate at which the main carbohydrate source, usually maltose, was utilized (Tasman & Brandwijk, 1940). In the present work glucose replaced maltose, but to obtain a prolonged phase of toxin production it was supplied at a

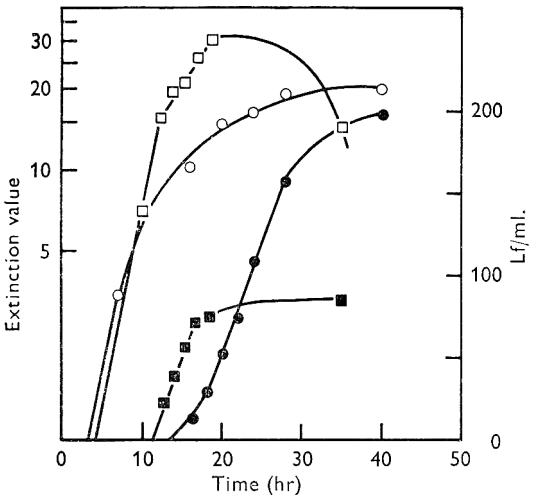

Fig. 1

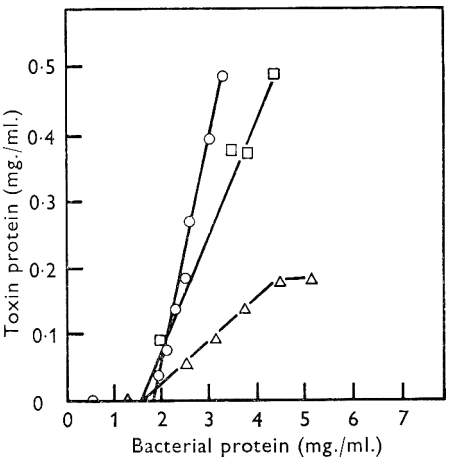

Fig. 2

Fig. 1. Growth and toxin production by *Corynebacterium diphtheriae* CN 200 on Linggood & Fenton (1947) medium with glucose in excess and with glucose-limited growth. □, Glucose excess (5 g./l. throughout culture period); ○, glucose-limited growth. Glucose was added to the culture slowly to maintain at pH 7·0 (van Hemert & van Wezel, 1966). Open symbols, extinction of culture; closed symbols, toxin concentration, Lf/ml.

Fig. 2. The amount of toxin produced by *C. diphtheriae* CN2000 at increasing bacterial concentrations in batch cultures and steady state chemostat cultures. ○, Batch culture to which glucose was fed at a growth-limiting rate (data from Fig. 1); △, batch culture in which the glucose concentration was 5 g./l. throughout (data from Fig. 1); □, glucose-limited chemostat culture. Each point represents a steady state obtained at a different concentration of glucose in the medium, and at a dilution rate of 0·051 hr^{-1} (At D = 0·051 hr^{-1} 9 g. glucose gave 1 g. bacterial protein; the medium without added glucose gave 1·5 g. bacterial protein/l.)

slow growth-limiting rate (van Hemert & van Wezel, 1966). When, instead, the glucose concentration was maintained at a high value (> 5 g./l.) throughout the incubation period, growth at the maximum rate continued for longer but slowed suddenly and toxin synthesis terminated early (Fig. 1). The maximum specific growth rate of *Corynebacterium diphtheriae* CN2000 on the Linggood & Fenton medium used was 0·35 hr^{-1} (doubling time, 120 min.). At high glucose concentrations, though respiration was not diminished, the organism fermented the sugar rapidly, resulting in the extracellular accumulation of acids; hence addition of considerable quantities of alkali was required to maintain the culture at pH 7·3.

The addition of iron (90 μg. atom/l.) to a culture completely inhibited toxinogenesis

but did not influence the final bacterial concentration; thus iron was not growth-limiting. It is possible, however, that the decrease of the intracellular iron concentration below a critical value is necessary for toxin synthesis, as suggested by Pappenheimer (1955). If this is so, then toxin will not be synthesized until the cell concentration is high enough to decrease the iron concentration to the critical value. In Fig. 2 the cell concentration is related to the toxin concentration. In both glucose-limited and excess glucose cultures no significant quantities of toxin (detectable by flocculation with anti-toxin) were produced before the cell concentration rose above 1·6 g. bacterial protein/l., at which time the iron content of the bacteria was 2·5 to 3·0 μg. atom Fe/g. bacterial protein.

The culture grown with excess glucose, where the growth rate was high during most of the toxin production phase, gave a lower yield of toxin per increment of cell concentration (0·06 g. toxin protein/g. bacterial protein) than in the slower growing glucose-limited cultures (0·30 g. toxin protein/g. bacterial protein).

Toxin synthesis in chemostat cultures

In chemostat cultures the growth rate is determined by the rate at which medium is fed to the culture, one component of the medium being used to limit the total amount of cell material produced. In the steady state the specific growth rate is equal to the dilution rate (the flow rate of medium into the culture divided by the volume of the culture). Since glucose could be used to limit the growth rate in batch cultures of *Corynebacterium diphtheriae* whilst allowing a high rate of toxin synthesis, it was used as the growth limiting substrate in chemostat cultures. A preliminary description of the continuous culture system has been given elsewhere (Righelato & van Hemert, 1969).

The chemostat cultures were done at growth rates between 0·100 and 0·027 hr^{-1}. The highest yields of toxin and the highest proportion of toxin protein to cell protein was obtained at the growth rate 0·051 hr^{-1}. This growth rate was used for the experiments described below. The steady-state at the dilution rate 0·051 hr^{-1} with 15·5 g. glucose/l. limiting growth has been repeated on several occasions giving a bacterial concentration of 3·15 g. protein/l., an extracellular protein concentration of 1·11 g./l of which 0·31 g./l. was toxin ($= 155 \pm 15$ Lf/ml.). Bacterial concentration, extracellular protein and toxin titre remained stable over several weeks of continuous cultivation.

Figure 2 shows the relationship between toxin titre and the cell concentration in batch and chemostat cultures. The results indicate that, in chemostat cultures as in batch cultures, below a minimum cell concentration toxin was not produced. The minimum cell concentration corresponded to an intracellular concentration of iron of 2·7 μg. atom/g. bacterial protein (Fig. 3). The concentration of intracellular non-heme iron and of three iron-containing enzymes (expressed per unit of culture volume instead of per unit of cell mass) did not vary with cell concentration (Table 1). Thus it would appear that the concentration of these enzymes was in fact limited by the low iron concentration.

As in batch cultures, the addition to the culture medium of 90 μg. atom Fe/l. completely inhibited toxin synthesis (Table 2). Immunodiffusion tests of supernatant fluids of the steady-state excess-iron culture and the steady-state restricted-iron

culture were made with a monospecific antidiphtherial serum (kindly supplied by Professor M. Raynaud) and a crude antidiphtherial serum which gave 7 to 8 precipitation lines with the culture supernatant fluid, the heaviest being the toxin. The toxin precipitation line could only be found in the restricted-iron culture samples; the other lines were present in both restricted-iron and excess-iron samples. The antigens of the culture supernatant fluids, other than the toxin, were also found, in higher concentrations, in bacterial extracts; their presence in the culture supernatant fluids may have been due to lysis of some of the bacteria. The higher concentration of non-toxic

Table 1. *The relationship between bacterial concentration and iron and some iron-containing enzymes of Corynebacterium diphtheriae* CN2000 *in glucose-limited chemostat culture*

Cell protein* g./l.	Intracellular non-haeme Fe^{2+} μg. atoms/l. culture	Bacterial Cytochrome b extinction (units/l. culture)	Succinate dehydrogenase mμ mole/ml./min.	Catalase $K_{obs.}$ sec.$^{-1}$
2·0	4·1	8	—	—
3·30	3·0	9	140	10·4
3·85	4·0	10	190	12·2
4·50	3·3	8	140	12·2

* Cell protein concentration was varied by varying the concentration of glucose in the medium, 9 g. glucose gave 1 g. bacterial protein. The medium without added glucose gave 1·5 g. bacterial protein/l.

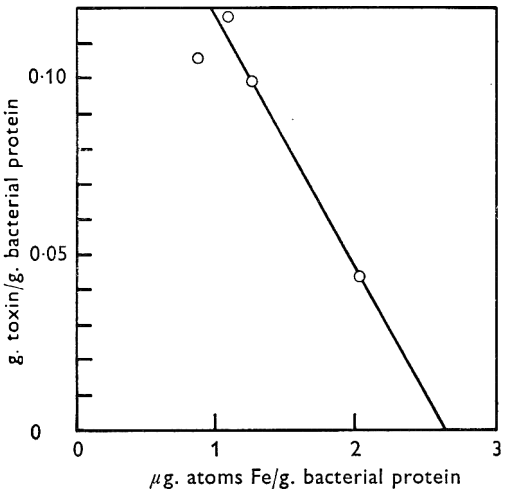

Fig. 3

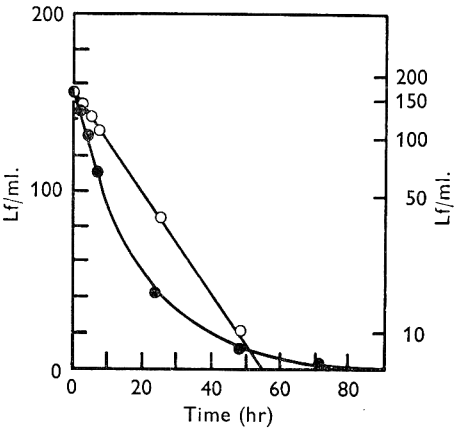

Fig. 4

Fig. 3. The relationship between intracellular iron concentration and toxin synthesis by *Corynebacterium diphtheriae* CN2000 in glucose-limited chemostat cultures. The medium contained 6 μg. atom Fe/l. The concentration of iron in the bacteria was varied by varying the bacterial concentration by changing the concentration of glucose in the medium; 9 g. glucose gave 1 g. bacterial protein. The medium without added glucose gave 1·5 g. bacterial protein/l.

Fig. 4. The dilution out of toxin from glucose-limited chemostat cultures of *C. diphtheriae* CN2000 after inhibition of toxinogenesis by iron. At O hr the iron concentration in the vessel and in the medium was increased from 7 μM to 97 μM. Dilution rate 0·051 hr^{-1}. ●, Linear scale; O, logarithmic scale.

extracellular protein in the restricted-iron cultures, described here and also observed by Alouf (1958) may reflect an increased liability of the iron-deficient bacteria to lysis.

Table 2 shows that iron addition, besides inhibiting toxin synthesis, brought about an increase in the concentration of diphtherial cytochrome b_{559} the major haeme-containing enzyme of the PW8 (PD) strain of *Corynebacterium diphtheriae* (Pappenheimer & Hendee, 1947).

Table 2. *The effect of iron on the steady-state cell concentration and extracellular protein formation by Corynebacterium diphtheriae* CN 2000 *in glucose-limited chemostat culture**

Fe in medium μg. atom/l.:	7	97
Cell protein g/l.:	3·85	4·33
Non-toxin extracellular protein* g./l.:	0·94	0·38
Toxin protein† g./l.:	0·32	0
Cell non-haeme Fe μg. atom/l.:	4·0	60
Cyt. *b* absorbtion units/l.:	10·8	40

* Total trichloroacetic acid precipitable protein in culture supernatant fluid less toxin protein.

† Calculated, assuming 1 Lf = 2·44 μg. protein.

The kinetics of disappearance of toxin from the culture indicate the rapidity with which toxin release was inhibited. In continuous culture the formation of a cell product at a constant rate results, in the steady state, in a constant concentration of the product (C_0) in the vessel. A step change may be applied to the culture, for instance the addition of an inhibitor, and the product present in the vessel before the application of the change is washed out exponentially by the continuous addition of fresh medium:

$$C_t = C_0\, e^{-Dt} \tag{1}$$

$$\text{and } \ln C_t = \ln C_0 - Dt, \tag{2}$$

where C_t is the product concentration t hours after imposition of the step change and D the dilution rate. If washout is to follow these kinetics the time for the inhibitor to exert maximum inhibition will be small compared to the mean residence time ($1/D$) of the culture.

The disappearance of toxin from the culture following the addition of iron is shown in Fig. 4 on a linear scale and plotted according to equation (2). The natural logarithm of the toxin concentration (C_t) falls linearly with a slope equal to $-$D and intercepts the ordinate at the zero time toxin concentration ($\ln C_0$). The inhibition of toxin synthesis thus was complete and immediate, to within the accuracy of measurement, upon the addition of the inhibitor.

DISCUSSION

Toxin synthesis by *Corynebacterium diphtheriae* CN 2000 in glucose-limited batch cultures and in steady-state glucose-limited chemostat cultures followed similar kinetics insofar as toxin release was proportional to the bacterial concentration above a minimum value. The limitation of growth by glucose, or the slowly hydrolysed sugar maltose, was necessary to obtain a high ratio of toxin protein to bacterial protein. During the toxinogenic phase of iron-deficient glucose-limited batch cultures

the rate of toxin production was 0·30 g. toxin protein/g. bacterial protein synthesized, whereas in excess of glucose toxin release occurred at 0·06 g./g. bacterial protein synthesized. Under glucose-limited growth conditions, therefore, a single protein, the toxin, represented a large proportion of the protein synthesized by the bacteria.

Whereas toxin synthesis in batch cultures ceased after about 30 hr of slow growth, the glucose-limited continuous cultures could be maintained for weeks without decrease in the rate of toxin synthesis or change in bacterial concentration. Similar stable continuous cultures of toxin-producing *Clostridium tetani* have been reported by Zaccharias & Bjorklund (1968). Pirt, Thackeray & Harris-Smith (1961), however, who were concerned with a cell-bound antigen of *Pasteurella pestis*, reported a progressive decrease in titre during continuous culture.

Significant quantities of diphtheria toxin were not released in batch cultures until the intracellular iron concentration had fallen below about 3·0 μg. atom/g. cell protein. A similar minimum intracellular concentration of iron below which toxin synthesis did not occur in chemostat cultures was found to be 2·7 μg. atom Fe/g. cell protein. Edwards & Seamer (1960), who worked with the same strain and a similar medium, found, by varying the iron concentration in their batch culture medium, that the intracellular iron concentration had to fall below 3 μg. atom Fe/g. bacterial dry weight for toxin synthesis to occur. In our glucose-limited cultures the cell protein:dry weight ratio was 0·48:1·0; thus Edwards & Seamer's critical intracellular iron concentration was approximately 50% of that found in the present work.

In steady-state chemostat culture at a low iron concentration (6 μg. atom/l.) the concentrations of several iron-containing enzymes did not increase with bacterial concentration. It appears, then, that the concentrations of iron-containing cell components were limited by the low iron concentration of the medium and that the bacteria can be regarded as iron deficient. Under these conditions the amount of toxin synthesized by the bacteria was lower at the lower bacterial concentrations, the amount of iron per unit of bacterial mass being higher.

A high concentration of iron added to a steady-state toxin-producing chemostat culture was completely and immediately inhibitory to toxin synthesis, as suggested by Barksdale *et al.* (1961). These authors found that iron added to u.v.-induced toxinogenic *Corynebacterium diphtheriae* at various times after induction decreased the yield of toxin to that given by the iron-free control at the time of addition.

The mechanism of the inhibition of toxinogenesis by iron remains to be established. The addition of iron to iron-exhausted toxinogenic bacteria caused, in addition to inhibition of toxinogenesis, an increase in the concentration of cytochrome *b*, the main haeme-containing enzyme. If the mM extinction coefficient of diphtherial cytochrome *b* is of the same magnitude as that of cytochrome *b* from other sources (about 30) the cytochrome concentration is about 0·3 μM in the toxin-producing cultures described here and 1·4 μM in the iron-inhibited culture. Since the intracellular iron concentrations in the low and high iron cultures were 4 and 60 μg. atom/l., respectively, cytochrome *b* accounts for only a small proportion of the iron in the cells, most being present as non-haeme iron. The distribution of iron in *Corynebacterium diphtheriae* and the changes in haeme and non-haeme iron-containing enzymes concomitant with the inhibition of toxinogenesis have been studied and will be reported elsewhere (Righelato, 1969).

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The Distribution of Iron in Iron-deficient Toxin-synthesizing and in Excess-iron Non-toxin-synthesizing *Corynebacterium diphtheriae*

By R. C. RIGHELATO*

Rijks Instituut voor de Volksgezondheid, Sterrenbos, 1, Utrecht, The Netherlands

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SUMMARY

In the presence of excess iron diphtherial toxin was not produced by *Corynebacterium diphtheriae* CN2000. 2·8% of the intracellular iron was present in the soluble fraction of washed bacteria; the remainder was bound on the respiratory particles, 4% as haeme and the rest as non-haeme iron. Succinate but not NADH reduced the cytochromes and a small proportion of the non-haeme iron.

Toxin was synthesized at a high rate by bacteria containing 6·5% of the iron present in excess-iron bacteria. Under such iron-deficient conditions the iron-containing components of the respiratory particles were present in decreased quantities. NADH oxidation, which proceeded via a soluble flavoprotein, was not affected by iron-deficiency, nor could reduction of non-haeme iron by NADH be demonstrated. The transition between the iron-deficient and the excess-iron states was studied.

INTRODUCTION

Diphtherial toxin is released by *Corynebacterium diphtheriae* PW8 (PD) in large quantities only when iron is exhausted from the medium (Pappenheimer, 1955); under such conditions, growth of the bacteria occurs without increase in the concentration of some iron-containing enzymes (Righelato & van Hemert, 1969). Addition of 90 μg. atom Fe/l. to the culture medium immediately completely and specifically inhibited toxin release. Edwards & Seamer (1960) found that iron in the medium up to 60 μg. atom/l. was almost entirely taken up by the bacteria, and Yoneda & Ishihara (1960) showed that iron was readily bound by iron-deficient *C. diphtheriae* organisms, no detectable extraction being observed on repeated washing with buffer at pH 6·8. Treatment with 10% (w/v) dithionite released almost all of the bacteria-bound iron. These authors did not, however, investigate the iron bound as haeme. Moreover, the location of iron within the cell has not been investigated in detail. It is known that addition of iron to iron-deficient toxin-producing cultures of the PW8 strain results in an increase in the concentrations of catalase and cytochrome in addition to the inhibition of toxin and coproporphyrin release (Pappenheimer & Hendee, 1947). These observations led to the hypothesis that the toxin was an apoenzyme of diphtherial cytochrome *b* (Pappenheimer, 1947). More recently it has been found that the electron transport pathways of the PW8 (PD) strain differ from those of other strains

* Present address: Glaxo Laboratories Limited, Ulverston, Lancs.

of *C. diphtheriae* which produce less toxin (Pappenheimer, Howland & Miller, 1962). The present work investigates the distribution and function of haeme iron and non-haeme iron in iron-deficient toxinogenic bacteria and in bacteria whose toxin release has been inhibited by growth in excess of iron.

METHODS

Organism. Corynebacterium diphtheriae CN2000 was used; it has been identified as a $PW8_S$(PD) strain (Barksdale, Garmise & Rivera, 1961) by Professor W. L. Barksdale.

Culture, media and apparatus are those described by Righelato & van Hemert (1969) for glucose-limited continuous culture of *C. diphtheriae*.

Preparation of bacterial extracts. Bacteria were harvested from cultures, by centrifugation for 20 min. at 8000 *g*, washed twice in 0·05 M-phosphate (pH 7·3) and resuspended in the buffer at 10 times the concentration of the cultures. For all assays the concentrated bacterial suspension was treated in a 20 Kc/s MSE sonic oscillator for 30 min. The broken-cell suspension so produced was used for determination of the quantities of protein, iron, cytochrome *b*, catalase and succinate dehydrogenase. All operations were done at 0 to 5°.

Bacterial protein was measured by the biuret method of Stickland (1951).

Respiratory assays. NADH oxidase and succinate oxidase were measured by the oxygen uptake rate at 150 mm. Hg oxygen partial pressure in an oxygen-electrode cell (Rank Ltd., Cambridge) at 35°.

The broken-cell suspension exhibited a high endogenous metabolism. Removal of whole bacteria by centrifugation at 3000 *g* for 10 min. followed by passage over G50 Sephadex almost eliminated the endogenous metabolism, whilst 80 to 90% of the protein, iron and catalase and succinic dehydrogenase activities was recovered.

Total haeme was extracted with HCl + acetone and measured as the pyridine hemochrome (Jacobs & Wolin, 1963).

Cytochrome b concentration in the disrupted bacteria was measured by dithionite-reduced minus ferricyanide-oxidized difference spectra, on a Beckman DB spectrophotometer. The difference between the 559 mμ peak and the 577 mμ trough was recorded and an mM extinction coefficient of 28·5 $cm.^{-1}$ found for mitochondrial cytochrome *b*, was used in estimating cytochrome *b* haeme concentrations (Rieske, 1967). The bacterial concentration used for this assay was equiv. 5 to 10 mg. bacterial protein/ml.

Succinate dehydrogenase (succinate-ferricyanide oxido-reductase) activity was measured by the method of Pappenheimer *et al.* (1962).

Catalase activity was measured by the method of Herbert (1955). Catalase haeme was calculated from the activity, assuming four haeme groups/mole, mol. wt of $2·3 \times 10^5$, and an activity of pure catalase $5·3 \times 10^7$/M/sec. (White, 1962).

Iron assays. The iron content of the medium and of disrupted bacteria was measured by the method of Kurup & Brodie (1967*a*). Samples (2 ml.) of disrupted bacteria (5 to 10 mg. protein/ml.) were put in two matched cuvettes, one oxidized with 0·05 ml. 0·1 M- $K_3Fe(CN)_6$ and the other reduced by the addition of a few mg. dithionite; the difference spectrum between 560 mμ and 480 mμ was recorded. One-tenth ml. 40 mM *o*-phenanthroline in 10% (w/v) ethanol in water was added to each cuvette. After rotating to mix and standing for 5 min. the difference spectrum was taken again; a

peak at 510 mμ corresponded to the ferrous-*o*-phenanthroline complex. A millimolar extinction coefficient of 7·9 cm.$^{-1}$ was used to calculate iron concentration.

Coproporphyrin in the cultures fluid was adsorbed on calcium phosphate, washed, eluted with 0·5 N-HCl and measured spectrophotometrically (Falk, 1964).

RESULTS

For the production of significant quantities of toxin in glucose-limited chemostat cultures of *Corynebacterium diphtheriae* CN2000 an intracellular iron concentration of less than 2·7 μg. atom/g. bacterial protein was necessary (Righelato & van Hemert, 1969); 0·10 g. toxin protein/g. bacterial protein were obtained in glucose-limited chemostat cultures when the intracellular iron concentration was about 1·0 μg. atom/g. bacterial protein. The addition of 90 μg. atom Fe/l. to such a culture resulted in complete and immediate inhibition of toxin release.

When a high concentration of iron was added to a chemostat culture supplied with a low iron medium the iron was rapidly taken up by the bacteria (Fig. 1). Within 10 min. of adding iron an increase in the non-haeme iron content of the bacteria was observed and a maximum intracellular concentration was reached within 90 min. at which time 20% of the iron in the medium remained in the culture supernatant fluid.

Table 1. *The effect of iron on toxin and porphyrin release and on intracellular iron concentrations in steady-state glucose-limited chemostat cultures of Corynebacterium diphtheriae* CN2000

	Fe in medium (μg. atom/l.)	
	7	97
Intracellular non-haeme Fe (μg. atom/g bacterial protein)	1·03	15
Intracellular haeme Fe (μmole/g. bacterial protein)	0·16	0·58
Extracellular coproporphyrin (μmol/g. bacterial protein)	1·67	0·38
Extracellular toxin (μmole/g. bacterial protein)	1·51	0

* Calculated from the value 2·44 μg. protein/Lf and assuming a molecular weight of 64,500 (Relyveld *et al.* 1964).

Table 1 shows the effect of the excess iron concentration in the medium on the steady-state intracellular haeme and non-haeme iron concentrations, and on toxin and coproporphyrin syntheses, both of which are known to be inhibited by iron (Pappenheimer & Hendee, 1947). Of the iron taken up by the restricted-iron toxin-synthesising bacteria 13% was recovered as haeme. At the high iron concentration, although the total haeme content of the bacteria increased 3·5-fold it constituted only 3·7% of the bacterial iron, a greater proportion of the iron being recovered as non-haeme iron. It is clear from Table 1 that the inhibition of coproporphyrin release cannot simply be accounted for by the channelling of coproporphyrinogen to protoporphyrin IX which is incorporated into haeme.

Iron is involved in many oxidation-reduction reactions in the cell, particularly in the respiratory enzymes, as both haeme and non-haeme iron. It was shown by Righelato & van Hemert (1969) that the concentrations of two haeme-containing enzymes and succinate dehydrogenase, which contains non-haeme iron, are limited in the medium used for toxin production by *Corynebacterium diphtheriae*. Table 2 shows the activities

Table 2. *The effect of iron on the concentration of some respiratory enzymes of Corynebacterium diphtheriae* CN 2000

		Fe in medium (μg. atoms/l.) 7	97	Fe-deficient/ Fe-excess (%)
Succinate oxidase activity	(μmole/g. bacterial protein/min.)	3·4	8·3	41
Succinate dehydrogenase activity		50	152	33
NADH oxidase activity		3·8	4·5	84
Catalase haeme concentration	(μmole/g. bacterial protein)	0·0021	0·020	10·5
Cytochrome *b* haeme concentration		0·10	0·31	32

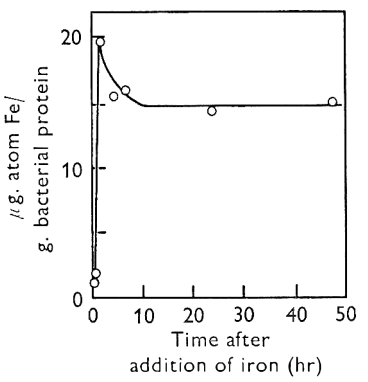

Fig. 1

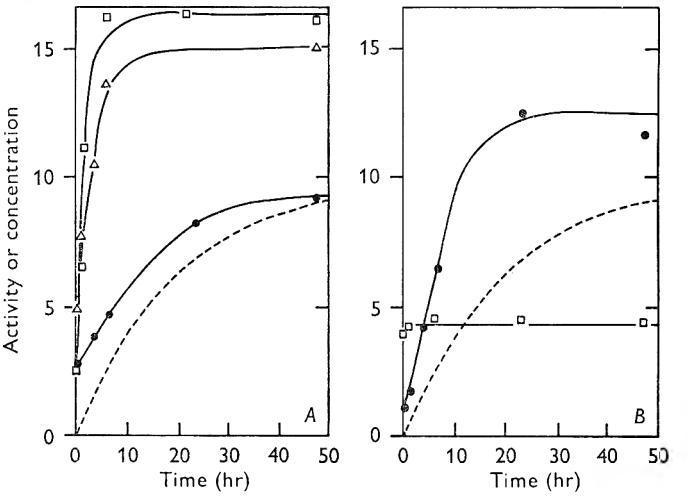

Fig. 2

Fig. 1. The uptake of iron by iron-deficient *Corynebacterium diphtheriae* CN 2000. At 0 hr the iron concentration in a steady-state glucose-limited chemostat culture was raised from 7 to 97 μg. atom/l. The culture contained 3·85 g. bacterial protein/l. Dilution rate = 0·051 hr^{-1}.

Fig. 2. The effect of iron addition to iron-deficient *Corynebacterium diphtheriae* CN 2000 on the concentrations of some respiratory enzymes. At 0 hr the iron concentration in a steady-state glucose-limited chemostat culture was raised from 7 to 97 μg. atom/l. The culture contained 3·85 g. bacterial protein/l. Dilution rate = 0·051 hr^{-1}. *A*. Components of the particulate fraction: □, Succinate oxidase, μmole succinate/g. bacterial protein/min.; △, succinate dehydrogenase, μmole succinate/g. bacterial protein/min.; ●, cytochrome *b*, absorbtion/g. bacterial protein × 10. *B*. Components of the soluble fraction: □, NADH oxidase, μmole NADH/g. bacterial protein/min.; ●, catalase haeme, μmole/g. bacterial protein × 500. The net synthesis of bacterial protein after the addition of iron is shown by the broken line. It was calculated from the expression $1\text{-}e^{-Dt}$, where D is the dilution rate of the culture and t the time since addition of iron. It is a measure of new growth and does not take into account protein turnover. (mg. new protein/mg. protein × 10).

of two respiratory processes and the concentrations of two haeme-containing enzymes in the steady-state chemostat cultures at low and high iron concentrations. With the exception of the NADH oxidase system, a soluble flavoprotein in the PW8 (PD) strain (Pappenheimer *et al.* 1962), large increases were observed in all the enzymes measured. Smaller quantities of cytochromes *c* and *a* were also present in the bacteria and

increased after addition of iron (see below, Fig. 3). Thus in the toxin-producing iron-restricted bacteria the concentrations of the cytochromes, catalase and succinate dehydrogenase appear to be limited by the low iron concentration.

The rates at which the concentrations of the enzymes shown in Table 2 changed after addition of iron to a steady-state glucose-limited chemostat culture are compared in Fig. 2 with the quantity of bacterial protein in the culture synthesised after the addition of iron. Succinate dehydrogenase and succinate oxidase increased most rapidly and approximately in parallel, suggesting that succinate oxidation was rate-limited by the dehydrogenase step, rather than by the oxidation of the cytochrome(s) involved. Cytochrome *b* increased in concentration approximately in parallel with new growth, whilst catalase concentration increased at a rate intermediate between that of cytochrome *b* and succinate dehydrogenase. The NADH oxidase system showed very little change after addition of iron.

The distribution of iron in the bacteria was investigated by differential centrifugation of the disrupted bacteria. After removal of unbroken bacteria and other heavy material by centrifugation at 3000 *g* for 10 min. the suspension was centrifuged at 120,000 *g* for 120 min. The supernatant fluid from this centrifugation is termed the soluble fraction (S) and the deposit, after twice washing in buffer, the particulate fraction (P).

Table 3. *The distribution of haeme and non-haeme iron in subcellular fractions of Corynebacterium diphtheriae* CN2000 *from glucose-limited chemostat cultures in restricted-iron and excess-iron media*

Bacterial fraction		Fe-restricted (7 μg. atom/l.)	Fe-excess (97 μg. atom/l.)	Fe-restricted as % of excess Fe
Broken bacteria				
Non-haeme iron		1·03	15	6·7
Total haeme iron		0·16	0·58	28
Catalase haeme iron		0·0021	0·020	10
Soluble fraction				
Non-haeme iron	μg. atom Fe/g. bacterial protein	0·28	0·44	62
Total haeme iron		ND	ND	—
Catalase haeme iron		0·0021	0·020	10
Respiratory particles (P_R)*				
Non-haeme iron		0·65	16	4
Total haeme iron		0·15	0·49	30
Catalase haeme iron		ND	ND	—

* The ratio of cytochrome *b* in the broken bacteria to the cytochrome *b* in the P_R fraction was used to calculate the amount of particle-bound iron in the bacteria:

$$\text{Fe (Bacteria)} = Fe\ (P_R) \times \frac{\text{Cyt. } b \text{ (bacteria)}}{\text{Cyt } b\ (P_R)}.$$

The soluble fraction contained 90 to 100% of the catalase activity of the whole bacterial preparation, indicating a high degree of disruption of the bacteria by the ultrasonic treatment. The fraction contained no dithionite-reducible haeme though small amounts of haeme were present as catalase. There was a low concentration of non-haeme iron in the preparations from both iron-resistricted and excess-iron bacteria (Table 3). No reduction of the non-heme iron by succinate and by NADH was detectable by the method of Kurup & Brodie (1967*b*), though NADH brought about reduction of a soluble flavoprotein. Pappenheimer *et al.* (1962) suggested that a soluble

flavoprotein was responsible for NADH oxidation in the PW8 (PD) strain of *Corynebacterium diphtheriae*, since NADH dehydrogenase activity was restricted to the soluble fraction.

The greater part of the non-haeme iron present in the bacteria was recovered in the particulate fraction P which also contained 70 to 90% of the cytochromes and succinate dehydrogenase after two washes in 0·05 M-phosphate (pH 7·5). By centrifugation of a suspension of the P fraction at 80,000 *g* for 90 min. a suspension of the repiratory particles (P_R) was obtained, although not all of the cytochrome could be extracted from the P fraction in this way. This suspension, after centrifugation at 120,000 *g* for 120 min., gave a homogeneous translucent red pellet. The pellet obtained from the excess-iron bacterial extract had a deeper red colour than that from the

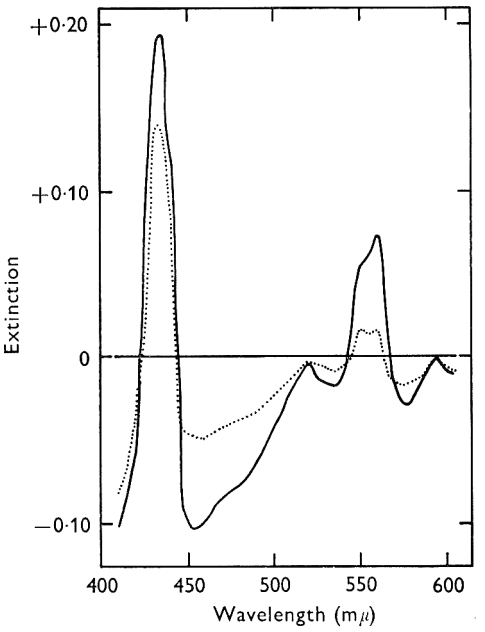

Fig. 3

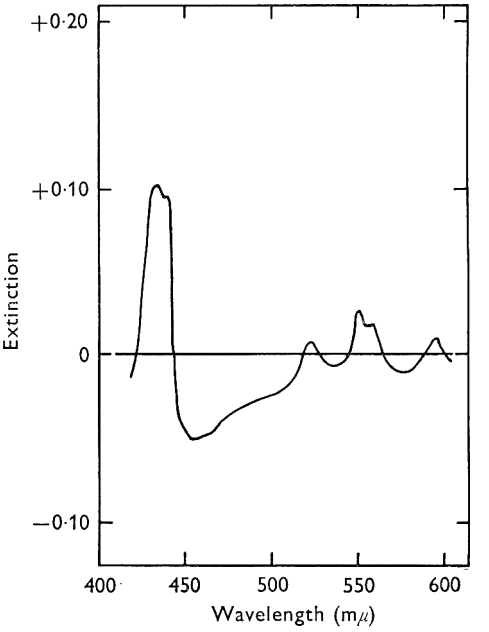

Fig. 4

Fig. 3. Dithionite-reduced minus oxidised difference spectra of the respiratory particle fractions of iron-deficient and excess-iron *Corynebacterium diphtheriae* CN 2000. Unbroken line, bacteria contained 16 μg. atom Fe/g. protein; dotted line, bacteria contained 1·2 μg. atom Fe/g. protein; zero line, oxidised minus oxidized samples. The reaction mixture contained about 3 mg. particle protein/ml.; PO_4, 0·05 M; Mg, 0·02 M; tris, 0·05 M; pH 7·4. A few mg. sodium dithionite were added to one cuvette. The concentrations of respiratory particles were adjusted to give the quantity of cytochrome *b* present in 10 mg. bacterial protein.

Fig. 4. Succinate-reduced minus oxidized difference spectrum of respiratory particle fraction of *Corynebacterium diphtheriae* CN 2000. Bacteria contained 16 μg. atom Fe/g. protein/ml.; PO_4, 0·05 M; Mg, 0·02 M; tris, 0·05 M; pH 7·4. A few mg. succinate were added to one cuvette at 25°.

iron-restricted bacteria. The concentration of haeme and non-haeme iron in the P_R fraction was about three times higher than the P fraction. The ratios of haeme:non-haeme iron were similar for the P and P_R fractions. It was concluded that virtually all

of the haeme and non-haeme iron present was associated with particles of the type recovered in the P_R fraction.

Table 3 shows the distribution of iron in the fractionated bacteria. Clearly the P_R fraction was the main site of action of iron, where it was found as both haeme and non-haeme iron. Of the iron taken up by the bacteria grown in excess iron only 4% was found as haeme whilst about 94% was firmly bound to the P_R fraction as non-haeme iron.

The P_R fraction was obtained fully oxidised after the washing procedure described above. Dithionite-reduced minus oxidised difference spectra showed that the P_R fraction contained flavoprotein, cytochrome b_{559} and smaller amounts of cytochromes c_{551} and a (Fig. 3). The concentrations of all of the components was higher in the preparations from excess-iron bacteria. Fig. 4 shows the difference spectrum obtained by using succinate to reduce the sample; in this case cytochromes c_{551} and a were fully reduced but b_{559} was only partially reduced, demonstrating clearly the c_{551} component. Succinate also reduced much of the flavoprotein and 0·7% of the non-haeme iron. Succinate oxidase activity of the P_R fraction was 30% inhibited by 3 mM KCN. NADH produced no cytochrome difference spectrum, with or without the soluble fraction, nor was there any NADH oxidase activity in the P_R fraction.

DISCUSSION

In the presence of an excess of iron in the medium, giving an intracellular concentration of 15·6 μg. atom Fe/g. bacterial protein and an extracellular concentration of 20 μg. atom/l. medium, diphtheria toxin was not synthesised by *Corynebacterium diphtheriae* CN 2000 in glucose-limited chemostat culture. Toxin was released at a high rate when the intracellular iron concentration was 1·2 μg. atom/g. bacterial protein and the extracellular concentration 0·5 μg. atom/l. Of the extra iron present in the excess-iron bacteria 97% was firmly bound to the respiratory particles, 3% as haeme and the rest as non-haeme iron. Concentrations of particle-bound non-haeme iron similar to those found in the present work with *C. diphtheriae*, have been found on the respiratory particles of *Mycobacterium phlei*, apparently bound to thiol groups (Kurup & Brodie, 1967*a*). The function of the non-haeme iron is not clear; 0·7% was reducible by succinate, whilst 30% of the cytochrome *b*, a similar proportion of the flavin, and most of the cytochrome *c* and a/a_3 components were reduced.

The rapid increase of succinate dehydrogenase activity after adding iron to a restricted-iron culture (Fig. 2) may indicate that the enzyme was iron-deficient. The increase in activity may have resulted from the attachment of iron to the enzyme, since the activity of succinate dehydrogenase as measured by ferricyanide reduction is related to the iron content of the enzyme (Singer, Kearney & Massey, 1957). After addition of iron to an iron-deficient culture the cytochrome concentration increased slowly, approximately in parallel with the bacterial growth. So, although the cytochromes and succinate dehydrogenase are probably located together as a functional group on the respiratory particles, the mechanisms of their limitation by iron-deficiency appear to be dissimilar.

The particle-bound haeme was mostly present in cytochrome b_{559} with smaller buantities of cytochrome c_{551} and a/a_3. Succinoxidase activity was 30% inhibited by 3 mM-KCN, suggesting that a terminal oxidase in addition to cytochrome oxidase was

involved, perhaps cytochrome *b*. Pappenheimer *et al.* (1962) found that cytochrome b_{559} acted as a terminal oxidase during succinate oxidation in the PW8 (PD) strain they used; however, their strain lacked cytochrome *c*.

NADH oxidation was not linked to the respiratory particles but proceeded via a soluble flavoprotein without the reduction of measurable quantities of non-haeme iron. NADH oxidase activities were similar in both restricted-iron and excess-iron bacteria. Thus the involvement of iron in this activity has not been demonstrated.

Jacobs, Maclosky & Jacobs (1967) studied the inhibition of haeme synthesis in *Staphylococcus epidermidis* in the absence of molecular oxygen which is required for the oxidation of coproporphyrinogen to protoporphyrin. On exposure of the staphylococci to oxygen in the presence of chloramphenicol rapid large increases in catalase and respiratory activity were observed. Jacobs *et al.* (1967) concluded that the apoenzymes of the haeme-containing enzymes were synthesized in the absence of haeme. The suggestion that the diphtherial toxin protein, released under conditions of iron-deficiency, is, in the presence of excess iron, bound to a respiratory enzyme was first put forward by Pappenheimer (1947). There is also some evidence that toxin is released from the membrane fraction (which includes the respiratory particles) of the iron-deficient bacteria (Uchida & Yoneda, 1967). Whilst the present work offers no direct evidence for the above theory, the main site of action of iron has been shown to be the respiratory particles and the concentrations of particle-bound iron and the flavin and haeme components have been shown to be effected by iron-deficiency.

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SHORT COMMUNICATIONS

Characterization of DNA of Colicinogenic Factor E_1 in a Providence Strain

By A. J. VAN RENSBURG
Department of Microbiology, University of Pretoria
AND N. HUGO
Department of Life Sciences, Atomic Energy Board, Pretoria, South Africa

(*Accepted for publication 2 July 1969*)

Episomes may be characterized by transfer to organisms in which the DNA differs markedly from that of the original strain (Marmur *et al.* 1961). Of the E_1 colicinogenic factors, only the one derived from *Escherichia coli* K-30 has been characterized in this way. In *Proteus mirabilis* this *Col* E_1 DNA has a buoyant density of $1{\cdot}710 \pm 0{\cdot}002$ g./cm.^{3} and displays circular forms of three contour lengths with corresponding molecular weights of 4·2, 8·5 and $12{\cdot}7 \times 10^6$ (DeWitt & Helinski, 1965; Bazaral & Helinski, 1968*a*). The higher circular forms in *P. mirabilis* resulted from a possible imbalance in the formation or concentration of enzymes responsible for the duplication of *Col* E_1 DNA and not from random recombination of monomers (Goebel & Helinski, 1968). In the original *E. coli* strain the *Col* E_1 DNA is almost exclusively in the monomer form (Bazaral & Helinski, 1968*b*).

Colicin J (CA-62) (Fredericq, 1948) was subsequently classified as an E_1 colicin. Coetzee (1964) transferred this colicinogenic factor to a Providence strain, NCTC 9295. This communication reports the nature of this E_1 colicinogenic factor in the Providence host.

The Providence strain carrying *Col* E_1 (Providence *Col* E_1^+) and the non-colicinogenic strain were grown overnight in nutrient broth. DNA was extracted by the phenol technique and fractionated on a MAK column (Roth & Helinski, 1967). Analytical equilibrium centrifugation in a ceasium chloride gradient was performed by the method of Meselson, Stahl & Vinograd (1957). *Pseudomonas aeruginosa* ^{15}N-DNA (density = 1·742 g./cm.^{3}) was used as reference. Preparative equilibrium sedimentation was done according to DeWitt & Helinski (1965). DNA for electron microscopy was taken directly from the MAK column and mixed with 0·4% cytochrome *c* in 0·1 M-ammonium acetate to 2 to 4 μg./ml. DNA was mounted as described by Kleinschmidt & Zahn (1959). Grids were shadowed with 40% palladium-gold and examined in a Philips EM 200 electron microscope. Electron micrographs were enlarged and the contour lengths of the DNA molecules measured. Molecular weights were calculated from the contour lengths.

Unfractionated DNA isolated from Providence *Col* E_1^+ and the non-colicinogenic strain was examined in CsCl density gradients. Microdensitometer tracings of the DNA profile obtained from both these strains displayed a single DNA component

which banded at 1·700 g./cm.^{3} (41 % G+C) which corresponded to the chromosomal DNA of the Providence host. Ceasium chloride density-gradient analysis of early DNA fractions from the MAK column revealed an additional DNA species with a density of 1·710 ± 0·001 g./cm.^{3} This DNA was only present in the colicinogenic strain and was assumed to be *Col* E_1 DNA. Alkaline denaturation and neutralization of these MAK fractions resulted in denaturation of the Providence DNA while the *Col* DNA resisted permanent denaturation.

Electron microscopy of the early fractions from the colicinogenic strain revealed open and supercoiled circular DNA molecules (Plate 1). Contour lengths of 65 of these yielded three classes, 2·12 ± 0·06, 4·25 ± 0·11 and 6·38 ± 0·16 μ, with a predominance of monomer and dimer molecules. Molecular weights of 4·16, 8·33 and 12·5 × 10^6 were calculated from the contour lengths.

The E_1 colicins share a common receptor site and the colicinogenic factors confer cross-immunity to the colicins. Support for this classification is now provided in that colicinogenic factors isolated from *Escherichia coli* K-30 and paracolon CA-62 are similar in morphology and molecular weight. The demonstration of multiple-length circular *Col* E_1 (CA-62) in the Providence strain agrees with findings for *Col* E_1 (K-30) in *Proteus mirabilis* and may suggest a similar defect in enzymes responsible for *Col* E_1 duplication in these hosts (Goebel & Helinski, 1968).

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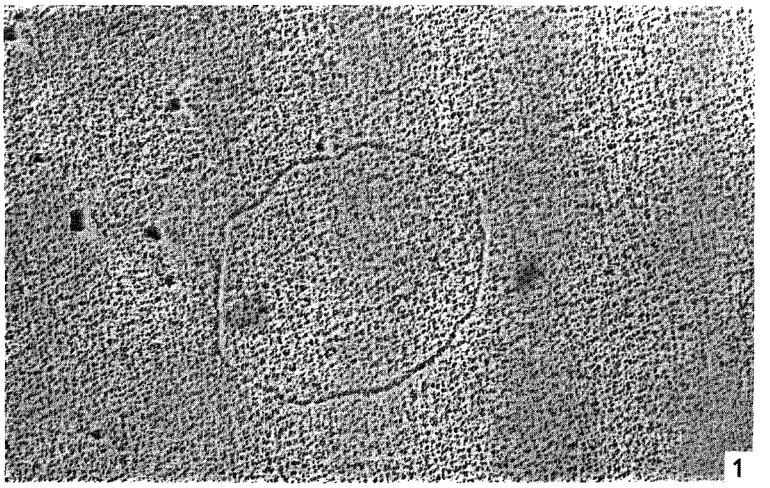
EXPLANATION OF PLATE

Electron micrographs of *Col* E_1 DNA isolated from Providence strain 9295 *Col* E_1^+. (Bar represents 0·5μ.).

Fig. 1. Monomeric open circular DNA (2·12μ).

Fig. 2. Supercoiled circular dimer DNA (4·25μ).

Fig. 3. Supercoiled circular trimer DNA (6·38μ).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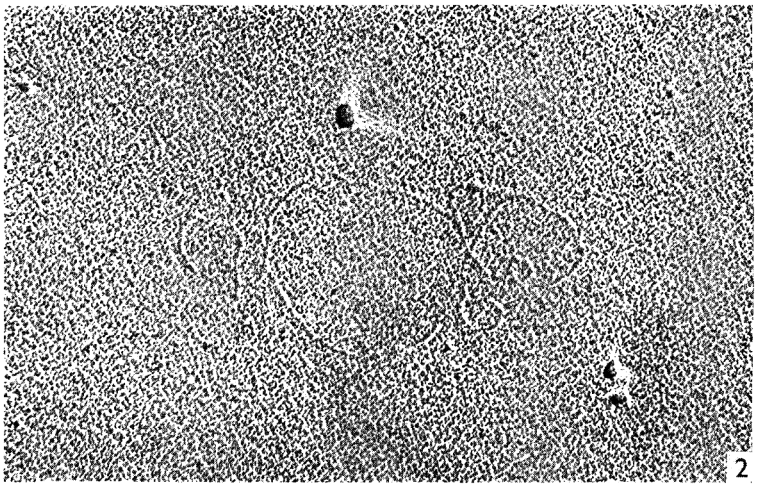
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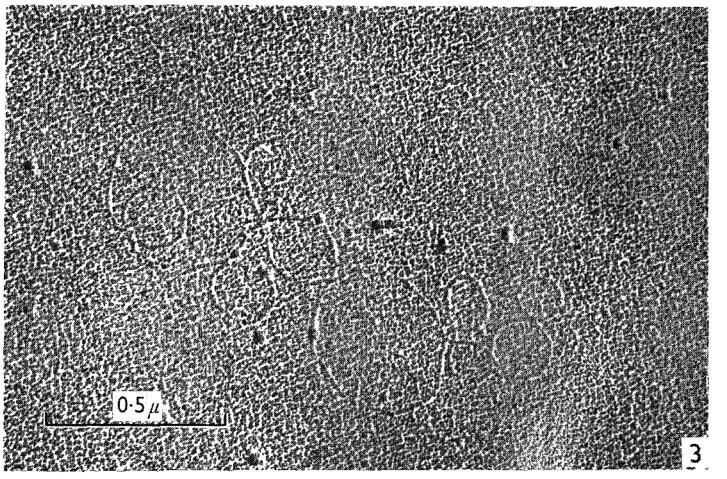
0·5 μ
3

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Microbial Culture Preservation With Silica Gel

By A. R. GRIVELL AND J. F. JACKSON
Department of Agricultural Biochemistry and Soil Science, Waite Agricultural Research Institute, University of Adelaide, South Australia

(*Accepted for publication 10 July 1969*)

Cessation of cellular metabolism is a prerequisite for the preservation and long-term storage of a microbial culture in a state essentially free from the accumulation of morphological and physiological variants (Reusser, 1963). The method of lyophilization (Heckley, 1961) is the most universally used procedure for stock culture preservation.

Hunt, Gourevitch & Lein (1958) and Perkins (1962) described methods for preserving microbial cultures by dehydration with anhydrous silica gel. The method of Hunt *et al.*, where silica gel and culture are held apart, has been used to preserve fungi (Lange & Boyd, 1968) and a range of bacterial species (Hunt *et al.* 1958; Norris, 1963). The technique reported by Perkins, in which a suspension of the micro-organism in skim-milk is added directly to the anhydrous silica gel, has been applied to stocks of the fungi *Neurospora crassa*, *Ustilago maydis*, strains of yeast (Perkins, 1962), cyst-forming slime moulds (Reinhardt, 1966) and *Claviceps paspali* (Mizrahi & Miller, 1968). This technique is inexpensive, rapid, extremely simple to use and, as reported in this communication, can also be applied to micro-organisms other than fungi.

METHODS

The experimental procedure used was that described by Perkins (1962) for fungal species. Each of the microbial species tested was grown in the appropriate medium specified in Table 1. For *Azotobacter vinelandii*, the medium contained (g./l. double-distilled water): sucrose, 10; K_2HPO_4, 2; $MgSO_4.7H_2O$, 0·4; Na-citrate, 0·2; NaCl, 0·1; $CaCl_2$, 0·05; $FeSO_4.7H_2O$, 0·01; $Na_2MoO_4.2H_2O$, 0·002; sufficient HCl to adjust the pH to 7·6; NH_4Cl, 0·1 was added for stock reactivation only. With the exceptions of *Aspergillus nidulans* and *Pseudomonas denitrificans*, the organisms were cultured in aerated liquid media and collected by centrifuging at 5000 *g* for 5 to 10 min. in sterile plastic centrifuge tubes sealed with screw caps. The cells were resuspended in reconstituted skim-milk (15%, w/v; Bonlac obtained from Trufood, Glenormiston, Victoria), previously autoclaved at 121° for 10 min. *A. nidulans* conidia and *P. dinitrificans* cells were suspended in milk (7·5%, w/v) added to cultures growing on agar slopes. It may be that the presence of the skim-milk is a requirement for the successful preservation of bacterial strains by this method (see Hunt *et al.* 1958; Reinhardt, 1966)

Cotton-wool stoppered 13 × 100 mm. Pyrex glass tubes were half filled with silica gel (grade as specified by Perkins, 1962) and dry-sterilized at 175° for 1·5 to 2 hr. Pre-cooled suspension (0·5 ml.) was added dropwise to each chilled tube of anhydrous

silica gel. These were held at 0° for a further 10 to 15 min., then kept for one week in a desiccator containing activated silica gel, prior to a viability test. Finally, each tube was sealed with Parafilm and stored over self-indicating silica gel in a sealed jar at 2° to 4°.

Reactivation of a dehydrated culture was accomplished by incubating several granules of the silica gel stock in the appropriate liquid medium, the remaining dehydrated material being resealed and stored as described. Thus, each tube of dehydrated culture may be used repeatedly. The medium used to reactivate each culture was the same as that in which it had been grown initially except in the case of *Azotobacter vinelandii* (above). No estimates were made of relative viabilities.

The metabolic characteristics of the bacteria were tested as described by the following authors: Brownell & Nicholas (1967) for nitrogen fixation by intact organisms; Radcliffe & Nicholas (1968) and Lam & Nicholas (1969) for nitrate and nitrite dissimilation; Adams (1950) for bacteriophage sensitivity.

Table 1. *Culture conditions and periods of survival of micro-organisms successfully preserved on anhydrous silica gel*

Species	Strain	Period of survival at last testing (weeks)	Culture medium and temperature
Saccharomyces cerevisiae	DELFT 171	46	YEPD medium (Manney, 1964); 30°
Aspergillus nidulans	bi-1	37	Minimal medium (Cove, 1966) + 1 g./l., KNO_3; 30°
Anabaena cylindrica	—	107	Nitrate medium (Brownell & Nicholas, 1967); 25–28°
Azotobacter vinelandii	ATCC 13705	103	See text; 30°
Escherichia coli	B	46	Hershey's Nutrient Broth (Chase & Doermann, 1958) + 0·5 g./l. extra glucose; 37°
	BB	45	
	HS	45	
	B-185	45	
	$K_{12}\lambda$	66	
	830	45	
Pseudomonas denitrificans	ATCC 13867	111	Nutrient agar (Radcliffe & Nicholas, 1968); 37°
Micrococcus denitrificans	NCIB 8944	61	Nutrient agar (Radcliffe & Nicholas, 1968); 37°
Thiobacillus concretivorus	NCIB 9514	13	Thio-oxidans medium (Vishniac & Santer, 1957) with trace elements diluted ten times; 30°

RESULTS AND DISCUSSION

Table 1 lists the microbial species which survived dehydration satisfactorily on anhydrous silica gel, and includes details of the culture conditions. To date, we have been unable to recover viable cells from dehydrated cultures of *Thiobacillus thiparus*, *Chlamydomonas eugametos* and *Euglena gracilis*.

All reactivated cultures had the expected macroscopic and microscopic appearance

and, when tested, the bacterial strains retained characteristic metabolic properties. Thus, *Azotobacter vinelandii* intact cells fixed nitrogen gas, *Pseudomonas denitrificans* and *Micrococcus denitrificans* dissimilated inorganic nitrogen compounds, *Thiobacillus concretivorus* lowered the pH of thiosulphate medium to less than 2, and the several strains of *Escherichia coli* (excepting the T_4-resistant strain, 830) were susceptible to infection by bacteriophage T_4.

This technique of stock culture preservation was particularly useful in simplifying basic microbiological manipulations in an essentially biochemical laboratory. It is recommended for its extreme simplicity and apparent reliability.

The authors thank Professor D. J. D. Nicholas for his interest in this work and Miss Marlene Short for skilled technical assistance. Financial assistance in the form of a CSIRO Postgraduate Studentship to A.R.G. is gratefully acknowledged.

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THE SOCIETY FOR GENERAL MICROBIOLOGY

The Society for General Microbiology held its fifty-fifth General Meeting at University College, London, on Monday, 31 March, and Tuesday and Wednesday, 1 and 2 April, 1969.
The following communications will be made:

ORIGINAL PAPERS

SYMPOSIUM ON MICROBIAL GROWTH

Session A

Subunit Structure and Thermolability of a Growth-limiting Enzyme. By NEIL L. MALCOLM (*Sub-Department of Chemical Microbiology, Department of Biochemistry, University of Cambridge*)

The growth inhibition of the obligate psychrophile, *Micrococcus cryophilus*, at moderate temperatures is, in part, a consequence of the thermolability of one of its activating enzymes, glutamyl-tRNA synthetase (Malcolm, N. L. (1969), *Nature, Lond.* **221**, 1031). A temperature-resistant mutant, TMR 9, capable of growth at 30°, has been isolated and subsequently characterized following ultraviolet irradiation of the psychrophile. This mutant differs from the wild-type in that cell extracts contain thermostable glutamyl-tRNA synthetase activity. An isolation procedure, yielding approximately 200-fold purification of the mutant enzyme, has been devised.

Molecular-weight determinations of glutamyl-tRNA synthetase activity in both psychrophile and TMR 9 cell extracts have been carried out by gel filtration on Sephadex G-200 and by sucrose density gradient centrifugation. Enzyme activity was found to be associated in both extracts primarily with a position corresponding to a molecular weight of 190,000, but also with a minor peak at about half that weight. Immunological analysis of cell extracts by means of antiserum prepared against purified TMR 9 glutamyl-tRNA synthetase showed the presence of cross-reacting material at positions equivalent to those noted for the presence of enzyme activity. In addition, the elution profiles for the wild-type extract included some cross-reacting material, but no corresponding activity, with an apparent molecular weight of about 50,000. Heating psychrophile extracts at 30° prior to chromatography led to a marked increase in this latter, inactive fraction, this accumulation being correlated with a loss of activity and cross-reacting material from the 190,000 and 100,000 molecular-weight positions. No re-association of these small subunits was observed. The mutant enzyme could also be broken down to inactive subunits of similar molecular weight, but only under extreme reducing conditions.

A Kinetic Study of the Mode of Growth of Surface Colonies of Fungi. By A. P. J. TRINCI (*Microbiology Department, Queen Elizabeth College, London*)

The growth kinetics of *Aspergillus nidulans*, *Mucor hiemalis* and *Penicillium chrysogenum* on solid media were studied. Growth of *A. nidulans* colonies can be divided into the four phases of lag, exponential, deceleration and constant growth rate. The effects of colony radial growth rate, of glucose concentration, medium depth, oxygen partial pressure and temperature were studied. The radial growth rate of glucose limited colonies of *A. nidulans* increased linearly with the log. of the initial glucose concentration. The effect of glucose concentration

on internode length, hyphal density and hyphal diameter were also studied. At glucose concentrations above 1% (w/v) there was an inverse relation between the radial growth rate of *A. nidulans* colonies and their peripheral hyphal density.

The relations between colony radial growth rate (*Kr*) and germ-tube specific growth rate (αg) on solid media and specific growth rate in submerged culture (αs) were investigated. Direct proportionality between *Kr* and αs of *Aspergillus nidulans* was found by varying specific growth rate by temperature changes. The germ tubes of *A. nidulans* conidia grew exponentially at a rate which was 2·3 times as great as the specific growth rate of the organism in submerged culture. The colony radial growth rates of the three moulds could not be used as a measure of their relative specific growth rates in submerged culture.

Population Studies of an Autochthonous Soil Bacterium in Model Systems. By H. T. TRIBE and PAMELA A. WILLIAMS (*School of Agriculture, University of Cambridge*)

Ecologists envisage micro-organisms growing in nature in habitats termed ecological niches. Experimental model systems may be set up to simulate natural niches and may be analysed into four components, viz. organisms, substrate, environment and time. A system based on these principles was devised to simulate one aspect of the soil habitat. Autoclaved yeast cells were used as substrate. They were placed in an environment of sand particles amongst which mineral salt solution was evenly distributed. Certain studies on growth of single and mixed cultures of soil bacteria in this system have already been described (Tribe, H. T. & Williams, P. A. (1967), *Can. J. Microbiol.* **13**, 467).

Populations attained in this system by a soil bacterium classed as a representative of Winogradsky's autochthonous grouping of soil organisms (NCIB 9736, of uncertain generic status) are compared over reasonably long periods of time with those found in two other kinds of culture interpreted as model systems. These were glucose substrate in shake flasks of mineral nutrient and glucose distributed in mineral salt amongst sand particles. Activity in all systems was estimated by carbon dioxide measurements. Population levels in the yeast cell system were maintained for much longer periods than in the glucose systems. The validity of comparisons between the different systems is examined.

It is considered likely that a main facet of autochthonous behaviour is ability of an organism to grow under certain circumstances at a very low rate, but the autochthonous condition poses many problems in the field of bacterial physiology.

Klebsiella Mutants Showing Temperature-dependent Polysaccharide Synthesis. By I. W. SUTHERLAND and MARY NORVAL (*Department of General Microbiology, Edinburgh University*)

The isolation of temperature-sensitive mutants of bacteria or bacteriophage has frequently been reported. The properties of such mutants have been ascribed to alterations in the structure of enzyme or repressor proteins resulting in instability at normal incubation temperatures. During studies on exopolysaccharide biosynthesis, numerous non-mucoid mutants of *Klebsiella aerogenes* were isolated from several different parent strains. Certain other mutants with unusual colonial appearance and apparently altered polysaccharide-synthesizing capabilities were also obtained. Their occurrence was much less frequent than that of the non-mucoid strains (O strains) and they were only isolated following aminopurine mutagenesis of three parental types. These crenated (CR) colonies were detectable only at lowered incubation temperature (20–25°) reverting to the parental type at 35°. The CR colonies gave rise to autoagglutinable cultures in liquid media at 20–25° but showed the normal uniform turbidity at 35°. Polysaccharide production was greatly reduced at low temperature. The exopolysaccharide slime or capsule had the same composition in mutant and parent cultures, as determined by chemical analysis, partial acid hydrolysis, and, where applicable, enzymic hydrolysis. The lipopolysaccharide extractable with aqueous phenol also appeared to be identical in both groups of cultures, being apparently a galactan-type with little or no glucose (Sutherland, I. W. & Wilkinson, J. F. (1966), *Biochim. biophys. Acta* **117**, 261; Koeltzow, D. E., Epley, J. D. & Conrad, H. E. (1968), *Biochemistry* **7**, 2920). However, at 20–25° differences in phage sensitivity patterns were detectable.

Whereas reversion of non-mucoid (O) mutants was never detected the CR mutants reverted to the parental type with frequencies of 0·05–0·4% when different mutagens were applied. It also proved possible to isolate a double mutant which we have designated CR-O type with some characteristics common to both CR and non-mucoid cell lines. Assays of certain of the enzymes involved in polysaccharide synthesis have so far revealed only slight differences between the enzyme levels of wild type cells, CR and O mutants grown under comparable conditions.

Poly-β-Hydroxybutyrate Synthesis in *Azotobacter beijerinkii*; the Non-involvement of Acyl Carrier Protein. By G. A. F. RITCHIE, P. J. SENIOR and E. A. DAWES (*Department of Biochemistry, University of Hull*)

Poly-β-hydroxybutyrate (PHB) biosynthesis has been investigated with *Azotobacter beijerinckii*, an organism which deposits massive reserves of the polymer (about 75% of cell dry weight) when fixing nitrogen in batch culture on glucose-salts medium (Stockdale, H., Ribbons, D. W. & Dawes, E. A. (1968), *J. Bact.* **95**, 1798). A fundamental problem posed is whether biosynthesis involves the thiol esters of an acyl carrier protein (ACP) or coenzyme A thiol esters of the intermediates.

An ACP has been isolated from the organism, purified and found to possess 3 moles of phosphate per mole of protein, a molecular weight of approximately 10,000 and to be of similar amino acid content to the ACP of *Escherichia coli*. Thiol esters of this ACP proved inactive in PHB synthesis in contrast with the coenzyme A derivatives.

The specific activities of β-ketothiolase and acetoacetyl CoA reductase, two key enzymes of PHB synthesis, reached a peak during exponential growth, immediately preceding a marked increase in PHB content which coincided with a rapid fall to zero of the oxygen tension of the medium.

Acetoacetyl CoA reductase was shown to be NADP-specific; cell extracts displayed high NADH-oxidase activity and an NAD-NADP transhydrogenase of about equivalent activity. It was additionally demonstrated that the triose phosphate dehydrogenase is relatively NADP-specific (cf. *Azotobacter agilis*; Johnson, E. J. & Johnson, M. K. (1961), *Proc. Soc. exp. biol. Med.* **108**, 728).

Thus during growth in batch culture, as the oxygen tension falls to zero, acetyl CoA, unable to be oxidized via the tricarboxylic acid cycle, is increasingly channelled to synthesis of acetoacetyl CoA, reduction of which to D(−)β-hydroxybutyryl CoA (the substrate for PHB synthetase) is coupled with the oxidation of NADPH generated in glucose metabolism. Under these conditions massive deposition of PHB occurs.

The Amino Acid Pools of Yeasts. By C. M. BROWN (*Department of Microbiology, The University, Newcastle upon Tyne, NE1 7RU*)

Amino acid pools in micro-organisms serve as precursers for protein synthesis and may have a role in the regulation of RNA synthesis. The amino acid pools of *Candida utilis* were studied in a chemostat (Dawson, P. S. S. (1965), *Biochim. biophys. Acta* **110**, 51) and found to vary both in size and composition depending on the growth rate and the energy source of the medium. In *C. utilis* (NCYC321) with NH_4^+ as source of nitrogen, Brown & Rose (1969), (*J. Bact.* **97**, 261) have shown that the pool size (amino nitrogen extracted with hot water/mg. dry wt) was lower in NH_4^+ limited cells than when glucose or phosphate were limiting. In cells grown under NH_4^+ limitation the pool size was greater with glycerol than with glucose as carbon source.

In *Candida utilis* and *Saccharomyces cerevisiae* the pool size increased with a decrease in oxygen tension (growth rate and temperature constant) and this increase was not associated with an increase in the RNA content of the cells. The pool size increased if the growth rate was raised (constant temperature) or if the temperature was lowered (constant growth rate) and these changes were observed together with increases in cell RNA. There were no observable changes in the types of amino acids present—the principle components of the pool from *S. cerevisiae* being glutamic acid, alanine, arginine, valine and cystine with smaller amounts of aspartic acid, leucine, amino butyric acid and an unidentified peptide containing arginine.

Experiments involving an increase in growth rate have established that the first major constituent of the cell to be altered in concentration by such changes in environment was RNA. In these experiments it has been found that pool size increases together with the increase in RNA thus suggesting a close involvement of the amino acid pool in the regulation of metabolism.

Aspects of Membrane Synthesis during the Cell cycle of *Bacillus megaterium* and *Escherichia coli*. By M. J. DANIELS (*Microbiology Unit, Department of Biochemistry, University of Oxford*)

The importance of the membrane in effecting chromosome segregation and cell division in bacteria suggested that a study of lipid synthesis during the cell cycle might reveal fluctuations reflecting the growth of the membrane. *Bacillus megaterium* KM and *Escherichia coli* 3/62 were synchronized by amino acid starvation, and lipid synthesis was measured by the incorporation of radioactive glycerol. The rate of incorporation per unit weight of cells was approximately constant between divisions, but showed a large transient increase over the period of cell division.

It is possible that the synchronization process causes some distortion of the normal patterns of balanced growth and an alternative approach was therefore employed. Exponentially growing unsynchronized cultures of *Escherichia coli* were 'pulse labelled' with glycerol and the cells were fractionated according to size by sucrose gradient centrifugation. The greatest incorporation per unit weight was observed in the largest and the smallest cells, that is in those undergoing division at the time of labelling.

The procedure of Tremblay, Daniels & Schaechter ((1969), *J. molec. Biol.* **40**, 65) was used to obtain a fraction of the membrane containing the attachment point of the DNA. Preliminary results suggest that this portion of the membrane is synthesized during the period of cell division.

These results will be discussed in terms of possible models of membrane growth.

Lability of *Iso*citrate Dehydrogenase in *Escherichia coli*. By P. M. BENNETT and W. H. HOLMS (*Department of Biochemistry, University of Glasgow, Scotland*)

Batch cultures of *Escherichia coli* ML308 were grown aerobically (Harvey, N. L., Fewson, C. A. & Holms, W. H. (1968), *Lab. Pract.* **17**, 1134) in simple salts media with either glycerol (4 mM) or glucose (2 mM) as the limiting component. The levels of malate dehydrogenase and α-ketoglutarate dehydrogenase remained constant when growth ceased. The activity of *iso*citrate dehydrogenase was maintained after growth on glycerol, but after growth on glucose it fell to 20% in 90 min. and then recovered to 75% over the following 150 min. After growth on glucose the cultures continued to evolve carbon dioxide for much longer than after growth on glycerol. The work of R. Britten ((1954), *Science, N.Y.* **119**, 578) suggested that carbon dioxide could result from metabolism of acetate accumulated during growth on glucose. When small amounts (0·6 mM) of acetate were added to cultures after limited growth on glycerol the *iso*citrate dehydrogenase activity decayed and recovered in a manner similar to that found in the glucose cultures without acetate addition.

Chloramphenicol (100 μg./ml.) added just at the end of growth on glucose prevented decay of *iso*citrate dehydrogenase but added at the point of maximal decay did not prevent recovery.

A combination of glyoxylate and oxalacetate profoundly inhibited the *iso*citrate dehydrogenase of our strain although higher concentrations were required than in some others (Shiio, I. & Ozaki, H. (1968), *J. Biochem.* **64**, 45). The loss of activity cannot be attributed to these or other dissociable inhibitors as neither dialysis nor gel filtration increased the activity of cell extracts.

The decay and recovery of *iso*citrate dehydrogenase is apparently associated with acetate metabolism but is not due to accumulation of inhibitors. The question remains as to whether the phenomenon involves enzyme destruction and resynthesis or some simpler mechanism.

Session B

Growth of *Arthrobacter citreus* in a Chemically-defined Medium and Its Requirement for Chelating Agents with Schizokinen Activity. By PHYLLIS SEIDMAN and E. C. S. CHAN *Department of Microbiology and Immunology, McGill University, Montreal, Quebec, Canada*)

Previous studies by many workers on the physiology of *Arthrobacter citreus* were carried out with either complex or semi-synthetic media. In order to study more definitely the roles played by cations following the finding that certain metallic ions promote growth of the organism at elevated temperatures (Wong, P. T. S., Chan, E. C. S. & Page, O. T. (1966), *Can. J. Microbiol.* **12**, 663) it was decided to determine the exact nutritional requirements of *A. citreus.*

Initial experiments showed that *Arthrobacter citreus* grew at 25° in a glucose-salts medium supplemented with 1 % (w/v) casein hydrolysate (Difco Casamino Acids) and thiamine hydrochloride (0·2 μg./ml.). However, this growth was characterized by a long lag phase of 3–4 days. The lag could not be reduced by supplementation with other vitamins, purines and pyrimidines, or ashed nutrient broth (BBL Trypticase Soy Broth). Subsequently, it was found that the addition to the medium of various sideramines (Bickel, H. *et al.* (1960), *Experientia* **16**, 129), such as ferrichrome and mycobactin, shortened the lag phase and increased the total cell crop.

It was also discovered that the sideramine requirement of *Arthrobacter citreus* could be replaced by a number of unrelated and synthetic metal chelators in low concentrations. The probable roles of these schizokinens or cell-division factors (Lankford, C. E. *et al.* (1966), *J. Bact.* **91**, 1070) in growth initiation and maintenance of cell division will be discussed.

Upon replacing the casein hydrolysate with a vitamin-free preparation or a mixture of 18 amino acids, a requirement for additional vitamins was evident. The essential vitamins and amino acids for growth of *Arthrobacter citreus* in a glucose-salts medium have now been determined to be thiamine, biotin, nicotinic acid, tyrosine, methionine, glutamic acid and cystine.

Column Chromatography of Ribosomal RNA, Rapidly Labelled RNA and DNA on Benzoylated DEAE-Cellulose. By I. H. MAXWELL (*Chester Beatty Research Institute, Institute of Cancer Research, Royal Cancer Hospital, London, S.W.3*)

Chromatography on benzoylated DEAE-Cellulose (BD-cellulose) has proved a useful method for fractionation of transfer RNA (Gillam, I., Millward, S., Blew, D., von Tigerstrom, M., Wimmer, E. & Tener, G. M. (1967), *Biochemistry* **6**, 3043). This material has not previously been used for chromatography of RNA of higher molecular weight, although benzoylated, naphthoylated DEAE-cellulose has been used for this purpose (Sedat, J. W., Kelly, R. B. & Sinsheimer, R. L. (1967), *J. molec. Biol.* **26**, 537).

Elution of ribosomal RNA from BD-cellulose with sodium chloride alone requires salt concentrations of at least 0·9 M. It has now been found that inclusion of sodium perchlorate at low concentration (around 0·02 M) in the eluent allows elution of ribosomal RNA at considerably lower concentrations of sodium chloride. This observation has been applied to the fractionation of RNA extracted from polyribosomes of *Bacillus megaterium.* Separation of much of the rapidly labelled RNA from ribosomal RNA was obtained. The possibility of fractionating rapidly labelled RNA by elution with sodium perchlorate at increasing concentrations was also indicated.

In general, the less secondary structure a nucleic acid has, the more tightly it adsorbs to BD-cellulose. Sheared DNA from *Escherichia coli* was eluted from BD-cellulose by sodium chloride at a concentration of 0·6 M. The same DNA after denaturation was not eluted by sodium chloride at concentrations up to 1·0 M. This property of BD-cellulose has been used in purifying several types of DNA which renature readily, including *E. coli* DNA cross-linked by reaction with sulphur mustard and component I DNA of polyoma virus. The replicative form of phage ØX-174 DNA has been purified in a similar way using benzoylated, naphthoylated DEAE-cellulose (Komano, T. & Sinsheimer, R. L. (1968), *Biochim. biophys. Acta* **155**, 295).

Transductional Analysis of Colicin E2 Refractory Mutants of *Escherichia coli* K12 in which Refractivity is Associated with Recombination Deficiency. By E. J. THRELFALL and I. B. HOLLAND (*Department of Genetics, University of Leicester*)

Mutants refractory to colicin E2 (designated *ref*II) may be isolated from certain strains of *Escherichia coli* K12, and the *refII* locus was previously shown, by interrupted mating experiments, to map in a position 3 min. to the left of *thr* (Holland, I. B. & Threlfall, E. J. (January, 1969) *J. Bact.*) Further mapping studies with the transducing phage P1 show this locus to be cotransducible with *ser*B, and to be closely linked to the HSP (host controlled modification) locus. *Ref*II mutants have been obtained in some wild-type strains, but not in others, and only the former can be transduced for the *ref*II locus. Evidence from transduction studies now suggests mutation in a second gene closely linked to *thr* is required for the phenotypic expression of E2 refractivity. It has been found that 10% of *ref*II mutants show increased sensitivity to ultraviolet irradiation, and give reduced numbers of prototrophic recombinants in crosses with male strains. The *rec*$^-$ character of these strains has been investigated by F′ transfer and beta-galactosidase induction in merozygotes. The results show that F′ and chromosomal transfer in these mutants does not differ from that of the parental strain, and it is suggested that these *uv*^{s} *ref*II mutants do indeed constitute a new class of *rec*$^-$ mutants. At least some of these *rec*$^-$ strains show poor growth rates in complex medium, and increased sensitivity to EDTA and deoxycholate. When ultraviolet resistant revertants of these *ref*II (*uv*^{s} *rec*$^-$) strains are selected they are found to have also reverted to colicin E2 sensitivity. They also appear to be *rec*$^+$ and to have regained normal EDTA and growth characteristics. The probability that these characters arise as the pleiotropic effects a single gene concerned with the integrity of some membrane protein, rather than the result of a deletion covering several genes will be discussed.

Mucopeptide Synthesis by Protoplast Membrane Preparations of *Bacillus megaterium*. By P. E. REYNOLDS (*Sub-Department of Chemical Microbiology, Department of Biochemistry, University of Cambridge*)

Membrane preparations obtained by osmotic lysis of protoplasts of *Bacillus megaterium* strain KM were active in incorporating radioactivity from UDP-*N*-acetylmuramyl-ala-glu-[^{3}H]diaminopimelic acid-[^{14}C]ala-[^{14}C]ala into mucopeptide material in the presence of UDP-*N*-acetyl-glucosamine and 30 mM-Mg^{2+}. The activity of these membrane preparations was enhanced by incubating the protoplasts prior to lysis in a peptone/glucose/salts medium containing 10% sucrose until the turbidity of the suspension was increasing exponentially.

Bacitracin (10^{-5} M) inhibited the incorporation of radioactivity into mucopeptide by 50% and into the lipid intermediates by 25%. Maximum inhibition of mucopeptide synthesis (70%) remained constant over a tenfold increase in bacitracin concentration suggesting that approximately three cycles of mucopeptide synthesis were occurring in this system if the mechanism of action of bacitracin proposed by Siewert & Strominger ((1967), *Proc. natn. Acad. Sci. U.S.* **57**, 767) is correct.

Vancomycin also inhibited the incorporation of radioactive substrate into polymerized material. The membrane preparations used contained less than 0·1% of the original mucopeptide so it appears unlikely that vancomycin inhibits the reaction as a result of binding to the mucopeptide that presumably functions as acceptor. Vancomycin binds to one of the substrates (Perkins, H. R. (1968), *Biochem. J.* **106**, 35P) but the removal of equimolar amounts of UDP-*N*-acetyl-muramyl-pentapeptide by any particular concentration of vancomycin is insufficient to account for the extent of the inhibition observed. Since the antibiotic did not affect the formation of the lipid intermediates it appears that vancomycin affects directly the enzyme involved in the polymerisation of cell-wall material. Both vancomycin and bacitracin prevented the release of alanine as a result of the inhibition of mucopeptide synthesis. Penicillin, however, inhibited the release of alanine directly since the antibiotic slightly stimulated the incorporation of radioactivity into mucopeptide material. The ratio of incorporation of [^{14}C]ala/[^{3}H]diaminopimelic acid was consistently higher in the presence of penicillin than in the control incubation and this is in agreement with the proposed mechanism of action of penicillin.

The Isolation and Characterisation of Mesosome Material from *Micrococcus lysodeikticus*. By D. J. ELLAR and J. H. FREER (*Sub-Department of Chemical Microbiology, Department of Biochemistry, University of Cambridge and Microbiology Department, University of Glasgow*)

The discovery of membranous invaginations of varying complexity in the cytoplasm of many bacterial species stimulated considerable speculation as to their function in prokaryotic cells. These structures which were named 'mesosomes' by Fitz-James ((1960), *J. Biophys. Biochem. Cytol.* **8**, 507) comprise an intracellular system of tubular or vesicular membranes bounded by an invagination of the plasma membrane. Electron microscopic data has not defined the role of mesosomes with any certainty, but has furnished suggestive evidence for their involvement in such cellular processes as transverse septum synthesis (Ellar, D. J. *et al.* (1967), *J. Bact.* **94**, 1189), genome replication (Ryter, A. & Jacob, F. (1964), *Ann. Inst. Pasteur* **107**, 384), membrane synthesis (Lampen, J. O. (1965), *Soc. gen. Microbiol. Symp.* **15**, 115) and electron transport (Ferrandes, B. *et al.* (1966), *C. r. hebd Séanc. Acad. Sci., Paris* **263**, 1632). Recent attempts to isolate mesosomes (Reavely, D. A. (1968), *Biochem. Biophys. Res. Comm.* **30**, 649) constitute a more direct approach to determining their function.

Exponentially growing *Micrococcus lysodeikticus* was harvested and washed twice in 0·1 M-Tris-HCl containing 40 mM $MgCl_2$ at 4°. The washed cells were resuspended in the same buffer made 2 M with sucrose and held for 1 hr. The suspension was then made 1 M with respect to sucrose by dilution with 0·1 M Tris-HCl containing 40 mM-$MgCl_2$ and lysozyme added (200 μg./ml.). During protoplast formation the membrane bounding the mesosome evaginates, expelling the contents of this structure which in the presence of 40 mM-$MgCl_2$ adhere to the outer surface of the protoplast membrane.

Protoplasts with adherent mesosome material were sedimented by centrifugation and resuspended in 0·1 M-Tris-HCl containing 20 mM-$MgCl_2$ and made 0·8 M with respect to sucrose. Under these conditions the mesosomes detach and can be recovered in the supernatant when the protoplasts are sedimented by centrifugation at 12,000 *g*. Experiments indicated that throughout these manipulations the protoplasts remained intact with no significant leakage of cytoplasmic material. Mesosomes were obtained from the supernatant by centrifugation at 100,000*g* and washed several times in 0·1 M-Tris-HCl containing 20 mM-$MgCl_2$. Upon discontinuous sucrose gradient centrifugation, mesosomes sediment as a single band with a density markedly lower than the plasma membrane. Analyses indicate a higher proportion of lipid in mesosomes compared to plasma membrane. Protein composition, enzymic activity and cytochrome content of mesosomes and plasma membranes have been compared. Marked differences in structure between these two membrane preparations are apparent in the electron microscope.

Uncoupling of Bacterial Growth by Pantothenate Starvation. By J. P. BÉLAICH, P. SIMONPIÉTRI and A. BÉLAICH (*Laboratoire de Chimie Bactérienne, C.N.R.S., Marseilles, France*)

Pantothenic acid is a growth factor for *Zymomonas mobilis* (Bélaich, J. P., Senez, J. C. (1965), *J. Bact.* **89**, 1195). This micro-organism is able to grow in anaerobiosis on a synthetic medium containing the usual amino acids as nitrogen and carbon source and glucose as energy source, or on a glucose-containing minimal medium with NH_4Cl as nitrogen source.

With pantothenic acid as limiting factor in the medium (concentration between 5×10^{-5} and 5×10^{-8} mg./ml.) the Neperian growth rate is reduced from 0·391 to 157 hr^{-1} in synthetic medium and from 0·300 hr^{-1} to 0·100 hr^{-1} in minimal medium. The molecular growth yield Yg (Bauchop, T. & Elsden, S. R. (1960), *J. gen. Microbiol.* **23**, 457) is simultaneously reduced from 6·5 to 2·5 in synthetic medium and from 4·7 to 1·7 in minimal medium. The fermentation balance for glucose remains the same in all cases (ethanolic fermentation) and the cellular level of catabolic activity remains constant and equal to about 1 mmole of glucose catabolized per min. per g. of cells (dry weight).

These experiments show that for *Zymomonas mobilis*, as for *Streptococcus faecalis* (Rosenberg, R. F. & Elsden, S. R. (1960), *J. gen. Microbiol.* **22**, 727) anabolic processes do not control catabolic activity.

The Metabolism of Phenol Analogues by Bacterium N.C.I.B. 8250. By E. G. BEVERIDGE and D. TALL (*School of Pharmacy, Sunderland Polytechnic*)

Bacterium N.C.I.B. 8250 metabolizes phenol via catechol and β-keto-adipic acid, catechol 1,2-oxygenase (EC 1.13.1.1) promoting ring fission. The effect of a second substituent group in the phenol molecule upon aspects of this metabolism was investigated, nitro-, carboxy-, fluoro-, chloro-, bromo-, methyl-, methoxy-, hydroxy-, and amino- substituents being studied.

Only phenol, catechol and 2-, and 4-carboxyphenols supported growth in a mineral salts medium. In the respirometer succinate grown cells oxidized phenol, catechol, 3-, and 4-fluorophenols, and 2-, and 4-carboxy-phenols after varying lag periods. Phenol adapted cells showed high substrate specificity oxidizing only phenol, catechol, 3-, and 4- fluorophenols and 4-chlorophenol without a lag. Almost complete liberation of fluorine as fluoride ions occurred during the metabolism of 3-, and 4-fluorophenols. With higher concentrations of the fluorophenols and 4-chlorophenol metabolism rapidly ceased suggesting accumulation of toxic metabolites. Phenol oxidation rate was reduced by many analogues, halogenated phenols being particularly inhibitory in the order bromophenols > chlorophenols > fluorophenols. Also several analogues apparently produced uncoupling of phenol oxidation.

Only cells grown in the presence of phenol or the fluorophenols were adapted to phenol metabolism. 2-Fluorophenol gratuitously induced high levels of phenol oxidizing activity and of catechol 1,2-oxygenase contrasting with suggestions that cis-cis muconate is the inducer for catechol 1,2-oxygenase in this and very closely related bacteria (Farr, D. R. and Cain, R. B. (1968), *Biochem. J.* **106**, 876; Canovas, J. L., Ornston, L. N. & Stanier, R. Y. (1967), *Science, N.Y.* **156**, 1695). Cells grown in the presence of some analogues, while not being adapted to phenol metabolism, subsequently oxidised phenol after shorter lag periods than did succinate grown cells. With the exception of catechol and 2-carboxyphenol induction of catechol 1,2-oxygenase or increased β-ketoadipate oxidizing activity had not occurred and some form of general preconditioning is suggested to explain the phenomenon.

The Effects of Antibiotics on the Interaction of T-factor, Aminoacyl-tRNA and Ribosomes. By R. N. HILL (*Sub-Department of Chemical Microbiology, Department of Biochemistry, University of Cambridge*)

The supernatant factor T of *Escherichia coli* extracts (Nishizuka, Y. & Lipmann, F. (1966), *Proc. natn. Acad. Sci. U.S.A.* **55**, 212) has been shown to stimulate the poly U-directed binding of phenylalanyl-tRNA to washed ribosomes in the presence of GTP (Lucas-Lenard, J. & Haenni, A-L. (1968), *Proc. natn. Acad. Sci. U.S.A.* **59**, 554).

In the studies reported here, [^{14}C] or [^{3}H]phenylalanyl-tRNA was incubated with washed *Escherichia coli* B ribosomes, in the presence of poly U, GTP and 0·01 M-Mg^{2+}. After completion of a 'non-enzymic' binding, addition of T-factor promoted a further 'enzymic' interaction of phenylalanyl t-RNA with the ribosomes, leading to a three-fold increase in binding. While some diphenylalanine formation accompanied the enzymic binding, no polyphenylalanine was detected. The synthesis of polyphenylalanine was entirely dependent on the addition of T and the complementary supernatant factor G to the incubations.

Tetracycline, at a concentration that completely inhibited polyphenylalanine synthesis, inhibited the enzymic binding *ca.* 90 %, while significantly stimulating the non-enzymic step. The latter result contrasts with the inhibition of non-enzymic binding reported by Vazquez, D. & Monro, R. E. ((1967), *Biochim. biophys. Acta* **142**, 155), working at 0·02 M-Mg^{2+}. It was confirmed that tetracycline only inhibits non-enzymic binding at Mg^{2+} concentrations greater than 0·01 M.

Similarly, enzymic binding was inhibited over 90 % by streptogramin A, and 75 % by spiramycin III, at concentrations of these antibiotics that only very weakly inhibited non-enzymic binding.

Erythromycin, amicetin and pactamycin weakly inhibited the enzymic, rather than the non-enzymic, binding, while puromycin and sparsomycin had little effect on the extent of either binding.

Session C

Fine Structure and Taxonomic Position of Neisseria haemolysans (THJØTTA & BØE, 1938). By A. REYN (*Neisseria Department, Statens Seruminstitut, 80 Amager Boulevard, 2300 Copenhagen S, Denmark*)

In 1938, Thjøtta & Bøe described a Gram-negative diplococcus which 'gave fermentation reactions as stated for *Neisseria sicca*' from which species it differed by being haemolytic especially on rabbit blood agar. They proposed the name *Neisseria haemolysans* as the species name under the generic name *Neisseria*. In 1961 Berger U. (*Int. Bull. bact. Nomencl.* **11**, 17) felt that *N. haemolysans* deviated to such an extent from the aerobic, oxidase-positive *Neisseria* as defined in *Bergey's Manual* (7th edition) that it could not be retained in this genus. A new genus was proposed to which the generic name *Gemella* was given (Berger, U. (1966), *Z. Hyg. Infekt.-Kr.* **146**, 253). *Gemella* was described as an aerobic, oxidase-negative genus within the Neisseriaceae. By gas chromatography, Yamakawa, T. & Ueta, N. ((1964), *Jap. J. exp. Med.* **34**, 361) found that the composition of *N. haemolysans* (ATCC 10379) differed from other *Neisseria* in both fatty acids and sugars.

With the electron microscope it was recently demonstrated (Reyn, A., Birch-Andersen, A. & Lapage, S. P. (1966), *Can. J. Microbiol.* **12**, 1125) that the Gram-variable bacterial culture labelled *Neisseria haemolysans* (ATCC 10379) had a Gram-positive type of cell wall. Also the inner structure and the mode of division resembled that of typical Gram-positive organisms. In collaboration with Birch-Andersen thin sections of six additional cultures labelled either *N. haemolysans* or *Gemella haemolysans* were studied with the electron microscope and all found closely to resemble ATCC 10379. For control, the cultures were checked serologically and bacteriologically by U. Berger. The buoyant density of their DNA (isolated by B. W. Catlin) was analysed for mole % GC by M. Mandel. The mole % GC was 33·5 (see Schildkraut, C. L., Marmur, J. & Doty, P. (1962), *J. molec. Biol.* **4**, 430).

It is concluded that *Neisseria haemolysans* or *Gemella haemolysans* is not related to Neisseriaceae. The taxonomic position is uncertain, and the organism may be assigned to a family of Gram-positive micro-organisms possibly Lactobacillaceae in the tribe Streptococceae.

Fine Structure of Group D Streptococcal L-form Colonies. By PAULINE S. CORFIELD and D. G. SMITH (*Department of Botany and Microbiology, University College, London*)

L-form sp. of group D streptococci were propagated on media of high osmotic pressure (7·5 atmospheres) containing penicillin to prevent reversion to the bacterial form (Hijmans, W. (1962), *J. gen. Microbiol.* **28**, 177). The L-form colonies had a typical central mass of small cells under the agar, extending on to the surface as large pleomorphic cells.

Two strains studied in particular were *Streptococcus faecalis* var. *zymogenes* H69D5 and a motile unidentified strain B74. L-form strain H69D5 became stable after 12 months propagation, whereas strain B74, upon removal of the penicillin, failed to revert after 5 months continued subculture. The 'stable' L-forms could be reverted to the bacterial form by substituting 30 % gelatin for the agar.

Whole colonies were fixed for electron microscopy using three different fixatives; osmium, acrolein/osmium or gluteraldehyde/osmium.

The central part of the colony contained cells varying in size from 3 μ down to 100 mμ in diameter. The ultrastructure of young, unstable L-colonies was compared with that of older, stable L-colonies.

After isolation, young colonies showed more structural variation than the older cultures; the cells having filamentous extensions of the plasmalemma, and often whirls of membranes, suggesting disorganized membrane synthesis.

In unstable (9-month) colonies of strain H69D5 some cells showed invaginations of the plasmalemma—or microtubular-like structures—with an external diameter of 250 Å (Corfield, P. S. & Smith, D. G. (1968), *Archiv Mikrobiol.* **63**, 356). These are of unknown significance.

Apparent membranous invaginations were also observed in all young unstable colonies of strain B74, these had an external diameter of approximately 130 Å and were often closely packed.

Colonies of stable, 18 month cultures of strain H69D5 revealed cells with structures that resembled the rigid 'cores' reported by McCandless, R. G., Cohen, M., Kalmanson, G. M. & Guze, L. B. ((1968), *J. Bact.* **96**, 1400) in strains of *S. faecalis* var. *zymogenes* bacterial cells. The L-form 'cores' lacked detail and were varied in size and shape.

Changes in the Levels and Distribution of Certain Enzymes During Encystment in *Hartmanella castellanii*. By S. M. BOWEN and A. J. GRIFFITHS (*Department of Microbiology, University College of Wales and Monmouthshire, Cardiff, CF1 3NR*)

During the differentiation of amoebae to cysts in replacement medium (Griffiths, A. J. & Hughes, D. E. (1969), *J. Protozool* (in the Press)) various changes in enzymic levels and distribution have been observed. For example, hydrolytic enzymes which appear to be associated with sedimentable subcellular particles, show definite changes in their activities and distribution during encystment. It has been suggested (Tomlinson, G. (1967), *J. Protozool.* **14**, 114) that the glyoxylate cycle is involved in the conversion of vegetative cell lipid to cyst-wall cellulose. Experiments carried out here have shown that one of the key enzymes of the pathway isocitrate lyase is not present in the vegetative form but appears during encystment. There are changes in the levels of malate synthetase as encystment progresses. Fractionation of subcellular particles by zonal centrifugation is now in progress with the view to establishing the changes occurring in the subcellular distribution of these and other enzymes during encystment: it appears that the glyoxylate system is in the peroxisomes.

Inhibitors such as Tetracycline which inhibits protein synthesis in amoebae, Actinomycin D and inhibitors of encystment (azide, arsenite) have been added and removed at various times during encystment as a preliminary to the study of the regulatory pattern of enzyme formation and disappearance during encystment.

Mycological peptone, glumatic acid or histidine inhibit encystment if added up to 10 hr after incubation in the encystment medium; after 24 hr there is no inhibition. These results and those obtained with inhibitors support the idea that after 24 hr, at the most, the cells are completely committed to encystment. Inhibition by histidine and glutamate leads to death but mycological peptone reverses the changes initiated earlier and vegetative cells result.

Streptococcal taxonomy. By D. B. DRUCKER and T. H. MELVILLE (*Dental School, University of Wales, Cardiff, and School of Dental Surgery, Liverpool*)

The characteristic appearance of Streptococcus makes this genus instantly recognizable. The morphological homogeneity of the genus is deceptive, however, for it encompasses a vast array of strains having differing properties, which were not satisfactorily classified until the work of Sherman, whose scheme is still the basis of the existing classification of Streptococcus (Breed, R. S., Murray, E. G. D. & Smith, N. R. (1957), *Bergey's Manual of Determinative Bacteriology*, 7th ed. (London: Bailliere, Tindall and Cox Ltd.)). New discoveries have rendered this classification obsolescent; for example, certain members of the viridans group of streptococci have been shown to possess a Lancefield antigen more usually associated with the pyogenic group (Williams, R. E. O. (1956), *J. Path. Bact.* **72**, 15). In order to overcome this problem, modern methods of bacterial classification were utilized. Some 178 characteristics were noted for each of 252 strains of streptococci. The collection of isolates included 63 type strains as nomenifers. The large number of results obtained had to be handled by an electronic computer, which clustered those micro-organisms which shared combinations of characteristics, which were statistically significant.

The final analysis and classification were performed manually. The programme used was a development of one previously applied to streptococcal classification by Colman, G. ((1968), *J. gen. Microbiol.* **50**, 149). The computer listed no fewer than 45 clusters, although some of these were identical, being a single cluster which formed at more than one significance level. After analysis of the results, 12 phenons were constructed. Four of those phenons corresponded

to the definition of *Streptococcus faecalis* in Bergey's manual, although an examination of the whole range of their characteristics showed that they were in fact not identical one to another. The tendency using the existing tests for enterococci to cluster heterogeneous strains has previously been observed by Raj, H. & Colwell, R. R. ((1966), *Can. J. Microbiol.* **12**, 353). A phenon which included *S. bovis* as a nomenifer was formed; together with a phenon of very high significance which consisted simply of cariogenic streptococci for which the name *S. mutans* has been tentatively suggested. A phenon corresponding to the accepted definition of *S. salivarius* was observed, and other less well-defined phenons represented *Streptococcus* species, *mitis*, *anginosus* and *sanguis*. Two other phenons were produced which are currently being examined. No clusters were formed corresponding to the well-documented species, *S. pyogenes* and *S. agalactiae*, but this was probably due to the fact that a minimum cluster size of five strains was employed and fewer than five strains of both *S. pyogenes* and *S. agalactiae* were examined. Although the results do not substantiate previous findings, they probably complement them since a different range of organisms has been studied.

Changes in E_h, pH and pO_2 during growth of *Escherichia coli*. By B. SANYAL and C. RUSSELL (*Department of Bacteriology and Virology and Turner Dental School, Manchester University*)

The relationship between E_h, pH and pO_2 in growing bacterial cultures has only recently been investigated in any detail (Hewitt, L. F. (1950), *Oxidation-Reduction Potentials in Bacteriology and Biochemistry* (Edinburgh; E. and S. Livingstone); Tengerdy, R. P. (1961) *J. Biochem. Microbiol. Technol. Eng.* **3**, 241–253). In the present work these parameters were monitored in both batch and continuous cultures of *Escherichia coli*, using both complex and synthetic media. The apparatus used involved a multi-channel recorder which also selected the parameter to be measured.

In stirred aerobic batch culture, initial pH 6·5 and population size 10^6 organisms/ml., pO_2 fell to zero after *ca.* 2 hr. E_h reached zero some time later, continued to fall, passed through a minimum value and remained negative for 12 hr. E_h then rose to a positive value, although pO_2 remained zero. If pO_2 was maintained above zero by bubbling in O_2, E_h never became negative. Variation in initial population between 10^2 and 10^6 organisms/ml. did not affect the point at which E_h fell sharply, which always occurred at 10^7 organisms/ml., i.e. the differences in numbers of organisms was not reflected in the E_h, in spite of the organisms being in a similar metabolic state (cf. Tengerdy, R. P. *et al.* (1967) *Appl. Microbiol.* **15**, 959–5). pH stat experiments showed that the higher the pH the more rapid was the decrease in E_h and the lower were the minimum and final (24 hr) values reached. It was also found that manual alternation of the pH level gave a concomitant alternation in E_h. This phenomenon was observed in continuous culture, when apparently self-induced fluctuations in E_h and pH were seen. This behaviour was most pronounced in non-limiting synthetic medium with low pH-buffering capacity.

Growth of *Blakesleea trispora* relative to carotene, sterol, and cofactor production. By J. D. BU'LOCK, D. DRAKE and D. J. WINSTANLEY (*Microbial Chemistry Laboratory, Department of Chemistry, The University, Manchester, M13 9PL*)

Correlation of the mating history of *Blakesleea trispora* cultures with the production of, and response to, trisporic acids (which promote carotene and sterol synthesis) is complicated by varied behaviour on different media. Using either synthetic medium (glucose-asparagine-phosphate-thiamine) (*A*) or malt extract (*B*) the total growth was comparable, but whereas on *A* a phase of linear dry-weight increase with slow N uptake from 30 to 70 hr followed P exhaustion, on *B* the dry-weight curve was sigmoid, with balanced N and P uptake halting simultaneously at 24 hr. On both media, pH rose during rapid growth and fell thereafter, and ergosterol content (10–20 mg./g. dry wt) was steady throughout, but whereas on *B* the β-carotene content increased steadily, on *A* this increase stopped at 60 hr. Up to 200 mg./l. of trisporic acids accumulated on *B*, whereas levels on *A* were far lower. Mixing the (+) and (−) strains during growth of the inocula, instead of at inoculation, allowed maximal β-

carotene and trisporic acid production on *B* but the cultures on *A* were not affected. Using a malt-extract medium supplemented with phosphate and ammonium succinate led to an intermediate situation: following rapid balanced N and P uptake, further slow uptake occurred; after β-carotene synthesis halted, as on *A*, it was resumed, and final trisporic acid levels were also higher than on *A*, though both were still lower than on *B*. On this supplemented medium, a possible trisporic acid precursor appeared transiently at *ca.* 40 hr. Clearly full manifestation of the trisporic-mediated phenomena occurring in mixed (+, −) cultures requires optimal nutritional conditions.

Sensitivity of *Pseudomonas aeruginosa* to Polymyxin B sulphate and EDTA: Role of the Divalent Cation of the Growth Medium. By M. R. W. BROWN and J. MELLING (*Pharmaceutical Microbiology Group, School of Pharmacy, Bath University, Somerset*)

Pseudomonas aeruginosa NCTC 6750 grown in batch culture in simple salts medium under conditions of Mg-limitation lost sensitivity to EDTA (Brown, M. R. W. & Melling, J. (1968), *J. gen. Microbiol.* **54**, 439).

Using similar techniques, the effect of such conditions on the sensitivity of this organism to polymyxin B sulphate has been examined. Several divalent cations were examined for their ability to substitute for Mg in its role concerned with the sensitivity of *Pseudomonas aeruginosa* to polymyxin or EDTA. Mg-limited organisms lost sensitivity to polymyxin: criteria were lysis as indicated by drop in E_{470}, drop in colony count and leakage of 260 mμ absorbing material (membrane filtration: Brown, M. R. W., Farwell, J. A. & Rosenbluth, S. A. (1969), *Anal. Biochem.* **27**, 484). Furthermore, Mg was added to Mg-limited cultures at intervals before growth ceased due to depletion of glucose, and the cultures tested for sensitivity to polymyxin and EDTA. The results indicated that growth in a Mg-plentiful medium for a minimum period of about a generation time was needed even partially to restore sensitivity, and several generation times needed fully to restore sensitivity to polymyxin or EDTA. The divalent cations Ca, Ba, Sr, Be and Zn were able to substitute to a varying extent for Mg in its role in the sensitivity of *P. aeruginosa* to polymyxin and EDTA. The order of effectiveness of these cations in substituting for Mg was different for the two agents.

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The Glyoxylate Cycle as an Important Pathway in Fungal Morphogenesis. By J. C. GALBRAITH (*Department of Biology, Paisley College of Technology*) and J. E. SMITH (*Department of Applied Microbiology, Strathclyde University, Glasgow*)

A shift from the tricarboxylic acid cycle (TCA) to the glyoxylic acid cycle (GLC) is known to accompany differentiation of the resistant sporangia of *Blastocladiella*, male gametangia of *Allomyces* (Cantino, E. C. (1966), *The Fungi*, vol. II), Academic Press), and conidia of *Neurospora crassa* (Turian, G. (1966), *The Fungi*, vol. II, Academic Press). Differentiation of all these forms is characterized by synthesis of glyoxylate from isocitrate, and transamination of the glyoxylate to glycine by glycine-alanine transaminase.

Parallel changes have been found to precede conidiogenesis in *Aspergillus niger*. Enzyme activities were assayed at intervals throughout the growth of sporing and sterile control mycelia grown in identical conditions excepting the nitrogen source. Prior to the first morphological signs of sporulation, there was a marked increase in the activities of isocitrate dehydrogenase (carboxylating), isocitrate lyase and glycine-alanine transaminase. There was no increase in the sterile mycelium. The activity of malate synthetase remained low throughout growth. α-Oxoglutarate dehydrogenase could not be detected in either type of mycelium, although malate dehydrogenase activity was high in both throughout the growth period. The requirement for glyoxylate for spore formation was further shown by the induction of sporulation on the addition of exogenous glyoxylate to an otherwise non-sporing medium.

The causal relationship between the proposed metabolic changes and differentiation has still to be proved in all four fungi. The importance of the GLC appears to be not as an anaplerotic pathway, but in the provision of a source of glyoxylate. The function of glyoxylate

has still to be demonstrated. The evidence favours its transamination to glycine, which may be important as a purine precursor in the nucleic acid synthesis necessary for translation of the genes active in differentiation.

Oscillation of Generation Times and the Control of Cell Division. By L. B. QUESNEL (*Department of Bacteriology and Virology, University of Manchester*)

Examination of published graphs recording the growth of synchronized cultures studied for three or more cycles of division suggests that the interval times between division rounds follows an alternating sequence from short-to-long-to-short or long-to-short-to-long (e.g. Abbo, F. E. & Pardee, A. B. (1960), *Biochim. Biophys. Acta* **39**, 478; Hunter-Szybalska, M., Szybalski, W. & DeLamater, E. D. (1956), *J. Bact.* **71**, 17). This oscillation is especially evident in cultures synchronized by the stationary-phase method of Cutler, R. G. & Evans, J. E. ((1966) *J. Bact.* **91**, 469). Direct recording (phase-contrast microscopy) of the individual cell generation times during the outgrowth of *Escherichia coli* populations on transfer to fresh synthetic medium (in a continuous flow chamber) reveals that in about 60 % of developing clones the consecutive mean generation times in the 2nd, 3rd and 4th generations showed a damped oscillation pattern (Quesnel, L. B. (1963), *J. appl. Bact.* **26**, 127). Maximum individual cell size in batch culture is encountered at 1st division (e.g. Takebe, I. & Yanagita, T. (1957), *Ann. Rep. Inst. Fd. Microbiol., Chiba Univ.* **10**, 27), and the size of the daughter cell at its inception may determine the time elapsing before its subsequent division. It appears that the condition of the cell at fission, if 'abnormal' for the conditions of culture, contributes negative feedback information tending to correct the abnormality producing the 'signal'. It is commonly observed that the oscillations die away as the clones grow into log phase. Oscillating generation times are observed in single-cell lines, in clonal means and in populations comprising many clones. In some individual-line cases oscillation has been observed for six generations.

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