A Haematin-Independent Mutant of *Haemophilus influenzae*

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SUMMARY

A mutant of *Haemophilus influenzae* has been isolated and found to be independent of haematin. Its microscopic morphology and growth properties were found to be similar to those of a strain of *H. parainfluenzae* and not to those of its parent. The use of non-synthetic media in assessing the haematin requirements of haemophilus strains is discussed.

INTRODUCTION

Organisms which have been given the generic name Haemophilus are grouped together because of their requirement for blood for growth in culture media. It was found that two growth factors present in blood were involved and these were termed the X and V factors. Fildes (1921) showed that the X factor could be replaced by haematin, and Lwoff & Lwoff (1936, 1937) identified the V factor as either di- or tri-phosphopyridine nucleotide. Within the group strains have been isolated from natural sources requiring both the X and V factors, and others needing only one of them. Those requiring both factors have been termed *Haemophilus influenzae* and those requiring only the V factor have been termed *H. parainfluenzae*. The microscopic appearance of these two subgroups also tends to differ. *H. influenzae* appears predominantly as very small cocco-bacilli 1–1·5 by 0·3–0·4 μ (*Topley & Wilson's Principles*, 1955), known as the 'typical' morphology, although the organism is pleomorphic and longer forms may occur, either as a small minority amongst the 'typical' cells, or as a majority under changed cultural conditions. On the other hand, *H. parainfluenzae* appears predominantly as well-defined rods. However, within the *H. influenzae* subgroup there is a continuous gradation as regards the degree of requirement for haematin since some strains require less haematin than others (Smith, Hale & O'Callaghan, 1953; Biberstein & Gills, 1961). The present paper describes some properties of a mutant of *H. influenzae*, produced in the laboratory and exhibiting complete independence of haematin.

MATERIALS AND METHODS

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

Tryptone agar (TA). Tryptone (Difco) was dissolved in glass-distilled water at 20 g. together with NaCl at 10 g./l. The pH was brought to about 3 with HCl and activated charcoal added to give a 0·2% (w/v) suspension. The mixture was allowed to stand with occasional shaking for 15 min. and then filtered. The filtrate was adjusted to pH 7·5 and agar (Davis) added to give a 2% (w/v) solution. The medium was stored at this stage. When required, the molten tryptone agar was dispensed in 20 ml. volumes into 25 ml. screw-capped bottles and cooled to 56° in a water bath. Half a millilitre of a 4% (w/v) solution of crystalline bovine albumin was then added and the mixture poured as plates.

Tryptone blood agar (TBA). The molten tryptone agar was dispensed in 20 ml. volumes into 25 ml. screw-capped bottles, cooled to 56° in a water bath, and oxalated horse blood added to give a 5% (v/v) suspension. The mixture was then poured as plates.

Chocolate tryptone agar (CTA). To the molten tryptone agar cooled to about 80° was added oxalated horse blood to give a 5% (v/v) suspension, and the mixture allowed to turn to a chocolate colour, with heating further in a water bath if required. The medium was then dispensed as plates or as slopes in cotton-wool plugged tubes.

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

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

Haematin solution. A stock solution of haematin (Roche Products Ltd.) was prepared as follows: 50 mg. haematin was mixed with 12·6 ml. $0·5M$-Na_2HPO_4, heated to dissolve, and 86 ml. glass-distilled water + 1·6 ml. M-KH_2PO_4 added. The solution was sterilized by autoclaving at 115° for 10–15 min.

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

Synthetic medium (SM). This was a chemically defined medium with the composition previously described (Butler, 1962).

Evaluation of growth requirements. Suspensions of the organisms were made in normal saline and a loopful spread over a segment of a plate of medium. After incubation at 37° for 24 and 40 hr., a visual assessment was made of the density and homogeneity of growth and of colony size.

Serological reactions. Antisera were prepared in rabbits by giving a course of injections of a suspension of organisms in physiological saline at an approximate concentration of 10^9 cells/ml. Two injections, 6 days apart, of 0·2 ml. of the suspension into the vein of the ear, were followed at 6-day intervals by three further injections of 0·4 ml. The rabbits were bled 5 days after the last injection and the serum separated and kept at 4° until tested.

Tube agglutination was carried out in 6 × 1 cm. glass test tubes. An initial serum dilution in saline of 1/10 was made, and from this serial doubling dilutions in saline were set up using 0·5 ml. volumes. To each tube of serum dilution was added an

equal volume of a saline suspension containing about 10^8 bacteria/ml. The range of final serum dilutions was 1/20 to 1/5,120. Each titration included a negative control consisting of a tube containing the bacterial suspension with an equal volume of saline only. All dilutions and controls were incubated for 18 hr. at 56° followed by 1 hr. at 4°. The presence of granular agglutination was then observed visually with the aid of a concave mirror. The titre of a serum was recorded as the reciprocal of the final dilution containing the least amount showing clear-cut agglutination.

Table 1. *Composition of medium for the isolation of strain Rd1, the haematin-independent mutant of the* Haemophilus influenzae *strain Rd*

Substance	mg.	pH of stock solution	
Glycine	0·96	—	
DL-Alanine	0·96	—	
DL-Valine	2·88	6·9	
L-Leucine	2·88	6·9	
DL-Isoleucine	1·6	6·9	
DL-Serine	2·88	—	
DL-Threonine	1·44	—	
DL-Aspartic acid	2·88	6·9	Dissolved in
L-Glutamic acid	4·8	6·9	glass distilled
L-Arginine HCl	0·96	6·9	H_2O to give total
L-Lysine $(HCl)_2$	2·88	—	volume 6·0 ml.
L-Cysteine HCl*	2·5	—	
DL-Methionine	1·44	—	
Glutathione*	5·0	—	
DL-β-Phenylalanine	1·44	6·9	
L-Tyrosine*	1·6	6·9	
L-Tryptophane*	0·48	6·9	
L-Histidine HCl	1·19	6·9	
L-Proline	2·88	—	
Crystalline bovine albumin*	40·0	—	
	ml.		
Yeast extract solution	1·0		
Glucose-salt solution	0·8		
Haematin solution	0·3		
Coenzyme I solution*	0·06		
2 % (w/v) N.Z. (Davis) agar in glass distilled H_2O	13·0		

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

RESULTS

Production and isolation of the mutant Rd1. During the early stages of development of the chemically defined medium (SM) for *Haemophilus influenzae* reported previously (Butler, 1962), a yeast-extract-containing medium (YEM) of the composition shown in Table 1 was used. Segments of plates of this medium were inoculated with strains Rd, RdTr and FID and spread over the surface. After 4 days incubation at 37°, strain Rd had given rise to several colonies, densely white in the centre and with a translucent rim; under them a white deposit appeared in the medium. The colonies were subcultured on to stock CTA and later streaked on to TA, TBA, TA + DPN, together with spot inocula of staphylococci, and also on to YEM and

CTA. On the two latter media the organisms grew along the full length of the streaks as small colonies down to the lowest dilution, but growth was potentiated around the staphylococci. On TA, satellitism was shown around the staphylococci but the colonies were small, and on TA + DPN good growth occurred over the whole inoculum. The strains RdTr, FID and the parent organism Rd, were also streaked on to these media (except YEM and CTA) for comparison. All three strains grew well on the blood medium, giving small colonies over the whole inocula, and satellitism. On TA, strain FID showed satellitism but neither strains Rd nor RdTr grew, and with added DPN, both strains FID and RdTr grew but no growth was obtained with strain Rd. These results are summarized in Table 2. It seemed clear that a mutant of strain Rd had been isolated which was less dependent on added haematin, and it was designated strain Rd 1. It was subsequently maintained on slopes of CTA.

Table 2. *Comparison of the capacity of various media to support growth of strains Rd, RdTr, FID and the mutant Rd 1*

Strain	1* TBA	2* TA	3* TA+ DPN	4 CTA	5 YEM	6 SM + haematin (mg./100 ml. medium)			
						0	1·2	1·8	2·4
Mutant (Rd 1)	G+S	S	G	G	G	G	G	G	G
Rd	G+S	O	O	—	—	O	G	G	G
RdTr	G+S	O	G	—	—	O	G	G	G
FID	G+S	S	G	—	—	G	G	G	G

* Media 1, 2 and 3 were streaked with *Staphylococcus aureus* to demonstrate satellitism.

G = growth   TBA = tryptone blood agar
S = satellitism   TA = tryptone agar
G + S = growth enhanced around staphylococci   CTA = chocolate tryptone agar
O = no growth   YEM = yeast extract medium
                    DPN = diphosphopyridine nucleotide
                    SM = synthetic medium

Haematin requirement. The haematin requirement was ascertained with the use of SM by varying the amount of haematin in that medium. The final concentrations of haematin used were 0, 1·2, 1·8 and 2·4 mg./100 ml. medium. All strains grew at concentrations down to 1·2 mg./100 ml., but only strains FID and Rd 1 grew without haematin. The growth was equal to that on the haematin-containing plates. These results are also included in Table 2. It was concluded, therefore, that strain Rd 1 was haematin-independent.

Microscopic morphology. Smears made from 24 hr. stock slopes of strains Rd, RdTr, FID and Rd 1 were Gram stained using dilute carbol fuchsin as counter-stain. Strain Rd had the 'typical' appearance, being predominantly cocco-bacillary with a few long forms; strain RdTr was similar to its parent strain Rd. Strain FID had the 'atypical' appearance, with mainly well-defined rods and only a few, if any, other forms; and strain Rd 1 resembled strain FID exactly.

Growth requirements. Table 3 summarizes the results obtained concerning the purine, pyrimidine, amino acid and mineral salt requirements of strain Rd 1, obtained during the development of SM previously reported. The growth requirements of strains Rd, RdTr, and FID were ascertained at the same time and have been reported elsewhere (Butler, 1962). It will be noted that strain Rd 1 has an

essential requirement for uracil, pyridoxine, isoleucine and valine. The uracil could be replaced by guanine + hypoxanthine. A reduction in the concentration of phosphates (not mentioned in Table 3) below the level normally included in SM resulted in reduced growth of strain Rd 1. Strains Rd, RdTr and FID required albumin or polyvinyl alcohol in the medium and exhibited a peak of maximum growth when subjected to varying concentrations of these substances. Strain Rd 1 behaved similarly, resembling strain FID in its behaviour with albumin, and strain RdTr in respect of polyvinyl alcohol. Sodium oleate could replace albumin or polyvinyl alcohol, as found for strains Rd, RdTr and FID.

Table 3. *Purine and pyrimidine, vitamin, amino acid and mineral salt requirements of the mutant Rd 1*

Type of substance	Essential	Stimulatory	Not required
Purines and pyrimidines	Uracil (replaceable by guanine + hypoxanthine)	Adenine	Thymine, cytosine, hypoxanthine, guanine
Vitamins	Pyridoxine	Ca pantothenate, putrescine (aneurin in absence of Ca pantothenate)	Biotin, folic acid, aneurin, glutamine
Amino acids	Isoleucine, valine	Tryptophane, cysteine or glutathione, histidine, tyrosine, arginine, aspartic acid, glutamic acid, leucine, phenylalanine	Lysine, glutathione or cysteine, methionine, alanine, glycine, proline, threonine, serine
Mineral salts	$ZnSO_4$	Na acetate, $NaHCO_3$, $MgSO_4$, $FeSO_4$	$CaCl_2$, $MnCl_2$

Repetition of isolation of the mutant

In view of the fact that the mutant designated Rd 1 was isolated in the course of investigations in which the strains Rd, RdTr and FID were often inoculated on different segments of one plate of medium and the remarkable resemblance between the properties of strains Rd 1 and FID, it was thought desirable to attempt another isolation of a similar mutant from the parent strain Rd. On repeating the experiment in the same way as in the original isolation, colonies were obtained in the segment inoculated with strain Rd, which, after first subculturing on to CTA, were found to be haematin-independent. Subculture *directly* from the YEM plate on to SM without haematin gave no growth, and on to TA with 'spot' inocula of staphylococci gave only barely detectable satellite growth. Colonies also appeared on the segment of the YEM plate inoculated with strain RdTr, but they did not cause a precipitation to occur in the medium below the colonies, nor were the organisms found to be haematin-independent.

Serological reactions. The titres obtained by tube agglutination of the cell suspension of the parent strain Rd and the mutant strain Rd 1 with their antisera are shown in Table 4. Satisfactory homologous reactions were observed, strain Rd 1 producing higher titres than did strain Rd. Cross-agglutination, however, was seen in only one direction. Cells of the parent strain Rd were agglutinated by the serum

of the mutant strain, but no agglutination was detected between cells of the mutant strain and the antiserum to the parent strain. When the heterologous cells in the cross-tests were removed by centrifugation and the *homologous* cells added to the supernatants, agglutination was obtained after further incubation, to the same titres as had already been found in the conventional homologous titrations, showing that the negative reaction (anti-Rd with Rd1 cells) was not due to the presence of a soluble inhibitor in the Rd1 bacillary suspension.

Table 4. *Titres of agglutination of cells of parent strain Rd and of haematin-independent mutant Rd1 by their antisera and by supernatants from mixtures of antisera with heterologous cells*

Cells	Antisera		Supernatants	
	Rd	Rd1	Rd*	Rd1†
Rd	320	320†	320	
Rd1	< 10*	2560		2560

*† After recording the titres obtained, the cells of the heterologous tests were removed by centrifugation and the supernatants mixed with the homologous cells and agglutination titres noted after further incubation.

Table 5. *Comparison of the properties of strain Rd1 with those of strains Rd and FID*

Predominantly FID-like properties	Predominantly Rd-like properties	Intermediate properties	Neither Rd- nor FID-like properties
Haematin independent	Stimulation by NaHCO₃	Purine and pyrimidine requirements	Stimulation by Na acetate
Microscopic morphology			
Stimulation by amino acids on 'Zeocarb' treated yeast extract			
Vitamin requirements			
Amino acid requirements			
Behaviour with albumin			

Strain Rd = *Haemophilus influenzae* (rough from type d).
Strain Rd1 = haematin-independent mutant of strain Rd.
Strain FID = *H. parainfluenzae*.

DISCUSSION

The organism Rd1 is an example of a mutant, derived by artificial selection from a haematin-dependent *Haemophilus influenzae*, which has become completely haematin-independent. Such an organism would be classified as a strain of *H. parainfluenzae* according to the criteria commonly used in identifying members of the genus *Haemophilus*. The curious features of the isolation of the mutant were that its colonies appeared on a medium (YEM) which contained at least 0·7 mg. haematin per 100 ml. medium, but which was unable to maintain normal growth of the parent strain Rd, and that these colonies gave rise to a culture growing well on SM without haematin, and on other media lacking haematin, but only after subculture on a complete medium (CTA). On the other hand, no similar mutant was obtained by a direct method—i.e. by plating out large inocula of strain Rd on SM without haematin.

This procedure was carried out several times, since it formed part of the routine check for purity of the stock cultures of strain Rd. The inocula of this strain so tested were as large as, or even larger than, those from which the mutant was obtained on YEM. These findings suggest that there may have been a multiple-step process of mutation to haematin-independence, also involving a stage of growth on complete medium for phenotypic expression to occur; for example, the initial phase of selection on YEM may have been towards independence of some simpler constituent (e.g. adenine, which is required by the parent but not by the mutant), and after this change had become phenotypically expressed, haematin-independence became conferred on the organism. However, since the exact composition of the yeast extract was not determined, and in the absence of sufficient knowledge of the metabolic pathways involved, it is not possible to decide what may be the correct explanation of the phenomenon.

The results given in Table 2 with respect to the undefined media would suggest that strain RdTr might be haematin-independent also, since it grew on medium containing only tryptone agar + DPN, but it gave no growth on the defined medium without haematin. These facts emphasize the following points:

(1) The extent of haematin independence can only be determined by the use of a defined medium. The tryptone used above obviously contained a small (and almost certainly variable) amount of haematin or haematin-like compound. The presence of haematin or similar substances had been suspected in such media by others (e.g. Biberstein & Gills, 1961), and Gilder & Granick (1947) also reported the presence of haematin in yeast extract. It is pertinent to note, therefore, that all results referring to the need or otherwise of haematin obtained by using an undefined medium must be viewed with some reservation. For example, the reported haematin independence of *H. influenzae* growing anaerobically should be re-assessed. The finding of Gilder & Granick that haematin is still required for anaerobic growth but in smaller amounts is quite likely, but these authors used a proteose-peptone base which may well have contained some haematin so that the actual requirement could not have been determined.

(2) The haematin content of the tryptone was sufficient to allow growth of strain RdTr but not of strain Rd. This supports the view that there is a gradient of haematin requirement amongst the haematin-dependent strains (Smith *et al.* 1953; Biberstein & Gills, 1961). This may account for the unsuccessful attempts to isolate a haematin-independent mutant of strain RdTr using exactly the same conditions as for strain Rd. It may be that as strain RdTr appears normally to be able to grow with lower concentrations of haematin than strain Rd, the haematin content of the 'mutation medium' may have been too high to have a selective influence. However, this point was not further investigated.

It is interesting to compare the properties of strain Rd1 with those of its parent strain Rd and those of strain FID. Smears of growth of the same age taken from CTA slopes show predominantly well-defined rods for strain Rd1 and FID, while strain Rd is predominantly cocco-bacillary. On a medium containing Zeo-carb treated yeast extract (Butler, 1962) strain Rd would not grow, but strains Rd1 and FID gave reduced growth which was stimulated in both organisms by the addition of amino acids. The vitamin and amino acid requirements were exactly those of strain FID, both requiring pyridoxine, isoleucine, and valine as essential

metabolites, and both stimulated by the same vitamins and amino acids, whilst strain Rd showed no absolute requirement for any one vitamin or amino acid. The behaviour with albumin was similar to that of strain FID, although the behaviour with polyvinyl alcohol was similar to that of strain RdTr, the other mutant derived from the same parent. The purine and pyrimidine requirement was intermediate between strains Rd and FID. Strain Rd1 required uracil and was stimulated by adenine, although the uracil could be replaced by guanine + hypoxanthine. Strain Rd had an essential requirement for both uracil and adenine, whilst strain FID required guanine and was stimulated by uracil and adenine. Most of the mineral salt requirements were shared with both strains Rd and FID, with two exceptions, viz. NaHCO₃ stimulated strain Rd1, Rd (and RdTr) but not strain FID, and sodium acetate stimulated strain Rd1 only. These comparisons are summarized in Table 5 from which it can be seen that the properties of strain Rd1 are predominantly similar to those of strain FID. It is therefore tempting to suggest that as both strains Rd1 and FID require valine, it may be that this amino acid is required in the pathway leading to the synthesis of haematin or in an alternative pathway by-passing the need for haematin altogether. It may also be of interest to note that both strains Rd1 and FID are independent of adenine (required by strain Rd), although strain Rd1 retained its parental requirement for uracil.

It is necessary to consider the possibility of contamination by *Haemophilus parainfluenzae*:

(1) *Contamination of stock culture.* As stated above, the stock culture of strain Rd was maintained on slopes of CTA. These were subcultured twice weekly, and were periodically inoculated on to plates of TA and checked for satellitism, and also on to SM not containing haematin. No growth was obtained in either case. Stock cultures were never taken from plates also containing strain FID. It is also felt that a *Haemophilus parainfluenzae* contaminant would not require an initial sub-culture on CTA before the expression of its haematin-independence, since this character would already have been expressed.

(2) *Contamination of plate.* Colonies have been obtained on YEM plates inoculated with strain Rd, but not also inoculated with strain FID, which have resembled the strain Rd1. Again, it is felt that the comment made above concerning the lag in the expression of the haematin independence is relevant. Also it may be significant that the colonies showing haematin-independence always appeared on segments inoculated with strain Rd and not on those inoculated with strain RdTr.

No attempt was made to obtain the mutant from cultures grown from single-cell isolates of strain Rd: as pointed out by Hilson & Elek (1959), it is doubtful whether this procedure affords any significantly greater protection against contamination than conventional methods of obtaining and handling pure cultures.

The property of haematin-dependence is heavily relied on to distinguish between *Haemophilus influenzae* and *H. parinfluenzae*. The isolation of a haematin-independent mutant of *H. influenzae* shows that this character is not stable on a medium, which although unfavourable to the general growth of the strain used, was not deliberately devised for the selection of haematin-independent mutants. These results suggest that workers who are involved in the isolation, identification and maintenance of *H. influenzae* strains should take particular care not only that the medium used for their culture is complete, but also that the cultures should be

subjected to the least number of subcultures through various media. Such precautions should minimize the risk of any change in characters which have hitherto generally been considered sufficiently stable for use in taxonomy.

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Amino Acids, Amino Sugars and Sugars Present in the Cell Wall of some Strains of *Streptococcus pyogenes*

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SUMMARY

Hydrolysates of the cell walls of 26 strains of group A streptococci were examined by paper chromatography. In all cases lysine, glutamic acid, alanine, glucosamine, muramic acid and rhamnose were found as the principal components. The mean relative proportion of lysine:glutamic acid:alanine was 1·1:1:4. Thus even when a number of strains of a particular bacterial species was tested, there was good uniformity in the cell-wall composition. Samples of cell-wall material which remained undigested after treatment with proteolytic enzymes all contained small amounts of several amino acids. It seems probable that a small amount of polypeptide or protein remained firmly attached to the cell walls examined.

INTRODUCTION

Cummins & Harris (1956) examined the amino acids, amino sugars and sugars present in the cell walls of a large number of bacterial strains. It appeared that the amino acids present in relatively large quantities were characteristic of the genus, while the sugars and amino sugars characterized the species within a genus. Other investigations (summarized by Cummins, 1956; Salton, 1956, 1960; Work, 1957) tended to support this conclusion. In most of these studies, however, only a few strains of any one species were tested. Little quantitative information is available about the relative amounts of the principal amino acids present in the cell walls of different organisms. The main purpose of the present work was to see whether, in a larger number of strains of a species than tested by previous workers, there was a constancy in the principal amino acids of the cell wall and in their relative molecular proportions. Salton (1953) and Cummins & Harris (1956) commented on the presence of small quantities of many amino acids in hydrolysates of cell-wall material from group A streptococci. These workers had subjected the cell walls before hydrolysis to digestion by trypsin or by trypsin + pepsin. Schwab, Cromartie & Roberson (1959) reported that papain was more effective than trypsin, chymotrypsin, or pepsin, in that it removed a greater proportion of nitrogen-containing

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substances from such cell-wall material. Accordingly, a comparison was made of the amino acids present in small amount in samples of cell-wall preparations of group A streptococci which had been treated with different proteolytic enzymes.

METHODS

Organisms. Strains of group A streptococci were selected from stocks held at the Streptococcus Reference Laboratory (Colindale, London) and at the Department of Rheumatology, the University Hospital, Leiden, Netherlands. The designation or origin of the strains were as follows (the NCTC number is given for those strains which are included in the National Collection of Type Cultures, Colindale, England): strains carrying an M antigen: types 1 (NCTC 8198), 2 (NCTC 8322), 3 (NCTC 9994), 4 (NCTC 8326) 5, 8 (NCTC 8324), 9, 11, 12, 14, 17 (NCTC 8304), 23 (NCTC 8301), 24 (NCTC 8305), 25, 27, 30. The type strain 12 was isolated from a case of nephritis. Strains identified by the presence of T antigen: types 2 (NCTC 8322, glossy), 3/13, 5/27; a strain-carrying type 12 M antigen and a type 10 T antigen (designated 12/10); a strain-carrying type 14 M antigen alone (14/Lowe, NCTC 8199) and a variant of this strain carrying both type 14 and type 51 M antigens (designated 14/51, Wiley & Wilson, 1961); strains AED (type 12 T antigen), GL 8 (type 19 M), ADA (type 14 M) obtained from Dr L. Dienes (Boston U.S.A.) and strains B6 (typing pattern 11/27/44) isolated locally in Leiden, Netherlands. As controls, two strains of group C streptococci, one of human origin and carrying a T antigen demonstrated by type 2T antiserum (C/2T) and the other of animal origin (C anim) and a group E streptococcus, were analysed.

Isolation of cell walls. All the strains were grown for 48 hr. at 37° in 2 l. of Oxoid Nutrient Broth. The cultures were checked for purity, centrifuged, the organisms washed twice with 100 ml. distilled water and finally resuspended in 25 ml. distilled water. A 1 ml. sample was removed and the weight of organisms dried over P_2O_5 was determined. Volumes (6 ml.) of suspension were then mixed with an equal volume of ballotini beads (grade 12) in the cup of a Mickle (1948) disintegrator. Tributyl phosphate (0·04 ml.) was added to prevent frothing. After 30 min. disintegration a smear of the disrupted organisms was stained by the Gram method. In most instances a high degree of cell breakage was observed microscopically; intact Gram-positive cocci were rarely seen. Occasionally it was necessary to continue the disruptive procedure for a further 15–30 min. After completion of the disintegration, the cell-wall suspension was decanted from the beads; these were then washed twice in 6 ml. distilled water. The cell walls were sedimented from the combined suspensions and washings, at 10,000 rev./min. (11,730 *g*) for 30 min. and washed twice in distilled water. A sample of insoluble cell-wall material was removed and the dry weight determined. The yields of streptococci from the different strains were in the range of 135–687 mg./2 l. culture, with a mean value of 412 mg. The yield of insoluble cell-wall material represented about 26 % of the dry weight of the streptococci.

In a separate investigation strain AED was grown in 6 l. of Todd–Hewitt broth (Difco) for 24 hr. at 37°. The cell walls were isolated as before, except that the streptococci were washed more thoroughly with distilled water (5 times) and the sedimented walls washed 5 times with distilled water, once in saline and once in 0·05 M-phosphate buffer (pH 7·8). At each stage in the washing procedure the lower

dark-coloured layer was discarded. The sedimented material retained at each stage was examined by phase contrast and electron microscopy. In the final preparation no intact organisms were seen.

Enzyme treatment of the isolated cell walls. The isolated cell-wall preparations obtained from all the streptococcal strains were resuspended in 25 ml. 0·1 M-phosphate buffer (pH 7·8) and after the addition of crystalline ribonuclease (1 mg./ml.) incubated for 3 hr. at 37°. Then crystalline trypsin (1 mg./ml.) was added and the incubation continued for a further 3 hr. The insoluble material remaining after digestion was sedimented in a high-speed centrifuge 30 min. at 22,500 *g*, washed three times with distilled water and dried over P_2O_5 *in vacuo*. Before drying, the cell-wall preparation obtained from the separate investigation with strain AED was divided into three portions; one portion was immediately dried over P_2O_5, the other two portions were subjected to digestion with pepsin or papain for 17 and 5 hr., respectively, in the appropriate buffer solution. After this further digestion the samples were washed and dried over P_2O_5.

Hydrolysis conditions. For chromatographic analysis of the amino acids and amino sugars 7·5 mg. dry weight of the wall material of each strain were heated with 5 ml. 6N-HCl in sealed tubes at 108° for 16 hr. After cooling, the contents of the tubes were transferred to small Petri dishes. The hydrolysates were then taken to dryness on a steam bath to remove HCl, redissolved in a small amount of water, filtered to remove humin, dried twice over NaOH *in vacuo* and finally dissolved in 0·4 ml. distilled water and stored frozen. For the chromatographic analysis of sugars 20 mg. dry weight of the wall material from each strain were heated with 2 ml. 0·5N-H_2SO_4 in a sealed tube at 100° for 2 hr. After cooling, the hydrolysates were neutralized with a saturated solution of $Ba(OH)_2$, centrifuged and the sediment washed twice. The original supernatant fluid and the washings were mixed, dried over P_2O_5 *in vacuo*, dissolved in 0·25 ml. distilled water and stored frozen.

Chromatography. Amino acids, amino sugars and sugars were separated by two-dimensional descending chromatography on Whatman no. 1 paper (40 × 30 cm.). *n*-Butanol + acetic acid + water (60 + 10 + 20 by vol.) and 2/4–2/5 lutidine (British Drug Houses, Ltd.) + water (65 + 35 by vol.) were used as solvents, each developed for 20 hr. For the detection of amino acids and amino sugars 0·01 and 0·02 ml., respectively, of the appropriate hydrolysates were placed on the papers. After drying of the chromatograms, amino acids and amino sugars were developed with ninhydrin and the sugars with aniline hydrogen phthalate (Partridge, 1949). The latter reagent also revealed the presence of amino sugars and N-acetylamino sugars. The identity of most of the spots was established by using as markers pure substances subjected to the same chromatographic conditions, separately and after admixture with the unknown sample.

Quantitative estimations of lysine, glutamic acid and alanine were performed according to the method of Kay, Harris & Entenman (1956). Duplicate chromatograms were developed from 6 μl. samples of hydrolysate. The percentage standard deviation of the mean values for known quantities of lysine, glutamic acid and alanine were ± 5·7, ± 5·9 and ± 3 %, respectively. The results are expressed as relative molecular ratios, taking the value for glutamic acid as one unit.

RESULTS

Amino acids and amino sugars. All the cell-wall hydrolysates of the strains tested contained lysine, glutamic acid, alanine, glucosamine and muramic acid; this finding is in keeping with other reports (Cummins & Harris, 1956). When it was seen that in addition to these amino acids a few other ninhydrin-positive minor spots were also present on some of the paper chromatograms, several hydrolysates were retested with three times as much material applied to the chromatograms. These chromatograms gave a pattern in which eight out of the eleven minor spots were identified as glycine, serine, aspartic acid, arginine, cysteine, threonine, valine, leucine and/or isoleucine. The identity of the remaining spots was not established. The number of additional minor spots differed with different strains.

Table 1. *Relative molecular ratios of the three principal amino acids present in the cell walls of some streptococci*

Streptococcus		Relative proportion of amino acids (glutamic acid = 1)		
		Lysine	Glutamic acid	Alanine
Group A type	1	1·1	1	3·8
	2	1	1	4
	2T	1·2	1	4·2
	3	1·1	1	4
	3/13T	1·1	1	4
	4	1·1	1	4·2
	5	1·1	1	3·9
	5/27T	1·1	1	4·1
	8	1·1	1	3·8
	9	1	1	4·2
	11	1·1	1	4·3
	12	1·2	1	4
	12/10	1·1	1	4·1
	14	1·1	1	3·8
	14 Lowe	1·2	1	4
	14/51	1	1	4·1
	17	1	1	3·8
	23	1·1	1	3·9
	24	1	1	4
	25	1·1	1	4·1
	27	1·1	1	3·9
	30	1·1	1	4·1
AED		1·1	1	3·7
GL 8		1	1	3·7
ADA		1·1	1	3·9
B6		1	1	3·6
Group C anim.		1·2	1	3·8
type 2T		1·2	1	4·2
Group E		1·1	1	4·3

During the initial isolation of the separate samples of the cell wall of strain AED (see methods) it appeared that little of the cytoplasmic material which contaminated the cell walls was removed. However, after treatment with ribonuclease and trypsin, the material was purified to such a degree that further treatment with pepsin or papain did not influence the appearance of the cell-wall preparations in the electron

microscope. The chromatographic pattern of the hydrolysates of the three different enzyme-treated portions of the cell-wall preparations of strain AED was indistinguishable from that obtained with other strains. Arginine was absent from all chromatograms of strain AED in both preparations.

Hydrolysates (6 μl.) of group C and group E streptococci showed the same pattern of major components as that of group A streptococci except that a spot corresponding to galactosamine was found in both group C strains. No efforts were made to identify the additional minor amino acid components in these hydrolysates.

The relative molecular ratio of lysine, glutamic acid and alanine in the cell-wall hydrolysates from the strains examined is given in Table 1. The mean ratios for the 26 strains of group A streptococci examined were 1·1:1:4. The number of group C strains examined was too small to permit conclusions to be drawn about the relative lysine contents of their cell walls.

Sugars. The chromatograms for the sugars present in the cell-wall hydrolysates of the 26 strains of group A streptococci showed three distinct spots corresponding to rhamnose, glucosamine and *N*-acetylglucosamine. Glucosamine was reported to be present in cell walls in its acetylated form by McCarty (1952). The relatively mild conditions of hydrolysis used here for the detection of sugars would explain why glucosamine was found partly in its original form. The group C and group E strains showed spots corresponding to glucose, and to glucose and mannose, respectively. The absence of the muramic acid spot from these chromatograms and also of the galactosamine spot from both group C strains was probably related to the insensitivity of their reactions with alanine hydrogen phthalate.

DISCUSSION

The results reported here about the amino acids, amino sugars and sugars in the cell walls of 26 strains of group A streptococci suggest an uniformity in the major components of the cell walls. Final judgement will be possible only when information is available about the amounts of all the components. The information about the relative molecular ratios of the principal amino acids argues however in favour of the uniformity of the cell-wall composition of group A streptococci. Other unidentified components of the basal streptococcal cell wall probably exist. For example, polyolphosphates have been demonstrated in the cell walls of several bacterial species (Armstrong *et al.* 1959; Ikawa, 1961). It is not yet known whether these components occur in group A streptococci. Hayashi & Barkulis (1959) reported the following composition of the cell wall of group A streptococci: amino acids 53 %, hexosamines 24 %, rhamnose 22 %. On the basis of these figures and taking into account the low phosphorus content of the cell wall of group A streptococci (0·7–0·8 %; McCarty, 1952), it seems that little space is left for teichoic acids as major cell-wall components.

Despite the general similarity of the cell-wall structure of the strains here examined, several strains have been recognized which have certain peculiarities. Roberts & Stewart (1961) described one strain of group A streptococci which in addition to rhamnose contained glucose and galactose in the cell wall; this pattern is usually found only in group D and group E streptococci. One of us (H.G.) recently investigated an unusual bile-tolerant non-haemolytic streptococcus which

carries group A polysaccharide hapten in its cell wall. Chromatographic analysis of the sugar components present in enzyme-treated cell-wall preparations revealed glucose in addition to rhamnose. Quantitative variation in the composition of group A streptococcal cell walls is known. McCarty & Lancefield (1955) analysed the cell wall of a strain of this species which had lost group specificity after repeated mouse passage. In consequence of a decrease in the glucosamine content of the C polysaccharide, the ratio of rhamnose to glucosamine in the cell wall in this variant was 4·8–6, whereas the same ratio in the parent strain was 1·5–2. Nevertheless, even when these minor quantitative and qualitative differences are considered it appears that the major components of the cell walls of *Streptococcus pyogenes* strains are a reliable guide to the taxonomic homogeneity of this group of organisms. The original suggestion of Cummins & Harris (1956) that a study of cell-wall composition among micro-organisms might have taxonomic significance is supported by the present work. It is interesting that the hydrolysates of cell-wall material, purified in different ways, always contained small amounts of many amino acids. This suggests that a polypeptide resistant to the action of proteolytic enzymes is firmly attached to the basal cell wall. Such a polypeptide may be related to the streptococcal T antigen normally found on the cell wall. Trypsin digestion of whole streptococci is effective in releasing T antigen from some, though not all strains of streptococci (Lancefield, 1954). The isolated T antigen of type 1 streptococci is resistant to proteolytic digestion (Lancefield & Dole, 1946). Differences in the retention of T antigen on the samples of cell walls studied in the present work may have occurred. Ikawa (1961) reported the presence of protein in the cell walls of three other species of Gram-positive bacteria; this protein was resistant to digestion by proteolytic enzymes and represented 6·5–9 % of the dry weight of cell wall.

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Relative Humidity and the Killing of Bacteria: the Variation of Cellular Water Content with External Relative Humidity or Osmolality

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SUMMARY

The paper deals with the relationship betweeen the water content of washed *Serratia marcescens* and the thermodynamic activity of water, a_w, over the range $0 < a_w < 1·0$. Two overlapping types of measurement are used: osmotic water exchange and water-vapour sorption. The data are given in terms of the equation $w = Bm^{-r}$ where w is water content, m is external osmolality (actual or effective, depending upon the technique used), B is a constant and r has values which can be interpreted in terms of the type of process involved: e.g. in the initial stages of osmotic dehydration, r of unity would indicate ideal solution behaviour; in the final stages of drying, any constant value of r would conform to an adsorption function like that of Halsey (1948). The data obtained by the two techniques used fall on a single curve with several zones including (a) non-ideal solution behaviour at high a_w, (b) two intersecting 'co-operative adsorption' zones at very low a_w, and (c) a region of variable r where processes such as dissolution of small molecules and 'saturated' swelling of interlinked polymeric structures are presumed to occur. It is thought that detailed interpretation of the unified water uptake curves would be valuable in understanding mechanisms of humidity-dependent loss of viability, in particular the zones of exceptionally rapid attenuation.

INTRODUCTION

This paper presents information on the water content of bacteria at external relative humidities (*RH*) from 100 % to 'zero' as part of a study of humidity-dependent losses of viability. It was done partly to fill a need for a means of converting measured 'equilibrium' moisture content values for washed cells to the corresponding *RH*. Further, water transfer curves provide an overall picture which is useful when considering factors of possible significance in cell survival studies.

METHODS

Biological methods

Bacterial cell suspensions used in sorption measurements: five ml. portions of a 24 hr. broth culture of *Serratia marcescens* strain 8 UK were used to inoculate tryptose agar in Roux bottles. The cells were harvested in distilled water after

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24 hr. growth at 31°, centrifuged and resuspended in distilled water to give a suspension having about 10^{11} viable cells and 67 mg. dry residue/ml. Suspensions used for osmotic volume determinations: these were prepared from frozen cells stored at $-20°$, which had been grown in tryptose broth at 30° and harvested after 28 hr. by continuous process centrifuging. They were resuspended in an equal weight of growth medium and frozen in pellets. The pellets, when thawed, had a viable count of about 7×10^{11}/g.

The *Escherichia coli* B on which osmotic measurements were made was prepared from stock stored at $-20°$. The cells had been grown in nutrient broth at pH 8·1 and harvested at 18 hr. by continuous process centrifuging. After one washing with distilled water they were sealed into small tins and frozen. Before freezing, the total count was 5×10^{12}, the viable count about 3×10^{12} cells/g. dry solids. A single freezing and thawing cycle had negligible effect on viability.

Equilibration of bacterial cells with water vapour

The experiments were done with the silica spring sorption balance (McBain & Bakr, 1926) shown in Fig. 1. Freeze-drying and subsequent equilibration with water vapour took place within the same sealed chamber under precisely controlled conditions.

Procedure: (a) Freeze-drying of cells: 1·5 ml. of stock suspension was placed in an aluminium foil bucket and frozen in a closed vessel immersed in solid CO_2 and alcohol for 30 min. The cold trap in the sorption apparatus, containing water, was immersed in solid CO_2 and alcohol. The spring suspension frame, spring, and frozen sample were placed in position (Fig. 1) and the apparatus evacuated. Drying was continued to constant weight, 16 hr. being sufficient as a rule. (b) Sorption: the vacuum line was closed and the water reservoir warmed in stages, sufficient time being allowed at each reservoir temperature for constant weight to be reached. At the lower water-vapour pressures this took about $1\frac{1}{2}$ hr., increasing at higher pressures to about 6 hr. because of 'collapse' of the fluffy freeze-dried material and also because of the larger absolute amounts of water to be transferred. Constancy of weight, once reached, was maintained for at least 40 hr. (c) Reversibility: loss of water by the rehydrated freeze-dried samples was followed by reversing the sequence of changes of reservoir temperature. (d) Test of performance: the water sorption curve of a specimen of solid egg albumin (not dissolved and freeze-dried) followed very closely the course described by Bull (1944).

Measurement of water inaccessible to solutes

Total cell volume V (Fig. 2) was determined by measuring the dilution of a solution of dextran by the intercellular water. Dextran concentrations and leakage from the cells were determined refractometrically (Brice & Halwer, 1951).

Osmotic water transfer was determined by measuring that portion of a cell pad which was inaccessible to sucrose or phosphate added in sufficient quantity to bring about the desired water movement. In two experiments sucrose and phosphate were added simultaneously, and concordant values for the inaccessible cell volume were obtained from independent analyses for the two substances. The experimental procedure was as follows: cell pads of volume V_c containing g gram of dry cells were prepared by centrifuging washed cells (10 ml. distilled water to 1 g. wet cells)

at 10,000 g. Each pad was resuspended in volume V_a of a solution of concentration c_a of one of the above solutes. The bacteria were removed by centrifuging and the supernatant of concentration c_f, containing the original added solution diluted by water in the cell pad, was decanted. In absence of leakage of intracellular solutes, the ratio c_a/c_f would be simply related to the amount of water in the pellet available to dilute the added solution. There was always a little leakage, and control runs were done by adding V_a ml. of water to another cell pad. In separate experiments
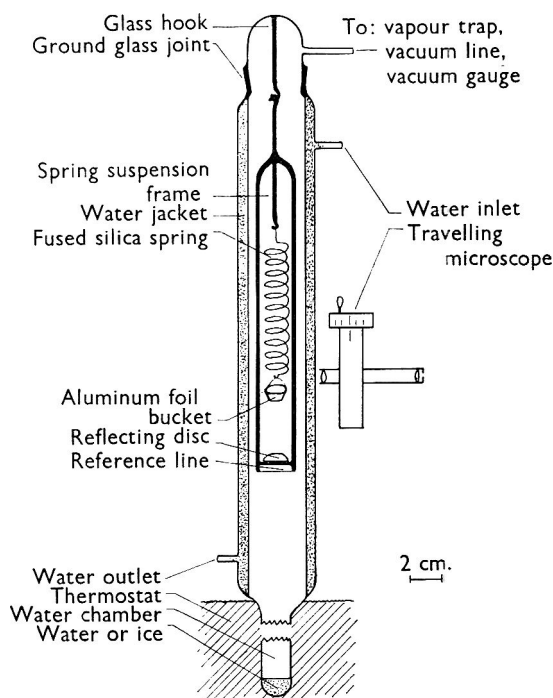
Fig. 1. Silica-spring sorption balance used to measure equilibration of bacterial cells with water vapour. Bacterial sample is in aluminium foil bucket. Changes in weight of the sample are measured by observing movement of the bucket with respect to a thin reference wire on the spring suspension frame, using a travelling microscope independently mounted. Changes of 0·03 mg could be detected. Absolute vapour pressure of water p above sample is determined by temperature of water or ice in cold trap at bottom of apparatus, while relative vapour pressure or water activity is determined by the ratio of p to the saturation vapour pressure of water p_0 at the temperature of the jacket surrounding the sample chamber. Direct thermal exchange between sample and cold trap is minimized by having constant temperature water flowing *downward* through jacket, and by a reflecting disk below sample.

it was found that the leakage of substances containing phosphate from the bacteria was a function of the ionic strength of the environment. Thus when phosphate was being used to measure inaccessible volume, the control run was done with a KCl solution of initial concentration c_a instead of pure water. If the concentration c_0 of the supernatant from the sample represents the increment in c_f caused by leakage, the true dilution factor is $c_a/(c_f - c_0)$ and the volume inaccessible to the solute in question, V' ml./g. dry cells, is given by

$$gV' = V_c + V_a(c_f - c_0 - c_a)/(c_f - c_0). \tag{1}$$

For dextran, c_0 varied from 0·08 to 0·5 c_f as the salt concentration was increased; for sucrose, c_0 varied from 0·06 to 0·03 c_f as the sucrose concentration was increased; for phosphate, c_0 was 0·01 to 0·06 c_f.

Sucrose analyses were made in the differential refractometer at 5461 Å. Phosphate was determined by the Fiske–SubbaRow method as modified by Boltz & Mellon (1947). In converting the analytical data for the equilibrium continuous phase to external osmolalities, use was made of the osmotic coefficient values of Stokes (1945) for phosphate and Scatchard, Hamer & Wood (1938) for sucrose.

THEORY

The cell model

Representing the bacterial cell by an osmotically sensitive protoplast and cell wall, the volume subdivisions are portrayed in Fig. 2.
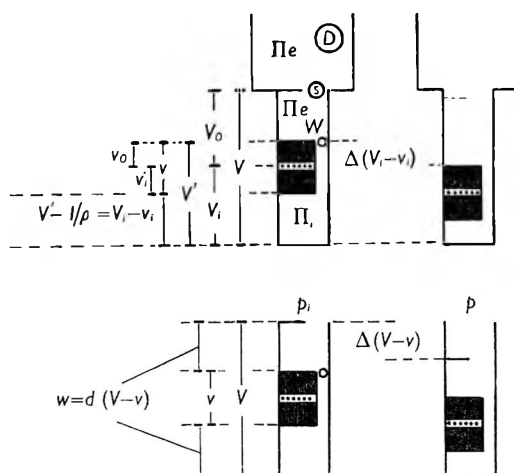

Fig. 2. Volume subdivisions of bacterial cell (not to scale). Upper left, fully hydrated cell immersed in solution of osmotic pressure Π_e. Upper right, cell partly dehydrated osmotically. Lower left, fully hydrated cell free of extracellular liquid equilibrated with water vapour at partial pressure p_1 close to saturation. Lower right, cell partially dehydrated by equilibration with water vapour at partial pressure p equivalent osmotically to the external solution used to produce osmotic dehydration in upper right. In all diagrams horizontal dots represent cytoplasmic membrane and black areas are cell solids of total weight 1 g. Solids above membrane belong to cell wall, those below membrane to cell interior. The corresponding volumes of solids and aqueous subdivisions are shown on the left and are self-explanatory. In upper left diagram, the wide area above the 'organism' represents a suspending medium containing dextran, D, sucrose, S, and water, W. The 'gateway' to the cell wall is large enough to admit molecules of S but D is excluded. The gateway to the osmotically sensitive part of the organisms, the channel to the right of the cell solids, will not admit S or D but is freely permeable to W. The symbols in the middle, $\Delta(V - v_i)$ and $\Delta(V - v)$, represent the volume changes actually measured by osmotic water distribution measurement and by water sorption measurements, respectively.

Notation

The following symbols will be used in addition to those in Fig. 2.

a, constants in the BET, Halsey, and logarithmic isotherms; a_w, water activity or relative vapour pressure p/p_0; B, constant in equation (18); c, constants in the

BET and logarithmic isotherms; d, density of water; f, a factor used in comparing water sorption data with osmotic volume changes, equation (16); m, molality, moles/1000 g. water. Subscript e refers to external medium, i to phase within cell osmometer; m', osmolality, $\nu\phi m$ for electrolytes, ϕm for simple non-electrolytes; M_w, molecular weight of water; n_i, moles of osmotically active solute in osmotically sensitive volume V_i (Fig. 2); p, p_0, vapour pressures of water over an aqueous solution and over pure water, respectively; r, mole ratio; moles of a solute per mole of water (equation 6), r, a constant in Halsey's isotherm and in equation (18); R, gas constant, 8·3144 joule deg.$^{-1}$ mole^{-1}; RH, relative humidity, 100 a_w; T, absolute temperature; w, water content of washed bacteria equilibrated with a vapour phase, g./g. dry cells; θ, fraction of water adsorption sites occupied by water; Π, osmotic pressure; ρ, density of dry bacterial cell solids; ϕ, molal osmotic coefficient of solvent, equations (2), (7); ν, number of ions into which a strong electrolyte dissociates.

Osmotic water transfer

The osmotic pressure equations (see Dick, 1959) for the osmotically responsive portion of the cell and for the ambient solution of reference substance, respectively, are

$$\Pi_i(V_i - v_i) = n_i\,\phi_i RT, \tag{2}$$

$$\Pi_e = m_e\phi_e dRT/1000 = m'_e RT/1000. \tag{3}$$

Assuming osmotic equilibrium between the interior and exterior of the osmometer and the absence of any hydrostatic pressure difference,

$$m'_e = m'_i, \tag{4}$$

$$\log(V_i - v_i) = \log\phi_i n_i - \log m'_e + 3. \tag{5}$$

Ideal behaviour would be represented by a straight line of unit slope, while lines of any other slope or of varying slope would indicate variation of $\phi_i n_i$ with external osmolality, failure to achieve osmotic equilibrium, or intrusion of a hydrostatic pressure term.

A sorption isotherm equivalent to equation (5)

A sorption isotherm is usually a plot of water content w against water activity a_w. In order to describe the whole course of cell water exchange a convenient parameter is the osmolality m'_e since this can be given a formal value for any water activity by way of Bjerrum's definition of the osmotic coefficient (Guggenheim, 1957)

$$\ln a_w = -\phi\Sigma r. \tag{6}$$

It follows that

$$\ln a_w = -10^{-3}\phi m M_w = -0.01806\,m'_e \text{ and } m'_e = -127.83\log a_w. \tag{7}$$

The description of sorption data by an equation equivalent to (5) would be the same as using an isotherm of the form

$$\log(V - v) = A - \log(-\log a_w), \tag{8}$$

$$A = \log\phi_i n_i + 0.8934. \tag{9}$$

A modified equation (5) *for sorption data*

The so-called logarithmic adsorption isotherm (De Boer, 1956)

$$\ln a_w = \theta/c'RT \qquad (10)$$

has been widely used for chemisorption, while for physical adsorption Halsey's (1948) equation can be written

$$\ln a_w = -a/RT\theta^{1/r}. \qquad (11)$$

Using the subscript 1 to mean completion of a monolayer of water ($\theta = 1$), we can put

$$-a/RT = \ln (a_w)_1 \quad \text{and} \quad \theta = (V-v)/(V-v)_1 \qquad (12)$$

giving

$$\ln a_w = \theta^{-1/r} \ln (a_w)_1 \qquad (13)$$

or, from (7),

$$m'_e = \theta^{-1/r} (m_e)_1 \qquad (14)$$

and

$$\log (V-v) = -r \log m'_e + r \log (m'_e)_1 + \log (V-v)_1. \qquad (15)$$

The corresponding equation for osmotic volume change is (5), which can be applied to the water volume of the whole cell by assuming

$$V-v = f(V_i - v_i) \qquad (16)$$

so that

$$\log (V-v) = -\log m'_e + \log \phi_i\, n_i + \log 1000\, f, \qquad (17)$$

where ϕ_i is a function of m'_e. Comparing (15) and (17),

$$\log (V-v) = -r \log m'_e + \log B, \qquad (18)$$

$$B = (m'_e)_1^r (V-v)_1, \qquad (19)$$

$$\phi_i n_i = Bm'_e{}^{(1-r)}/1000\, f. \qquad (20)$$

Equation (18) is applicable wherever a straight line is obtained by plotting $\log (V-v)$ against $\log (m'_e)$. With unit slope ($r = 1$), ideal solution behaviour is indicated; otherwise, $\phi_i n_i$ varies according to equation (20). In the 'osmotic' region this can be attributed to increase of osmotic coefficient with increasing m'_e if $r < 1$, rather than to a change of n_i. If the line is curved, equation (18) does not hold, because (*a*) the functional dependence of ϕ_i upon m'_e does not follow equation (20), or (*b*) n_i varies with m'_e because of leakage, precipitation, or dissolution, or (*c*) a hydrostatic term is omitted. The behaviour of r in the sorption region is discussed later.

Application of equation (18) *to inaccessible volume data*

The 'osmotic' cell volume V' is measured by a dilution technique. It comprises water within the osmometer and the entire dry volume of the cell, and reference to Fig. 2 will show that $(V_i - v_i)$ is not directly comparable to $(V-v)$. To reconcile osmotic and sorption data, the assumption is made (equation 16) that the water fraction and osmotic coefficient are the same for the osmotically responsive part of the cell as for the whole cell. The factor f can be obtained from dextran and sucrose impermeable volumes for standard conditions (subscript s); by inspection of Fig. 2,

$$f = (V_s\rho - 1)/(V'_s\rho - 1). \qquad (21)$$

RESULTS

Sorption experiments

A sorption cycle taken at 20° is illustrated in Fig. 3; the characteristic hysteresis will be noted. Any progressive change in the specimen during the lengthy experiment was ruled out by checking a few individual points with fresh cell samples. The points at the highest *RH* values (p/p_0 around 0·99) are of course of little significance, for maintenance of an *RH* of 99 % at 20° in these experiments depended upon maintaining a steady temperature difference between the main chamber and reservoir of only 0·15°. The results of experiments at 36° resemble those at 20° in accuracy and general appearance. The data refer to essentially non-viable cells; the fractional viable recovery after freeze-drying was about 10^{-1}, that at the end of a 14-day experiment about 10^{-10}.
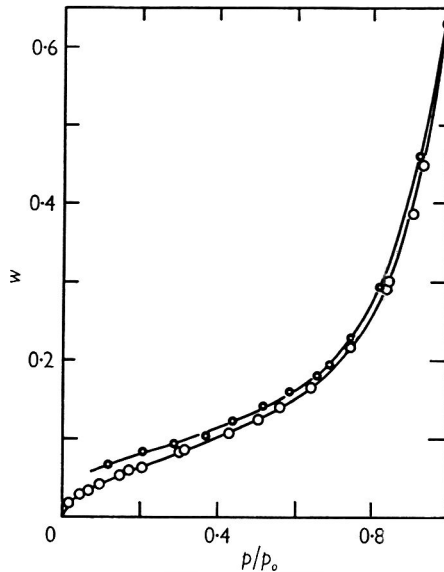

Fig. 3. Water sorption and desorption curves of *Serratia marcescens* at 20°. Abscissa, relative aqueous vapour pressure or water activity. Ordinate, grams sorbed water per gram dry weight of organisms. Open circles, water uptake; small heavy circles, water loss.

The data conform over the usual range (*RH* 10 to 45 %) to the BET equation (Brunauer, Emmett & Teller, 1938)

$$a_w/w(1-a_w) = (1+ca_w-a_w)/ac. \tag{22}$$

Values of the constants *a* and *c* are given in Table 1.

Osmotic shrinkage of cells in sucrose and phosphate solutions

The dilution measurements gave values of the cell volume inaccessible to the added solute, *V'* ml./g. dry solids. The cell water of *Serratia marcescens* and *Escherichia coli* B inaccessible to the solute was taken to be $V'-0·763$, assuming the density of the dry residue to be 1·31. This was converted by equation (16) into

Table 1. *BET constants* for bacteria and proteins*

See text equation (22). The constant a is usually interpreted as the weight of a complete monolayer of water in grams per gram dry material. The constant c is dimensionless and is related exponentially to the heat of adsorption of the water monolayer expressed in multiples of RT.

		D1		S1		D2	
		a	c	a	c	a	c
Serratia marcescens	20°	—	—	0·069	10·5	0·069	52·4
Pseudomonas aeruginosa†	30°	0·064	35·5	—	—	—	—
Staphylococcus aureus†	30°	0·054	48·5	—	—	—	—

	a	c
Egg albumin‡	0·0615	11·60
Stretched nylon‡	0·0176	5·97
Collagen‡	0·0952	17·80

* Brunauer, Emmett & Teller (1938).
† Imelik (1951), desorption of wet cells.
‡ Bull (1944), average of adsorption and desorption at 25 and 40°.

D1 indicates first desorption of wet cells. S1 is the first sorption of water by freeze-dried cells and D2 is the succeeding desorption.
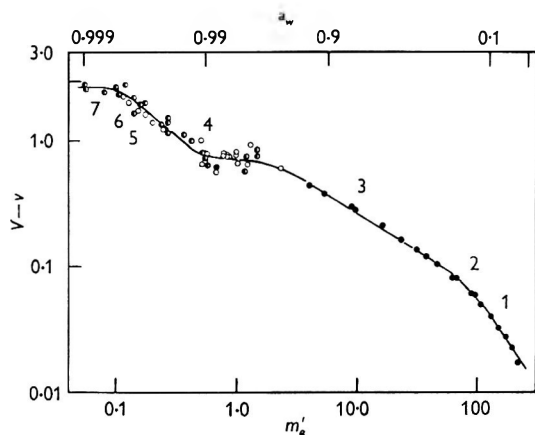

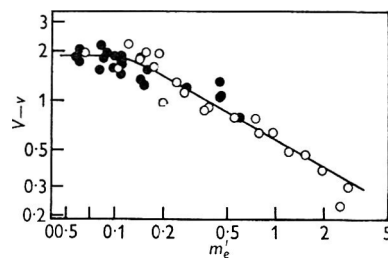Fig. 4   Fig. 5

Fig. 4. Water volume of one gram dry *Serratia marcescens* as a function of environmental osmolality. Lower scale of abscissae: actual or effective external osmolality. Upper scale, water activity or relative humidity × 1/100. In osmotic shrinkage experiments the actual external osmolality is determined by the nature and concentration of added solutes, and the recorded water volumes are corrected for the presence of the cell wall, which does not participate in the osmotic shrinkage (see text). In water sorption experiments the cell water is measured directly as a function of water activity in the vapour phase, and the corresponding effective osmolalities are calculated by text equation (7). Open circles: data obtained with added sucrose. Half-closed circles: with added sodium dihydrogen phosphate. Closed circles: sorbed water.

Fig. 5. Water volume of 1 g. dry *Escherichia coli* as a function of environmental osmolality. Open circles: data using added sucrose; closed circles: added sodium dihydrogen phosphate. See also legend to Fig. 4.

total cell water, $(V-v)$, per g. dry residue, using for V_s the volume inaccessible to dextran (for *S. marcescens*, 3·38 and for *E. coli* 2·65 ml./g.) and for V'_s the volume inaccessible to sucrose (for *S. marcescens*, 2·49, for *E. coli* 2·00 ml./g.). Application of equation (21), with ρ equal to 1·31, gives $f = 1·52$ for both cell types.

Combined osmotic and sorption data

The data for *Serratia marcescens* are given in Fig. 4. There appear to be three distinct regions of approximate conformity to equation (18), for which the appropriate values of the 'constants' r and B are assembled in Table 2. The osmotic shrinkage data for *Escherichia coli* are similarly plotted in Fig. 5 while the corresponding numerical data are included in Table 2.

Table 2. *'Constants' and limits of linear water exchange curves of bacteria*

See text equations (18) and (20) and Fig. 4. Column (1) identifies the linear region in Fig. 4, corresponding to slopes given in column (4). These zones extend over the range of external osmolalities in column (3) and the corresponding ranges of water volume are shown in column (2). Column (5) gives the values of the 'constant' B used in calculating the osmotic coefficients ϕ_i of column (7) and the apparent number of moles of osmotically active intracellular solutes per gram.

(1) Zone	(2) $(V-v)$ ml./g. dry cells	(3) m'_e	(4) r	(5) B	(6) $10^3 n_i$	(7) ϕ_i
Serratia marcescens						
1, monolayer sorption	0·02–0·05	230–100	1·20	16	—	—
3, multilayer sorption	0·1–0·5	53–4	0·60	1·07	—	—
5, osmotic shrinkage	1·0–2·2	0·46–0·1	0·82	0·44	—	—
	0·8	0·46	0·82	0·44	0·207	1·23
	2·2	0·145	0·82	0·44	0·207	(1·00)
Escherichia coli						
5, osmotic shrinkage	0·3–1·5	3·0–0·18	0·55	0·57	—	—
	0·3	3·0	0·55	0·57	0·176	3·54
	1·5	0·18	0·55	0·57	0·176	(1·00)
*E. coli**						
5, osmotic shrinkage	1·2–3·55	3·0–0·3	0·50	2·2	—	—
	1·2	3·0	0·50	2·2	0·80	3·16
	3·55	0·3	0·50	2·2	0·80	(1·00)
*Staphylococcus aureus**						
5, osmotic shrinkage	0·76–2·24	3·0–1·0	1·00	2·24	1·49	1·00

* Calculated from data of Mitchell & Moyle (1956). See text.

DISCUSSION

Continuity of osmotic and sorption data. Figure 4 represents both osmotic shrinkage and water sorption of washed *Serratia marcescens*. Since there is no discontinuity, our assumption that the water relationships of the osmotically responsive portion are not greatly different from those of the organism as a whole is probably a reasonable one. The compatibility of the two sets of data also suggests that at the highest humidities equilibrium was attained, despite the difficulty of maintaining nearly saturated atmospheres (Ashpole, 1952).

Zones of water transfer. The following zones can be recognized in Fig. 4: (1) water monolayer formation; (2) transition to multilayer adsorption; (3) multilayer water uptake; (4) transition to osmotic swelling; (5) osmotic swelling; (6) transition to constant cell volume; (7) limiting cell volume.

Zone (1). Formation of a monolayer. This is a steep linear region terminating when about 0·063 g. water has been taken up per g. dry cells. Similar initial phases have been noted in other processes, for example, in the adsorption of water on anatase (Halsey, 1948). The inflexion presumably signals completion of a monolayer of water, since the value 0·063 agrees quite well with the BET constant a (Table 1). Similar values of a have been obtained by Imelik (1951) for other micro-organisms (Table 1). They are fairly typical of an average protein; indeed our *Serratia marcescens* curves are scarcely distinguishable from that of egg albumin. It is therefore likely that all the cell solids are accessible to water vapour and that the sorption curves at low *RH* values are essentially those of the bacterial protein and nucleic acid which together comprise perhaps 90 % of the dry cell mass. If there are significant contributions from proteins of more extreme types, such as collagen and silk, these must average out. The sorbed monolayer is not readily removed (Fig. 3) as seen in the different BET constants c for sorption and desorption (Table 1), the latter having values comparable to those of Imelik (1951) for the initial drying of wet cells.

Zones (2) and (3). Multilayer adsorption. After a short curved transition, there is a straight line of reduced slope ($r \sim 0·6$) which extends to a_w about 0·92. This covers the range of the cases illustrated by Halsey (1948) and interpreted by him in terms of co-operative (multimolecular) adsorption upon a non-uniform surface.

Zone (4). Transition from co-operative adsorption to osmotic swelling. With continued application of equation (18) θ would approach ∞, with gross condensation of water. Instead, the slope r decreases, perhaps because of an intermolecular structure which limits the expansion of the cell solids. This would be equivalent to a decrease in the effective area of adsorbent.

This region of the sorption curve overlaps the curve of osmotic dehydration. Evidently, at *RH* values high enough to distend the hydrophilic cell structures to their maximum extent, some or all of the small molecular solutes have dissolved, and the further course of events with increasing *RH* is best considered in terms of the osmotic coefficient ϕ_i. As the *RH* increases from 80 to 99, the slope continues to decrease somewhat, with an average r of about 0·21. Thus the variation of $n_i\phi_i$ with external osmolality follows equation (20) roughly, increasing about 12-fold as m'_e increases from 0·5 to 12·5. Since some solutes may be precipitated at the lowest *RH* values within this region, the osmotic coefficient must increase by a larger factor than 12 and the increase must be due mainly to interactions involving the polymeric components and the non-solvent water (hydration).

Zones (5) to (7). Limited osmotic swelling leading to constant cell volume. When $m'_e \sim 0·6$ there is a fairly abrupt transition to a third linear region with $r \sim 0·82$. This is the region of osmotic swelling. The behaviour deviates appreciably from the Boyle–Van't Hoff equation (Lucké & McCutcheon, 1932), with r differing from unity. If the osmotic coefficient is unity at the 'dilute' end of this linear zone, then it rises to 1·23 when the external osmolality is increased to 0·46 (Table 2).

If equation (18) were followed at external osmolalities below 0·1, the line in Fig. 4 would be extended until lysis occurred, perhaps with a shift in slope towards ideal behaviour ($\phi_i = 1$). *Serratia marcescens* and many other bacteria, however, are not lysed even by distilled water. Fig. 4 shows that there is a rather sharp

transition from osmotic swelling to the maintenance of large internal hydrostatic pressures at constant volume.

The existence of the zone of constant cell volume was deduced by Mitchell & Moyle (1956) from values of the volume of *Escherichia coli* inaccessible to inorganic phosphate in presence of different concentrations of NaCl. The transition appeared to commence when m'_e was about 0·4, but this conclusion rested upon a single experimental point; a recalculation shows that equation (18) is followed throughout, with $r \sim 0·5$ (Fig. 6). Our own measurements on *E. coli* (Fig. 5), agree with the recalculated Mitchell & Moyle results and show also that the external osmolality must be reduced to about 0·18 before the slope of the line begins to fall off. The cell volume is constant when m'_e is less than 0·1.
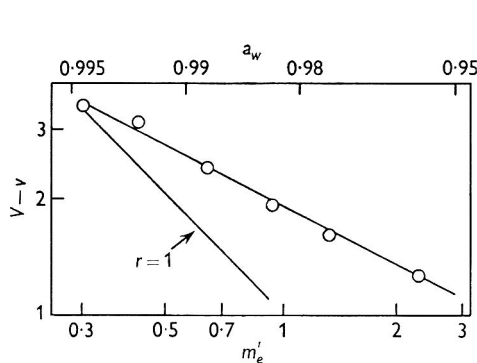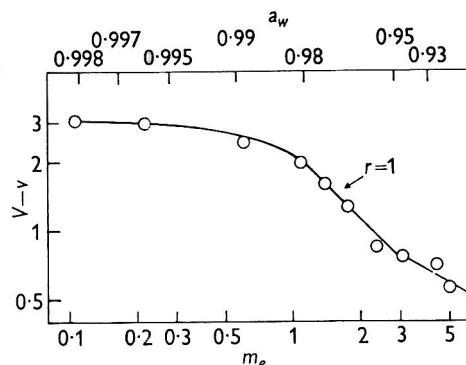

Fig. 6   Fig. 7

Fig. 6. Water volume of one gram dry *Escherichia coli* as a function of environmental osmolality, from volume measurements in NaCl solutions using inorganic phosphate as tracer. Values of phosphate impermeable volume estimated from Mitchell & Moyle's (1956) graph, their Fig. 4, and corrected for the fact that the cell wall contributes to the dry weight but does not participate in the osmotic movement of water. The correction factor *f* (equation 16) was taken to be 1·52, determined as described in this paper.

Fig. 7. Water volume of one gram dry *Staphylococcus aureus* at different relative humidities. Data of Mitchell & Moyle (1956), given in their Fig. 1 as a plot of weight of cell sample against reciprocal of the molality of the sucrose solution used to control the *RH*. Values read from this curve were corrected for dry weight of cell sample, taken as 47 mg, and for osmotic coefficient of water in sucrose solutions (Scatchard *et al.* 1938).

Limiting cell volume in vapour phase equilibration. In vapour phase equilibration of osmotically resistant cells, the increase of internal hydrostatic pressure must always be such as to bring the vapour pressure of the cell into equilibrium with the atmosphere; the water content must remain nearly constant until the atmosphere becomes fully saturated. At this point the cell is in equilibrium with both liquid water and water vapour and the equilibrium water content of the system is indeterminate. In the presence of a trace of external solute, however, it is infinite, and uptake of water in a saturated or slightly undersaturated atmosphere will proceed indefinitely. The predicted levelling-off of water content in vapour phase equilibration has been found for *Staphylococcus aureus* by Mitchell & Moyle (1956), starting when m'_e is about 0·6 (Fig. 7). At lower *RH* values there is, as with *Serratia marcescens*, an ideal region succeeded by one of increasing osmotic coefficient.

Solute content of bacteria. The values of n_i (Table 2) indicate the amounts of

osmotically active solutes per g. dry cells, while the values of m'_e at the limiting (maximum) cell volume give the internal osmolality. It is of interest that Mitchell & Moyle's *Escherichia coli*, grown in a salt-glucose medium, contained four times as much dissolved substance as ours, grown in nutrient broth of low salt content. *Staphylococcus aureus* contains much more dissolved material than *E. coli* or *Serratia marcescens*—1·49 m-mole/g., and a limiting internal osmolality of 1·0. It is surprising that the apparent osmotic coefficient remains constant over a three-fold range of m'_e (1·0 to 3·0).

Relative humidity and the stability of air-borne bacteria. At several points the interpretation of the water uptake curve of *Serratia marcescens* is speculative and at others incomplete. Nevertheless, there is relevance to the problem of stability of air-borne bacteria: (1) The existence of inflexions in the curve invites more detailed interpretation and comparison with stability data (Monk, McCaffrey & Davis, 1957). (2) The hysteresis-like retention of water during desorption suggests that bacterial stability may depend upon the history of the particle, especially upon the direction of water transfer. A more detailed study might reveal slow irreversible changes in the dehydrated organisms or the existence of potential energy barriers, e.g. super-saturation, failure to dissolve, formation of cross-linked structures which do not expand reversibly on wetting, capillary effects of the type discussed by Hill (1952). R. M. Izatt & T. Hatch (to be published) have shown clearly the effects of relatively gross amounts of hygroscopic additives in modifying the water uptake curves. (3) Clearly the curve of Fig. 4 does not represent a series of true equilibria, for metabolic processes continue (Monk *et al.* 1957) and viability is gradually lost. Despite the enormous retardation of these processes, however, there must be zones of water content within which the diffusion rates of intracellular molecules or polymeric submolecules become sharply dependent upon *RH*; one such region presumably would be within the overlapping zones of non-ideal solution and of failing co-operative multilayer adsorption. Here one might well seek special biological effects, arising perhaps from random translocations of specific molecules present in statistically small numbers. (4) Comprehensive data such as those in Fig. 4 are required in any theoretical study of air-borne organisms since rates of 'equilibration' with an atmosphere will depend partly upon the amounts of water to be transferred for a given change of *RH*. The differential amounts, dV/da_w, vary by more than 2 orders of magnitude between *RH* 0 and 100%.

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Biochemical Activity of Micro-organisms Isolated from Various Regions of the World Ocean

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SUMMARY

There were found considerably fewer biochemically active micro-organisms among heterotrophs inhabiting the equatorial–tropical zone of the World Ocean than in the high latitude area. Many micro-organisms with many-sided enzymic activities were present in the microbial population of the near-polar area, determining more profound transformations of organic matter in the waters of these areas as compared with the tropical area and consequently an increased concentration of biogenic substances. It is suggested that there is a unique exchange between the areas of low latitudes and those of high latitudes in the World Ocean. Currents driving equatorial–tropical waters to the north and south carry with them organic matter, chiefly allochthonous in origin; this material is more completely decomposed by the microbial species which inhabit high latitudes. The liberated biogenic substances are carried by currents into the depths of other geographic areas, thus increasing the reserve of substances taking part in the primary production of organic matter in these areas of the World Ocean.

INTRODUCTION

Microbiological investigations in the Indian, Atlantic and Pacific Oceans, in the Central Arctic, Norwegian and Greenland Seas and in the seas of the Antarctic, made it possible to accumulate data on the quantitative distribution (Kriss, Abyzov, Lebedeva, Mishustina & Mitskevitch, 1960) and species composition of the microbial population in the World Ocean.

The ultrafiltration method (Kriss, 1959) was used to obtain cultures of micro-organisms inhabiting the seas and oceans examined. The primary treatment of the water and mud samples was done in the microbiological laboratory on board ship immediately after collection. Water samples were filtered through membrane filters which subsequently were placed on nutrient agar (tryptic hydrolysate of fish flour, 50 g.; ocean water, 1000·0 ml.). Mud suspensions were inoculated on this agar in appropriate dilutions. The colonies were transferred to agar slopes of the same composition; often representatives of dissimilar colonies were taken. The test tubes were kept in the ship's laboratory until visible growth appeared; after this they were stored in a refrigerator (+2° to +4°) till the expedition came to an end.

Thus a collection of over 4000 strains of heterotrophic micro-organisms was obtained. This collection reflected the composition of the microbial population in various places of the World Oceans, i.e. from the North Pole to the Antarctic coast and at different depths, from the surface down to the bottom. The whole collection,

with the exception of strains which did not survive, was investigated from the standpoint of the capacity of strains to metabolize proteins and carbohydrates and to use bound oxygen and inorganic nitrogen compounds. This was of significance not only for the species characteristics of the microbial forms found in the World Ocean, and which can grow in laboratory conditions on artificial nutrient media of organic or inorganic composition, but also for obtaining some idea of the biochemical activity of these microbial species in decomposing readily assimilable forms of organic matter in seas and oceans.

METHODS

Proteolytic activity was studied on meat-peptone gelatine, meat-peptone broth, and milk. During 15 days after inoculation, gelatine liquefaction, milk coagulation and peptonization, and ammonia and hydrogen sulphide formation in broth were recorded. Ammonia was detected by a litmus paper strip and hydrogen sulphide by the blackening of a lead acetate paper. The two paper strips were fixed to a cotton-wool stopper of a test tube.

The reddening of peptone water + azolitmin (peptone, 1 %; azolitmin, 0·02 %) containing the following carbohydrates (1 %): glucose, sucrose, maltose, lactose, mannitol, was taken to indicate fermentation. Uncoloured zones around the streak on potato agar, after treatment with Lugol's iodine solution, pointed to the ability of a strain to decompose starch. Czapek's medium (g.:K_2HPO_4, 0·5; $MgSO_4$, 0·5; NaCl, 0·5; KNO_3, 1·0; $CaCO_3$, 2; sucrose, 20·0; agar, 20·0; $FeSO_4$, trace; distilled water, 1000·0 ml.) was used to establish whether mineral sources of nitrogen were utilized and whether nitrates were reduced to nitrites.

When growth was not observed, sea salt was added to meat-peptone gelatine, milk, peptone water with carbohydrates, potato agar, or Czapek's medium with an inorganic source of nitrogen, the content of sea salt in a medium then being 2 %.

RESULTS

Over 500 strains (17 % of the collection) brought about clear hydrolysis of protein (gelatine, milk proteins; Table 1). Much decomposition of protein with the formation of hydrogen sulphide was observed in 4 % of cultures. According to ZoBell & Upham (1944) from 60 strains isolated from the Pacific Ocean water and mud off the Californian coast, 30 hydrolysed casein and 47 liquefied gelatine. The greater percentage of proteolytic strains in ZoBell & Upham's investigations might be due to their organisms having been isolated from the coastal zone of the Pacific Ocean. The same reasons may have been responsible for the predominance of strains liquefying gelatine (451 of the total of 733 cultures) in the collection of micro-organisms isolated from the Black Sea (Kriss, Markianovich & Rukina, 1954). The Black Sea is an intracontinental basin with a considerable continental effluent.

Table 1 shows the activity of bacterial cultures obtained in ocean areas with regard to carbohydrate dissimilation with the formation of acid. The highest percentage of strains (26·4 %) fermented glucose, acidifying the medium. The formation of acid in the media with sucrose or maltose was observed in about the same proportion of cultures (18–19 %). Then followed mannitol (15 %) and lactose (10 %). These data indicate only the capacity of the cultures from the oceanic collection to ferment some carbohydrates and mannitol with the formation of acids. The overwhelming majority of strains (> 80 %) grew in media with glucose, sucrose,

maltose, lactose, or mannitol. Similar results were obtained in the medium with starch as carbon source. However, the percentage of strains hydrolysing starch was comparatively great (63·3 %).

ZoBell & Upham (1944) reported that 75 % of strains in their collection fermented glucose with acid formation. Among the Black Sea cultures the percentage decomposing glucose, maltose, sucrose, lactose or mannitol giving acid was also considerably greater than among strains in the oceanic collection.

Of 3158 strains of micro-organisms found in various ocean areas 2873 grew on the medium with a mineral source of nitrogen (Table 1). However, only 40 % of the strains reduced nitrate. The percentage of Black Sea strains which reduced nitrate to nitrite was about the same: about 30 % of the whole collection.

Differences in the numbers of microbial forms from the Atlantic, Indian and Pacific Oceans, which hydrolysed protein and dissimilated carbohydrate are noteworthy. The percentage of strains which hydrolysed gelatine or casein, decomposed protein to give hydrogen sulphide, and fermented glucose, sucrose, maltose or mannitol with acid formation, was highest in the Atlantic Ocean and lowest in the Pacific Ocean. In this respect the Indian Ocean samples were intermediate. Table 1 shows the range of these quantitative differences. In the Pacific Ocean the percentage of strains which liquefied gelatine, formed hydrogen sulphide from meat-peptone broth, and peptonized milk coagulation was 4 to 5 times lower than in the Atlantic Ocean. The percentage of strains forming acid from carbohydrates and mannitol was 2 or 3 times to 5 times higher in the Atlantic Ocean as compared with the Pacific Ocean.

Since nearly all the water samples were collected far from land, at various depths in open areas of the Atlantic, Indian, and Pacific Oceans, it is suggested that the differences reflect not the microbiological specificity of these parts of the World Ocean but are influenced by geographic factors.

The map (Fig. 1) shows that in the Atlantic Ocean the studies were made mainly in high latitudes including areas adjacent to the Arctic Ocean, while in the Pacific Ocean the stations were only in the subtropical, tropical, and equatorial areas. Microbiological sections crossed all geographic zones from the north tropical region to the Antarctic region in the Indian Ocean.

A more detailed study clearly showed geographical regularities in the distribution of biochemically active and inactive species of heterotrophs in the World Ocean. In the Indian Ocean, the greatest percentage of proteolytic cultures was found in the high latitude areas (Table 2). The percentage of cultures which liquefied gelatine and peptonized and decomposed protein with hydrogen sulphide formation was a few times higher in the Antarctic and sub-Antarctic areas than in the equatorial zone. The same correlation was observed in the percentage of strains which fermented carbohydrates among the cultures from the Antarctic seas and from tropical zones.

Similar results were obtained with samples from the Atlantic Ocean: there was a greater percentage of strains which hydrolysed protein and fermented carbohydrates in the sub-Arctic and arctic area samples than in the tropical samples (Table 3).

The biochemical activity of the great majority of cultures in the Pacific Ocean collection was not high. Investigations in the Pacific Ocean proved that the proteolytic and fermentative activities of the microbial population were comparatively weak in the low latitude areas of the World Ocean. It is noteworthy that the scarcity

Table 1. *Biochemical activity of heterotrophic micro-organisms isolated from various regions of the World Ocean*

Numbers show the following: numerator = number of strains examined; denominator = number of strains growing on a certain medium; numbers in parentheses = % of strains growing capable of a certain enzymic process.

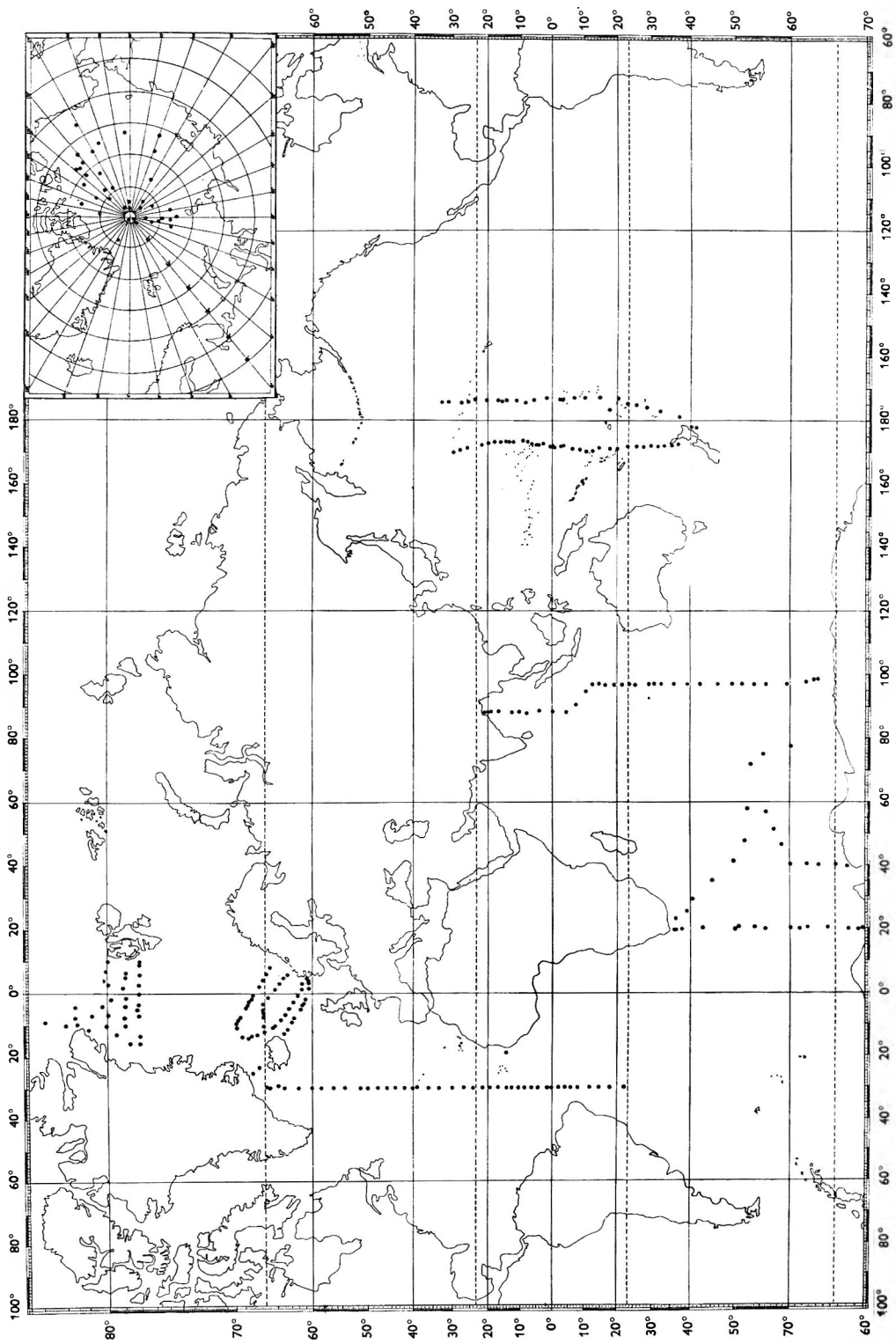|	Gelatine hydrolysis	H$_2$S formation	Peptonization of milk proteins	Peptone water acid with					Starch hydrolysis	Utilization of mineral nitrogen	Denitrification
				Mannitol	Glucose	Sucrose	Maltose	Lactose			
Atlantic Ocean, Greenland and Norwegian seas, Central Arctic	1089 / 1052 (31·2)	1068 / 961 (7·7)	1021 / 1021 (29·5)	1067 / 790 (25·6)	1084 / 886 (47·6)	1093 / 850 (30·9)	1074 / 827 (31·9)	1077 / 772 (14·6)	1091 / 989 (46·8)	1092 / 1001 (91·7)	1092 / 503 (46·1)
Indian Ocean and seas of Antarctic	900 / 889 (14·7)	864 / 790 (3·5)	898 / 898 (15·2)	883 / 805 (17·1)	899 / 852 (21·1)	897 / 822 (19·0)	880 / 828 (18·4)	883 / 797 (8·8)	901 / 820 (72·7)	893 / 843 (94·4)	893 / 351 (39·3)
Pacific Ocean	1202 / 1155 (7·6)	1184 / 1097 (1·3)	1203 / 1203 (8·2)	1179 / 1068 (5·2)	1191 / 1099 (13·3)	1197 / 1155 (9·5)	1184 / 1107 (11·7)	1192 / 1061 (7·3)	1203 / 1102 (71·0)	1173 / 1104 (94·1)	1173 / 419 (35·6)
Total in the World Ocean	3200 / 3096 (17·7)	3116 / 2848 (4·1)	3122 / 3122 (17·1)	3129 / 2663 (14·9)	3174 / 2837 (26·4)	3187 / 2827 (18·7)	3138 / 2762 (19·7)	3152 / 2630 (9·9)	3195 / 2911 (63·3)	3158 / 2873 (90·97)	3158 / 1273 (40·3)

Fig. 1. Microbiological Stations in the World Ocean.

Table 2. *Biochemical activity of heterotrophic micro-organisms isolated from various geographic zones of the Indian Ocean*

Numbers show the following: without parentheses = number of strains growing on a certain medium; in parentheses = % of strains capable of a certain enzymic process.

	Gelatine hydrolysis	H$_2$S formation	Peptoniza-tion of milk proteins	Peptone water acid with					Starch hydrolysis	Utilization of mineral nitrogen*	Denitrifi-cation*
				Mannitol	Glucose	Sucrose	Maltose	Lactose			
Antarctic area, 70° S.–50° S.	274 (19·3)	221 (5·9)	277 (16·6)	219 (29·7)	259 (28·6)	233 (28·8)	239 (27·2)	227 (12·3)	234 (57·7)	282 (91·1)	282 (40·8)
Sub-Antarctic area, 50° S.–40° S.	111 (20·7)	91 (7·9)	111 (24·3)	104 (19·2)	106 (34·0)	98 (32·7)	100 (25·0)	105 (8·6)	100 (63·0)	106 (94·3)	106 (37·7)
Southern subtropical area, 40° S.–23° S.	163 (21·5)	143 (4·2)	164 (23·2)	154 (21·4)	158 (23·4)	156 (18·6)	159 (20·1)	134 (11·2)	154 (67·5)	162 (95·1)	162 (22·2)
Southern tropical area, 23° S.–10° S.	134 (5·9)	132 (0·8)	136 (8·0)	126 (7·1)	128 (11·6)	132 (0·8)	130 (10·0)	129 (5·4)	134 (87·3)	134 (97·0)	134 (61·1)
Equatorial area, 10° S.–10° N.	131 (5·3)	128 (0·8)	133 (6·7)	127 (3·1)	126 (8·7)	130 (6·9)	127 (6·3)	127 (3·9)	122 (89·3)	132 (99·2)	132 (43·9)
Northern tropical area, 10° N.–23° N.	76 (6·6)	75 (0·0)	77 (5·2)	75 (9·3)	75 (12·0)	73 (13·7)	73 (12·3)	75 (8·0)	76 (89·5)	77 (98·7)	77 (26·0)

* In this column numbers without parentheses show the number of strains examined.

Table 3. *Biochemical activity of heterotrophic micro-organisms isolated from various geographic zones of the Atlantic Ocean*

Numbers show the following: without parentheses = number of strains growing on a certain medium; in parentheses = % of strains capable of a certain enzymic process.

	Gelatine hydrolysis	H₂S formation	Peptonization of milk proteins	Peptone water acid with					Starch hydrolysis	Utilization of mineral nitrogen*	Denitrification*
				Mannitol	Glucose	Sucrose	Maltose	Lactose			
10° S.–28° S.	86 (0·0)	83 (0·0)	86 (4·7)	71 (5·6)	83 (27·7)	81 (6·2)	75 (6·7)	56 (1·8)	79 (70·9)	86 (96·5)	86 (66·3)
10° S.–10° N.	99 (5·1)	98 (0·0)	100 (8·0)	79 (7·0)	95 (20·0)	89 (7·9)	88 (8·0)	53 (11·3)	90 (84·4)	100 (94·0)	100 (58·0)
10° N.–28° N.	123 (8·9)	118 (0·0)	123 (0·8)	78 (2·6)	117 (6·8)	97 (3·1)	115 (0·0)	95 (3·2)	118 (83·1)	123 (98·4)	123 (46·3)
23° N.–40° N.	77 (88·8)	72 (0·0)	77 (2·6)	55 (0·0)	63 (20·6)	62 (8·2)	65 (1·5)	63 (8·2)	76 (77·6)	78 (93·6)	78 (42·3)
40° N.–60° N.	32 (65·6)	29 (6·9)	32 (56·3)	24 (54·2)	26 (78·1)	25 (08·0)	23 (78·0)	26 (42·3)	32 (25·0)	36 (86·1)	36 (41·7)
60° N.–80° N.	416 (89·4)	356 (17·7)	379 (45·9)	273 (38·1)	287 (61·0)	277 (49·1)	246 (52·4)	266 (21·1)	383 (28·5)	440 (87·0)	440 (38·4)
80° N.–to the north	219 (40·1)	205 (4·4)	224 (44·2)	210 (34·8)	215 (76·7)	219 (42·5)	215 (48·8)	213 (16·0)	211 (27·0)	229 (94·3)	229 (49·8)

* In this column figures without parentheses show the number of strains examined.

of biochemically active microbial species separates the northern subtropical zone in the section along 172° E. (Table 4) from the northern subtropical zone in the section along 174° W. (Table 5) and from the southern subtropical zones in both sections. In the northern subtropical zone, the microbiological stations were worked in the area of the powerful Kuroshio current which is a branch of the north-equatorial current, poor in biochemically active heterotrophs.

Most of the microbial cultures from the equatorial–tropic zone hydrolysed starch. The percentage of cultures which hydrolysed starch was lower in the high latitudes of the Indian and Atlantic Oceans.

The differences in the number of denitrifying micro-organisms in various geographic zones of the World Ocean were negligible. The data obtained contradict Brandt's hypothesis (Brandt, 1904) which suggests the activities of micro-organisms which reduce nitrate and nitrite as the reason for scarce plant life in the tropical areas. The percentage of denitrifiers found in the equatorial–tropical area was not greater than in the subarctic and subantarctic areas where the water is most rich in phytoplankton.

The considerable percentage of strains which utilize inorganic nitrogen and which are about equal in all the geographic zones of the World Ocean is noteworthy. Of the cultures examined 86–99 % developed well on media with an inorganic nitrogen source.

Since microbiological sections crossed the equatorial–tropical area four times (twice in the Pacific Ocean, once in the Indian Ocean, and once in the Atlantic Ocean), the northern subtropical zone three times (twice in the Pacific Ocean and once in the Atlantic Ocean) and the southern subtropical zone three times (twice in the Pacific Ocean and once in the Indian Ocean), it was of interest to compare the biochemical activity of heterotrophs in various geographic zones of the whole World Ocean. Table 6 shows that the equatorial–tropical zone of the World Ocean is distinguished by very few microbial species which decompose protein and ferment carbohydrate. In the Arctic, sub-Arctic, Antarctic, and sub-Antarctic areas the percentage of strains which decompose protein is 4 to 60 times greater, and the percentage of strains which ferment mannitol, glucose, sucrose, maltose or lactose 2 to 11 times greater than in the equatorial zone. These data show that the processes of organic matter decomposition and the liberation of biogenic elements are more vigorous in high latitudes. Although the number of heterotrophs there is not as great as near the equator, and their biochemical activity is influenced by low temperatures, the presence of comparatively great numbers of microbial forms with many-sided enzymic activity determines, however, more profound transformations of organic matter in high latitudes than in tropical areas. Hence, the increased content of biogenic substances needed for the development of marine plant life in the Arctic, sub-Arctic, and sub-Antarctic areas of the World Ocean. In fact, considerable phytoplankton biomasses, which are many times greater than those in the tropical areas, are observed in these geographic zones. Evidently, a relative abundance of microbial forms in high latitude waters, producing versatile reactions in the decomposition of organic matter, results in increased concentrations of biogenic substances not only in the waters of these latitudes. These products of organic matter decomposition and transformation fill the depths of other areas of the World Ocean by virtue of the deep currents which penetrate to the lower latitudes.

Table 4. *Biochemical activity of heterotrophic micro-organisms isolated from various geographic zones of the Pacific Ocean, in the section along 172° E.*

Numbers show the following: without parentheses = number of strains growing on a certain medium; in parentheses = % of strains capable of a certain enzymic process.

	Gelatine hydrolysis	H₂S formation	Peptoniza-tion of milk proteins	Peptone water acid with					Starch hydrolysis	Utilization of mineral nitrogen*	Denitrifi-cation*
				Mannitol	Glucose	Sucrose	Maltose	Lactose			
40° S.–23° S.	190 (15·8)	174 (1·7)	195 (14·4)	178 (7·3)	174 (23·0)	188 (14·9)	183 (24·0)	174 (12·6)	178 (59·6)	193 (08·3)	193 (43·0)
23° S.–10° S.	139 (18·7)	136 (2·2)	149 (16·1)	128 (8·6)	137 (24·1)	144 (20·8)	136 (24·3)	137 (18·98)	125 (63·2)	136 (89·0)	136 (30·2)
10° S.–10° N.	222 (3·2)	224 (0·0)	227 (2·6)	201 (2·5)	206 (5·8)	224 (3·1)	218 (3·7)	218 (2·8)	213 (79·8)	221 (96·4)	221 (28·5)
10° N.–23° N.	137 (0·0)	126 (0·8)	141 (5·0)	130 (0·8)	137 (2·9)	137 (2·9)	129 (1·6)	134 (0·0)	128 (79·7)	136 (97·1)	136 (44·9)
23° N.–40° N.	71 (1·4)	67 (0·0)	75 (1·3)	72 (0·0)	69 (7·3)	74 (0·0)	70 (2·9)	72 (1·4)	67 (82·1)	73 (97·3)	73 (52·1)

* In this column numbers without parentheses show the number of strains examined.

Table 5. *Biochemical activity of heterotrophic micro-organisms isolated from various geographic zones of the Pacific Ocean, in the section along 174° W.*

Numbers show the following: without parentheses = number of strains growing on a certain medium; in parentheses = % of strains capable of a certain enzymic process.

	Gelatine hydrolysis	H₂S formation	Peptonization of milk proteins	Peptone water acid with					Starch hydrolysis	Utilization of mineral nitrogen*	Denitrification*
				Mannitol	Glucose	Sucrose	Maltose	Lactose			
40° S.–23° S.	94 (11·7)	90 (2·2)	97 (8·3)	88 (9·1)	87 (13·8)	92 (10·9)	79 (10·1)	85 (8·2)	93 (66·7)	95 (89·5)	95 (32·6)
23° S.–10° S.	74 (2·7)	73 (1·4)	76 (7·9)	75 (2·7)	69 (11·6)	71 (8·5)	73 (5·5)	61 (1·6)	73 (75·3)	75 (98·7)	75 (29·3)
10° S.–10° N.	137 (2·9)	128 (0·0)	143 (4·9)	121 (8·3)	132 (12·1)	139 (8·6)	136 (8·8)	105 (0·95)	137 (76·6)	144 (94·4)	144 (24·3)
10° N.–23° N.	53 (7·6)	50 (0·0)	57 (10·5)	46 (6·5)	51 (17·7)	52 (17·3)	47 (19·2)	44 (15·9)	48 (62·5)	57 (93·0)	57 (40·4)
23° N.–40° N.	38 (7·9)	29 (18·8)	43 (11·6)	29 (10·3)	37 (13·5)	34 (11·8)	36 (19·4)	31 (9·7)	40 (45·0)	43 (90·7)	43 (51·2)

* In this column numbers without parentheses show the number of strains examined.

Table 6. *Biochemical activity of heterotrophic micro-organisms isolated from various geographic zones of the World Ocean*

Numbers show the % of strains capable of a certain enzymic process.

	Gelatine hydrolysis	H₂S formation	Peptonization of milk proteins	Peptone water acid with					Starch hydrolysis	Utilization of mineral nitrogen	Denitrification
				Mannitol	Glucose	Sucrose	Maltose	Lactose			
70° S.–50° S.	19·3	5·9	16·6	20·7	28·6	28·8	27·2	12·3	57·7	91·1	40·8
50°–40° S.	20·7	7·9	24·3	19·2	34·0	32·7	25·0	8·6	63·0	94·3	37·7
40° S.–28° S.	17·0	2·7	16·2	12·9	21·2	15·4	19·9	11·2	64·0	93·1	38·3
28° S.–10° S.	6·7	1·2	10·3	6·5	18·4	11·7	13·5	9·1	74·7	94·8	43·4
10° S.–10° N.	3·9	0·2	4·9	5·8	10·4	7·2	6·1	4·3	81·8	96·0	38·6
10° N.–23° N.	5·1	0·3	4·5	3·95	7·9	7·2	5·2	4·6	80·5	97·2	41·0
23° N.–40° N.	16·1	2·4	4·1	1·9	14·8	3·5	5·9	3·6	72·1	94·3	47·9
40° N.–60° N.	65·6	6·9	56·3	54·2	73·1	68·0	73·9	42·3	25·0	86·1	41·7
60° N.–80° N.	39·4	17·7	45·9	38·1	61·0	49·1	52·4	21·1	28·5	87·0	38·4
North Arctic 80° N. and to the north	46·1	4·4	44·2	34·8	76·7	42·5	48·8	16·0	27·0	94·3	40·8

Table 7. *Survival of heterotrophic micro-organisms isolated from various geographic zones of the World Ocean (cultivated during a year in Moscow)*

	Atlantic Ocean, Norwegian and Greenland Seas			Indian Ocean and seas of the Antarctic			Pacific Ocean		
	No. of strains isolated	No. of strains not surviving	% of strains not surviving	No. of strains isolated	No. of strains not surviving	% of strains not surviving	No. of strains isolated	No. of strains not surviving	% of strains not surviving
70° S.–50° S.	.	.	.	479	142	29·6	.	.	.
50° S.–40° S.	.	.	.	165	57	34·5	.	.	.
40° S.–28° S.	.	.	.	269	95	35·7	538	248	46·6
23° S.–10° S.	89	4	4·5	146	12	8·2	330	112	34·0
10° S.–10° N.	148	5	3·4	137	2	1·5	469	97	20·7
10° N.–23° N.	109	3	2·8	90	2	2·2	300	95	31·6
23° N.–40° N.	73	9	12·3	.	.	.	261	141	54·0
40° N.–60° N.	61	18	29·5	.	.	.	.	.	.
60° N.–80° N.	1018	471	46·5	.	.	.	.	.	.

So far, the reasons for the scarcity of biochemically active microbial forms in tropical waters and their increased concentration in high latitude waters have not been elucidated. Possibly, a relatively high concentration of allochthonous organic matter in the tropical waters, which is easily assimilable by micro-organisms and which is the reason for a considerable density of heterotrophs in the equatorial-tropic zone of the World Ocean, does not necessarily bring about enzymic reactions that would guarantee a full utilization of the organic matter. The scarcity of food resources in the high latitude waters may result in the adaptation of heterotrophs which allows a more economic use of organic substances at the expense of their more profound decomposition and transformation.

There was a notable connexion between the survival of marine heterotrophs under laboratory conditions and the geographic position from which the cultures were obtained. Table 7 shows data on the survival of heterotrophs obtained from various geographic zones of the Atlantic, Pacific and Indian Oceans. Taking into account that the time of cultivation in the laboratory was not the same for strains from the various areas of the World Ocean, only data from the first year's cultivation were taken for comparison. The percentage of death among strains from high latitudes was a few times higher than among cultures obtained in the equatorial-tropical zone. In the Atlantic Ocean collection the percentage of dead cultures from the sub-Arctic and Arctic areas (Norwegian and Greenland Seas) was 6 to 15 times greater than among cultures obtained in low latitudes. About the same correlation was observed in comparing the percentage of deaths of strains from the equatorial-tropical zone of the Indian Ocean and from the Antarctic seas. The percentage of cultures obtained in the equatorial zone that did not survive was about half those from subtropical areas of the Pacific Ocean.

Taking into consideration that the collection of heterotrophs from various areas of the World Ocean was made up, to a great extent, of micro-organisms inhabiting depths with about the same temperature in all geographic zones, it is hardly possible to suppose that a greater survival of strains from low latitudes was due to their better adaptation to the temperature conditions of the laboratory. It seems that a comparatively high percentage of the deaths of the strains from high latitudes was due to the fact that in the cultures of these heterotrophs, because of their many-sided enzymic activities, there accumulated metabolic products more harmful to microbes than those present in the less biochemically active cultures of hetero-trophs from the equatorial–tropical zone.

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The Survival of Starved Bacteria

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SUMMARY

Samples of populations of *Aerobacter aerogenes* grown in continuous culture on a limited supply of glycerol died linearly with time without significant cryptic growth when aerated in buffered physiological saline at their growth pH and temperature. Death was uninfluenced by atmospheres varying from pure O_2 to 2 % O_2 in N_2 but was accelerated under N_2 in media of various E_h values. Death was accelerated in environments of higher or lower tonicity, in unbuffered media, at pH values above 7, temperature above 40°, by strong illumination. Within limits, lower pH values or temperatures prolonged survival. Death was not immediately accompanied by breakdown of the osmotic barrier. Populations more dense than equiv. 20 μg. dry wt./ml. survived longer, sparser ones died more rapidly. The death rates of the populations studied were not influenced by 30 % i-erythritol or by certain high molecular weight materials. Several metabolic inhibitors were tested; most of them accelerated death or had no effect; three protected transiently. Mg^{2+}, Ca^{2+} or Fe^{3+} ions delayed death. A variety of trace elements, inorganic ions, growth factors, or an amino acid mixture, had no significant action; glycerol or intermediate compounds accelerated death. Dying populations showed rapid initial breakdown of intracellular RNA with release of phosphate and base fragments into the medium; most of the ribose was metabolized. Intracellular protein was degraded after a lag; intracellular polysaccharide and DNA were scarcely degraded at all. Endocellular reserves declined much more rapidly than did viability; the Q_{O_2} with glycerol and 'glycerol dehydrogenase' activity declined in parallel with viability. Populations permitted to grow at different rates died more rapidly the slower the growth rate; steady states were obtained by slow continuous culture in which constant proportions of the organisms were dead.

For a given growth rate the nature of the limiting nutrient influenced the form of the survival curve: carbon, phosphorus, sulphur or oxygen limitations gave almost linear curves, N-limitation gave a sigmoid curve and Mg-limitation a concave form. In some instances the growth rate influenced the form of the survival curve and the death rate; Mg-limited organisms apparently died faster the faster they grew. The survival of stationary phase populations derived from batch cultures depended markedly on which chemical component of the environment limited growth; 'logarithmic phase' and 'stationary phase' C-limited organisms differed insignificantly in death rates. Partially synchronized populations from batch cultures died marginally more rapidly when harvested just after division ceased.

INTRODUCTION

The survival of bacteria stored in aqueous suspension and subject to no overt stress has been studied by many workers, mostly at the beginning of this century. Shearer (1917) reported that physiological saline was more toxic than distilled water or 1·5 % NaCl to meningococci; Ca or K antagonized this toxicity. Cohen (1922) cited earlier studies and showed that 'Bacterium coli' and 'Bacterium typhosum' in distilled water or dilute buffer died most rapidly at elevated temperatures and high pH values. The two species had different pH optima for survival; in distilled water their death was erratic and 'coincided with apparently insignificant pH variations'. Winslow & Falk (1923a) observed that 'B. coli' survived longer in physiological saline than in distilled water and that the pH optima for survival were different in the two media; Winslow & Falk (1923b) studied the effect of calcium and sodium ions on the viability of such populations. Winslow & Donoff (1928) and Winslow & Haywood (1931) were concerned with survival in nutrient media in which certain cations prevented growth. Morrison, El Bagoury & Fletcher (1956) observed that certain concentrations of chloramphenicol delayed the death of *Escherichia coli* stored in broth. Cook & Wills (1958) showed that washed buffered populations of *E. coli* maintained high viabilities at room temperature, compared with unwashed or unbuffered aqueous suspension; survival times were influenced by the growth conditions of their populations. Ryan (1959) recorded exponential death of histidineless *E. coli* in spent medium, though during this period the organisms had an appreciable mutation rate. Harrison (1960) reported that below a certain limit dense populations of *Aerobacter aerogenes* taken from logarithmic phase cultures survived longer than sparse populations in a non-nutrient buffer; centrifuged suspensions in which organisms had died contained materials that prolonged the lives of fresh populations. Strange, Dark & Ness (1961) published a study of the survival of stationary phase populations of the same strain of *A. aerogenes* in non-nutrient buffer. They showed that the composition of the growth medium, the phase of growth and the period during which the organisms had been in the stationary phase influenced the survival characteristics of their populations. Death of the populations was preceded and accompanied by degradation of polymeric cell constituents (protein, ribonucleic acid, polysaccharide) and excretion of fragments of these polymers. The mean contents of these polymers within the organisms were influenced by the composition of the medium from which the populations were harvested; so were the rates at which dying populations degraded these polymers, the order in which they were degraded, and the death rates. Strange (1961) reported that these reactions decreased the ability of the organisms to form adaptive enzymes without affecting their viability or ATP content. Ryan (1959) showed that glucose accelerated the death of his histidineless mutants; Strange *et al.* (1961) observed that addition of glucose to moribund populations accelerated their death rate. Harrison (1960) in contrast, reported that traces of glucose prolonged survival.

Studies on survival at ordinary temperatures in non-nutrient aqueous solutions are complicated by three factors. (1) Buffers prepared from highly purified reagents contain impurities which permit limited growth of bacteria. Garvie (1955) showed that this phenomenon could account for apparent 're-activation' of killed bacteria;

Strange *et al.* (1961) encountered a similar effect during their studies. (2) As soon as a portion of the population dies, nutrients may be released into the medium enabling the survivors to multiply. This phenomenon is well known in the ageing of bacterial cultures (e.g. see *Topley & Wilson's Principles* 1955) and was apparent in the work of Winslow & Falk (1923*a*, see Table 1). Ryan (1955) termed it 'cryptic growth' and concluded that it did not occur to any significant extent in non-multiplying populations of histidineless *Escherichia coli* (Ryan, 1959). Strange *et al.* (1961) called it 'regrowth' and showed that it could be prevented by dialysing the suspension or renewing the suspending fluid after filtration. Harrison (1960) invoked the phenomenon (termed 'cannibalism') to account for the increased survival time of dense bacterial suspensions as compared with more dilute ones, but he included within the term maintenance of viability (without growth) at the expense of materials released by dead organisms. (3) The third factor was briefly alluded to by Harrison (1960) but dismissed by him: laboratory glassware, reagents and distilled water may contain materials actively toxic to bacteria.

The aim of the work reported here has been to study the influence of environmental and historical factors on the survival of bacteria in starvation conditions, taking into account these complications as far as practicable. Misleading results due to cryptic growth were avoided by: (*a*) choosing conditions which provided a rapidly dying population capable of supporting only limited cryptic growth; (*b*) concentrating on the death of the first 90 % of that population. Multiplication of the stored populations at the expense of nutrient impurities in the storage media was rendered unimportant by: (*a*) choosing a simple medium (essentially physiological saline with a minimal concentration of a non-nutrient buffer); (*b*) using initial cell populations sufficiently dense that such residual growth as did occur would contribute negligibly to the apparent viability of the population. Toxicity due to impurities was avoided by: (*a*) demonstrating that one such toxic agent was metallic in nature and that its action could be eliminated by adding traces of a chelating agent; (*b*) choosing to work with as dense populations as the earlier considerations permitted so that the proportionate effect of traces of toxic material should be as small as possible.

Variations in survival characteristics due to the phase of growth and cultural conditions were reduced to a minimum by studying steadily growing populations from a continuous culture apparatus in which the growth rate was limited by the supply of energy source. These populations resembled those harvested just at the end of the logarithmic phase of growth of a batch culture. The culture was permitted to grow for many generations at a constant growth rate: variations consequent on transfer from batch culture to continuous culture might thus be expected to have taken place before the work recorded here was done; the mean physiological state of the populations studied should thus have been reasonably constant. Evidence that changes of this character did take place is presented later.

METHODS

Organism. *Aerobacter aerogenes* (NCTC 418) from Professor Sir Cyril Hinshelwood's laboratory was maintained in a continuous culture apparatus designed by Dr D. Herbert of this laboratory. This strain of *A. aerogenes* was incorrectly

designated No. 417 by Postgate, Crumpton & Hunter (1961) owing to a confusion of laboratory records. It was used by Strange *et al.* (1961) and Postgate & Hunter (1961) and is probably that used by Harrison (1960, 1961). The culture vessel contained 100 ml. culture and an air space of roughly equal volume. A defined medium of the following composition was pumped in at an appropriate rate: $NaH_2PO_4.2H_2O$ (5 mM), $(NH_4)_2HPO_4$ (45 mM), K_2SO_4 (10 mM), glycerol (2 g./l.); trace element mixture to give MgO (1·25 mM), $CaCO_3$ (0·1 mM), $FeCl_3$ (0·1 mM), ZnO (25 μM), $MnCl_2$ (25 μM), $CuCl_2$ (5 μM), $CoCl_2$ (5 μM), H_3BO_3 (5 μM) Na_2MoO_4 (10 μM), HCl (2·9 mM), sodium ethylenediaminetetra-acetate (EDTA; 1·59 mM); NaOH (about 5 mM) to pH 7·8 when prepared; the pH value fell to 7·4 after autoclaving in batches of 10 l. for 30 min. at 121°. The temperature during growth was maintained at 40° by a thermostatically controlled 150 watt infra-red lamp; the usual dilution rate (proportion of culture volume replaced/hr.) used was $0.25 \pm 0.01/hr^{-1}$; in these conditions the culture was at pH 7.0 ± 0.1 and the bacterial population equivalent to about 1 mg. dry wt./ml.; bacterial numbers were 2·3 to 2.5×10^9 organisms/ml. as determined by the method of Norris & Powell (1961). Aeration was provided by flowing air at 10 l. hr. over a vortex produced by stirring the culture magnetically; other work here had shown that in these conditions aeration was adequate and that the bacterial population of the culture depended on the glycerol concentration of the influent medium. All data reported here refer to the strain between 4 and 24 months in continuous culture in these conditions. Brief interruptions occurred during the period owing to holidays or trivial failures of the culture device; a reserve of organisms frozen in 10 % (v/v) glycerol and stored at $-20°$ (Postgate & Hunter, 1961), renewed monthly so that the viability did not fall below 90 %, was kept to reinoculate the continuous culture in case of interruption. After such episodes cultivation was continued until the survival curve of the population was normal before sample populations were used for further experiment.

Before the work reported here the strain underwent spontaneous alteration of at least three characters:

(i) The mean length of the organism increased from about 2·5 to about 5 μ, and the scatter of individual lengths became wider than that of the parent culture. On transfer to tryptic meat broth the population regained its original size.

(iii) The minimum mean generation time of the population on transfer to batch culture in the glycerol medium was 55 ± 3 min. as compared with 44 ± 2 min. for the original stock.

(iii) The organisms developed a long lag when plated on tryptic meat agar; in certain circumstances up to 70 % of the organisms did not divide. The original stock maintained on this medium grew rapidly.

The colonial form was uniform; periodic checks on the fermentation properties of the strain showed that it retained the diagnostic reactions characteristic of *Aerobacter aerogenes*. Tests on its antigenicity showed that it remained immunologically similar to the parent stock.

Storage. Bacteria were prepared for survival studies as follows. A sample (usually 5 ml.) was removed from the continuous culture vessel and the organisms washed twice in NaCl (0·85 g./l.) by centrifugation, re-suspended in distilled water to avoid carry-over of salt when special environments were tested, and 1 ml. added to 50 ml. saline-tris buffer (see below) in nominally 8 in. × 1·5 in. hard glass

tubes plugged with cotton wool. These were incubated in a water bath at 40°
and gently aerated with 25 to 50 ml. damp air/min. through a Pasteur pipette
pushed through the cotton-wool plug. For protracted survival curves (e.g. Fig. 1),
when condensate might leach nutrients from the cotton wool, a rubber seal ('Suba-
seal') replaced the cotton-wool plug. The evaporation rate from such suspensions
was 1·5–2 ml. in 24 hr.; in experiments lasting longer than this appropriate volumes
of sterile distilled water were added to the suspensions. Occasionally 25 or 30 ml.
volumes of suspension in 8 in. × 1¼ in. tubes were studied. The saline-tris buffer
consisted of 9 vol. 0·137 M-NaCl + 1 vol. 0·048 M 2-amino-2-hydroxymethylpropane
1:3 diol ('tris') + EDTA to 0·316 mM. This amount of buffer maintained the pH
value unchanged during aeration for 24 hr. at 40°. Populations stored in this
manner contained about 4×10^7 organisms/ml. (equiv. about 20 μg. dry wt. bacteria/
ml.; some loss of organisms occurred during washing) having an initial viability
of 95–99 %. Samples were taken at intervals to determine viability; where the effect
of a growth inhibitor was being examined, populations were centrifuged and re-
suspended in saline before the viability determination. This procedure had no
influence on the viability of the population though there was a further loss of
bacteria during centrifugation. In an experiment on this topic a population of
$7·17 \times 10^7$ viable organism/ml. had only $6·27 \times 10^7$/ml. after one centrifugation;
$3·3 \times 10^6$ organisms/ml. were accounted for in the supernatant fluid.

Filtration. During the work reported in the next section it became clear that
Millipore filter disks (Oxoid Ltd.) contained materials that could act as energy
sources for growth and which in certain circumstances could accelerate the death of
starved populations. Such filters were therefore set up, autoclaved and then washed
with 400 ml. distilled water followed by 50 ml. saline-tris.

Measurement of viability: The percentage viable organisms in stored populations
was measured by slide culture (Postgate *et al.* 1961). The medium was that pre-
scribed above supplemented with Difco yeast extract (1 g./l.); Difco casamino-
acids (1 g./l.); Douglas's digest broth (10 %, v/v, Medical Research Council, 1931),
Difco agar-agar (15 g./l.). It was filtered hot through a Millipore filter. In experi-
ments with small population densities conventional plate counts on a similar medium
were used.

Chemical analyses. Protein was determined by the biuret reaction (Stickland,
1951), boiling the alkaline suspension; bovine serum albumin, fraction 5 (Armour
Laboratories) was used as standard. Free amino acids were determined by treat-
ment of equiv. about 20 mg. dry wt. bacteria with acetic acid (100 g./l.; Mattick,
Cheeseman, Berridge & Bottazzi, 1956), neutralization of the supernatant fluid with
2 N-NaOH and analysis for primary amino groups with β-naphthaquinonesul-
phonate (Frame, Russell & Wilhelmi, 1943); Difco 'Casamino-acids' solutions were
used as standard amino acid mixtures. Polysaccharide was determined by a modi-
fication of the anthrone reaction devised by Mr P. J. Phipps (this laboratory;
personal communication) with glucose as standard. Ribonucleic acid was measured
by the orcinol reaction (Morse & Carter, 1949) with ribonucleic acid (Boehringer and
Son) as standard. Deoxyribonucleic acid was measured by the diphenylamine
reaction with calf thymus nucleic acid (Gulland, Jordan & Threlfall, 1947) as stand-
ard. Polymeric cell constituents were measured on whole organisms without
preliminary treatment and thus include diffusible derivatives of the materials

sought. Phosphate was determined by a version of the phosphomolybdate reaction (King, 1946). Colorimetric measurements were made with a Spekker absorptiometer (A. Hilger Ltd., London) or a Unicam spectrophotometer (Cambridge Instrument Co. Ltd., Cambridge). Fluorimetric measurements were made with the Locarte fluorimeter using exciting light of 340–380 mμ and recording wavelengths longer than 470 mμ (maker's filters LF2 and LF6 respectively). Bacterial dry weights were deduced from the optical densities of bacterial suspensions measured in the Spekker with a filter transmitting maximally at 540 mμ. and calibrated with suspensions of known dry wt./ml. from our continuous culture. Determinations of enzyme activity were performed with conventional Warburg manometers at 40°; 'glycerol dehydrogenase' activity was determined by the procedure of Fahmy & Walsh (1952) with the modification that the suspensions were incubated in Thunberg tubes under nitrogen at 40° for 20 min. Fahmy & Walsh used tubes open to air; in our tests incubation under N_2 was found to be necessary with enzyme preparations from the bacteria, and was therefore considered preferable for testing moribund populations.

Materials. Reagents of analytical grade were used when available. Water was distilled and then de-ionized by passage through a mixed bed ion-exchange resin.

RESULTS

Standard survival curves. Populations (equiv. 20 μg. dry wt. *Aerobacter aerogenes*/ ml.) harvested from steady growth at a dilution rate of 0·25 hr.$^{-1}$ and allowed to die in aerated buffer at 40° and pH 7 showed a linear survival curve in which a constant % of the initial population died per hr. over the first 7–10 hr. A statistically analysed curve with such populations was quoted by Postgate *et al.* (1961); this showed a mean death rate of about 8 %/hr. with empirical 95 % confidence limits of ± 9 % for the technique of viability determination used. Most of the survival curves obtained during this work resembled this; the death rate, over the period when the first 70 % of the population died (about 5 hr.), was usually between 6 % and 12 %/hr., though extremes as low as 4·6 %/hr. and as high as 17 %/hr. were encountered. The reasons for these fluctuations are not known. The survival curve of the population showed considerable reproducibility from day to day for several weeks; sometimes it changed over 2 or 3 days; during the 20 months in which the culture was studied the population showed a trend towards faster death rates. Occasionally survival curves of a slightly sigmoid character were observed, in which an initially descending portion was followed by a steeper linear decline. In all cases the curve flattened off, when after 7–10 hr., the viability had fallen below 30 %; by 24 hr. the viable population had fallen below 2 %. Because of the occasional variability of the survival curves all experiments reported here included as control a population dying in aerated buffer.

Cryptic growth. This phenomenon leads to an increase in the total count of a population during its death. However, the systematic and operational errors in making a conventional total count are such that small percentage increases in bacterial numbers might be missed (Norris & Powell, 1961). It may be detected by comparing survival curves with and without a non-bactericidal growth inhibitor such as penicillin (see Ryan, 1959); if these are identical it follows that cryptic

growth has not occurred in the absence of bacteriostatic agent. Ordinary cultural experiments showed that penicillin G (100 units/ml.) inhibited growth of equiv. 20 μg./ml. inocula in the glycerol medium for 17 hr. and that chloramphenicol (100 μg./ml.) inhibited multiplication for 2 days. The latter observation was confirmed by slide culture on agar containing chloramphenicol; none of 600 organisms watched divided over 24 hr. Table 2 records experiments showing that cryptic growth did not occur in the conditions we used routinely: the survival curves over the first few hours with and without chloromycetin or penicillin were identical.

On prolonged incubation cryptic growth was readily detected (Fig. 1). The maximum cryptic growth of which our populations were capable was determined by experiments of which the following is typical. A suspension of about twice the customary population density (6.75×10^7 viable bacteria/ml.) was left to die at $40°$ with aeration for 24 hr. in the presence of 100 u. penicillin G/ml. to prevent cryptic growth. By then the viability was 1.3%. The suspension was filtered through a Millipore membrane and the filtrate assayed with a population from continuous culture which has been made resistant to penicillin by two batch subcultures (about 11 generations) with 200 and 100 u./ml. successively. For assay, 5 vol. filtrate were diluted with 4 vol. Millipore-filtered saline-tris buffer plus 1 vol. culture medium made up without glycerol so as to bring substrates leached from dying organisms to concentrations comparable with those to be expected in the suspensions we routinely studied; the portion of basal medium supplied an excess of mineral nutrients. The diluted filtrate was inoculated with 10^4 penicillin-resistant bacteria/ml. Growth was followed by plate counts and compared with that in a control buffer supplemented with basal medium. The filtrate supported a maximum excess growth of 7.2×10^5 organisms/ml. over the control; these grew as a result of the death of about 3.3×10^7 organisms/ml. Hence the maximum cryptic growth of which our populations were capable was one new organism at the expense of about forty-seven dead ones.

Growth on impurities. Direct assays of the extent of growth permitted by impurities in our reagents and on our glassware were made by inoculating 30 ml. lots of buffer with about 4×10^3 organisms from the continuous culture. Saline-tris buffer supported from 2×10^2 to 8×10^4 viable organisms/ml. as the maximum viable population. Addition of 0.1 vol. of the basal medium (without glycerol) to supply mineral nutrients augmented growth to 2.8–3.5×10^5 organisms/ml. Garvie (1955) and Strange *et al.* (1961) obtained values of about 10^6 organisms/ml. for residual growth on impurities in phosphate buffers.

Manipulation of organisms. The populations studied were subject to centrifugation and changes of temperature and chemical environment during preparation for storage. Organisms washed once, twice or three times in saline gave essentially similar survival curves of slope 7.8%/hr. and initial viabilities of 90–95 %. A repetition of Gossling's (1958) experiments using buffers prepared according to his prescriptions in the manner represented schematically below showed negligible effects of chemical environment on viability.

A temperature decrease to 18–$20°$ was unavoidable and this 'cold shock' (Hegarty & Weeks, 1940) may have caused the 1–8 % of dead organisms usually found after preparation for survival curves. A decrease to $4°$ did not alter the viability; a decrease to $0°$ obtained by squirting the suspension on to ice caused a small but

significant kill. Viabilities in an experiment on this topic were 99 % at room tem-
perature, 98 % at 4° and 94·5 % at 0°; freezing in solid CO_2 reduced the viability to
below 1 % (see Postgate & Hunter, 1961).

```
                                                            Viability
                                                              (%)
                                           ┌─→M/15 phosphate   95·3
                   ┌─→M/15 phosphate───────┤
                   │                        └─→Ringer          92·7
        Culture──┤
                   │                        ┌─→M/15 phosphate   95·5
                   └─→Ringer────────────────┤
                                            └─→Ringer          26·7

   Centrifugations:     1st                 2nd
```

Prior to storage the bacteria spent 3–5 min. in distilled water. This treatment
affected the subsequent death rate though it did not measurably influence the initial
viability of the population. In a typical experiment organisms suspended in dis-
tilled water before storage in buffer died at 10·1 %/hr. compared with 5 %/hr. for
those suspended in saline-tris buffer. Exposure to M-NaCl before storage led to a
survival curve similar to that obtained after exposure to distilled water.

Need for buffer. The viabilities of populations suspended in unbuffered physio-
logical saline declined to 15 % in 2 hr. and 5 % in 3 hr. Death was accompanied by
a change from pH 6·0 to 4·9. In distilled de-ionized water the viability fell to 13 %
after 2 hr.

Need for ethylenediaminetetra-acetate. Early in this work the slopes of the survival
curves were erratic; they showed an abrupt change on one occasion when a new
batch of saline-tris buffer was used; in this batch the organisms all died within 2 hr.
and the death rate returned to about 8 %/hr. when EDTA was added. This suggested
the buffer might contain a toxic metal. Extraction of 200 ml. of buffer with
8-hydroxyquinoline in $CHCl_3$ gave a chloroform solution which by spark spectro-
scopy showed an unusual amount of copper (Dr L. C. Thomas, personal communica-
tion). Laboratory-distilled and de-ionized water contained about 2×10^{-7}M-Cu^{2+};
its effect was neutralized by EDTA. The routine practice was adopted of adding to
the buffer 0·316 mM-EDTA (three times the concentration necessary to neutralize
the most toxic batches encountered).

Effect of recovery medium. The medium used for slide culture contained nitro-
genous supplements (casein hydrolysate, yeast extract, tryptic meat broth) to reduce
the scatter of division lags among the organisms counted. A medium without these
supplements gave less accurate viabilities due to overgrowth of late starters by
organisms of short lag (Postgate *et al.* 1961). The rich medium gave greater survivals
with dying populations: tests with conventional plate counts on the rich medium
and the minimal medium gave comparable counts on the two media when the
populations tested were more than about 50 % viable; below this value the minimal
medium gave consistently lower counts. For example, a 40 % viable population
gave a count of $5·8 \times 10^6$ organisms/ml. on the rich medium and $3·7 \times 10^6$ ml. on
minimal medium; a 27 % viable population gave counts of $5·2 \times 10^6$/ml. and
$2·9 \times 10^6$/ml. respectively. Replica plating from rich to minimal agar showed that
these differences were not due to the appearance of nutritional mutants in the
population counted, and we conclude that more of the population died during the
lag phase on minimal agar than died during this period on rich agar.

Effect of light. To determine survival curves the bacterial suspensions were stored in a thermostat illuminated by indirect daylight. The illumination at the surface of the water ranged from 4 to 14 ft.-c. according to the weather; appropriate tests showed that this degree of illumination had no killing effect on our populations. For example, a population died at 9·2 %/hr. in our ordinary tubes and in tubes screened by wrapping in aluminium foil. By intensifying the illumination killing by visible light was observed: a population held at 40° in a transparent thermostat was all dead in 4 hr. when illuminated with 100 ft.c. from a 75 W. tungsten filament bulb at pH 6·9 ± 0·05; the survival curve showed a sigmoid form. The control, screened with aluminium foil, died linearly at 9·3 %/hr. and was about 60 % viable after 4 hr.
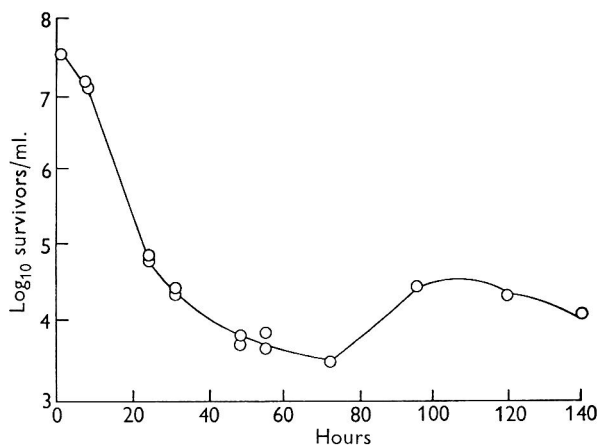
Fig. 1
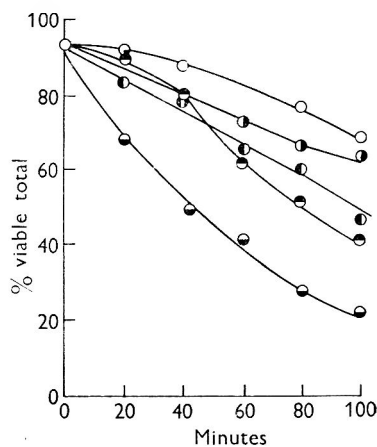
Fig. 2

Fig. 1. Survival curve of *Aerobacter aerogenes* showing cryptic growth. *A. aerogenes* harvested from continuous culture on glycerol at $D = 0·25 \text{ hr}^{-1}$ was washed twice by centrifugation, resuspended in distilled water, added to 'saline-tris' buffer at 20 μg. dry wt. organisms/ml. and allowed to die at 40° at pH 6·95 ± 0·05 under forced aeration. Points up to 24 hr. by slide culture; including plate counts at 0, 7 and 24 hr.; subsequent points by plate counts alone.
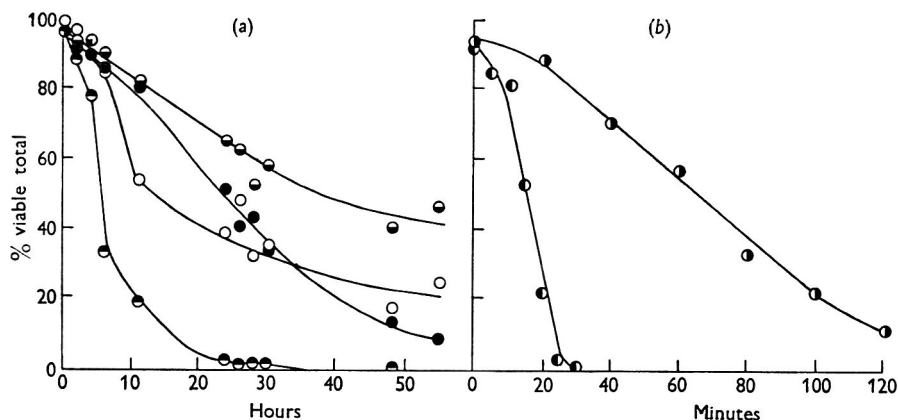
Fig. 2. Effect of population density on death of *Aerobacter aerogenes*. *A. aerogenes* suspensions prepared as for Fig. 1; added to saline-tris buffer and allowed to die at 40° and pH 7·1 ± 0·1 with forced aeration. Tube ○ contained 25 μg. dry wt. organisms/ml.; ◑ : 2·5 μg./ml.; ◐ : 0·25 μg./ml.; ◓ : 0·025 μg./ml.; ◒ ; 0·0025 μg./ml. In the first of these the viabilities were determined by slide culture, in the rest by plate counts assuming all the initial viabilities were similar.

Effect of population density on survival curve. Harrison (1960) showed that between populations of 10^5 and 10^8 organisms/ml. the death rate of *Aerobacter aerogenes* was slower the denser the population; at 10^9 organisms/ml. the survival curve became steeper again. Our experiments confirmed Harrison's: denser populations survived longer than sparser ones (Fig. 2); the most dense population we have tested contained about 4×10^8 organisms/ml., probably yet too sparse to show accelerated death.

Effect of temperature. The effect of temperature on the death rate is illustrated in Fig. 3. Death was slower at 30° and 20°; faster at 10, 45 and 50°. A small contribution to the shallower death curves at 20° and 30° may have been due to cryptic

growth. The organisms did not grow at 45° or above; at 60° more than 99 % of the organisms were dead by the time (about 3 min.) the environment had warmed up to this temperature.

The slide cultures prepared from populations dying at 10° and 20° showed many morphologically aberrant forms once death had started. Small bacteria sometimes became relatively large refractile spheres which burst, leaving convoluted forms or 'ghosts'. Slides watched for 7 hr. at 37° showed that most of these bodies did not form micro-colonies; they were recorded as dead.

Effect of deep freezing. Freezing the population in diethylene glycol solution (100 g./l.), which caused no change in viability, accelerated the death of the organisms after thawing. This experiment was described by Postgate & Hunter (1961) and illustrated in their Fig. 1.

Fig. 3. Effect of temperature on death of *Aerobacter aerogenes*. *A. aerogenes* suspension prepared as for Fig. 1; added to saline-tris buffer at 20 μg. dry wt./ml. and allowed to die at pH 7·0 to 7·1 with forced aeration. Part (*a*) tubes ◑ : 40°; ○: 30°; ◐ : 20°; ●: 10 ± 1°; part (*b*) ◑ : 45°, ◑ : 50°.

Effect of osmotic environment. The concentration of the buffered sodium chloride was that usually described as physiological, though such an environment is in fact hypotonic to the bacterial cytoplasm. The effects of adding materials that would alter this condition were studied.

(i) *Sodium chloride.* Organisms made sensitive to osmotic shock by Nakamura's (1923) procedure (incubation with lysozyme followed by exposure to pH 10) lysed in distilled water and in buffered saline; lysis was much decreased in buffer containing 1·15 M-NaCl. However, the death rate of the ordinary bacteria in buffer containing 1·15 M-NaCl (41·0 %/hr.) was greater than that in the control (5·2 %/hr.). An environment of marine salinity (0·05 M-NaCl buffered with tris) caused accelerated death (60 % died/hr. compared with 17·5 %/hr. in a control); sea water filtered and buffered at pH 7 with the usual concentration of tris was less toxic and the death rate was like that in the control buffer.

(ii) *Polyethyleneglycol or dextran.* High molecular weight substances protect organisms in freeze drying because their osmotic effect occurs at the cell wall (see Record & Taylor, 1960). Dextran (Glaxo Laboratories Ltd., mean M.W. 125,000) 50 g./l. and polyethyleneglycol (mean M.W. 10,000), 50 g./l., provided by Dr B. R.

Record, were tested in saline-tris buffer. Both slightly accelerated death and altered the shape of the survival curve; their effects were small and were not studied further.

(iii) *i-Erythritol*. This compound like glycerol penetrates the osmotic barriers of bacteria freely (Mitchell & Moyle, 1956) including our strain (Postgate & Hunter, 1961). Cultural tests showed that the organisms did not use it for growth. Survival curves in buffer supplemented with 300 g. *i*-erythritol/l., which may be expected to have diluted the cytoplasmic water considerably, were virtually indistinguishable (death rate: 5·2 %/hr.) from controls without this supplement (5·3 %/hr.).

Effect of pH *value.* Figure 4 illustrates that a pH value below 7·0, at which they had grown, was optimal for survival.

Effect of atmosphere. Removal of CO_2 from the influent air with NaOH did not significantly influence the death rate (12·5 %/hr. without CO_2, 12·4 %/hr. with). Replicate suspensions aerated with $O_2 + N_2$ mixtures ranging from pure O_2 to 2 % (v/v) O_2 died at similar rates. Replacement of air by commercial 'oxygen free' nitrogen (British Oxygen Company Ltd., O_2 below 5 p.p.m.) gave erratic death rates but usually accelerated death (e.g. 5 %/hr. in control; 21·5 %/hr. with N_2). The increased death rate was usually associated with a decline in pH value; e.g. in N_2 buffered suspensions fell from pH 7·0 to about 6·0 in 5 to 7 hr., reaching about pH 5·3 by 24 hr.; in air the pH value scarcely changed (7·0 to 6·9) over 24 hr. On one occasion the usual pH change did not occur in N_2 but death was nevertheless rapid.

Effect of reducing agents. The death rate in an atmosphere of N_2 was influenced by poising the E_h value of the environment with chemicals known to interact with biological systems. With sodium ascorbate (2×10^{-2} M) the death rate was 10·8 %/hr., compared with 21·5 %/hr. with sodium thioglycollate (2×10^{-2} M) and 21·5 %/hr. with N_2 in an unpoised buffer. The control suspension in air died at 5 %/hr. Acid was formed in all the instances in which the environment was anaerobic.

Effects of nutrients

Effect of trace elements. The culture medium used contained trace elements held in solution by a slight molar excess of EDTA. We are not certain that all these supplements were necessary for growth. The trace element mixture, when added to saline-tris buffer in the proportion used in preparing the growth medium, had a preservative effect (death rate 5·7 %/hr. compared with control 13 %/hr.). This phenomenon was analysed by testing the component elements singly at the concentrations used in the culture medium (Table 1). Ca, Mg, or to a slight extent Fe, protected the organisms; all the other elements were without effect when EDTA was present at 316 μM. The protective effect of calcium was complex: the death rate increased in a parabolic fashion for about 5 hr., then settled to a linear rate equal to that of the control. A test with a 'non-toxic' batch of buffer (see 'need for EDTA') without EDTA showed that Co, Mn, Cu and Zn were then toxic.

Effect of nitrogen. Addition of NH_4Cl (10 g./l.) did not significantly alter the death rate (control: 5·4 %/hr., $+ NH_4Cl$ 5·6 %/hr.). Vitamin-free casein hydrolysate 20 g./l. supported growth when the experiment was extended for 24 hr. Over the first 2 hr., however, the survival curve paralleled that of the control, suggesting that amino acids exerted no protective effect.

Effect of potassium and phosphate. Addition of KCl (M/150) accelerated death slightly (control: 6·4 % /hr., + KCl: 10 %/hr.) NaH_2PO_4 (1·25 mM) added to the buffer had no significant effect (control: 5 %/hr., + NaH_2PO_4: 6 %/hr.).

Death in the growth medium. Populations survived longer when incubated in the spent culture medium from which organisms had been removed (death rate 2·8 %/hr.). This was not due to protective materials excreted by the organisms because the same death rate was obtained in the basal medium prepared without a carbon source. The whole of the protective effect of spent or basal medium was attributable to its trace element content: the death rate in the basal medium (5·3 %/hr.) was somewhat greater than that in saline-tris buffer supplemented with Ca, Mg and Fe (3·7 %/hr.); 10·4 % of a control in ordinary saline-tris buffer died/hr.

Table 1. *Effect of trace elements on death of starved* Aerobacter aerogenes

Survival curves at the growth pH value (7) and temperature (40°) in saline-tris buffer with the supplements below were obtained as described in Methods.

Supplement	Concentration (μM)	Death rate (%/hr.)
$FeCl_3$	100	12·4
$ZnSO_4$	25	14
$MnSO_4$	25	14·7
$CuSO_4$	5	16
$CoSO_4$	5	14
H_3BO_3	5	15
$NaMoO_4$	10	14·8
$CaCl_2$	100	c. 5·0*
$MgCl_2$	1250	5·6
All†	—	5·4
None	—	15

* Curve with Ca non-linear; see text.
† Mixture contains Zn, Mn, Cu and Co as chlorides.

Effect of vitamins. Several compounds were tested at concentrations recorded in the literature as having growth factor effects for appropriately exigent organisms. None significantly influenced the death rate of our populations over the first 7 hr. The substances were (conc./ml.): adenine (10 μg.), adenosine (4 μg.), adenylic acid (4 μg.), p-aminobenzoic acid (0·1 μg.), biotin (1 mμg.), cyanocobalamin (1 mμg.), glutamine (2 μg.), haemin (c. 1 μg.), m-inositol (10 μg.), nicotinamide (0·1 μg.), sodium pantothenate (1 μg.), phthiocol (1 μg.), pteroylglutamic acid (1 mμg.), pyridoxal (2 μg.), pyridoxamine (2 μg.), pyridoxine (2 μg.), riboflavine (0·1 μg.), ribose (2 μg.), thiamine HCl (0·1 μg.), Tween-80 (10 μg.), uracil (10 μg.).

Effect of carbon source. Glycerol (10^{-2}M) accelerated death. This phenomenon was not due to toxic materials in the analytical grade glycerol used, since intermediates such as sodium pyruvate, oxaloacetate, α-ketoglutarate, succinate, citrate or DL-malate also accelerated death. Selected data illustrating this phenomenon are given in Fig. 5.

In this context we should record an experimental hazard. In certain experiments it was necessary to filter batches of saline-tris buffer through a Millipore membrane and the survival curves in filtered buffer were steeper (6·7 %/hr.) than the control (4·7 %/hr.). By extracting Millipore membranes with substrate-free growth medium

and testing the extracts microbiologically we showed that this phenomenon was not due to toxic materials in the filter but to carbon substrates which permitted growth in the basal medium and accelerated death in the buffer. Adequate washing of the membranes removed these materials.

Effect of metabolic inhibitors. Death in aqueous suspension is preceded by considerable endogenous metabolism (Strange *et al.* 1961) and it is reasonable to suppose that metabolic inhibitors, provided they are not bactericidal, might influence survival directly. Several known metabolic inhibitors were tested by incorporating them into the routine saline-tris buffer and comparing the death rates of populations in their presence with those in controls. Samples of the dying populations were
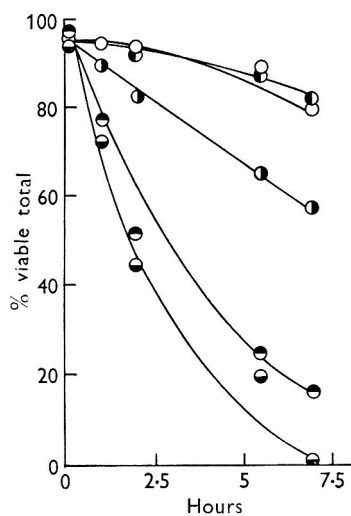
Fig. 4.
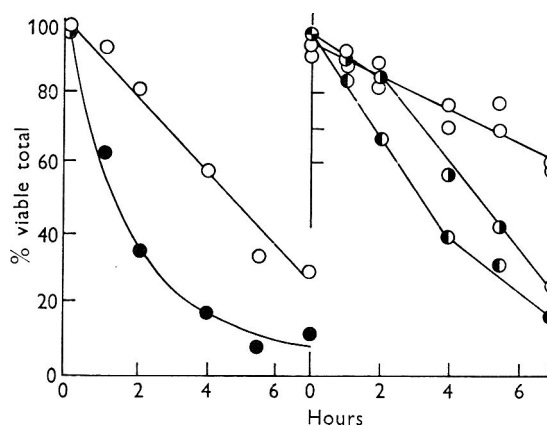
Fig. 5.

Fig. 4. Effect of pH value on death rate of *Aerobacter aerogenes*. *A. aerogenes* suspension prepared as for Fig. 1; added to saline-tris buffer at 20 μg./ml. and allowed to die at 40° under forced aeration at various pH values. ○: 6·1; ◑, 6·5; ◐ : 7·0; ◕ : 7·45; ◔ : 7·9; all pH values ± 0·05.

Fig. 5. Effect of carbon sources on death of *Aerobacter aerogenes*. Suspension of *A. aerogenes* prepared as for Fig. 1; added to saline-tris buffer at 20 μg. dry wt./ml. and allowed to die at 40° and pH 7·0 ± 0·1 under forced aeration. ○: controls; ●: plus glycerol; ◐ : plus sodium pyruvate; ◑ : plus sodium citrate (all supplements 10^{-2}M).

centrifuged and re-suspended in saline to remove the inhibitor before determining the viability by slide culture; as mentioned in 'Methods' this procedure had no effect on the viability of the control populations. The compounds were tested at concentrations judged from the literature to be likely to have a marked effect provided that the material reached its site of action. Compounds which showed an immediate bactericidal effect were examined by slide culture to find the maximum concentration that exerted no such effect (see Postgate *et al.* 1961, Table 3) and were tested at one half that concentration. The results are given in Table 2. Yttrium or lanthanum nitrates showed modest preservative effects; controls showed that these were not due to the nitrate ion. With lanthanum these effects were observed in four experiments; lanthanum at $5 \times 10^{+5}$M had no effect; 2×10^{-4}M-lanthanum behaved like

the concentration quoted in Table 2; 10^{-3}M-lanthanum had bactericidal effect leaving only 25 % viable organisms at the start of the survival curve. The death rate with pyridine sulphonate (10^4M) was not influenced by adding its metabolite analogue nicotinic acid to 10^{-6}M.

State of the osmotic barrier. Stored suspensions of some micro-organisms liberate purines into the external medium (Holden, 1958; Higuchi & Uemura, 1959).

Table 2. *Effect of metabolic inhibitors on the death rates of starved*
Aerobacter aerogenes

Death rates measured at growth temperature (40°) and pH value (7) in a saline-tris buffer; for details see text.

Inhibitor	Concentration (μg./ml.)	Death rates Control	Death rates Test	
4-Aminopteroylglutamic acid	50	7·1	7·4	
Aureomycin	20*	6·6	6·8	
Aza-adenine	100	5·4	5·3	
Aza-guanine	100	8·5	10·5	
Aza-uracil	100	5·0	5·0	
Aza-xanthine	100	8·5	8·4	
Chloramphenicol	100	5·6	4·8	
p-Fluorophenylalanine	100	7·1	7·7	Little or effect
*iso*Nicotinic acid hydrazide	100	7·1	7·0	
Penicillin G	100*	5·0	1·5	
Pyrithiamine	200	5·4	5·1	
Sodium azide	100	6·6	8·0	
Sodium fluoracetate	100	7·2	7·2	
Sodium fluoride	78	7·2	8·4	
Sulfathiazole	100	6·6	6·6	
Trypan blue	100	7·0	7·2	
Anilinonaphthalene-8-sulphonic acid	15†	9·6	9·8	
Beryllium nitrate (cryst.)	100	8·0	42	
Methylene blue	5‡	9·0	30	
Proflavine	20‡	9·0	31	Death rate accelerated
Sodium arsenate	640	9·5	13	
Sodium iodoacetate	208	7·2	28	
Sodium malonate§	1270	7·2	48	
Sodium pyridine-3-sulphonate	16‡	11·0	21·5	
Lanthanum nitrate (cryst.)	43	11	9·5	Death rate reduced
Yttrium nitrate (cryst.)	38	10·5	8·4	

* Units/ml.

† 50 mμ mole/ml.; see fig. 9.

‡ Concentrations double those quoted showed an immediate bactericidal effect on a portion of the population.

§ Dead organisms mainly spherical.

Strange *et al.* (1961) showed that these bases were products of endocellular metabolic processes which preceded death. Since breakdown of the osmotic barrier of cells also releases such materials from the cytoplasm it was of interest to discover the state of the osmotic barriers in moribund suspensions.

Mager, Kuczynski, Schatzberg & Avi-dor (1956) showed that, at low solute concentrations, the optical density of living bacterial suspensions depended on the tonicity

of the suspending medium. With *Pasteurella tularense* the optical density (OD) in 0·4 M-MgCl₂ was twice that in distilled water. They interpreted this phenomenon as due to a change in refractive index of the cells consequent upon an adjustment of the water content of the internal environment to some sort of equilibrium with the exterior; it did not occur with heat-killed or disinfectant-killed organisms, they termed this phenomenon the 'optical effect'. It provides a test of whether the osmotic barrier is functioning. With the culture of *Aerobacter aerogenes* used in this work, live organisms showed about 25 % greater OD in 0·4 M-NaCl compared with suspensions in distilled water (Fig. 6); equimolar Na₂SO₄, MgCl₂ or glucose showed smaller increments. Heat-killed organisms (10 min., 60°) showed a slight negative effect (OD in 0·4 M-NaCl about 90 % of that in water); mixtures of heat-killed + live bacteria showed an optical effect in proportion to the % live organisms over the range + 25 % to − 10 % of OD in water.
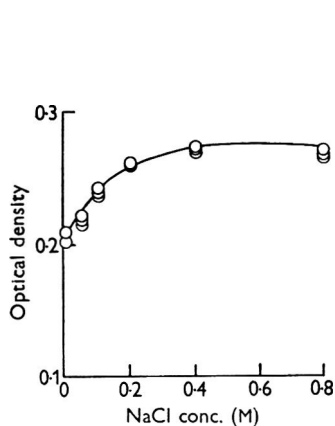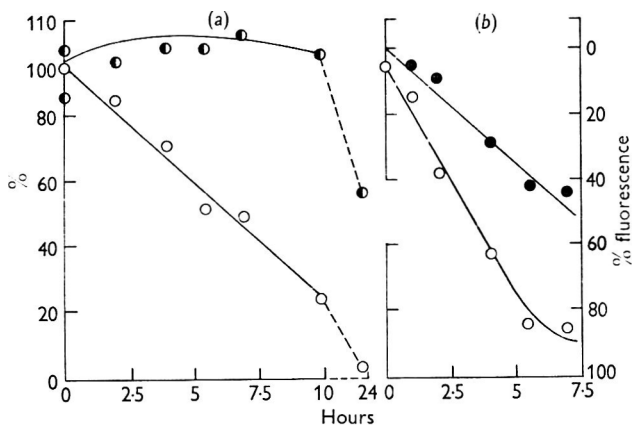
Fig. 6   Fig. 7

Fig. 6. The optical effect with logarithmic phase *Aerobacter aerogenes*. A strong washed suspension of *A. aerogenes* from continuous culture was added to NaCl solutions to give 0·12 mg. dry wt. organism/ml. and the optical densities were measured at 540 mμ. with a 1 cm. light path. Points taken at 2, 10 and 30 min. after preparation of suspensions.

Fig. 7. Permeability and viability of moribund *Aerobacter aerogenes*. *Part a.* Suspension of *A. aerogenes* prepared as for Fig. 1; added to saline-tris buffer at 150 μg. dry wt. organisms/ml. and allowed to die at 40° and pH 7·10 ± 0·05 with forced aeration. Samples centrifuged and re-suspended in distilled H₂O and 0·4 M NaCl for optical effect; diluted in saline for viability. Optical effect expressed as % of range between wholly live cells (+ 35 %) and cells killed by heating 5 min. at 60° (− 10 %). O: viability; ◐: optical effect. *Part b.* Suspension of *A. aerogenes* 20 μg./ml., pH 7·05 ± 0·05, otherwise similar to Part a. Viability determined as usual. Permeability to anilinonaphthalene-8-sulphonic acid determined fluorimetrically after addition of dye to 50 mμ mole/ml. Expressed as % of range between untreated suspension and one killed by exposure to 60° for 5 min. O: viability; ●: fluorescence.

The optical effect was examined with suspensions dying in buffer as usual, except that a ninefold greater concentration of organisms was used (initial OD = 0·22–0·25 in water in 1 cm. cuvette); the extent of the optical effect as % of maximum was compared with the viability. The results of one such experiment are shown in Fig. 7a. Though the figures for the optical effect show a fair scatter, there is no doubt that the optical effect persisted near its maximum extent when

nearly 80 % of the organisms had died. This curve includes a point observed after 24 hr., when over 98 % of the population was dead and the viability was outside the range of the slide-culture technique; nevertheless, over 50 % of the organisms showed the optical effect. It seems that the osmotic barrier was intact after 'death'. Independent support of this finding was provided by the demonstration of an unchanged 'amino acid pool' (see below) and by experiments with anilino-naph-thalene-8-sulphonic acid (similar to the toluidine dye used by Newton, 1954) which forms a fluorescent complex with these bacteria only when the osmotic barrier is destroyed. This dye was non-toxic at the concentration used (Table 2); Fig. 7*b* illustrates an experiment showing that organisms in moribund suspensions became permeable to the dye more slowly than the rate at which they died.

Mager (1959) showed that certain aliphatic diamines (notably spermine) delayed osmotic breakdown of sensitive bacteria and of spheroplasts. Since breakdown of the osmotic barrier did not normally accompany death of the organisms studied here one may predict that spermine ought not to influence the death curve. This was so: a population died at 6·6 %/hr. alone, 7·2 %/hr. with 10^{-4} M-spermine.

Biochemical changes accompanying death. Strange et al. (1961) showed that *Aerobacter aerogenes* harvested from the stationary phase of growth in a defined medium based on mannitol degraded endocellular ribonucleic acid before death; the catabolism of protein and polysaccharide was less extensive. Our populations, which differed in being harvested while steadily growing, behaved similarly in conditions resembling those used by Strange et al. Suspensions of equiv. 10 mg. dry wt. bacteria/ml. aerated in saline-tris at 40° and pH 7·0 ± 0·2 for 17 hr., during which period 98 % of the population died, lost about 50 % of their pentose and 15 % of their polysaccharide. About twice as much inorganic phosphate and about three times as much material which absorbed at about 260 mμ appeared in the medium as was released by heat-killing or treating with a quaternary detergent. About 20 % of the pentose lost by the organisms was detectable in the medium after death. Hence the phosphate and 260 mμ materials were generated by a metabolism which involved pentose fermentation and were not formed by osmotic leakage such as that observed by Mager (1959) from *Neisseria perflava* and *Pasteurella tularense* in media of low tonicity. The materials which absorbed about 260 mμ had a complex absorption spectrum: the supernatant fluid showed a main maximum at 254 mμ with subsidiary peaks at 247·5, 260 and 265 mμ. Chromatography by Mr H. E. Wade indicated hypoxanthine, uracil, guanine and an unidentified anionic material. Cytosine, cytidine, uracil and inosine were absent.

This population was some 500-times those used for our routine survival studies. Measurements on the rates of breakdown of bacterial constituents were therefore made with shorter term tests on populations concentrated by centrifugation for analysis. Typical figures for the initial composition of the organisms used are given in Table 4. RNA breakdown started immediately and was most rapid in the early stages of death of the population (Fig. 8*a*); in contrast, protein catabolism (Fig. 8*b*) was not detectable during the first few hours, though it ultimately took place. Figure 8*b* includes data in which the 'amino acid pool' was estimated during death; it remained unchanged for several hours. The polysaccharide content showed an initial decrease of about 20 %, then remained constant. Curves for DNA metabolism during death were not obtained because pilot experiments had shown virtu-

ally no change: in two experiments the viabilities of 30 μg./ml. suspensions fell
from 96–97 % to 10 and 29 %; the DNA contents of the organisms declined during
this period by 12·5 and 3·5 % respectively.

Enzymic changes during death. A few experiments were performed on the state
of the respiratory system of our population during death. Figure 9 a shows that the
endogenous Q_{O_2} declined rapidly during the first hour of storage and settled to a
low level during the main part of the survival curve. High population densities were
necessary for the measurement of the Q_{O_2}; for practical reasons a much more con-
centrated suspension was used than would ordinarily have been compatible with
the customary death rate. By coincidence the slope of the survival curve was much
as usual, possibly because the high population density was in the range where
the death rate accelerated with increasing density (Harrison, 1960). The upwards
inflexion of the Q_{O_2} value towards the end of the curve may be attributable to cryp-
tic growth.
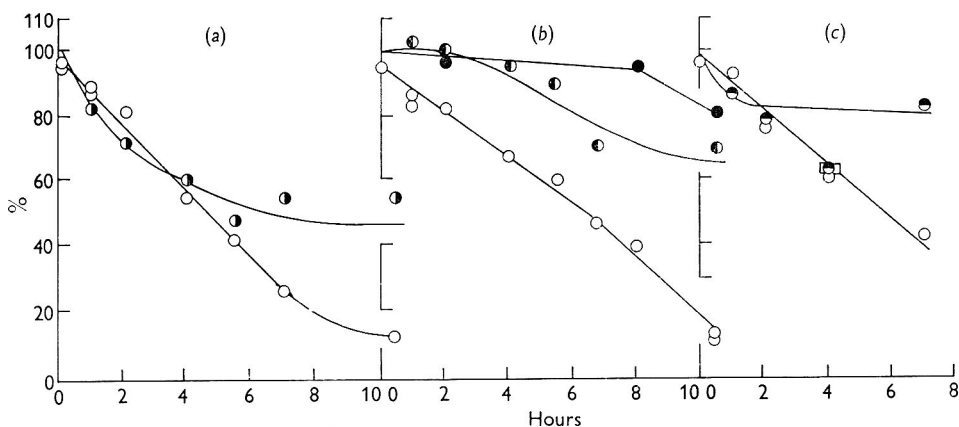
Fig. 8. Polymer content and viability of moribund *Aerobacter aerogenes*. *Part a.* Sus-
pensions of *A. aerogenes* prepared as for Fig. 1; added to saline-tris buffer at 36 μg. dry
wt./ml. and allowed to die at 40° and pH 7·2 ± 0·05 with forced aeration. 10 ml. samples
removed at intervals for viability determination and for pentose after centrifugation.
Curves incorporate data from three experiments. ○: Viability; ◑: pentose content.
Part b. Suspensions prepared as for Fig. 1; added to saline-tris buffer at 45 μg./ml. and
allowed to die at 40° and pH 7·0 ± 0·5 with forced aeration. 75 ml. portions concentrated
by centrifugation for protein analyses. Data also included for 'free amino-nitrogen'
contents of a similar suspension dying at 50 μg./ml. and pH 7·2. Curves incorporate
data from three experiments. ○: viability; ◐: protein content; ●: amino-N content.
Part c. Suspension prepared as for Fig. 1; added to saline-tris buffer at 60 μg. dry wt./
ml. and allowed to die at 40° and pH 7·0 ± 0·05 with forced aeration. 50 ml. samples
removed for duplicate polysaccharide determinations after concentration by centrifuga-
tion. ○: viability; ●: polysaccharide content.

Populations of the culture of *Aerobacter aerogenes* used in this work metabolized
glycerol incompletely in the Warburg manometer, taking up about 30 % of the
theoretical amount of O_2 for complete oxidation of the substrate before the rate of
oxygen uptake declined to about the endogenous value. No increase in the poly-
saccharide content of the organisms was found at the point at which the Q_{O_2} de-
clined; the fate of the substrate was not studied further. Initial Q_{O_2} values with
glycerol are compared with viability of a stored suspension in Fig. 9 b. The decline

in Q_{O_2} with the growth substrate paralleled the decline in viability. Figure 10 records the dehydrogenase activities of moribund populations with glycerol as hydrogen donor. The 'glycerol dehydrogenase' activity declined roughly in parallel with the viability throughout the experiment; hence the decline in Q_{O_2} with glycerol observed earlier can be attributed at least partly to the enzyme system involved in the initial attack of glycerol. Our strain is known to have not a simple dehydrogenase but a glycerol kinase and a glycerophosphate dehydrogenase (Mr D. Tempest, personal communication).
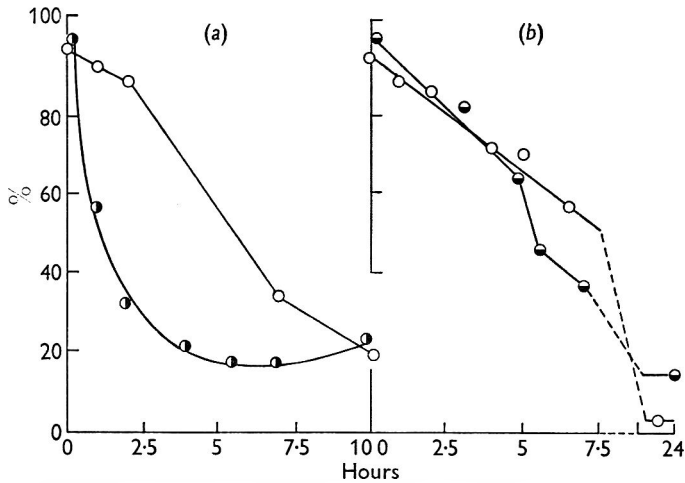
Fig. 9. Respiration of moribund *Aerobacter aerogenes*. *Part a*. *A. aerogenes* suspension prepared as for Fig. 1; added to saline-tris buffer in a Warburg manometer at 4·5 mg. dry wt./ml. and allowed to die at 40° and pH 6·95 ± 0·05 with shaking and with the apparatus open to air. Periodically the suspension was sampled for viability determination and immediately afterwards the endogenous Q_{O_2} was determined. ○: viability; ◑: endogenous Q_{O_2}. *Part b*. *A. aerogenes* suspension prepared as for Fig. 1; added to saline-tris buffer at 100 μg./ml. and allowed to die at 40° and pH 6·95 ± 0·05 with forced aeration. Periodically 20 ml. samples were centrifuged, the organisms transferred to Warburg manometer containing a saline phosphate buffer, and the Q_{O_2} with glycerol determined. ○: viability; ◒: Q_{O_2} with glycerol.

Survival curves of organisms grown in other conditions

Effect of growth rate on death rate. After over 18 months in continuous culture samples of our population were transferred to a second continuous culture apparatus in which the dilution rate was altered, causing corresponding changes in the imposed doubling time. Figure 11a illustrates the survival curves obtained after the populations had spent at least seven generations at a new dilution rate; it appears that the faster the population grew the slower it died. This generalization has been confirmed twice with carbon-limited populations of this organism: once in experiments not reported here in which survival curves were obtained in phosphate buffer, once in the series of experiments involving unusually slow growth rates (see below) illustrated in Fig. 11b.

The populations obtained at the slowest growth rates contained a proportion of organisms dead at the start, about 15 and 30 % at dilution rates of 0·12 and 0·06 hr^{-1} respectively (Table 4). This phenomenon occurred in several instances of slow
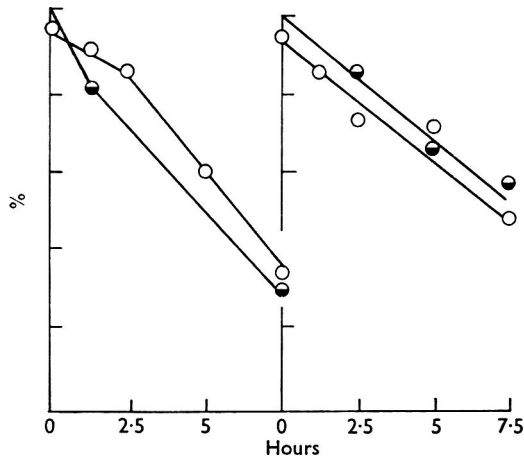
Fig. 10. 'Glycerol dehydrogenase' activity of moribund *Aerobacter aerogenes*. *A. aerogenes* suspension prepared as for Fig. 1; added to saline-tris buffer at 25 μg./ml. and allowed to die at 40° and pH 6·95 ± 0·05 with forced aeration. At intervals viabilities were determined and 40 ml. samples were centrifuged, the organisms re-suspended in a saline phosphate buffer and the dehydrogenase activity towards glycerol determined quantitatively with tetrazolium (see Methods; two experiments recorded). ○: viability; ◐ : 'glycerol dehydrogenase' activity.
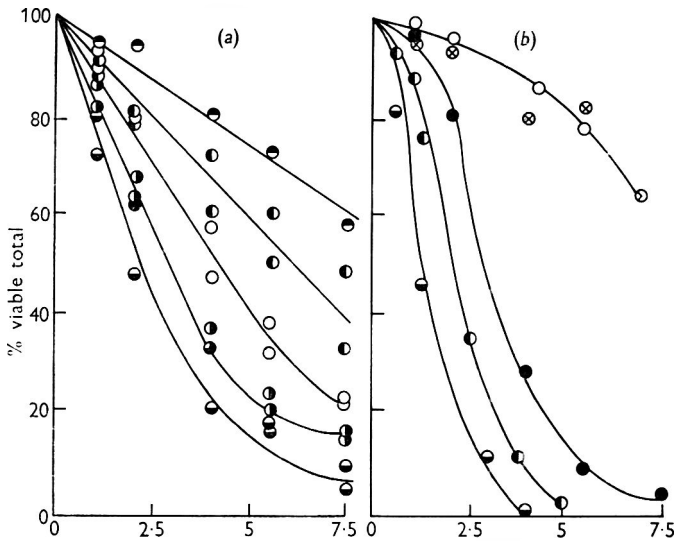
Fig. 11. Effect of growth rate upon death of *Aerobacter aerogenes*. Part a. *A. aerogenes* was grown in continuous culture (glycerol limiting growth) at different doubling times and the survival curves of 20 μg./ml. populations in saline-tris buffer at pH 7·0 ± 0·05 and 40° obtained (see text). The survival curves are corrected for organisms dead at the start of the experiments: the doubling times (t_d) refer to the viable proportion of the population in the continuous culture. The customary dilution rate was 0·25 hr.$^{-1}$. ◐ : t_d = 57 min.; ◑ : 1 hr., 18 min.; ○: 2 hr. 42 min.; ◑ : 5 hr.; ◒ : 10 hr. 6 min. (see also table 4). Part b. A similar experiment using a strain that had not undergone the alterations described in Methods. ○: t_d = 2 hr. 55 min.; ●: 5 hr. 56 min.; ◖ : 11 hr. 48 min.; ◒ : 21 hr.; ⊗: 2 hr. 53 min. after protracted growth at low dilution rates and viabilities (see table 3).

continuous culture (see Tables 3, 4); the survival curves in Fig. 11 are corrected for the organisms initially dead and refer only to the initially viable proportion of the populations tested. Similarly, the doubling times of the populations given in Tables 3 and 4 are corrected to take the dead organisms into account, since when a proportion of a steady state population is dead the doubling time of the multiplying organisms is no longer log 2/dilution rate.

Effect of cultural history. We recorded in Methods how our population of *Aerobacter aerogenes* underwent changes before the work reported in this paper was carried out. An experiment with Dr D. Herbert and Mr D. Tempest provided us with the opportunity to repeat and extend the experiment described in the previous paragraph with the unaltered parent strain of *A. aerogenes*. A continuous culture

Table 3. *Viability of populations of* Aerobacter aerogenes *in slow continuous culture*

The parent stock (see Methods) of *A. aerogenes* was grown in continuous culture at 37° with glycerol limiting growth (see text) and at intervals the dilution rate (proportion of the culture vessel replaced per hour) was halved. Some data from this experiment are included in fig. 11 *b*.

Dilution rate (hr.$^{-1}$)	Period (days)	Mean viability (%)	No. of determinations	Mean doubling time of viable organisms (hr.)
0·232	3	96·6	4	2·92
0·112	2	94·2	2	5·92
0·055	3	91·3	3	11·8
0·0295	5	86·1	8	21
0·0145	9	76·3	7	38·9
0·0074	24	57·5	19	61·2
0·0038	35	37·8	21	82·0
back to 0·226	4	92·6	4	2·89

was set up differing essentially in that the volume was 2 l., the glycerol concentration (still limiting) was 10 g./l. and the temperature was 37°. The viabilities of the populations at different dilution rates were determined, and in each new steady state duplicate survival curves of populations (equiv. 20 μg. dry wt./ml. at 40° and pH 7·00 ± 0·05) were obtained using a single batch of saline-tris buffer throughout. Some of the data obtained are collected in Table 3 and Fig. 11 *b*. Though the death rate was inversely related to the growth rate, in all instances the forms of the survival curves were different from those obtained with our customary population; instead of being arithmetically linear they gave an almost linear plot of probit against time. The steady-state viabilities for a given dilution rate of this population (Table 3) were higher, where they overlapped, than those obtained with our customary population (Table 4) grown in carbon-limiting conditions. Towards the end of the experiment, when the steady-state viability had been low for several divisions, the dilution rate was abruptly restored to the starting value of 0·25 hr.$^{-1}$. The steady-state viability rose rapidly to over 90 %, yet in spite of the powerful natural selection in favour of longevity that might be expected to have operated during the months of slow continuous culture, the survival curves then obtained did not differ significantly from those observed at the outset (Fig. 11 *b*).

Effect of enriching the growth medium. The medium was enriched with yeast

extract and casein hydrolysate; glycerol was still the main component supporting growth. Since these supplements increased the yield (wt. organisms/wt. substrate) the glycerol concentration was lowered to maintain the population in the continuous culture at about equiv. 1 mg. dry wt. organism/ml. The composition of the growth medium was that described in Methods but with 10 g. glycerol and 0·6 g. each Difco casamino acids and Difco yeast extract/l. Organisms grown in these conditions died somewhat faster (18 %/hr.) than the parent culture (16 %/hr.) after a similar number of divisions from the time of inoculation.

Effect of changing limiting nutrient. All the experiments so far recorded refer to populations derived from a continuous culture in which the concentration of glycerol controlled the population density. To study the effect of limitation by other

Table 4. *Influence of growth conditions on various properties of* Aerobacter aerogenes *populations in continuous culture*

A. aerogenes was grown in continuous culture at different rates and with various nutrients limiting growth. For details of procedures see text; analyses refer to % (w/w) washed, freeze-dried organisms; their accuracy was limited by relatively small amounts of material being available.

(i) Properties of cultures

Limiting nutrient	Dilution rate (hr.$^{-1}$)	Population density (mg. dry wt. organisms/ ml. culture)	Yield*	Mean viability (%)	t_d† (hr.)
C (21·7 mM glycerol)	0·72	0·92	1·17	98	0·97
	0·5	1·22	1·55	98	1·38
	0·25	1·08	1·38	97	2·75
	0·12	0·84	1·07	85	5·08
	0·06	0·7	0·89	7C	8·82
O$_2$	0·25	0·49	—	c. 100	2·76
N (5 mM NH$_4$Cl)	0·44	0·52	7·44	96	1·55
	0·24	0·52	7·44	96	2·77
	0·12	0·54	7·7	95	5·69
P (0·4 mM NaH$_2$PO$_4$)	0·45	0·64	51·5	97	1·53
	0·27	0·74	60	96	2·54
	0·12	0·88	71	98	5·77
S (0·31 mM Na$_2$SO$_4$)	0·41	0·20	200	97	1·68
	0·26	0·24	240	92·5	2·5
	0·12	0·22	220	9C	5·35
Mg (20 μM MgCl$_2$)	0·43	0·38	391	96	1·58
	0·25	0·57	587	c. 86	2·48
	0·11	0·72	741	62	4·37

(ii) Compositions of organisms

		Protein (%)	RNA (%)	DNA (%)	Polysaccharide (%)
C	0·25	68·6 ± 3·5	12 ± 0·6	3·50 ± 0·03	2·34 ± 0·06
N	0·24	61·3 ± 3·0	14·5 ± 0·7	3·90 ± 0·04	3·80 ± 0·10
P	0·27	77·5 ± 4·5	13·9 ± 0·8	4·43 ± 0·04	2·88 ± 0·07
S	0·26	57·6 ± 2·9	13·7 ± 0·7	3·53 ± 0·03	3·63 ± 0·10
Mg	0·25	74 ± 3·8	17 ± 0·9	4·17 ± 0·04	3·33 ± 0·09

* mg. dry wt organisms/mg. element.
† t_d: mean doubling time of the viable organisms in the culture.

nutrients, portions of our population were transferred to the second continuous culture apparatus and allowed to grow with different components of the medium limiting the population density. The ordinary carbon-limiting conditions gave a population density equiv. 1–1·1 mg. dry wt. organism/ml. Oxygen limitation was obtained by altering the air flow to 0·6 l./hr. and simultaneously decreasing the stirring rate; the population density declined to about half (Table 4). Nitrogen limitation was obtained by using K_2HPO_4 in place of $(NH_4)_2HPO_4$ in the recipe quoted in Methods and adding NH_4Cl to 5 mM. Magnesium limitation was obtained by altering the magnesium content to 20 μM; sulphur limitation by using KCl in place of K_2SO_4 and adding Na_2SO_4 to 0·31 mM. Phosphorus limitation was obtained by using $NaHCO_3 + NH_4HCO_3$ in place of the phosphates quoted in the recipe and adding NaH_2PO_4 to 0·4 mM; the medium was sterilized by filtration under positive pressure; the culture was buffered to pH 7·0 $\pm$ 0·1 by incorporating 30 % (v/v) of CO_2 in the gas phase. The organisms were allowed to grow at three dilution rates approximating to 0·12, 0·25 and 0·45 hr.$^{-1}$. Data are collected in Table 4. With C-limitation the yield increased with dilution rate until it passed a maximum where 'wash-out' began; with N- and S-limitation the yield was little influenced by flow rate over the range tested; with P-limitation the yield decreased somewhat at faster flow rates; with Mg-limitation the yield decreased markedly with increasing flow rate (cf. Herbert, 1958). The steady-state viabilities obtained with the S-limited culture probably signify a trend in the direction of the partially dead continuous cultures obtained with very slowly growing C-limited populations. The P- and N-limited culture did not show this phenomenon in the range tested; in other experiments we have observed it with N-limited organisms grown very slowly. The Mg-limited organisms showed the phenomenon to a marked extent.

Strong illumination with visible light accelerated the death of our stored suspensions (see above). The continuous cultures were illuminated with diffuse daylight in day-time and were subject to intermittent illumination by the infra-red heaters which, depending on the type of bulb used, sometimes included considerable amounts of white light. Killing by visible radiation might therefore have contributed to the low steady-state viabilities found in these experiments. A fresh continuous culture of Mg-limited organisms was set up from our customary population and run with strong visible illumination (about 1500 ft.-c.) at a dilution rate of 0·1 $\pm$ 0·01 hr.$^{-1}$. When the viability had settled at a steady value for a few days the infra-red heater was changed for one with a red filter (about 80 ft.-c.) for a few days; finally, the culture vessel was wrapped in blackened foil and the temperature maintained at 40° by a water jacket. Throughout this experiment the steady-state viability remained at 75 $\pm$ 5 %; we conclude that killing by visible light contributed negligibly to the steady-state viabilities obtained during this work.

The survival curves obtained with these populations showed marked differences. In Fig. 12 survival curves at about 0·25 hr.$^{-1}$ are collected; each curve is typical of at least four examples for a given nutrient limitation. In all instances the organisms had spent over 100 generations in their new growth condition because the experiments with Mg-limitation gave some suggestion that the form of the survival curve changed over the first forty or so generations. P- and O-limitation, like the ordinary C-limitation, gave virtually linear survival curves; those of O-limited bacteria were the least steep. S-limitation gave a nearly linear curve, particularly

when corrected for the 10 % of bacteria dead at its start. N-limitation gave a sigmoid curve: a period of slow death was followed by one of rapid decline. The N-limiting medium had a twofold excess of glycerol over that required to account for the population density at $D = 0.25$ hr.$^{-1}$ (Table 4); increasing this excess to fourfold did not significantly alter the survival curves obtained. Mg-limitation gave concave survival curves. All the survival curves flattened after 80–90 % of the population had died; a Mg+Ca mixture (concentrations as in Table 1) prolonged survival of N-, P-, S- and Mg-limited organisms; O-limited organisms were not tested.

The influence of growth rate on death rate in the various conditions of nutrient limitation tested are illustrated in Figs. 13 and 14, which are comparable with
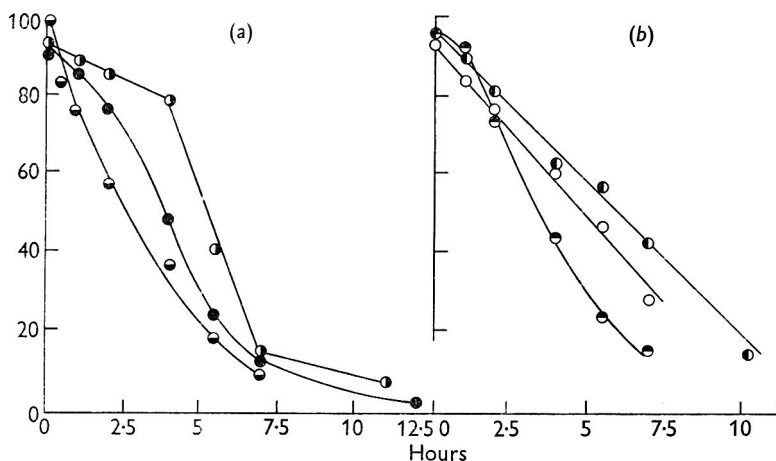
Fig. 12. Effect of nutritional state on death of *Aerobacter aerogenes*. *A. aerogenes* was grown in continuous culture at a dilution rate of 0·25 hr.$^{-1}$ with different components of the medium limiting the growth of the organisms. Survival curves of 20 μg./ml. populations in saline-tris buffer aerated at 40° and pH 7·00 ± 0·05 were obtained (see text). Part (a): ●: limited by S; ◑: by N; ◒: by Mg. Part (b): ◐: by O; ◓: by P; ○: by C.

Fig. 11a. The generalization that the faster the organisms had grown the slower they died applied to N-, P- and S-limited bacteria, though at the slower growth rates the differences were small compared with those obtained with C-limitation. With N- and P-limitation the survival pattern depended on the length of an initially flatter portion of a sigmoid survival curve; the slopes of the steeper portion did not change markedly among the three growth rates tested. The curves for P-limited organisms illustrate a case in which the flatter portion became undetectably small and the survival curve shifted from sigmoid to linear. The survival of Mg-limited organisms was complicated by the low steady-state viabilities of the populations tested. The actual curves obtained are shown in Fig. 14a, the corrected curves in 14b. The generalization previously observed was reversed in these conditions; the faster these organisms had grown the faster they died (see Discussion). The form of the survival curve shifted with increasing growth rate from sigmoid through linear to concave.

Comparison with stationary phase populations. A well aerated batch culture was inoculated from the continuously growing population and survival curves taken

(*a*) between the 8th and 9th generation of logarithmic growth, and (*b*) 100 min. after the population had become stationary due to exhaustion of glycerol. Contrary to expectation, the survival curves were closely similar (Fig. 15).

Some experiments were undertaken to determine the effect of different nutrient limitations on the survival patterns of stationary phase bacteria. Inocula were taken from the continuous culture and allowed to grow as batch cultures (usually under forced aeration) in conditions in which the C, N, Mg or O content of the environment limited growth. After the populations had been at their maximum optical densities for 4–5 hr. the death rates of washed samples were determined in
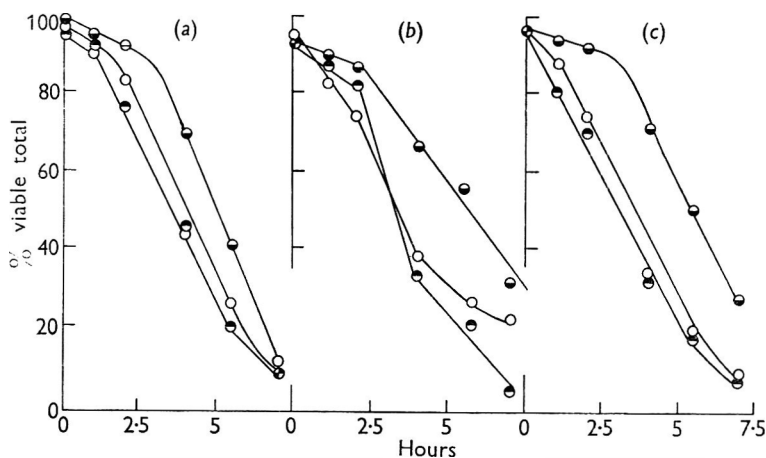
Fig. 13. Effect of growth rate on death rates of *Aerobacter aerogenes* in different nutritional states. *A. aerogenes* was grown in continuous culture at the dilution rates indicated with different components of the medium limiting the growth of the organisms. The survival curves of 20 μg./ml. populations in saline-tris buffer at pH $7·00 \pm 0·05$ and 40° were obtained (see text). Curves are typical of at least four tests in each condition. Part (*a*) N-limitation at dilution rates of ◒ : 0·44, ○: 0·24 and ◓ : 0·16 hr.$^{-1}$. Part (*b*) S-limitation at dilution rates of ◒ : 0·41, ○: 0·30 and ◓ : 0·12 hr.$^{-1}$. Part (*c*) P-limitation at dilution rates of ◒ : 0·42, ○: 0·27 and ◓ : 0·12 hr.$^{-1}$.

the usual way. The experiments described in this and the next paragraph used organisms grown at 37°; the temperature of the control continuous culture was 37° for their duration. The C-limited population died more rapidly than did a N-limited population, but the survival curve in the latter condition remained linear. Mg-limitation led to an L-shaped survival curve. Oxygen limitation, obtained by growing the organisms in a sealed flask containing a magnetic stirrer, provided the most long-lived population.

Death of synchronized populations. A modified culture apparatus was constructed to provide a continuous supply of synchronously dividing organisms. Its construction (to be described) was based on a principle used by Campbell (1957) to induce partial synchrony in yeasts by subjecting them to repeated short periods of starvation between generations. Figure 16 indicates that this treatment induced partial synchrony with our strain of *Aerobacter aerogenes*. Populations taken from four stages in the cycle showed small differences in their death rates; death was most rapid towards the end of division (70 min.) and at the beginning of the new cycle (10 min.).
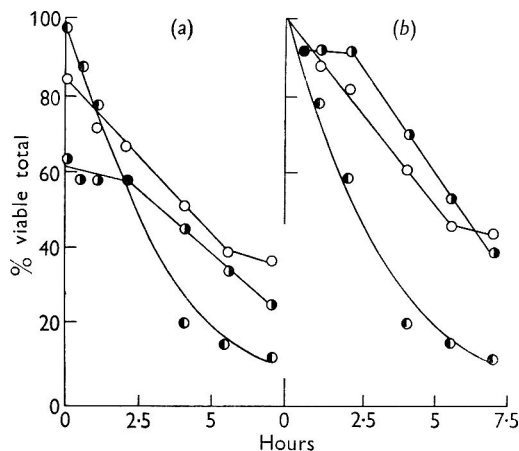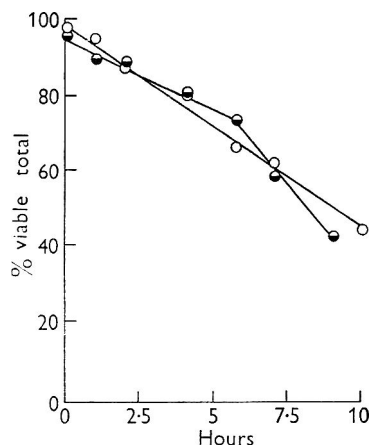
Fig. 14

Fig. 15

Fig. 14. Effect of growth rate on death rate of magnesium limited *Aerobacter aerogenes*. *A. aerogenes* was grown in continuous culture at the dilution rates indicated with 20 μM-$MgCl_2$ limiting growth. The survival curves of 20 μg./ml. population in saline-tris buffer at pH 7·00 ± 0·5 and 40° were obtained (see text). Curves are typical of at least four tests in each condition. Part (*a*) actual curves at dilution rates of ◑ : 0·43, ○: 0·25 and ◐ : 0·13 hr.⁻¹. Part (*b*) same curves corrected for organisms dead initially. Doubling times of viable organisms corresponding to the dilution rates quoted are ◑ : 0·16 hr., ○: 2·45 hr. and ◐ : 3·8 hr.

Fig. 15. Effect of phase of growth on death of *Aerobacter aerogenes*. *A. aerogenes* was inoculated into a well aerated batch culture and after 8 to 9 generations of 'free' logarithmic growth survival curves of a 20 μg./ml. population in saline-tris buffer at pH 7·00 ± 0·05 and 40° were obtained and compared with similar survival curves obtained later when the same population had completed logarithmic growth owing to exhaustion of glycerol and had been stationary for 100 min. For procedure see text. ○: logarithmic phase organism; ◒ : stationary phase organisms.
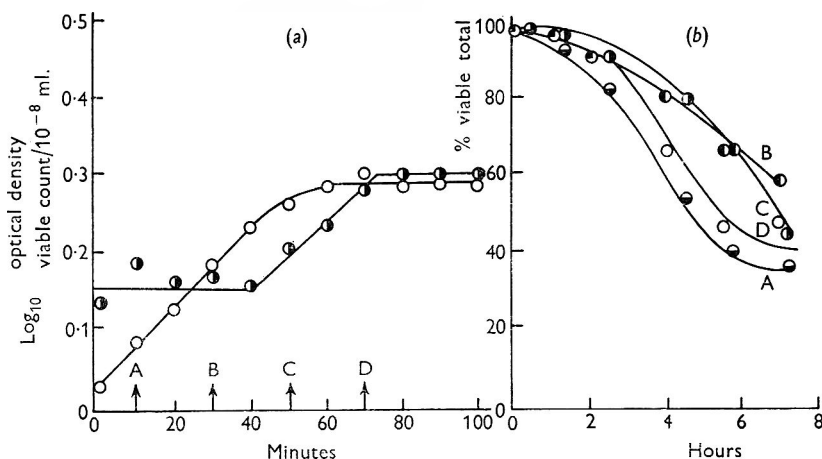

Fig. 16. Death of synchronized populations of *Aerobacter aerogenes*. *A. aerogenes* was grown at 37° in an apparatus that imposed synchrony on the population as evidenced by part (*a*); ○: optical density; ◑ : plate count (all samples 98 to 100 % viable). Survival curves of 20 μg./ml. populations in saline-tris buffer at pH 7·00 ± 0·05 and 37° were obtained with samples taken at intervals through the synchronous division cycle and are quoted in part (*b*); A: ◒ 10th min.; B: ◐ 30 min.; C: ◑ 50th min.; D: ○ 70th min.

DISCUSSION

Biological integrity of 'dead' micro-organisms. We have used the term 'dead' to describe bacteria that failed to multiply in the arbitrary favourable environment provided by our slide-culture medium. This is a legitimate usage, since microbiologists are usually concerned with the ability of the organisms to initiate a fresh population rather than its survival as an individual. Our organisms retained their osmotic barriers after death and may thus have been in some sense 'alive'; Razumovskaya & Osipova (1958) noticed *Acetobacter melanogenum* organisms which were impermeable to the vital stain acridine orange yet which did not form colonies in plate counts. It follows that the assumption commonly made that dead organisms are permeable to the medium and live organisms are not is not universally true. Since this assumption is the basis of several procedures for the rapid assay of viability (immersion refractometry, vital staining, optical effect, leakage of purine bases), results obtained with these procedures should be interpreted cautiously.

Analysis of stresses. Studies on microbial survival necessarily involve applying some intentional stress (drying, chilling, disinfection, etc.) to the population. Subsidiary stresses may be applied unintentionally. We studied starvation at their growth pH value and temperature as the stress likely (*a*) to cause least interference with the physiological organization of the organisms and (*b*) to entail a minimum of subsidiary stresses. Yet our procedure for submitting organisms to starvation proved to engender three subsidiary stresses. The overt stress was limitation of the supply of energy source during growth; subsidiary stresses were: (i) trace element deficiency during death (demonstrated by the protective effect of calcium, magnesium or iron), (ii) a stress due to the pH value of growth not being optimal for survival, (iii) a stress (presumably osmotic) caused by brief exposure to distilled water before storage which committed our populations to a faster death rate than they would otherwise have shown.

Cryptic growth. About fifty of our organisms needed to die to support division of one. Since we have concerned ourselves primarily with the survival of the first 80 % or so of our populations we can be confident that the individual organisms at the beginning and end of the survival curves were the same. This consideration only eliminates cryptic growth as a factor in the survival of population of our C-limited organisms in our routine conditions: equiv. 20 μg dry wt. organisms/ml. saline-tris buffer at 40°. Cryptic growth may have influenced the survival patterns of organisms studied at other temperatures, in other media or even at higher population densities. It undoubtedly influenced the survival pattern of the organisms when the survival curve was examined over several orders of magnitude (Fig. 1).

Toxicity of buffer. Though we identified copper and neutralized it with EDTA we were unable rigidly to exclude the possibility that a subsidiary stress due to another toxic material in the buffer influenced the survival curves. Three considerations make this possibility unlikely. First, cryptic growth in fact occurred provided one waited long enough; secondly, one would need to attribute rather special properties to the postulated toxic material to account for the variety of survival patterns which were obtained depending on the nutritional status of the population being tested; thirdly, the survival patterns obtained in our routine conditions were unchanged when a saline-phosphate buffer was used instead of saline-tris.

Physical conditions affecting survival. Illumination, though a potential hazard, did not influence our findings. Temperatures below the 40° imposed for growth prolonged survival, but there was a limit below which further cooling accelerated death. This finding is relevant to the custom of storing viable bacteria at refrigerator temperatures: it is possible that at room temperatures survival would be prolonged. The population effect, the dependence of death rate on the concentration of organisms, is a curious phenomenon earlier observed by Harrison (1960) which our experiments entirely confirm. We can add nothing to Harrison's discussion of it beyond the demonstration already mentioned that cryptic growth plays no part in it.

Chemical conditions affecting survival. A pH value lower than 7 favoured survival of our populations, consistent with earlier reports (Cohen, 1922; Winslow & Falk, 1923a; Strange *et al.* 1961). Anaerobic conditions accelerated death despite the change to more favourable pH values that usually occurred in these conditions. Inorganic components of the growth medium such as phosphate, potassium, ammonium, sulphate, etc., had no influence on survival though some of these ions have been implicated in the salt balance of microbes. Only certain trace cations, Mg, Ca and to some extent Fe, were protective. Mr R. Strange (personal communication) has observed protection of stationary phase *Aerobacter aerogenes* starved in phosphate buffer by Mg or Ca. The vitamins were unequivocally inactive, but the conclusion that amino acids were inactive must be regarded as tentative since the tests were complicated by growth.

Substrate-accelerated death. Glycerol, the energy source for growth, accelerated death. This observation is consistent with statements in the publications of Ryan (1959) and Strange *et al.* (1961). The phenomenon was not due to toxic impurities in the glycerol since known intermediates in glycerol metabolism also accelerated death; it is being studied further.

Biochemical changes during death. Our populations metabolized their endocellular polymers, thus resembling the organisms studied by Strange *et al.* (1961). Endocellular RNA was metabolized first, the ribose largely oxidized, adenine and cytosine (but not guanine) de-aminated and excreted, inorganic phosphate excreted. Ribose oxidation might well account for the rapid drop in endogenous Q_{O_2} that preceded death. Protein was metabolized later, at such a rate that the 'amino-acid pool' of the bacteria remained unchanged for several hours. Polysaccharide, of which the organisms contained little, was catabolized slightly at the start of the survival curve and then not at all; DNA was scarcely metabolized. Bacteria grown in conditions other than C-limitation may have shown different catabolic patterns (cf. Strange *et al.* 1961), but it seems that with our organisms RNA was the most expendable 'reserve' material.

Rahn & Schroeder (1941), from a study of the catalase and succinic dehydrogenase activities of *Bacillus cereus* near the threshold of thermal death, concluded that enzyme decay did not accompany death. In our experiments 'glycerol dehydrogenase' and 'glycerol oxidase' activities declined in parallel with viability. This parallelism could imply that the relevant enzymic activities remained unchanged until the organism died, when they became negligibly small. Or it may be fortuitous. In either case both this phenomenon and substrate-accelerated death (see above) have an obvious practical relevance to the state of bacteria which are shaken with substrates + buffer for long periods in Warburg manometers.

Effect of metabolic inhibitors. Despite the metabolism that accompanies death none of the metabolic inhibitors tested prolonged survival to any great extent. The protective effect such substances have on aerosols (Webb, 1959a, b) must be connected with supplementary stresses involved in aerial suspensions and recovery therefrom. Our failure to observe protection by chloramphenicol contrasts with the report of Morrison, El Bagoury & Fletcher (1956), but Professor R. B. Morrison has informed us that their phenomenon, which occurred in meat-extract broths, was due to inhibition by chloramphenicol of a decarboxylase system which caused the pH value to change to toxic values; our storage conditions did not provide substrates for such an enzyme. Lanthanum and yttrium showed slight protective effects which may have been connected with their anti-phosphatase activity (Clayton, 1959); alternatively, they may have had a sparing action on the organisms' reserves of active ions such as Mg or Ca.

Shapes of survival curves. Despite doubts dating back many years (e.g. Buchanan & Fulmer, 1928), survival curves of dying bacteria are widely expected to be exponential in form. The majority of the C-limited populations of *Aerobacter aerogenes* gave linear survival curves, though after about 80 % had died the curve flattened and could be held to approach an exponential form. For a given growth rate the shape of the survival curve depended on the nutritional status of the population: C-, O-, or S-limitation gave near linear curves, N-limitation gave sigmoid curves and only Mg-limitation gave concave curves approximating to the exponential from the outset.

Effect of biological history. Our strain had undergone the variations reported in Methods. The experiment recorded in Fig. 11b involved growing the original laboratory stock which had not changed; the survival curves obtained were smoothly sigmoid and gave linear plots of probit against time. We conclude that the biological history of the population influenced its survival characters, and expectation of this result was one of our reasons for choosing to study organisms that had spent many generations in steady continuous culture. However, our attempt deliberately to select for long-lived organisms was not successful; in contrast Harrison (1961) has briefly noted the isolation of 'starvation resistant' mutants.

Relation of death rate to growth rate. The faster C-limited organisms grew, the slower they died. *Aerobacter aerogenes* growing rapidly with glycerol contains more RNA than slowly growing organisms (Herbert, 1958; his Fig. 11), so this observation is consistent with the suggestion that the longevity of these bacteria was in part determined by their RNA content.

The generalization that the faster the bacteria grew the slower they died applied also to N-, P-, and S-limited populations. Harrison (1961) briefly mentioned a converse relation which (personal communication) applied to N-limited organisms. The only instance of a converse relation occurred with our Mg-limited populations and in this instance the position was complicated by proportions of dead organisms present at the start of the survival curve determination. It could be argued that the most mortal individuals were dead before their survival characters could be examined experimentally, and that the generalization was true in principle even with Mg-limited organisms.

Continuous culture of moribund populations. By decreasing the growth rate until the death rate made an appreciable contribution to the population dynamics, steady

states in which constant proportions of the population were dead were obtained with C-, S- or Mg-limited organisms; Mr D. Tempest has obtained comparable steady states with N-limited *Aerobacter aerogenes*. It seems that these bacteria were obliged either to multiply or die; we were unable to provide them with just sufficient nutrient to maintain themselves indefinitely without dividing. These observations have two consequences of importance. First, a further proportion of the organisms from a moribund population would die during the lag phase of slide culture or plating; hence the viabilities recorded by the techniques were those of the population at the end of its lag phase, not at the time of sampling. This fact probably accounts for the lower viabilities obtained when largely dead populations were plated on a minimal medium compared with those found with the customary 'rich' medium. Secondly, if it is generally true that slow continuous culture of bacteria leads to steady states in which substantial proportions of the populations are dead, then practical installations based on analogous principles (e.g. activated sludge plants; anaerobic digestion of sewage) and natural systems of a similar character (e.g. rumina of animals, sulphur springs) may well be largely populated by dead microbes.

Effect of growth phase. Populations harvested from the stationary phase of growth are often regarded as less fragile than those from the exponential phase (see review by Winslow & Walker, 1939) since they are more resistant to cold shock (Sherman & Albus, 1923; Hegarty & Weeks, 1940; Meynell, 1958; Gorrill & McNeil, 1960), heat shock (Ellicker & Frazier, 1938; Lemcke & White, 1959), decompression (Fraser 1951), desiccation (Lemcke, 1959), freeze-drying (Fry & Greaves, 1951) and other stresses. Our experiments with partly synchronized organisms, which died slightly more rapidly when harvested just before or after division, led us to expect that a proportion of the logarithmic phase organisms from batch cultures of our bacteria would be hypersensitive to death by starvation. In fact this was not so; we could detect no significant differences between the survival patterns of logarithmic phase and early stationary phase populations from batch cultures of our strain. This observation is not necessarily in conflict with the general fragility of other logarithmic phase bacteria, because the examples mentioned above involved different stresses from starvation. But there is a discrepancy between our finding and the report of Strange *et al.* (1961) that stationary phase *Aerobacter aerogenes* from batch culture died considerably less rapidly of starvation in phosphate buffer than did logarithmic phase organisms. Though there are differences in experimental detail between our studies and those of Strange *et al.* (1961), we are inclined to attribute difference in behaviour to differences in the biological histories of the sub-strains studied. Our organisms had spent many months in continuous culture before testing as batch cultures and may have remained physiologically homogeneous on transfer to batch culture compared with organisms subject to repeated batch culture. We suggest that, provided inheritable differences among the individuals in the inoculum are reduced to a minimum, the phase of growth of a population has only a small effect on its susceptibility to death by starvation. Generally speaking, the nutritional status of the population is the most important factor.

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Serological Tests of a Relationship between Rumen Selenomonads *in vitro* and *in vivo*

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SUMMARY

Fluorescent antisera to strains of small *Selenomonas ruminantium* var. *lactilyticas* grown *in vitro* from sheep rumen contents appeared to react specifically with some of the large selenomonads seen *in vivo* in rumen contents. It seems probable, therefore, that the selenomonads isolated by the usual cultural methods are those seen in rumen contents.

INTRODUCTION

In a previous paper (Hobson & Mann, 1961) the isolation from the sheep rumen of glycerol-fermenting selenomonads, identified as *Selenomonas ruminantium* var. *lactilyticas* (Bryant, 1956), was described. These bacteria were isolated by culturing dilutions of rumen fluid from a number of sheep in suitable media. However, the cells which grew in these cultures, although definitely selenomonads in morphology, never exceeded 3μ in length, and were generally smaller. Direct microscopic observation of rumen contents, however, shows that although there is some variation in size of bacteria morphologically resembling selenomonads, there are usually a number of selenomonads which are very large, being $5-8\mu$ in length. These obviously visible large organisms are usually those referred to when mention is made of selenomonads in descriptions of rumen contents (see, for instance, Moir & Masson, 1952). Although Bryant (1956) reported that some of his strains of *S. ruminantium* isolated from the bovine rumen were up to 7μ in length *in vitro*, none of our sheep rumen strains ever attained anything like this size. It is known that cultural conditions can influence the morphology of bacteria, and Herbert (1953) showed that the rate of growth can influence the size of cells of rod-like bacteria (e.g. *Aerobacter aerogenes*) in continuous culture. It was thought, therefore, that the large selenomonads seen in the rumen, which are growing under presumably optimum conditions in medium in what is, in effect, a continuous culture might be identical with the smaller selenomonads grown *in vitro* in 'batch' cultures under what might not be completely optimum conditions, and which certainly differ from those obtaining in the living rumen. A possible method of identifying the organisms *in vitro* and *in vivo* seemed to be the use of fluorescent antisera. Fluorescent antisera have been used in these laboratories in the study of rumen bacteria for a number of years (e.g. Hobson & Mann, 1957; Hobson, Mann & Oxford, 1958) and the necessary apparatus was readily available. This paper describes some results obtained during the experiments.

METHODS

Organisms. The isolation and properties of the Selenomonas strains 6 and 17 (from sheep on different diets) were described by Hobson & Mann (1961), and they had been kept as subcultures on slopes, as described in that paper, since isolation. Strains 6 and 17 were representatives of groups differing slightly in fermentations. The other bacteria used were from stock cultures or formolized suspensions kept in this laboratory, and the properties of many of them have been described in previous papers. We are indebted to Dr M. P. Bryant, U.S.D.A., Beltsville, Maryland, U.S.A., for cultures of bovine rumen strains of *Ruminococcus albus*, *Selenomonas ruminantium* (strain GA 192) and *S. ruminantium* var. *lactilyticas* (strain PC 18).

Preparation of antisera to sheep rumen strains of Selenomonas ruminantium. 'H' and 'O' antisera were prepared as follows. For 'O' antigens cultures were grown for 17 hr. in a casitone-yeast liquid medium containing 0·5 % (w/v) glucose similar in constitution to the media described previously (Hobson & Mann, 1961). A washed suspension of the cells in saline was heated at 100° for 2 hr., the cells were centrifuged off, washed twice in saline and finally resuspended in saline with 0·25 % formalin added as preservative. Rabbits were given two courses of six daily intravenous injections of this suspension starting with three injections of 0·2 ml. suspension of opacity 1 (opacity tubes, Burroughs Wellcome, Ltd.) and increasing the dose from 0·5 to 1 ml. of suspension of opacity 6 during the second week. One week rest was given between the courses, and one week after the final injection the rabbits were bled for preparation of antisera. Starting the injections with a dilute suspension was found necessary since more concentrated suspensions proved fatal in earlier experiments. For testing for 'O' agglutination alcohol-treated suspensions were prepared by suspending the washed bacteria in 50 % (v/v) ethanol/saline and keeping at 37° for 20 hr. The bacteria were then centrifuged off and finally suspended in formol-saline as above. For 'H' antigens cultures were grown for 17 hr. in a casitone–yeast medium with the glucose reduced to 0·1 % (w/v). Formalin, 0·25 % (v/v) was added to the culture and it was incubated overnight at 37°. The bacteria were centrifuged off and resuspended in 0·25 % (v/v) formol–saline. Antisera were produced by giving rabbits a course of inoculations similar to those used for preparation of 'O' antisera. All antigens and antisera were kept at 4°, the latter with a few crystals of Thiomersal B.P. (British Drug Houses, Poole, Dorset) added as preservative.

Conjugation of antisera with fluorescein isothiocyanate. In a previous work (Hobson & Mann, 1957) conjugation of antisera with fluorescein *iso*cyanate was used. As this procedure is somewhat long, involving the preparation of the *iso*cyanate from fluorescein amine, in these studies conjugation with fluorescein *iso*thiocyanate was used as this compound is commercially available. Using rumen contents, which contain fluorescing material of a number of colours, and under our conditions of viewing, we find that the green fluorescence of fluorescein is easier to distinguish than the red-orange fluorescence of dyes such as RB 200 (Chadwick, McEntegart & Nairn, 1958). The fluorescein *iso*thiocyanate was obtained from British Drug Houses. The material as obtained appears to be impure and probably contains fluorescein amine, as it is a brownish yellow colour and not completely soluble in acetone. No attempt was made to purify the material, but, in so far as it was

possible, an excess amount of *iso*thiocyanate dissolved in acetone was used for conjugation. Some preliminary conjugations established that, under the conditions described below, this resulted in a conjugate of sufficient fluorescence. The procedure is a modified form of that of Marshall, Eveland & Smith (1958). Whole serum, 2·5 ml., was stirred with 4·2 ml. 0·15 M-NaCl, and 1·7 ml. 0·5 M-carbonate/bicarbonate buffer, pH 9·2, at 2° in a cold room. One millilitre of an acetone solution containing 20 mg. fluorescein *iso*thiocyanate was added dropwise to the stirred serum solution from a hypodermic syringe fitted with a fine needle. After addition of the fluorescein solution the whole was left to stir at 2° for 18 hr. The resultant fluorescent solution was dialysed at 2° against 1 l. volumes of 0·15 M-NaCl buffered to pH 7·0 with 0·01 M-phosphate changed daily until the saline showed no further fluorescence in ultraviolet (UV) light. The dialysed solutions were then cleared by centrifuging at 22000*g* at 0° for 35 min. and the supernatants stored at 4° after the addition of a small amount of thiomersal. In the following descriptions 'conjugated antiserum' refers to the solution of antiserum in saline produced in this way. The conjugated antisera were then absorbed by shaking with liver powder as previously described (Hobson & Mann, 1957). For absorption of 17H conjugated antiserum with *Veillonella gazogenes* about 1·5 ml. of the antiserum was treated twice at 38° for 30 min. with a few drops of a thick saline suspension of the bacteria, and centrifuged at 22000*g* between each treatment and twice afterwards. Testing with a slide of *V. gazogenes* after this showed no reaction, but the reaction with the homologous organism remained.

Preparation of specimens. The slides used were of UV-transmitting glass (Shandon Scientific Co., Cromwell Place, London, S.W. 7) with Chance no. 1 coverslips. Immersion oil was Reichert non-fluorescing oil (Shandon Scientific Co.). Photographs were made on Ilford FP3 film with exposures of about 2–3 min. for UV and about 20 sec. for white light. (This light was of low intensity.) The microscope used was that described previously (Hobson & Mann, 1957). In the previous work (Hobson & Mann, 1957) wet preparations were used, as the methods of fixing tried all seemed to lead to increased non-specific fluorescence. However, the following method was finally used and this gives preparations which show no non-specific fluorescence of feedstuffs or protozoal contents when liver-absorbed sera are used and which remain stable for many months and can be examined many times. A thin smear of the bacteria or rumen contents was spread on a slide, any 'large' pieces of grass, etc., in the rumen contents being removed with a wire, and the film was allowed to dry at room temperature. The smear was then fixed by immersion in acetone for 20 min., the acetone allowed to evaporate and the slide immersed in buffered saline (as used for dialysis) for 1–2 min. The excess saline was removed by blotting gently and a small drop of the conjugated antiserum spread over the damp smear and the slide incubated at 38° for 30 min. in a Petri dish containing a piece of damp cotton wool to prevent evaporation. After this the slide was immersed in buffered saline for a few moments to remove excess antiserum, and washed by immersion in three lots of buffered saline for 3–5 min. each. The slide was gently blotted and a drop of redistilled glycerol containing about 10 % (v/v) buffered saline spotted on to the damp smear. This was covered by a cover glass and blotted as thin as possible. After examination, if the slide were to be kept, the excess immersion oil was removed by gentle wiping with a piece of tissue as the oil tends to become fluo-

rescent on exposure to air. Although the glycerol provides a non-fluorescent mountant a drawback is that it reduces the contrast in white light and so does not give sharp photographs.

Sheep and sampling procedures. Sheep 30 was fed on 900 g. hay and 450 g. grass cubes per day given in two feeds. This is the same diet as the sheep from which *Selenomonas ruminantium* strain 17 was obtained. Sheep 43 was fed on 900 g. hay and 450 g. concentrates (ground maize, crushed oats, bran) which is a similar diet to the sheep from which strain 6 was isolated. Samples were taken via a rumen cannula about 3 hr. after feeding.

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

Non-conjugated antisera. The antisera to *Selenomonas ruminantium* var. *lactilyticas* strains 6 and 17 were tested against the antigen suspensions prepared for 'H' and 'O' agglutinations. 'O' agglutination was recorded after 24 hr. at 50°, 'H' agglutination after 4 hr. at 50°. The 'O' antisera reacted to a titre of 1/2560 with the 'O' suspensions of the homologous organisms and the 'H' antisera reacted to a titre of 1/10240 in the case of strain 6 and to 1/5120 in the case of strain 17. It proved impossible, even by utilizing such techniques as the 'Craigie' tube, to obtain an inoculum which gave rise to a culture containing anything near 100 % motile, flagellated, bacteria and so the 'H' antisera always contained a high proportion of 'O' antibodies. The 'O' reaction could be decreased by absorption of the antisera, but never eliminated, and so there was cross-reaction between 'H' and 'O' antisera and suspensions. However, there was some indication that the 'H' antigens were specific to the strains, and that the 'O' antigen might be specific for *S. ruminantium* var. *lactilyticas*, as an agglutination reaction was obtained with 'O' suspensions of the bovine rumen strain of *S. ruminantium* var. *lactilyticas* but not with the bovine strain of *S. ruminantium*.

Conjugated antisera. The conjugated antisera were tested against organisms in dried films prepared as under 'Methods'. The results with the non-conjugated antisera mentioned above, although not particularly clear cut with respect to 'H' and 'O' agglutination, did show that antisera of sufficiently high titre for conjugation could be obtained and that these might be used for testing for the selenomonads in rumen contents. The 6O, 6H, 17O and 17H antisera were conjugated and liver absorbed as described above. Each of these antisera when tested against the corresponding antigen gave a good reaction, the 'H' antisera seeming to give a somewhat brighter reaction with the cell walls than the 'O' antisera, with many flagella reacting in the 'H' suspensions. The flagella could easily be seen on visual examination but were too small to be photographed successfully. Some flagellated cells were also seen in the 'O' suspensions. The 'H' sera also reacted when tested with the 'O' suspensions of the corresponding bacteria and vice versa. All four conjugated antisera were tested against suspensions of Gram-negative coccus LC (*Peptostreptococcus elsdenii*) (Hobson, Mann & Oxford, 1958); a rumen *Streptococcus bovis* (strain 18C6, Hobson & Mann, 1955); *Sarcina bakeri* (Mann, Masson & Oxford, 1954); a bovine rumen strain of *Ruminococcus albus*; a sheep rumen Butyrivibrio strain; *Bacteroides amylogenes* (Doetsch, Howard, Mann & Oxford, 1957) and a sheep rumen strain of *Veillonella gazogenes* (Hobson, Mann & Oxford, 1958). Some reaction was seen with a proportion (perhaps 60 %) of the cells of *Sarcina bakeri* and

a very faint reaction with a small proportion (perhaps 10 %) of the cells of the Butyrivibrio, but the only bright reaction, involving all cells, was with *V. gazogenes*. At the same time slides of rumen contents from sheep 30 and 43 were tested with all the antisera. These slides showed no non-specific reaction with food particles or protozoa, but some of the large selenomonads in the fields reacted well. In addition, in all cases a reaction with some small cocci and very occasionally some larger cocci was observed. No other bacteria reacted with the antisera. Since the reactions with rumen contents and individual bacteria both showed an apparently similar cross-reaction and all the conjugated antisera behaved similarly, 17H antiserum was absorbed with *V. gazogenes* until no fluorescent reaction was obtained. This anti-serum still reacted well with the corresponding 17 cells. When tested with rumen contents no reaction was observed other than with some selenomonad-like organisms. This absorbed antiserum was also tested against some other cultures of rumen bacteria which were available, including a lipolytic bacterium (Hobson & Mann, 1961) and no cross-reactions were found. Some photographs of this antiserum and 17O antiserum reacting with selenomonads in rumen contents are shown in Pl. 1. A conjugated normal rabbit serum absorbed with liver powder did not react with anything in rumen contents. The 17H serum absorbed with *V. gazogenes* was further absorbed with thick suspensions of 17H and 17O antigens until no reaction was obtained with 17 cells. No reacting selenomonads were seen in films of sheep 43 rumen contents treated with this serum.

DISCUSSION

In some of the fields examined a few reacting organisms were seen which appeared to be more coccoid, or straighter, than the normal crescentic selenomonad shape. A comparison of these fields with many fields of pure cultures of selenomonads reacting with fluorescent antisera made it seem most likely that these were actually selenomonads turned 'end on' or 'sideways' to the viewer. The tests of the antisera with pure cultures and rumen contents show that the antisera seem to be specific for certain selenomonads (not all selenomonads in the rumen contents reacted with the antisera), possibly those of the variety *lactilyticas*. Although the large seleno-monads (up to about 8μ long) were the most obvious cells reacting some of the reacting cells were considerably smaller, but not as small as the pure cultures. The evidence would thus suggest that the small selenomonads grown in pure culture are actually the large selenomonads seen in the rumen, and that even in the rumen there is some variation in size amongst serologically similar groups of selenomonads. No reaction in rumen contents was noted with anything resembling 'Quin's organism', a large bacterium which has occasionally been suggested to be a form of the sele-nomonads. This organism has not yet been cultured.

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EXPLANATION OF PLATE

PLATE 1

All photographs were of dry film preparations. Magnification in all cases × 880.

Fig. 1. Selenomonad in rumen contents of sheep 43 reacting with 17 'H' antiserum.

Fig. 2. Same field as fig. 1 in white light.

Fig. 3. Larger selenomonad in rumen contents of sheep 30 reacting with 17 'O' antiserum.

Fig. 4. Same field as fig. 3 in white light.

Fig. 5. Large selenomonad in rumen contents of sheep 30 reacting with 17 'O' antiserum. The two large objects are plant material fluorescing bright blue-white.

Fig. 6. Same field as fig. 5 in white light.

Fig. 7. Large selenomonad in rumen contents of sheep 30 reacting with 17 'O' antiserum. General background of blue-white fluorescing plant debris, and slight blue autofluorescence from some bacteria, e.g. the oscillospira in the top left.

Fig. 8. Same field as fig. 7 in white light.

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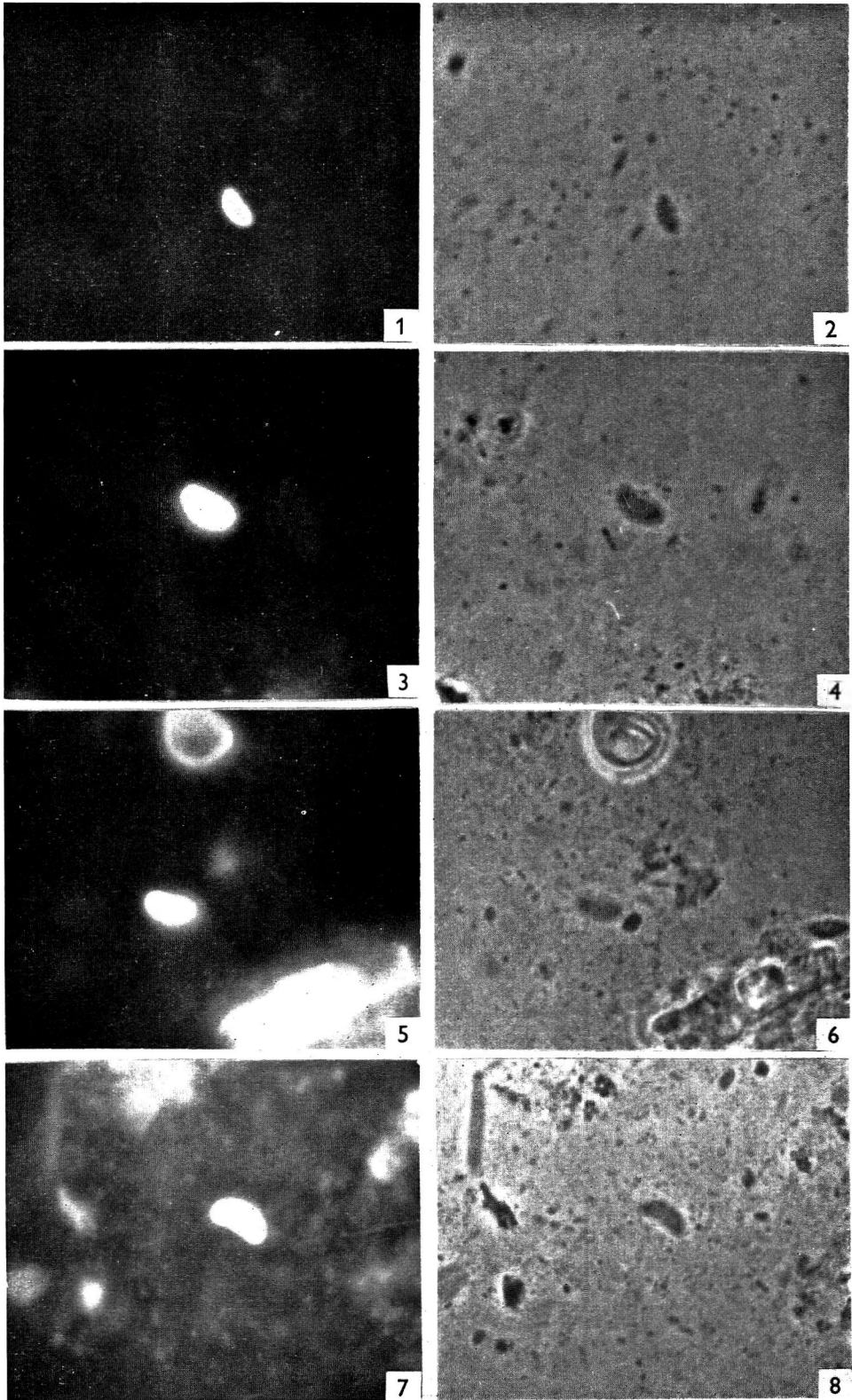
A Study of the Properties of Eaton's Primary Atypical Pneumonia Organism

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SUMMARY

Some of the recorded properties of a filterable organism (the PAP organism) isolated by Eaton and colleagues from cases of human primary atypical pneumonia suggest that it may be related biologically to the Mycoplasma (pleuropneumonia-like) group. In further investigation of this possibility now reported it was found that the organic gold salt, sodium aurothiomalate, which is known to inhibit certain mycoplasmas, would also inhibit infection with the PAP organism in the hamster and chick embryo lung. Some accepted viruses of a similar order of size to the PAP organism —namely, Nigg's pneumonitis virus of mice (psittacosis group), three strains of influenza virus A, vaccinia and variola viruses—were not inhibited by sodium aurothiomalate. On the other hand, grey lung virus of mice, an organism with some properties resembling those of the PAP organism, was strongly inhibited. Diethyl ether and the antibiotic kanamycin sulphate both inhibited the growth of the PAP organism in chick embryo lung. An intensified Giemsa-staining method revealed small red-purple coccobacillary bodies (later called elementary bodies, EB) in the lungs of chick embryos infected with three different strains of PAP organism isolated, respectively, in 1944, 1954 and 1960 in different parts of the United States. The EB were not seen in uninfected chick embryo lungs. The EB and the antigen of the PAP organism stained by the fluorescent antibody technique occupied essentially the same position in relation to the mesobronchial epithelial cells of chick embryo lung. The formation of both was suppressed in parallel by treatment of inoculum or eggs with diethyl ether, kanamycin sulphate or sodium aurothiomalate. There was a good but not absolute correlation between the presence or absence of EB and antigen in experiments on the growth or survival of the PAP organism in chick embryos, in cell-free media, and in titrations in eggs.

INTRODUCTION

Investigations by several groups of workers in the United States have shown convincingly that one of the varieties of human primary atypical pneumonia, namely that in which some patients develop serum agglutinins against the Streptococcus MG, or against human erythrocytes and active in the cold, is caused by a filterable organism (Eaton's agent or organism; the PAP organism) which can be grown in chick embryo lung or in certain tissue cultures, and produces small areas of pneumonia when inoculated intranasally into cotton rats and hamsters (Eaton,

Meiklejohn & Van Herick, 1944; Eaton, 1950a; Liu, Eaton & Heyl, 1956; Chanock et al. 1960; Clyde, Denny & Dingle, 1960; Cook et al. 1960; Gordon et al. 1960; Chanock et al. 1961). The handling of this organism in the laboratory was greatly eased by finding that its presence in sections of infected chick embryo lung could be demonstrated by the indirect fluorescent antibody technique with antisera either from convalescent patients or from rabbits immunized with infected chick embryo lung (Liu et al. 1956; Liu, 1957).

The nature and identity of the PAP organism has until recently been uncertain. Despite its large particle size (between 180 and 250 mμ as measured by filtration through Gradocol membranes (Eaton, 1950a)), it was not seen in smears or sections from infected tissues examined by light microscopy (Eaton & Liu, 1957). Its growth is not inhibited by sulphonamides and penicillin, but some strains are inhibited by streptomycin; tetracyclines, Carbomycin, erythromycin and aldehyde semicarbazones inhibit all strains so far tested (Eaton, 1950a; Eaton & Liu, 1957; Eaton, 1950b; Eaton, 1954–55; Eaton, Perry & Gocke, 1957). Although the size, antibiotic sensitivities and other properties of the organism resemble to some extent those of a rickettsia or a psittacosis group virus there are substantial reasons for supposing that it is neither. Thus: (a) sera from patients with antibody to the organism, or from hyperimmunized rabbits, do not fix complement either with psittacosis group antigen, or with group antigens from murine and epidemic typhus and rickettsialpox, or with phase 1 and 2 antigens from Rickettsia burneti; (b) sera containing antibody against the psittacosis group of viruses and R. burneti do not stain by immunofluorescence sections of chick embryo lung infected with the PAP organism; (c) examination of infected tissues from cotton rats, hamsters and chick embryos, including impression smears from yolk sacs infected with the PAP organism in Leeds, has not shown rickettsias or the characteristic elementary particles of the psittacosis group in preparations stained with ordinary Giemsa or rickettsial stains (Eaton, 1950a; Liu, 1957; Goodburn & Marmion, unpublished).

In 1961 we produced indirect evidence that the organism is not a virus but was related to the pleuropneumonia-like or Mycoplasma group of organisms (Marmion & Goodburn, 1961; Goodburn & Marmion, 1962). This view of the organism's identity was also considered as one of several possibilities by Eaton & Liu (1957). It has recently been substantiated by the cultivation, on cell-free media inoculated with tissue culture fluid infected with one of the standard strains of Eaton's organism, of a Mycoplasma which reacts specifically by immunofluorescence with sera known to contain antibody to Eaton's organism (Chanock, Hayflick & Barile, 1962).

A comparison of the properties of Eaton's organism and those of certain Mycoplasma is set out in Table 1. There is, of course, a wide variation of properties within Mycoplasma. The data given are derived from Edward (1947, 1954); Morton (1958), Pollock, Kenny & Syverton (1960) and Fogh & Hacker (1960); those on the PAP organism mainly from Eaton et al. (1944), Eaton, Meiklejohn, Van Herick & Corey (1945), Eaton (1950a, b), and the other papers already mentioned here. The evidence establishing the properties in brackets in Table 1, described in outline in earlier reports (Marmion & Goodburn, 1961; Goodburn & Marmion, 1962), is now presented in detail.

METHODS

Strains of Eaton's PAP organism. Three strains isolated in the U.S.A. were used: (1) The 'Hetter' (FH) strain, kindly given to us by Professor C. H. Stuart-Harris (Sheffield), was isolated in Boston in 1954 (Liu, 1957). It was in the form of infected chick embryo (CE) lung suspension and had had at least seven serial passages in

Table 1. *A comparison of various properties of Eaton's PAP organism with those of certain members of the Mycoplasma group*

	Eaton's PAP organism	Mycoplasma
Size	180–250 mμ	125–250 mμ
Light microscopy	('Elementary bodies') EB	Coccobacilliform or pleomorphic bodies
Sensitivity to chemicals		
Sulphonamide/penicillin:	–	–
Tetracyclines	+ +	+ +
Erythromycin	+	+ or –
Streptomycin	+ or –	+ or –
Kanamycin	(+ +)	+ +
Organic arsenic compound	(±)	+ or –
Organic gold salt	(+)	+
Diethyl ether	(+ +)	+ +
Type of pneumonia in animals	Consolidation rarely complete and very variable. Serial transmission very difficult*	Consolidation rarely complete and variable. Some difficulty in serial transmission†
Viability 20°	About 4 hr.	15 min. to 24 hr.‡
37°	About 6 hr.‡ (2 to 12 days)‡	15 min. to 24 hr.‡
56°	15 min.	2 min.
Lifeless media	Growth	Growth
Chick embryo and tissue culture	Growth	Variable growth

  – = Growth not inhibited; + +, +, ± = varying degrees of inhibition of growth; + or – = strains may be sensitive or resistant.
  * Lesion in hamster lung.
  † Lesion produced by *M. pulmonis* in mouse lung.
  ‡ Depends on the composition of the medium: see text.
  ( ) = Established by work described in this paper.

eggs, either in America or in England. (2) The classical 'Mac' strain (subculture Mac 73B) was obtained directly from Boston through the kindness of Dr M. D. Eaton. It was isolated from lung tissue in 1944 (Eaton *et al.* 1945) and was in the form of infected CE lung suspension; it had had seventy-three serial passages in eggs. (3) The 'Bethesda PI. 898' strain, recently isolated from a case of atypical pneumonia, was kindly given to us by Dr R. M. Chanock (National Institutes of Health, Bethesda, Md., U.S.A.). It had been maintained in primary monkey kidney tissue culture and had not been passed through chick embryos before it arrived in our laboratory. Infected material representing all three strains was stored in ampoules on solid carbon dioxide at −60° to −70° and was passaged from time to time in chick embryos or tissue culture.

Chick embryo techniques. Thirteen-day chick embryos were inoculated into the amnion under direct vision via the air sac (Taylor & Chialvo, 1942). After inoculation the eggs were incubated at 35° for 6 or 7 days. Without chilling, the embryos were then removed and the lungs harvested by opening the thorax from behind. A portion of each pair of lungs was cultured for bacteria, placed in a bijou bottle and rapidly frozen in a mixture of ethanol + solid carbon dioxide. The frozen lungs were then placed at $-40°$ for 24 or 48 hr. until the results of culture were known. Those proved sterile were pooled, ground with washed, sterile carborundum powder and mixed with a diluent in the proportion of 1·5 ml. for each pair of lungs from one embryo (= a '10%' suspension). The diluent (EO diluent) consisted of tryptose + phosphate broth (Difco) containing 1% (v/v) bovine serum albumin (fraction V, Armour), inactivated at 56° for 60 min., 0·3% (w/v) gelatin ('Oxoid' high grade bacteriological gelatin) and penicillin (1000 units/ml.). After light centrifugation (2000 rev./min., for 10 min.) the supernatant fluid was placed in ampoules, sealed, quick-frozen in a bath of ethanol + solid carbon-dioxide and stored at $-70°$. Lungs from three to six embryos in each batch were examined for infection by a fluorescent antibody technique (immunofluorescence) now described.

Fluorescent antibody technique with infected chick embryo lung. Frozen sections of lung were cut on a Leitz base-sledge microtome held in $-20°$ storage cabinet (Prestcold). The specimen-holder of the microtome was modified by replacing the tilting platform with a heavy split brass block into which could be clamped removable brass pegs on to which the CE lungs were frozen. Further cooling was provided by linking the brass pegs, by a copper strip, to a cylindrical brass container filled with chips of solid carbon-dioxide. In addition a perforated container filled with chips of solid carbon-dioxide was placed in the freezing cabinet below the microtome and in the path of a stream of cold air from an electric hair dryer; this cooled the air further and circulated it around the microtome knife and the modified specimen holder. Conditions were adjusted by switching the dryer on or off so as to maintain the required temperature of $-17°$ to $-20°$ around the knife.

Tissue blocks were prepared by harvesting one lung from a chick embryo, placing it hilum-side upwards in a drop of 15% (w/v) aqueous gelatin (Oxoid), buffered to pH 7·4, on the surface of a brass peg, protecting it with a collar of tin foil and quick-freezing by immersing the whole of the shaft of the peg in acetone + solid carbon-dioxide mixture. Sometimes, to save labour in infectivity titrations, up to three lungs from different embryos were orientated in the gelatin solution and then frozen to make a composite block. Sections were cut at about 5μ and rapidly melted on to microscope slides, previously dipped in 1% (w/v) aqueous gelatin (Oxoid, buffered to pH 7·4), exposed to formaldehyde overnight, and then placed in a current of cold air from the hair dryer for at least 2 hr. to remove traces of formaldehyde.

Some sections of CE lung were used unfixed, but most were fixed for 10 min. in Analar-grade acetone (previously held over anhydrous calcium sulphate) then dried on to the slides for 60 min. in a stream of cold air from the hair dryer and stored at 4° or $-20°$ until stained. In general the best results were obtained when lungs were harvested and cut on the same day. Single sera, or pools of human convalescent sera from local cases of atypical pneumonia which had a high or rising titre of Streptococcus MG agglutinins, or serum from a rabbit immunized by repeated

intraperitoneal injections of CE lung suspension infected with the Hetter strain, were used as the middle layer in the indirect fluorescent-antibody technique. For the detection of antigen in sections the sera were diluted, just before use, to a concentration 10 or 20 times greater than that just reacting in previous titrations on infected chick embryo lungs by immunofluorescence, then absorbed twice with liver and lung powder from mice and chick embryos. About two drops of this diluted serum were mixed with one drop of a 1/10 dilution of guinea-pig serum (fresh or preserved by Richardson's (1941) method) in saline (to provide the necessary co-factors for staining) and placed on the chick embryo lung sections so as to cover them completely and uniformly. Dilutions of serum for antibody titrations were made in saline containing 10 % (v/v) guinea-pig serum and 0·2 ml. of each serum dilution transferred to a series of sections. The slides were placed in a moist chamber at 37° for 60 min. and then washed with three changes of phosphate-buffered saline (pH 7·4). The sections were then covered with a suitable dilution, previously determined by titration on positive sections, of fluorescein-labelled antihuman or antirabbit globulin; obtained initially from Sylvana Chemical Co. N.J., U.S.A., or Microbiological Associates, Bethesda, Md., U.S.A., and later prepared here with fluorescein isothiocyanate from G. T. Gurr (London) and anti-globulins from Dr W. Goldie (Leeds) or from Burroughs Wellcome and Co. Ltd. (London). The diluted conjugates were absorbed twice with the liver powders and once with activated charcoal (about 5 mg./ml.).

After further incubation at 37° for 30 min. and three washes with phosphate-buffered saline the sections were mounted in glycerol (Analar) buffered to pH 7·4, and the coverslips sealed with colourless nail varnish. On examination in the ultra-violet microscope the characteristic intense fluorescence described by Liu *et al.* (1956) was seen in association with epithelial cells of the mesobronchi and, more rarely, in small patches in the parabronchi.

Ultraviolet microscope. The optical system was constructed here and consisted of a high-pressure mercury vapour lamp (Mazda ME/D, 250 W., 20,000 stilbs), a glass condensing lens, a 3 cm. thick Perspex box containing 5 % (w/v) aqueous copper sulphate ($CuSO_4.5H_2O$) and a 2 mm. thick Chance glass filter no. OX 1 (Pilkington Bros. Ltd., Wales). The light coming through this system was focused directly on to the substage of a Bactil microscope (Watson Ltd., London) with a reflecting dark-ground condenser. Non-fluorescent-immersion oil ('Fluor-free'; G. T. Gurr, London) was used between the condenser and the slide. Either a pale yellow gelatin filter, Aviol 102 (Ilford, Ltd., London) or a Chance glass OY 13 (Pilkington Bros. Ltd., Wales) was placed in a ×8 ocular held in a Bactil monocular tube.

Hamster inoculations. Infected chick embryo lung suspensions were also tested by intra-nasal inoculation of hamsters. Hamsters (60–90 g.) were lightly anaes-thetized with ether and 0·6 ml. of inoculum was instilled in two stages; first 0·2 or 0·4 ml., then fresh anaesthesia and the instillation of the residue. The hamsters were not very susceptible to Eaton's organism and the macroscopic lung lesions were relatively slight and showed great animal-to-animal variation by comparison with those, for example, produced by influenza virus. More impressive changes were seen on microscopical examination; for this purpose the lungs were fixed in 10 % (v/v) formaldehyde saline, sectioned in paraffin wax and stained with haematoxylin and eosin.

Mouse inoculations. White mice (Schofield 12/1A; 18–22 g.) were lightly anaesthetized with ether + chloroform and given 0·1 ml. of inoculum intranasally in the experiments with influenza, grey lung and Nigg's pneumonitis viruses.

RESULTS

Organic gold preparations are known to have an inhibitory effect on infections with some pleuropneumonia-like organisms; for example on pneumonia or arthritis in mice caused, respectively, by *Mycoplasma pulmonis* and *Mycoplasma arthritidis* (Edward, 1947; Findlay, Mackenzie & MacCallum, 1940; Sabin & Warren, 1940). Their action on virus infections does not seem to have been investigated very extensively, but Jungeblut (1930–31), Kolmer & Rule (1933–34) and Andrewes, King & van den Ende (1943) found that poliomyelitis virus in monkeys and influenza A and lymphogranuloma (LGV) viruses in mice were not inhibited. Bauer (1958) found that an inorganic gold salt did not inhibit neurovaccinia in mice. The effect of sodium aurothiomalate on infections with two strains of Eaton's PAP organism, and with certain large viruses of a similar size was investigated in hamsters, mice and chick embryos.

Effect of sodium aurothiomalate on Eaton's PAP organism

Experiments with the PAP organism in hamsters. Groups of hamsters were inoculated intra-nasally with '10 %' CE lung infected with the Hetter (FH) strain of PAP organism. About half the animals were given a daily intraperitoneal injection of 3·0 or 5·0 mg. of sodium aurothiomalate (Myocrysin; May and Baker) for 12 or 13 days. This was a dose of 4–8 mg., average of 5 mg./100 g. hamster body weight. The remaining animals were either not injected, or were injected with the same volume of distilled water containing 1/5000 phenylmercuric nitrate (a preservative present in the preparation of sodium aurothiomalate used). Twelve or thirteen days after inoculation the animals were killed with coal-gas and their lungs assessed for the extent of macroscopic and microscopic lesions. The range of macroscopic scores was 0·5 = a few small (about 1–2 mm. diam.) grey pneumonic areas in one or other lung; 1·0 = moderate numbers of grey areas in both lungs; 2·0 = numerous grey areas in both lungs; 3·0 = numerous grey areas in both lungs together with one larger area of frank red consolidation in either lung; 4·0 = numerous grey areas both lungs, together with two or more larger areas of red consolidation. The microscopic lesion scores depended on: (*a*) proportion of bronchi and vessels with cuffs of mononuclear cells seen in the section and was assessed in five units representing 10–100 % 'cuffing' of the bronchi and vessels; (*b*) a 3-unit assessment of the extent of alveolar consolidation. The sum of these two scores gave the total for the section, the maximum being 8·0. Average lesion scores were calculated by dividing the total score by the number of hamsters in the group.

A total of 56 hamsters (27 gold-treated; 29 controls) were used in four separate experiments with the Hetter strain. Table 2 shows the frequency distribution of the individual macroscopic and microscopic lesion scores in the total group of 56 hamsters and also the average lesion scores obtained in each of the four experiments. Despite the small extent of the visible lesions there was a clear-cut and significant difference between the overall results for the gold-treated and control groups, and

the suppression of lesions was consistent in each of the four experiments. Analysis showed that the differences between treated and untreated groups were unlikely to have arisen by chance alone; for macroscopic lesions $\chi^2 = 31.5$, D.F. $= 5, P < 0.001$ for microscopic lesions $\chi^2 = 53.3$, D.F. $= 8, P < 0.001$.

Table 2. *Intensity of lung lesions in gold-treated and untreated hamsters infected with Eaton's PAP organism*

	Number of hamsters with lung lesion score cf (macroscopic)						Total
	4	3	2	1	0·5	0	
Controls	2	4	9	7	5	2	29
Gold-treated	0	0	0	1	9	17	27

	Number of hamsters with lung lesion score cf (microscopic)								Total
	8	7	6	5	4	3	2	1	< 1	
Controls	3	3	5	12	5	1	0	0	0	29
Gold-treated	0	0	0	0	0	2	5	15	5	27

The average macroscopic (MC) and microscopic (mc) lesion scores in the four experiments were, respectively: controls MC = 1·5, 1·6, 1·7 and 1·6; mc = 4·6, 6·0, 5·8 and 4·9; gold-treated MC = 0·25, 0·14, 0·3 and 0·07; mc = 1·5, 1·0, 1·2 and 1·1.

Table 3. *Frequency distribution of the fluorescent intensities of specific antigen of Eaton's PAP organism in chick embryos treated with sodium aurothiomalate and their controls*

Material given via the yolk sac	Number of chick embryos with a specific fluorescence of an intensity					
	− or ±	+	+ +	+ + +	+ + + +	Total
Sodium aurothiomalate	32	3	2	1	0	38
Sterile diluent or phenyl-mercuric nitrate	5	14	18	8	2	47

Some chick embryos were inoculated via the yolk sac with a single dose of sodium aurothiomalate and others with inert diluent or phenylmercuric nitrate. The Hetter strain of PAP organism was inoculated into the amnion of all embryos. Six or seven days later lungs were stained with the human convalescent serum pool and fluorescein-labelled antihuman globulin. Variable numbers of mesobronchi were present in the lung sections and a mean value for the intensity was derived from them for the embryo as a whole.

A similar experiment with the Bethesda PI. 898 strain in hamsters also showed that sodium aurothiomalate inhibited the lung lesions. The average lesion scores, macroscopic (MC) and microscopic (mc) were: untreated MC = 2·1, me = 3·3; gold-treated MC = 0·39, mc = 0·8 and for animals given inoculum mixed with human antiserum to the PAP organism: MC = 0·46, mc = 0·5.

Sodium aurothiomalate appeared mainly to decrease the perivascular and peri-bronchial infiltration with mononuclear cells; small focal cellections of polymorpho-nuclear leucocytes were still present in the treated animals. As the gold salt might have limited the tissue reaction, rather than the multiplication of the PAP organism, attempts were made to find out whether gold salts would also act on the organism in the chick embryo, in which it grows without much tissue reaction.

Experiments with PAP organism in chick embryos. In these experiments 12- or 13-day chick embryos were inoculated in the yolk sac with a single dose of 25 or 30 mg. sodium aurothiomalate. On the same or next day, the eggs were inoculated amniotically with a 1/10 to 1/500 dilution of 10 % Hetter-infected CE lung suspension as appropriate. Control eggs received the same volume of sterile diluent or 1/5000 phenylmercuric nitrate into the yolk sac and then the infected CE lung suspension into the amnion. The treated and control eggs were then incubated for 6 or 7 days, the lungs harvested, sectioned and examined for antigen by the fluorescent antibody technique, and also stained by an intensified Giemsa staining technique described below.

There was a fairly high mortality among the chick embryos which had been given sodium aurothiomalate, and this made it necessary to inoculate large batches of eggs to ensure enough survivors for examination. Eight separate experiments were performed in which 85 embryos were examined, 38 in the group given gold and 47 in the controls. Six (15·8 %) of the 38 treated embryos and 42 (89·4 %) of the 47 controls had antigen detected by immunofluorescence in one or more mesobronchi at an intensity of + or greater ($\chi^2 = 43\cdot1$, D.F. = 1, $P < 0\cdot001$). Table 3 shows a further analysis of the distribution of the intensities of the antigen by immunofluorescence in the 85 treated and control embryos. Unstained or feebly staining mesobronchi were significantly more frequent in the lungs of gold-treated embryos. In two experiments the unsectioned embryo lungs were pooled in two appropriate groups and inoculated into hamsters intra-nasally to obtain independent confirmation of a difference in the content of PAP organism in treated and untreated embryos. The average macroscopic lung lesion scores were, respectively, 0·04 and 0·1 for suspensions from gold-treated embryos, and 1·2 and 2·0 for those from the untreated control embryos.

In vitro *effect of sodium aurothiomalate on the PAP organisms.* Portions of '10 %' CE lung suspension infected with the Hetter strain were mixed either with sodium aurothiomalate to a final concentration of 1·0 mg./ml. suspension, or with EO diluent. The mixtures were held at room temperature for 60 min. then subjected to two cycles of centrifugation at 10,000 rev./min. in the angle head of a refrigerated centrifuge. The deposits from the last centrifugation were suspended in half the original volume of EO diluent and tested in chick embryos by the standard method. Treatment with the gold salt diminished but did not abolish the capacity of the suspension to produce specific antigen in the chick embryo lung. It was concluded that the gold salt has only a slight direct effect on the organism or that contact over a long period is required for its action.

Effect of sodium aurothiomalate on other viruses

The significance of the effect of organic gold on the PAP organism would be enhanced if accepted viruses of a similar size were not inhibited when tested under our conditions. The organisms chosen were a virus of the psittacosis group causing pneumonia in mice (Nigg & Eaton, 1944), three strains of influenza virus A, vaccinia, and variola (alastrim) viruses. Grey lung virus of mice (Andrewes & Glover, 1945) was also examined because it is a large organism of uncertain identity and because it resembles the PAP organism in its sensitivity to tetracyclines. Also grey lung

virus has been shown to be inhibited by organic tri- and pentavalent arsenicals (Andrewes & Niven, 1953), a property shared with certain pathogenic bovine, ovine and caprine Mycoplasma organisms (Bridré, Donatien & Hilbert, 1928; Mornet, Orue & Marty, 1951). The results obtained were as follows.

Table 4. *Mortalities, mean day of death and mean lung lesion scores in groups of mice inoculated with mouse pneumonitis virus (Nigg) and treated with sodium aurothiomalate or left as controls*

	Gold-treated			Untreated		
Inoculum*	Mortality†	Mean day of death	Extent of lung lesion in survivors‡	Mortality†	Mean day of death	Extent of lung lesion in survivors‡
250	6/6	2·6	—	6/6	2 1	—
12·5	6/6	5·3	—	5/6	5 0	—
4·0	9/15	9·7	75	8/15	9 2	65

   * Dose in multiples of that producing a lesion involving 50 % or more of the lung in half the mice inoculated.
   † Number dead/total mice inoculated.
   ‡ Sum of individual lung lesion scores, assessed as percentage of lung consolidated at autopsy 12 days after inoculation, divided by the number of survivors in the group.

Table 5. *The effect of sodium aurothiomalate on the lung lesions produced by grey-lung virus of mice*

	Gold-treated			Controls		
Inoculum*	Lesion scores†	Mean score‡	Mean weight§	Lesion scores†	Mean score‡	Mean weight§
10,000	0, 0, 0, 0·5, 1, 2	0·5	246	2, 3, 3, 4, 4, 4	3·3	570
1,000	0, 0, 0, 0, 0·5, D, D, D	0·01	269	2, 2, 3, 3, 3, 3, 3, 4	2·8	610
100	0, 0, 0, 0, D, D	0·0	314	2, 3, 3, 4, 4, 4	3·3	612

   * Dose in multiples of that required to produce a lung lesion in half the mice inoculated.
   † Extent of pneumonia: 4 = typical lesion involving 90 % or more of the lungs; 0 = no visible lesion; 0·5–3 = intermediate degrees of involvement; D = mouse died during the experiment.
   ‡ Sum of lesion scores divided by total number examined.
   § Corrected mean weight (mg.) of lungs in the group: see text.

Nigg's mouse pneumonitis virus. Groups of mice were inoculated intra-nasally with dilutions of a lung suspension from mice infected with a strain of Nigg's virus. Some groups were given, daily for 12 days, intraperitoneal injections of 2·0 mg. sodium aurothiomalate; others were left as controls. The dose of sodium aurothiomalate was proportionately greater than that given to the hamsters (about 10 mg./ 100 g. body weight of mouse) but it did not cause death or lung lesions in uninoculated mice. The results (Table 4) show that treatment with gold did not significantly prolong the survival time, decrease the mortalities or alter the mean values for pneumonic consolidation in the mice which survived to the twelfth day after inoculation.

Grey lung virus. Groups of mice were inoculated intra-nasally with dilutions of lung suspension from mice infected with a strain of grey lung virus from the National Institute for Medical Research, Mill Hill. Some groups were given a daily intraperitoneal dose of 2 mg. sodium aurothiomalate, others a dose of phenyl-

mercuric nitrate equivalent to that in the solution of sodium aurothiomalate used. After 14 days treatment the mice were killed and their lungs examined. In the treated mice there was a striking decrease in the degree of consolidation, in the mean weights and the size of the lungs and also in the amount of fluid exuding from the trachea (Table 5). The pneumonia caused by the virus is diffuse and it is sometimes difficult to judge by eye minor degrees of difference between lungs. For this reason each pair of lungs was weighed to obtain an estimate of consolidation and contained fluid. The treated and untreated groups of mice had increased in total body weight at different rates during the 14 days after inoculation so the weights of the excised lungs were corrected to a standard body weight (20 g. mouse) and the mean values calculated for each group (Table 5). A repetition of the experiment gave the same results.

Influenza viruses. Experiments with mice were done with the mouse-adapted line of the Barratt strain of influenza virus A. Mice were inoculated intra-nasally with allantoic fluid infected with virus and some were given 2 mg. sodium aurothio-malate daily for 10 days. There were no striking effects on mortality or lung lesion score, or in prolonging survival time in the treated mice as compared with controls. Experiments in chick embryos were done with the WSE strain of influenza A (Burnet, 1936) and with a strain of influenza A2 (Asian) virus. Ten- or eleven-day chick embryos were given a single dose of 25 mg. sodium aurothiomalate into the yolk sac either 2 days before or at the same time as allantoic inoculation with the influenza virus. Three days later the allantoic fluids were harvested and the haem-agglutinins titrated. When very small doses of influenza virus (about 4 EID50 doses) were used there was some decrease of mean haemagglutinin titre in the allantoic fluids from the gold-treated embryos as compared with the controls, but this effect was not observed with larger inocula (e.g. 400 EID50 doses).

Vaccinia and variola viruses. Ten-day chick embryos received a single dose of 25 mg. sodium aurothiomalate, or of phenylmercuric nitrate, equivalent to that in the sodium aurothiomalate used into the yolk sac. One or two days later the embryos were inoculated on the chorioallantoic membrane with a dose of vaccinia or variola (alastrim) virus sufficient to produce 15 to 20 pocks. After further incu-bation (2 days for vaccinia, 3 days for variola) the chorioallantoic membranes were harvested and the pocks counted. There was no significant difference in pock counts between the gold-treated and control embryos with either virus.

Effect of miscellaneous substances on Eaton's PAP organism

Andrewes & Niven (1953) found that neoarsphenamine and tryparsamide suppressed the lesions of grey lung virus in mice but had no action on Nigg's virus. In view of our results with sodium aurothiomalate and grey lung virus, the action of neoarsphenamine (Novarsenobillon, May and Baker) on the PAP organism was ascertained. Three groups of hamsters were inoculated intra-nasally with the Hetter strain of PAP organism: one group was given eight daily intraperitoneal doses of neoarsphenamine (5·5 mg./100 g. body weight); the second was given sodium aurothiomalate (6·1 mg./100 g. body weight); the third was left as a control. Eleven days after inoculation the animals were killed and the lesion scores were: neoarsphenamine mean macroscopic (MC) score = 0·6, mean microscopic score

(mc) = 3·0; aurothiomalate, MC = 0·5, mc = 0·7; controls, MC = 2·0, mc = 4·7. There was, therefore, some inhibitory effect on the lung lesions with the neoarsphenamine, but it was not as striking as with the sodium aurothiomalate.

A '10 %' suspension of CE lung suspension infected with the Hetter strain was mixed with anaesthetic grade diethyl ether in a final concentration of 20 % (v/v) ether. The mixture and an untreated portion of suspension were held at room temperature for 1 hr. and then at reduced pressure to remove the ether. The suspensions were then inoculated into chick embryos in the usual way. Ether-treatment abolished the capacity of the suspension of PAP organism to form specific antigen as detected by immunofluorescence.

A single dose of kanamycin sulphate ('Kannasyn', Bayer), equivalent to 5·0 mg. kanamycin base, placed in the yolk sac of chick embryos one day before amniotic inoculation with the Hetter strain, completely inhibited specific antigen formation.

The elementary particle of Eaton's PAP organism

Despite the large size of the PAP organism, Eaton and colleagues were unable to demonstrate elementary bodies in smears or sections from various infected tissues by using a variety of stains and the light microscope (Eaton, 1950a; Eaton & Liu, 1957). Later Liu (1957) with a knowledge of the exact position of the antigen demonstrable by immunofluorescence found that in histological sections the cells of the chick embryo mesobronchial epithelium appeared to be normal with intact cilia. Donald & Liu (1959) examined ultra-thin sections of infected CE trachea in the electron microscope and found particles of a diameter of 150–250 mμ with thin envelopes of 300 mμ in diameter, and some larger structures inside about 10 % of the non-ciliated epithelial cells.

In the early stages of our work with the PAP organisms we stained, by the standard overnight Giesma method, frozen sections of CE lung prepared for fluorescent antibody staining. There was some faintly stained red particulate material close to the surface of the mesobronchial epithelial cells in these sections. To see more clearly we used an intensified Giemsa staining method, based on the observation of Henneguy (1891) that treatment of sections with a solution of permanganate greatly increased the absorption of any stain used subsequently; according to Professor A. W. Downie this technique was probably first applied to Giemsa staining by P. Bruce White. Frozen sections (about 5μ) of infected CE lung were fixed in dehydrated acetone for 10 min., removed and allowed to dry. They were then covered with freshly prepared potassium permanganate solution (10 g./l.) for 2 min., washed with distilled water and placed face downwards for 18 hr. in a solution of 1/25 Giemsa stain (Improved Giemsa stain, R 66, G. T. Gurr, London) in phosphate buffer pH 6·4. The heavily stained sections were differentiated with a dilute acetic acid (5 g./l. water) until there was a clear distinction under the microscope between the nuclei and cytoplasm of the epithelial cells in the mesobronchi. The sections were then washed in distilled water and mounted as wet preparations in a mixture of glycerol + phosphate buffer (pH 6·4); the coverslips were sealed with colourless nail varnish. Such preparations were best examined quickly; more permanent ones could be made by taking the sections through acetone, xylene, and then mounting in polystyrene (DePeX), but there was a greater proportion of

failures due to decolorization and distortion. This staining procedure revealed numerous minute pink and purple bodies in association with epithelial cells of the mesobronchi. The bodies were coccoid, or coccobacillary and resembled the cocco-bacilliform bodies found in exudates from avian infectious coryza. This morphological similarity is not enough, of course, to prove that they are the same sort of organism; at this stage the non-committal term elementary bodies (EB) will be used to denote them.

A few EB were seen lying free in the lumena of the mesobronchi and some were unequivocally within the basophilic cytoplasm of the epithelial cells, but the majority appeared to be extracellular and arranged in a compact layer on the surface of the epithelial cells and largely within a layer of eosinophilic mucus which overlies these cells. This mucous layer was identified by its property of staining blue with Alcian blue at pH 3·0 and red by the periodic acid Shiff celestin-blue haemalum method (Pearse, 1960; it was necessary to prolong the staining times). The bacillary shape of the EB often gave a striking and distinctive palisade-like appearance to the surface of the epithelial cells. The mesobronchial epithelial cells were sometimes fragmented and the cell cytoplasm more contracted and basophilic than that of uninfected controls, but there was no visual evidence either of nuclear inclusions or of organized intracytoplasmic microcolonies of EB. The EB appeared to occupy the same general position in the CE lung as did the specific antigen—that is, they were present in the greatest numbers at the surface of the epithelial cells of the meso-bronchi and were usually absent from the parabronchi. Pl. 1, figs. 1 and 2, show the location of EB and specific antigen in an invagination of a mesobronchus in adjacent section from a CE lung infected with the Hetter strain. The angle enclosed by the cell-lined inlet is not quite the same in the two sections because the two staining processes produce different physical effects. Nevertheless it will be seen that the positions of antigen and EB correspond very closely. Examination of adjacent serial sections by the two methods rather than staining the same section, first by immunofluorescence then with Giemsa, was necessary because the EB seemed to be very fragile. Variations in fixing and Giemsa-staining techniques were not investigated exhaustively, but the following points seem important: sections should be mounted on gelatinized slides not directly on glass; acetone, absolute ethanol and possibly Bouin's solution are satisfactory fixatives, but formol saline and Palade's osmium tetroxide cause great distortion and loss of EB; unfixed sections may be used, and treatment with stronger permanganate solution (20 g./l. water) may be advantageous. Different preparations of Giemsa stain varied in their ability to stain EB.

Typical palisades of EB on the mesobronchial epithelial cells were not seen in any of numerous sections of lung of uninoculated chick embryos (Pl. 1, fig. 3) or in those from an experiment in which nine serial blind lung-to-lung passages were made starting from embryos inoculated with sterile EO diluent. On the other hand, EB and antigen were found in many sections from eggs inoculated during routine passage of the Hetter strain of PAP organism. The results of some examinations of embryos, uninoculated or inoculated with negative material, and of infected embryos are combined in Table 6, section (a), which shows that there was a good but not absolute correlation between the presence or absence of the two entities. Sodium aurothiomalate, which decreased the amount of specific antigen in CE lung (Table 3)

also decreased the number of EB seen in lung sections. Table 6, section (*b*) summarizes the results from 67 gold-treated and untreated embryos examined by both methods; once again there was a good correlation between the values for antigen and EB. Mesobronchi in the lungs of gold-treated embryos sometimes contained clumps of organisms resembling EB but swollen into larger spheres or long rods. Similarly, examination of sections from embryos inoculated with the Hetter seed and treated with kanamycin, or inoculated with Hetter seed previously treated with diethyl ether (see above), showed that formation of EB was suppressed in parallel with that of specific antigen.

Table 6. *Correlations observed between the presence and intensity of specific antigen, and presence and numbers of EB found by intensified Giemsa staining in chick embryo lungs*

Section (*a*) of the table shows the results for a group of 150 embryos comprising uninoculated normals, those from serial passages of normal CE lung and material from tissue culture experiments and isolation specimens, and from routine serial passage of the Hetter strain of Eaton's PAP organism. Section (*b*) gives the results for 67 treated and control embryos in the 8 experiments on the effect of sodium aurothiomalate on the growth of the PAP organism in eggs.

In all experiments, replicate sections of CE lung were stained with a pool of human convalescent sera and fluorescein labelled anti-human globulin, or by the intensified Giemsa method.

Section (*a*). Embryos inoculated with various positive and negative materials excluding those in experiments on the effect of gold treatment.

Intensity of fluorescence of specific antigen in bronchi	Quantity of Giemsa-stained EB in bronchi		Totals
	− to ±	+ to + + + +	
− to ±	96	2	98
+ to + + + +	7	45	52
Totals	103	47	150

Section (*b*). Embryos inoculated in experiments on the effect of gold treatment in eggs.

Intensity of fluorescence of specific antigen in bronchi	Quantity of Giemsa-stained EB in bronchi		Totals
	− to ±	+ to + + + +	
− to ±	29	4	33
+ to + + + +	6	28	34
Totals	35	32	67

The time of formation of antigen and EB was determined by inoculating embryos with the Hetter strain and harvesting lungs at intervals. Neither antigen nor EB were present in lungs harvested 3 hr. after inoculation. At 3 days after inoculation a fine layer of antigen was present but EB were not identified with certainty although a few spherical particles larger than EB were seen. At 4 days antigen had increased and EB were definitely present; both increased in parallel up to the sixth day.

Direct proof that specific antigen and EB are two attributes of the same (PAP) organism would be afforded by neutralizing their formation in chick embryos by

mixing antiserum with the inoculum. However the results of attempts to do this were equivocal; antiserum did not substantially decrease antigen formation. Eaton et al. (1945) noted that it was difficult to neutralize the PAP organism in chick embryo lung in contrast to the ease with which lesions were neutralized in the hamster or cotton rat lung. However, in the present experiments EB in the lungs of embryos which had received the Hetter strain mixed with antiserum appeared to be swollen, elongated and more variable in size than those in the lungs of the controls; this effect was similar to, but not as striking as, that seen in the gold-treated embryos.

The conditions under which strains of PAP organism have to be propagated (serial egg passage in high concentrations of penicillin) are ideal for permitting contamination with the less pathogenic avian mycoplasma organisms or for inducing L forms of contaminating bacteria. Indeed Van Herick & Eaton (1945) describe one episode in which their chick embryo line of PAP organism was contaminated with a pathogenic avian Mycoplasma. The possibility was considered therefore that EB might be units of a contaminating Mycoplasma organism or of a bacterial L form which were being passaged along with the PAP organism. In a test of the possibility two portions of a 1/5 dilution of 10% CE lung suspension infected with the Hetter strain were mixed with (a) a 1/5 dilution of pooled convalescent sera from cases of pneumonia with a high titre of antibody to the PAP organism, and (b) 1/5 dilution of human serum which did not contain antibody to the organism. Both sera were diluted in saline containing 10% (v/v) guinea-pig serum to provide complement. The mixtures were incubated at 37° for 30 min. and then inoculated into sloppy agar medium containing 20% (v/v) inactivated human serum, yeast extract, thymus nucleic acid and other growth supplements for Mycoplasma organisms. This medium would be adequate for most Mycoplasma but not the organism recently isolated by Chanock et al. (1962). The tubes of medium were incubated at 37° for 2 days before samples were inoculated into chick embryos, the lungs of which were subsequently examined for specific antigen and for EB. If the inoculum had been a mixture of the PAP organism and an EB-producing Mycoplasm organism then a dissociation of the two properties, and an increase in concentration of EB, might have been expected by passage through the sloppy agar medium. Instead the material from the tube inoculated with PAP organism and convalescent serum provoked fewer EB and less antigen than that inoculated with the organism and the serum without antibody to the PAP organism.

Additional evidence that formation of EB is a property of the PAP organism was obtained by examining two other strains—the classical Mac strain and the recent isolate Bethesda PI. 898. These strains were inoculated into eggs and lung sections examined by immunofluorescence and by Giemsa staining. At the same time serial passage of uninfected CE lung was started in eggs taken at random from the batches used for the two strains of PAP organism and passaged in parallel with them. Lung sections from this uninfected series of embryos consistently failed to react by immunofluorescence with human convalescent serum and did not show EB up to the ninth blind passage. On the other hand, both strains of PAP organism showed EB closely resembling those seen with the Hetter strain. The Mac strain required one passage in the chick embryo before typical specific antigen and EB were clearly present. The Bethesda PI. 898 strain (tissue and fluid phase from third passage in

infected monkey kidney cell culture) gave typical specific antigen in mesobronchi on first inoculation into the chick embryo and small numbers of EB were seen in association with epithelial cells. In subsequent egg passages specific antigen was seen distributed through the lung sections, with a stained cellular exudate in the lumena of the mesobronchi. In this material very large numbers of EB were seen, not only on the surfaces of mesobronchial cells, but also in association with the collections of inflammatory cells lying free in the mesobronchial lumen and in the parabronchi. On other occasions numerous EB were present but specific antigen was only poorly revealed by immunofluorescence if at all. As this picture differed somewhat from that observed with the Hetter and Mac strains attempts were made to explain it in terms of contamination with bacteria or viruses (influenza, parainfluenza, Sabin B and other simian viruses). These attempts were all negative and in fact the generalized, atypical immunofluorescent staining seemed to be specific as it occurred only with the convalescent and not with acute-phase sera from cases of pneumonia previously shown to have rising antibody titres against the Hetter strain. Further analysis of the behaviour of the Bethesda PI. 898 strain suggested that it grew faster and to a higher titre in the CE lung as compared with the Mac and Hetter strains. Antigen and EB started off in the usual position at the surface of the mesobronchial epithelial cells 4 days after inoculation, but by 6 or 7 days after inoculation (the usual harvesting time) there was deterioration of antigen, general staining of the sections and a widespread distribution of EB and inflammatory cells. Plate 2, figs. 1 to 3, shows various aspects of lung sections from embryos infected with Bethesda PI. 898 strain of PAP organism.

Experiments with Eaton's PAP organism in tissue culture and in cell-free (lifeless) media

Tissue culture. Several types of tissue or cell culture were tested for their ability to support survival or growth of the PAP organism. They were inoculated with the chick embryo lung suspension infected with the Hetter strain, incubated at 35° for 7–12 days. Mixtures of the fluid and cellular phases of the cultures were inoculated into chick embryos and tested for antigen. Promising results were obtained with Maitland-type cultures of chick embryo lung and with fragments of infected chick embryo lung on nutrient agar slopes. Monolayer cultures of chick embryo lung and of the FL line of human amnion cells gave poor results, but monolayer cultures of chick entoderm and primary monkey kidney cells were better. No cytopathic effects were observed in these preparations of chick entoderm or monkey kidney cells. Similar results with monkey kidney and chick entoderm tissue cultures have been reported by Chanock *et al.* (1960) and Gordon *et al.* (1960).

Cell-free media. Eaton and colleagues (Eaton, 1950*a*; Eaton & Liu, 1957) reported failure to grow pleuropneumonia-like organisms from chick embryo material infected with PAP organism, using, presumably, nutrient broth or solid medium with a high serum content. Having found the PAP organism to contain EB and to be sensitive to aurothiomalate we also tried to grow it in sloppy agar and on solid media. The media were supplemented with 20 % (v/v) heat-inactivated horse, rabbit or human serum free from antibody to the PAP organism; with yeast extract, sodium deoxyribonucleate, staphylococcal extract; bovine serum albumin

and potassium phosphate (see Klieneberger-Nobel, 1962, for details). Some batches of basal media were obtained from Dr D. G. ff. Edward and were known to support the growth of Mycoplasma. The media were inoculated with suspensions of chick embryo lung infected with the Hetter, Mac or PI. 898 strains, incubated at 37° and, at various intervals, portions were titrated in chick embryos for their content of PAP organism. The results suggested that the organism was surviving much better at 37° than previously reported (Eaton *et al.* 1945)—it was recovered from cultures held at 37° for 12 days—but unequivocal evidence of multiplication was not obtained. The media used were very similar to those successfully employed by Chanock *et al.* (1962) except that the sera were inactivated before use. The variables are being investigated further.

DISCUSSION

The similarities which were already known to exist between Eaton's PAP organism and the Mycoplasma (pleuropneumonia-like) group of organisms are noted in the Introduction and in Table 1. To these may now be added sensitivity to diethyl ether, kanamycin sulphate and sodium aurothiomalate. The last property was not shared by certain viruses (Nigg's pneumonitis virus of mice, influenza A viruses, vaccinia, variola) which are slightly larger or smaller than the PAP organism. On the other hand, grey lung virus of mice was sensitive to sodium aurothiomalate and smears from mouse lung infected with this virus when stained by the intensified Giemsa method showed bodies similar to EB. Various serological investigations have not revealed so far any antigenic relationship between Eaton's organism and *Mycoplasma mycoides* var. *mycoides*, *M. mycoides* var. *capri*, *M. gallinarum*, *M. iners*, *M. gallisepticum*, *M. hominis* Type 1, *M. fermentans*, or *M. salivarium* (Newnham, Goodburn & Marmion, unpublished). Preliminary experiments in collaboration with Dr P. Whittlestone (Department of Veterinary Pathology, Cambridge) do not suggest an antigenic relationship between the organism of enzootic pneumonia of swine and the PAP organism.

The finding in infected chick embryo lung sections stained by the intensified Giemsa method, of small bodies (EB) of coccobacillary shape resembling that of the bodies seen in the inflammatory exudate associated with certain pathogenic mycoplasmas or their cultures requires discussion. The evidence suggests that EB and specific antigen detected by immunofluorescence are two attributes of the same PAP organism. They have been found in the same position in association with the mesobronchial epithelial cells in CE lungs infected with three different strains of the PAP organism (Mac, Hetter (FH) and Bethesda PI. 898) isolated in 1944, 1954 and 1960, respectively. (They have also been found recently in a freshly-isolated Dutch strain sent to us by Dr J. F. Ph. Hers, Leiden, Holland.) They have not been found in lung sections from uninfected chick embryos or from embryos in a series of lung-to-lung passages of uninfected material. With the Hetter strain EB and antigen appear in the CE mesobronchus at about the same time, namely 3–4 days after inoculation. Both are suppressed by treatment of the inoculum of the chick embryos with diethyl ether, kanamycin and aurothiomalate. However, a dissociation of the two properties was observed in two situations. When the Hetter strain was titrated in chick embryos it was found that the end-point at which EB were seen in the CE lung sections was 10- or 100-fold lower than that determined by immunofluorescence.

This might be simply a matter of the sensitivity of the two techniques—small numbers of EB are difficult to identify with certainty. On the other hand, the reverse was found with the Bethesda PI. 898 strain: EB were sometimes seen when antigen was absent. Probably this was because the antigen of the Bethesda PI. 898 strain deteriorated rapidly during the last 2 days of incubation of the embryos. The size of the EB appeared to be larger than that estimated from filtration of the PAP organism. It is possible therefore that EB are a swollen (perhaps degenerate) fraction of the total population of particles of the PAP organism, all of which are demonstrable by immunofluorescence.

There are several possible interpretations of the nature of EB. They might, for example, be protrusions of cytoplasm from cells damaged by virus growth. This is improbable because the cells show slight damage only. EB have the staining qualities of nuclear rather than cytoplasmic material, and similar structures were not seen in mesobronchial cells in the lungs of chick embryos infected with influenza virus. Now that a pleuropneumonia-like organism has been isolated from material infected with the PAP organism (Chanock *et al.* 1962) the simplest explanation is that EB are the small reproductive cells of the organism growing mainly in the layer of mucus on the surface of the CE mesobronchial cells; a location which offers the advantages of physical support, high external colloid osmotic pressure and a high concentration of diffusible growth factors from the cells.

There is a discrepancy between our findings with CE lung sections stained by Giemsa and those of Donald & Liu (1959) with thin sections of infected CE trachea in the electron-microscope. These workers saw particles of 150–250 mμ, sometimes with a thin envelope 350–500 mμ, inside about 10 % of the epithelial cells, mostly non-ciliated. Particles on the surface of cells were not noted. In comparable material examined in the fluorescence microscope they saw antigen associated with both ciliated and non-ciliated cells. This discrepancy might be resolved if the fluorescent material was mainly on the cell surface and easily removed or distorted during fixation and embedding, and if phagocytosis of material is mainly a function of non-ciliated cells. Clyde (1961) provides support for the view that the PAP organism grows on, rather than in cells.

The finding that the PAP organism is related to the Mycoplasma has other implications which might be investigated with advantage. For example, apart from the grey lung virus of mice, already mentioned, there are other large filterable organisms of uncertain nature that cause pneumonia in animals, infectious pneumonia of pigs (Gulrajani & Beveridge, 1951; Betts, 1952), virus pneumonia of rats (Vrolijk, Verlinde & Braams, 1957) which have features in common with the PAP organism. Tests for sensitivity to gold salts and the demonstration of EB and antigen by intensified Giesma staining and immunofluorescence might be useful initial methods for distinguishing from the true viruses a group of organisms with biological properties similar to those of Eaton's organism. Clinically, the behaviour of known Mycoplasma in producing chronic infection in the rodent lung might stimulate a search for persistent infection and chronic damage in the human lung infected with Eaton's PAP organism.

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. Medium power view of the epithelial cell layer and lumen in mesobronchus of chick embryo. Acetone-fixed frozen section, Hetter strain, stained with human convalescent serum and fluorescein-labelled globulin. Collections of brightly fluorescing particles are present inside or on the surfaces of the epithelial cells and a few particles are free in the lumen.

Fig. 2. Next serial section to that shown in Fig. 1. High-power view of epithelial cell layer and lumen in the same invagination of the chick embryo mesobronchus but in a Giemsa-stained section. Numerous EB are present in a similar position to the specific antigen in Fig. 1. Acetone-fixed frozen section. Intensified Giemsa staining. Polystyrene (DePeX) mount.

Fig. 3. High-power view of the epithelial cell layer in an uninfected chick embryo mesobronchus. A faint stippling marks the edge of the cell cytoplasm and the layer of mucus can be seen at the cell edge and beyond it. EB are not present. Acetone-fixed frozen section. Intensified Giemsa stain. Glycerol-buffer mount.

PLATE 2

Fig. 1. Low-power view of chick embryo lung mesobronchus infected with 'Bethesda PI. 898' strain of PAP organism (4th egg passage 10^{-2} diln.). Inflammatory cells are present in the lumen. Unfixed frozen section. Intensified Giemsa staining. Glycerol-buffer mount.

Fig. 2. Low-power view of the same mesobronchus as in Fig. 1, but in an adjacent section stained by immunofluorescence. The specific antigen is not only present in the vicinity of the surface of the mesobronchial epithelial cells but also apparently in the inflammatory cells lying free in the lumen. Acetone-fixed frozen section stained with human PAP serum pool and fluorescein-labelled anti-human globulin.

Fig. 3. High-power view of the epithelial cell edge of a chick embryo lung mesobronchus infected with the 'Bethesda PI. 898' strain of PAP organism (3rd egg passage). EB can be seen in a layer on the surface of the epithelial cells, lying free in the lumen and also within the cytoplasm of a cell which is part of the exudate in the mesobronchial lumen. Unfixed frozen section, intensified Giemsa staining, Glycerol-buffer mount.
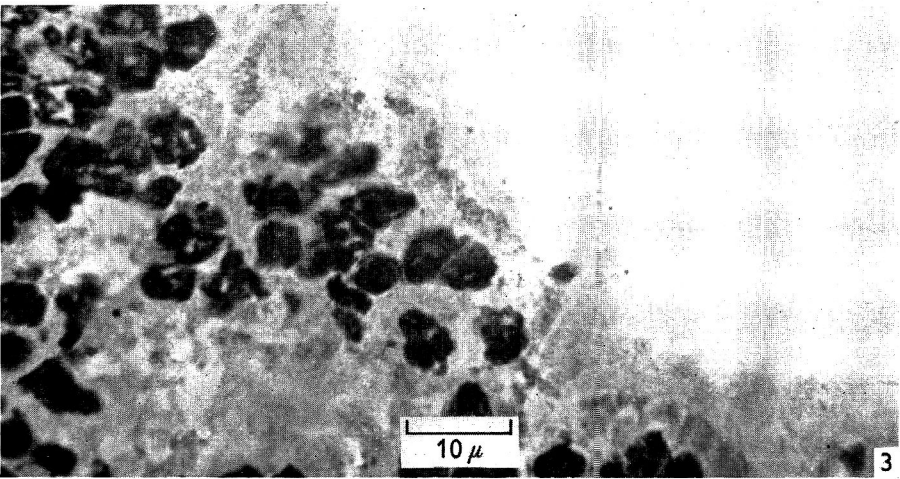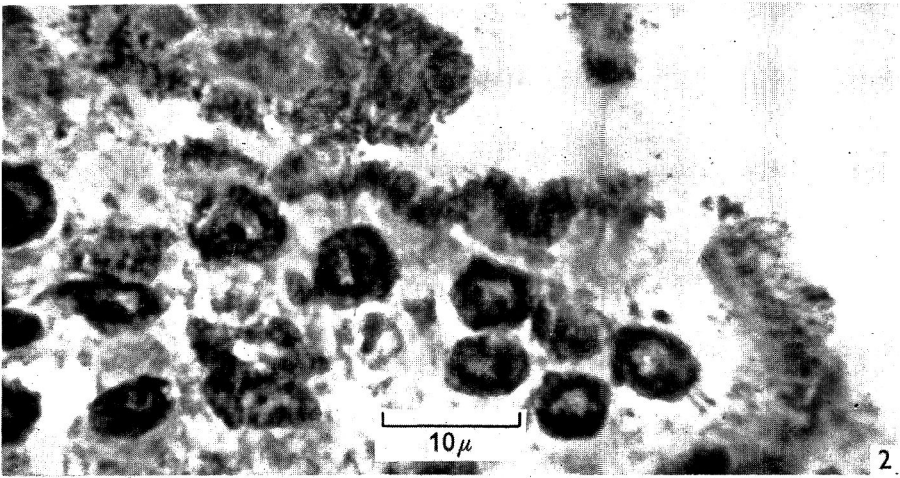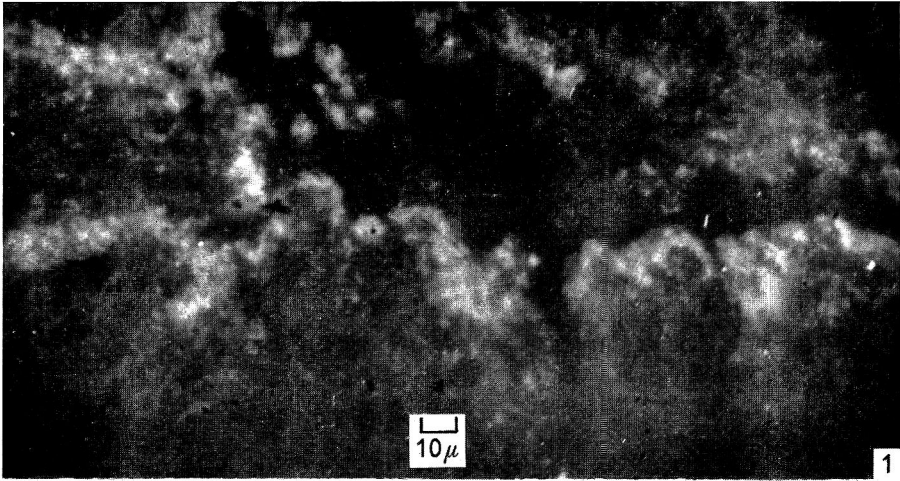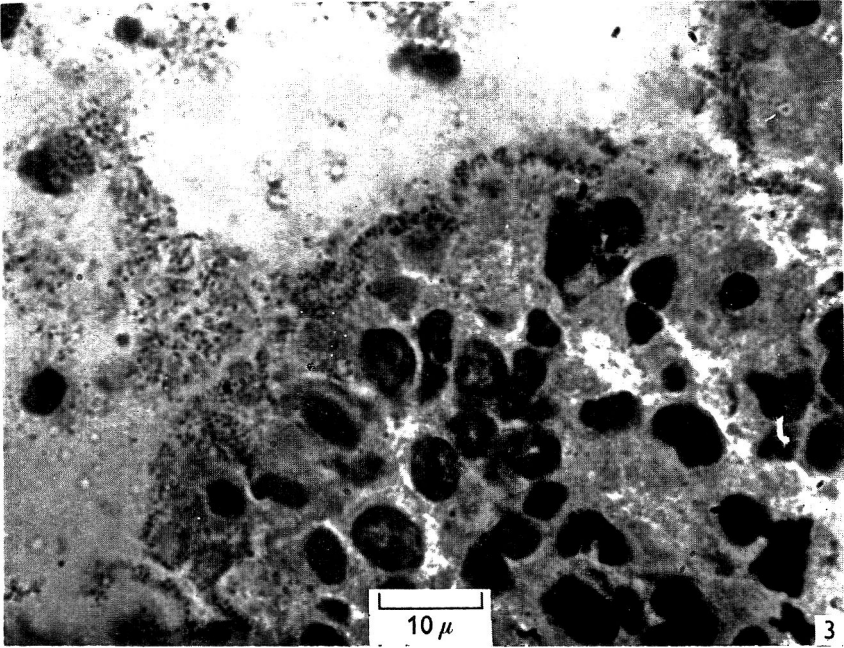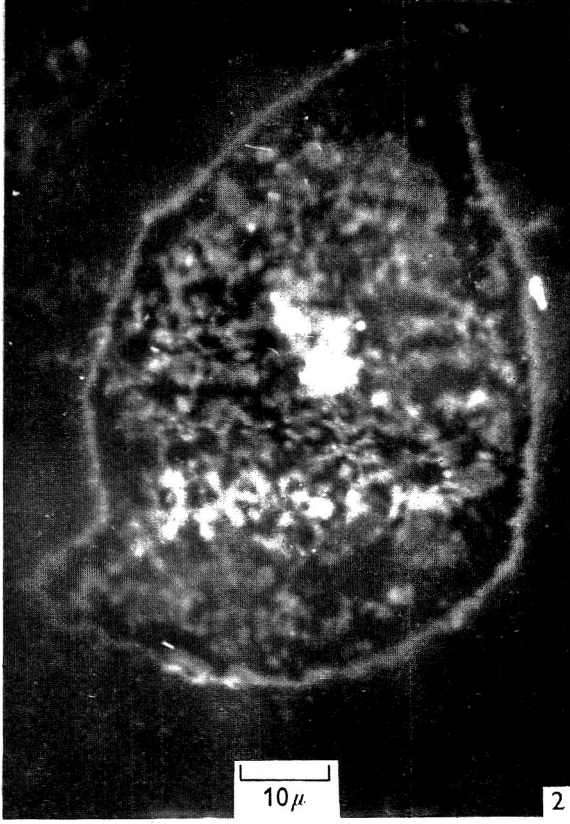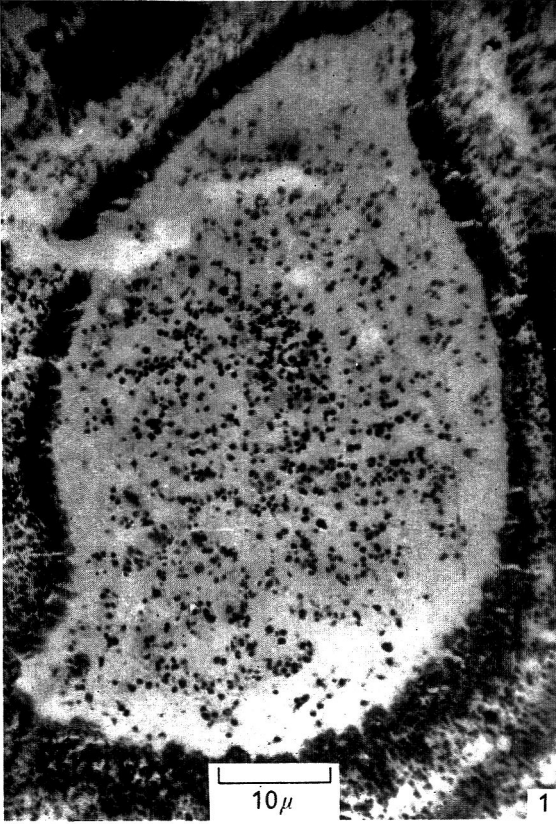
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Secondary Colony Formation by *Lactobacillus casei*

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SUMMARY

In addition to R outgrowths, strains of *Lactobacillus casei* growing on a carbohydrate-free medium also formed smooth secondary colonies situated on the primary colonies. These secondary colonies arose after about 6 days of incubation and were of two types: when centrally situated they formed papillae; when near the margin of the mother colony they often spilt over and formed fan-shaped outgrowths adjacent to the parent colony. Cultures of several different species of *Lactobacillus* were examined; secondary colony formation was confined almost exclusively to strains of *L. casei*. The fan-shaped outgrowths were pure cultures of organisms possessing a shorter generation time than those comprising the mother colony. An inverse relationship existed on crowded and sparsely inoculated plates between numbers of papillae and fan-shaped outgrowths/colony observed. The distribution of colonies bearing different numbers of secondary colonies did not follow a Poisson distribution; the random origin of fast-growing variants could not be established. On subculture the fast-growing variants produced colonies with a slightly different morphology from that of the wild-types. This difference was stable and persisted through numerous subcultures. In the presence of small quantities of fermentable carbohydrates secondary colony formation was not observed and this possibly explains how the wild type competes successfully with fast-growing variants in natural environments.

INTRODUCTION

In a study of the S–R variation in *Lactobacillus casei* (de Klerk & Coetzee, 1962) strains were plated on a carbohydrate-free medium. In addition to the R variants observed, secondary colonies in the form of smooth papillae often appeared on the surfaces of the colonies. A cursory examination of subcultures of the papillae on the same medium revealed colonies similar in appearance to the primary colony. Subsequently it was found that on crowded plates most of the smooth papillae which were situated peripherally continued to develop to form fan-shaped outgrowths which extended beyond the margins of the mother colony. The appearance of these outgrowths was remarkable (Pl. 1, fig. 1) and was encountered so frequently in subsequent work with the R variants that it was decided to investigate their nature.

METHODS

The media and the methods used for plating, testing biochemical reactions, making total counts of colony populations, and the phage and serological techniques were as described previously (de Klerk & Coetzee, 1962). Incubation was always at 37° unless otherwise stated.

Organisms. Three strains of *Lactobacillus casei* var. *casei* (C$_9$, 300, 316 used by de Klerk & Coetzee, 1962) were examined in detail, and 15 other strains of *L. casei* var. *casei*, 10 strains of *L. casei* var. *rhamnosus*, 5 of *L. plantarum*, 8 of *L. acidophilus*, 6 of *L. salivarius*, 10 of *L. fermenti* and 10 of *L. brevis* were examined for secondary colony formation. These strains had been isolated from human saliva and identified by the methods of Rogosa *et al.* (1953) and Wheater (1955).

Secondary colony formation. The effects of crowding on the development of papillae and fan-shaped outgrowths was studied by spreading 0·1 ml. of dilutions of overnight cultures grown in tomato glucose broth on to medium 1 agar (de Klerk & Coetzee, 1962). After 8 days of incubation the distribution of secondary colonies in the form of papillae and fan-shaped outgrowths on parent colonies was determined in areas of 3 cm.². The effect of the depth of medium on the incidence of the two types of smooth outgrowth was investigated by using different volumes of agar in similar Petri dishes. The effect of amino acids and vitamins on secondary colony formation was tested by incorporating vitamin-free pancreatic casein digest (Nutritional Biochemicals Corporation, Cleveland, Ohio, U.S.A.) at 10 g./l. into medium I. Ascorbic acid, nicotinic acid, thiamine hydrochloride, calcium D-pantothenate, riboflavin, *p*-aminobenzoic acid, DL-biotin, folic acid and vitamin B$_{12}$ (all obtained from Nutritional Biochemicals Corporation) were incorporated in medium I in the quantities and by the methods as used by Rogosa, Franklin & Perry (1961). The effect of addition of fermentable and non-fermentable carbohydrates was investigated as previously described (de Klerk & Coetzee, 1962). The carbohydrates used were glucose, cellobiose, rhamnose and melibiose.

Colony diameters. Suspensions were made of samples of organisms from fan-shaped outgrowths and from areas of the mother colonies which were devoid of secondary colonies. Suitable dilutions to yield about 50 colonies/plate were seeded on to medium I agar. Colonies derived from these two sources will subsequently be referred to as F variants and wild-types, respectively. The diameters of fifty well-separated colonies were measured after incubation for 48 hr. This was done with a micrometer eyepiece at ×41·25 magnification.

Generation times. Overnight cultures of single F variant and wild-type colonies in 5 ml. liquid medium I were transferred into 100 ml. quantities of fresh prewarmed liquid medium I. These cultures were maintained at 37° and at suitable intervals samples removed. The optical densities of these samples were read with a Zeiss spectrophotometer at 660 mμ. Total counts on the samples, suitably diluted in cold distilled water, were done with a Petroff–Hauser counting chamber and a phase-contrast microscope. Dilutions of the samples were also plated for viable counts.

Changes in pH value of cultures in medium I. Cultures of F variants and wild strains were grown in 250 ml. liquid medium I for 170 hr., sampled at intervals, and readings of pH values of the samples made with a Beckman pH meter.

RESULTS

Colonies of all strains of *Lactobacillus casei* var. *casei* and *L. casei* var. *rhamnosus* and one strain each of *L. fermenti* and *L. brevis* gave secondary colonies on medium I agar which appeared as papillae anywhere on their surfaces or margins. These out-

growths generally appeared between the fifth and seventh days of incubation. No secondary colony formation was ever seen on colonies of the other species examined. The number of secondary colonies formed by the single strains of *L. fermenti* and *L. brevis* mentioned was negligible in comparison with their numbers on colonies of the strains of *L. casei*. Secondary colony formation on this medium thus appeared to be a feature of strains of the latter species. The secondary colonies varied in size and were smooth and glistening (Pl. 1, figs. 2, 3). When situated centrally they soon stopped growing; but those at the periphery often spilt over the margin of the mother colony. Once in contact with the medium, they grew rapidly into large fan-shaped outgrowths, retaining their smooth glistening surfaces and entire edges (Pl. 1, fig. 4). Adjacent outgrowths did not coalesce (Pl. 1, fig. 5) as R variants tend to do (de Klerk & Coetzee, 1962). Forty-eight hr. colonies of organisms from the mother colony (wild-type) and the fan-shaped outgrowths (F variants) on medium I, were smooth, but differed in that the latter were larger and slightly more raised and opaque (Pl. 1, fig. 6). This morphological difference persisted through numerous subcultures. Subcultures from the fan-shaped outgrowths were homogeneous and yielded only F variants. Subcultures from papillae situated centrally usually yielded mixtures of two types of colony, corresponding to the descriptions of F variant and wild type given above. On other occasions only one or other of the colony types was recognized in subcultures from papillae. It was not possible to tell the composition of a papilla from its appearance. Microscopically, organisms from the mother colony and fan-shaped outgrowths or from subcultures of these on agar or in fluid media possessed identical morphologies. The organisms were arranged singly and in chains of 2 to 6 bacilli. The differences in size of the two colony types on medium I agar was verified by direct measurement: the mean diameter of colonies of the wild-type strain 300 was 0·46 mm.; the mean diameter of the F variant of this strain was 0·55 mm. Wild-type and F variant colonies of the other two strains showed similar differences. This difference in diameter was paralleled by results of total and viable counts. These results are presented in Table 1.

The tendency of these organisms to grow in chains of different lengths is one of the factors responsible for the viable counts being lower than the total counts. On further incubation of F variant and wild-type colonies on medium I agar, R outgrowths often appeared on both types of colony, but secondary colony formation was detected only on wild-type colonies. R outgrowths were occasionally seen on fan-shaped outgrowths (Pl. 1, fig. 7) and frequently a wild-type colony bore both fan-shaped outgrowths and R outgrowths (Pl. 1, fig. 8). No change in the incidence of secondary colony formation was detected on medium I supplemented with amino acids and/or vitamins. As with the R variants of *L. casei* (de Klerk & Coetzee, 1962) neither form of secondary colony appeared on medium I agar supplemented with glucose or cellobiose in excess of 0·5 % (w/v). Addition of rhamnose or melibiose (not normally fermented by *L. casei*) did not prevent secondary colony formation.

The morphological differences between the two types of colonies noted on medium I agar disappeared when these were subcultured on tomato glucose agar. Forty-eight hr. colonies of wild-types and F variants were circular, white, opaque, about 2 mm. in diameter, dome-shaped with an entire edge, with the surface very finely granular and glistening. The F variant of strain 300 differed from the R variant of this strain in that colonies of the R variant retained their rough appearance

on tomato glucose agar (de Klerk & Coetzee, 1962). The effect of inoculum-size on the formation of papillae and fan-shaped outgrowths by strain C_9 is shown in Table 2.

Table 1. *Total and viable counts of organisms in* 48 *hr.-colonies of wild strains and F variants of* Lactobacillus casei

Six whole colonies of each strain were cut out with the underlying agar and thoroughly emulsified in separate 0·25 ml. volumes of distilled water. The organisms present in samples were counted in a Petroff–Hauser chamber with a phase-contrast microscope. Suitable dilutions were plated on tomato glucose agar for viable counts. Colonies were enumerated after incubation for 48 hr. at 37°.

	Mean populations of six 48 hr. colonies	
Strain	Viable count (millions)	Total count (millions)
C_9 wild	1·2	4·3
C_9 variant F	2·9	8·0
300 wild	1·0	3·6
300 F variant	2·5	6·0
316 wild	3·3	5·0
316 F variant	4·4	7·5

Table 2. *The effect of crowding on the development of papillae and fan-shaped outgrowths of* Lactobacillus casei *strain* C_9

Samples (0·1 ml.) of 4 different dilutions of overnight growth of cultures in tomato glucose broth were spread on medium I in similar Petri dishes. These were incubated at 37° in an atmosphere of carbon dioxide for 8 days. Counts were done on 8 areas of 3 cm.² for each dilution.

		Mean no. of secondary colonies	
Dilution	No. of colonies/72 cm.²	Fan-shaped out-growths/colony	Papillae/colony
1×10^{-5}	1304	0·44	1·95
5×10^{-6}	488	0·28	3·24
1×10^{-6}	160	0·04	3·49
5×10^{-7}	56	0	5·72

The incidence of fan-shaped outgrowths/colony bore a direct relationship to the number of colonies/unit area. The number of papillae/colony on the other hand had an inverse relationship. Experiments with strains 300 and 316 yielded similar results. Distribution of colonies bearing different numbers of fan-shaped outgrowths or papillae, or the sum of the latter two did not approach Poisson distributions at any of the degrees of crowding used in the above experiments. Differences in the thickness of the layer of medium had no effect on secondary colony formation. F variants and wild-types had the same biochemical reactions and growth temperatures.

The results of agglutination and agglutinin absorption tests with sera prepared against wild-types and F variants of strains C_9 and 316 are shown in Table 3.

Unlike the R mutants of *Lactobacillus casei* previously described (de Klerk & Coetzee, 1962) F variants of strains C_9, 316 and 300 were found to be antigenically identical to the corresponding wild-types. *L. casei* strains 300 and 316 are susceptible

to lytic phages 300 and 316 respectively (Coetzee, de Klerk & Sacks, 1960). These phages had efficiencies of plating on the F variants of their hosts identical to those on the wild strains. R variants of strain 316 did not adsorb phage 316 (de Klerk & Coetzee, 1962). No phage active on strain C_9 was available. The changes in pH value in medium I liquid cultures of wild-types and F variants were identical and minimal; during incubation for 170 hr. there was a gradual change from pH 6·35 to 6·7. Measurement of the optical density of cultures growing in liquid medium I are

Table 3. *Results of agglutination and agglutinin absorption tests on antisera prepared against the wild-types and the F variants of* Lactobacillus casei *strains C_9 and 316*

Antigens were suspended in distilled water and serum dilutions made in 0·85 % NaCl solution. Equal volumes of antigen and antiserum were mixed and tests were kept in a water bath at 50° for 6 hr. and then at 4° overnight. Agglutinin absorptions were done by adding an excess of tightly packed heated antigen to a dilution of serum corresponding to thirty-two times the concentration of its titre. The contents of these tubes were well mixed and kept at 37° for 4 hr.

Antiserum	Serum absorbed with	Antigen	Titre
C_9 (wild-type)	—	C_9 (wild-type)	1/1024
	—	C_9 (F variant)	1/1024
	C_9 (F variant)	C_9 (F variant)	< 1/8
	C_9 (F variant)	C_9 (wild-type)	< 1/8
C_9 (F variant)	—	C_9 (F variant)	1/1024
	—	C_9 (wild-type)	1/512
	C_9 (wild-type)	C_9 (wild-type)	< 1/16
	C_9 (wild-type)	C_9 (F variant)	< 1/16
316 (wild-type)	—	316 (wild-type)	1/2048
	—	316 (F variant)	1/2048
	316 (F variant)	316 (F variant)	< 1/16
	316 (F variant)	316 (wild-type)	< 1/16
316 (F variant)	—	316 (F variant)	1/1024
	—	316 (wild-type)	1/1024
	316 (wild-type)	316 (wild-type)	< 1/8
	316 (wild-type)	316 (F variant)	< 1/8

Controls with 0·8 % (w/v) NaCl solution were satisfactory.

presented in Fig. 1. The slopes of these lines show that the F variants of *L. casei* grew more rapidly than the wild strains under the conditions of the experiment. Generation times of wild-types and F variants calculated from viable counts during the exponential period of growth always showed those of the F variant to be the shorter. The actual times obtained with any pair of strains were, however, so non-reproducible that this method of obtaining the absolute generation times was soon abandoned. This irregular behaviour was attributed to the fact that *L. casei* grew in chains of different lengths. Generation times of the wild-type and F variant of strain 300, calculated from total counts in six different experiments, varied from 173 to 185 min. for the wild-type and from 147 to 154 min. for the F variant. These results are considered to be reliable since organisms in chains could be enumerated individually and deaths during the period of the experiment (usually about 10 hr.) would be minimal. Experiments with wild-types and F variants of strains C_9, and 316 yielded similar results. These results confirm the indications obtained above that F variants have shorter generation times than wild-types.

Subcultures were made from centrally situated papillae of colonies of the three strains examined. A limited number of F variant-like colonies and wild-type-like colonies so obtained were examined by all the above methods. Organisms comprising the two types of colony were found to possess properties identical with those in F variant and wild-type colonies, respectively.
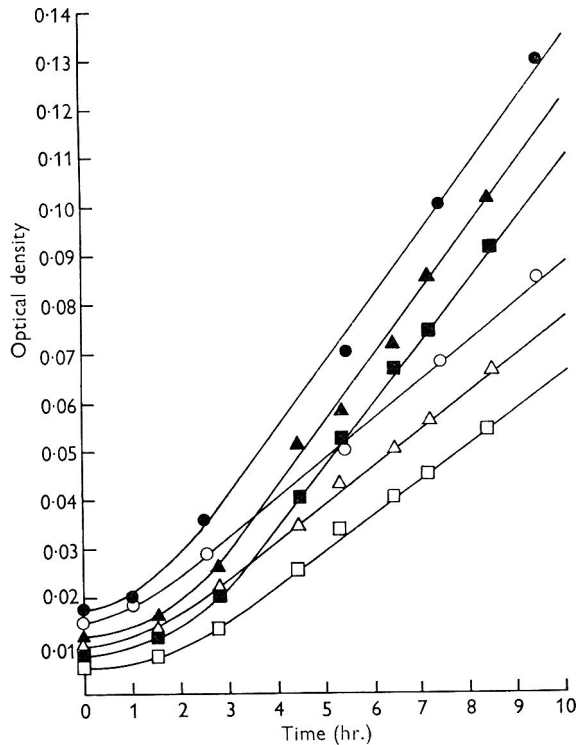

Fig. 1. Photometric determinations of growth of wild-types and F variants of strains C₉, 300 and 316. Overnight cultures of wild-type and F variant colonies in 6 ml. of medium I broth were transferred into 100 ml. volumes of fresh pre-warmed broth and maintained at 37° for 10 hr. At intervals samples were removed and optical densities read on a Zeiss spectrophotometer at 660 mμ. Strain 300 wild-type, ○; strain 300 F variant, ●. Strain C₉ wild-type, △; strain C₉ F variant, ▲. Strain 316 wild-type, □; strain 316 F variant, ■.

DISCUSSION

It is reasonable to suppose that organisms able to grow out above their relations in a colony must possess some selective advantage. This has been proved in many instances, see for example, the paper by Ryan, Schwartz & Fried (1955). These workers described the development of papillae arising on colonies of a histidineless strain of *Escherichia coli*, growing in the presence of subminimal quantities of histidine. The selective advantage enjoyed by the mutant organisms comprising these papillae was found to be due to the fact that they were histidine independent. Shah & Iyer (1960) showed that organisms in secondary colonies of *Bacillus subtilis* were favoured by their greater ability to neutralize the acids initially produced by fermentation in colonies on a medium containing glucose and peptone. The *Lacto-*

bacillus casei F variants encountered in the present work differed from the wild-types in that colonies of the former were larger, more opaque and contained more bacilli than the latter when growing on medium I. These differences may be associated with the observation that the F variants had relatively shorter generation times than the wild-types under the conditions of study. Absolute generation times could only be determined by means of total counts. The times obtained were much longer than those of *L. casei* and other lactobacilli determined under optimal conditions by a variety of methods (Mason, 1935). This was probably due to the poor growth-supporting qualities of medium I. It is interesting to note that *L. casei* has the shortest generation time of all the species of lactobacilli listed by Mason (1935). It might have been anticipated that fast-growing mutants of other species of lactobacilli which have longer generation times would possess a greater selective advantage and be encountered more frequently, yet secondary colony formation was practically confined to strains of *L. casei* var. *casei* and *L. casei* var. *rhamnosus*. Fast-growing variants of *E. coli* detected in continuous cultures have been reported by Novick & Szilard (1950) and by Moser (1955). In the present work the fan-shaped outgrowths were pure cultures of F variants but uncertainty still remains regarding the nature of many papillae. Most papillae apparently consisted of mixtures of F variant and wild-type organisms. Some consisted of pure cultures of F variant-producing organisms, but subculture of many papillae produced only wild-type colonies. It may be argued that a papilla is a dynamic structure and that it might, at a particular stage of its development, still be contaminated with parent organisms. Despite the limited means of identification available this could hardly explain the many instances where no F variant-like colonies were detected in subcultures of well-developed papillae. Loss of viability of F variants in papillae with survival of contaminating wild-type organisms was considered as an explanation of the latter finding. This appears to be unlikely. First, because there were no indications that F variant organisms tended to die off more rapidly than the wild-type, and secondly because F variants were often present in other papillae on the same colony. The fact that all secondary colonies failed to appear on media containing some carbohydrate is no argument for the basic homogeneity of these colonies. R mutants (de Klerk & Coetzee, 1962) also lose their selective advantage under these circumstances. Another argument in favour of the basic heterogeneity of secondary outgrowths is the fact that the distributions of colonies bearing homogeneous outgrowths and papillae often follow a Poisson distribution (Ryan *et al.* 1955; Coetzee, 1959; Coetzee & Sacks, 1960 *a*, *b*; de Klerk & Coetzee, 1962). The fact that the distribution of colonies of *L. casei* bearing varying numbers of secondary colonies did not follow such a distribution is another factor against the inherent identity of all these structures. However, an inverse relationship existed between numbers of papillae and fan-shaped outgrowths/colony on crowded and on sparsely inoculated plates. This might be explained on the basis that on crowded plates the colonies were smaller and secondary colonies which arose in the form of papillae thus stood a greater chance of spilling over the margins of colonies and developing into fan-shaped outgrowths. This could suggest that many papillae and fan-shaped outgrowths were of the same composition. Because other undetected phenotypic differences (and possible selective advantages) might be involved it was decided not to couple the single manifestation of fast growth with secondary colony formation. For

reasons mentioned above it has not been possible to obtain proof of the random origin of the F variants and to determine the mutation rates involved. The fan-shaped outgrowths, however, may be regarded as fast-growing variants of *L. casei* which possess a selective advantage under the austere conditions prevailing in growth on medium I. These variants maintained their characteristics through numerous platings and were stable in the environment tested. Like the R mutants, however (de Klerk & Coetzee, 1962), they lost their selective advantage and were not observed in the presence of small amounts of fermentable carbohydrates. This is a possible explanation why the wild-type maintains itself as the modal form.

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EXPLANATION OF PLATE

Colonial morphology of *Lactobacillus casei* strains on medium I agar.

Fig. 1. Crowded 6-day colonies of strain C_9 showing four fan-shaped outgrowths and numerous minute papillae. An R outgrowth is seen on one of the fan-shaped outgrowths. × 25.

Fig. 2. 7-day colonies of strain 300 showing numerous smooth papillae of different sizes. An R outgrowth and fan-shaped outgrowth are also present. × 40.

Fig. 3. 4-day colonies of strain 300 showing smooth papillae on their surfaces and at least two early fan-shaped outgrowths at the margins. × 25.
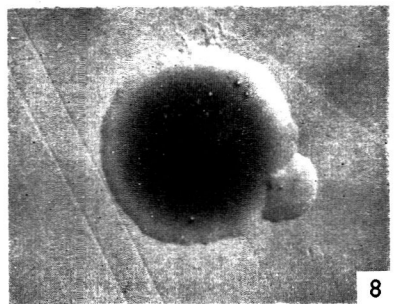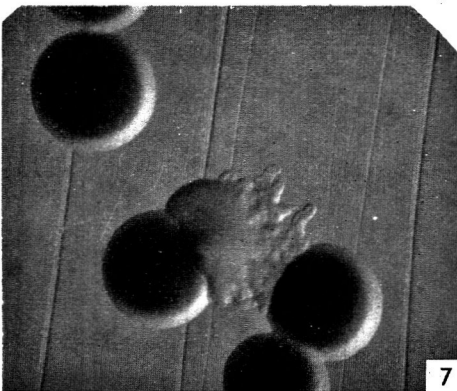
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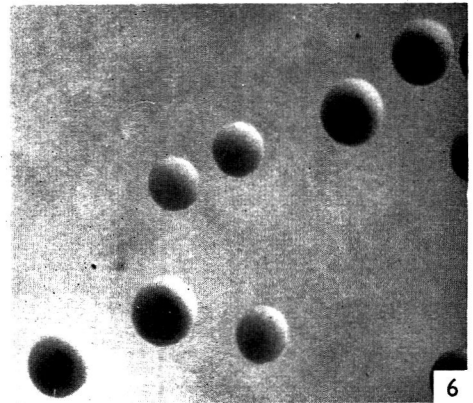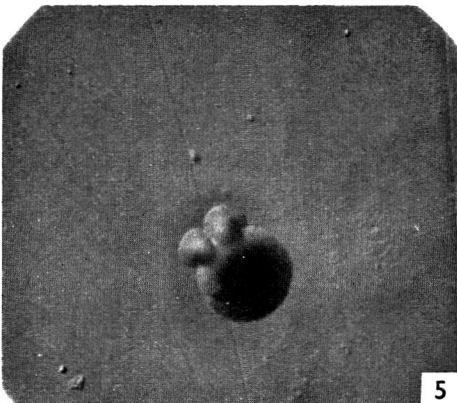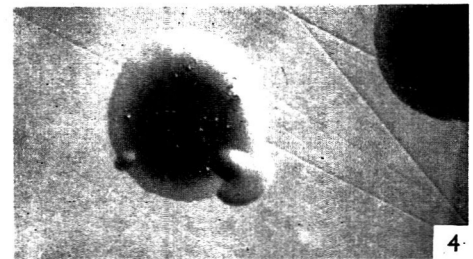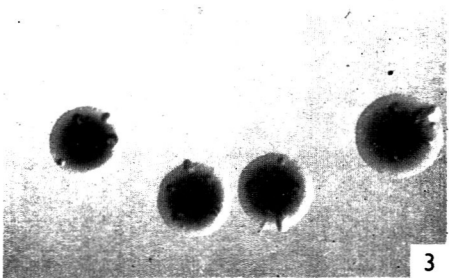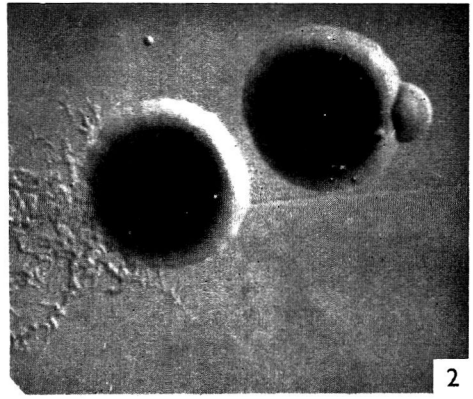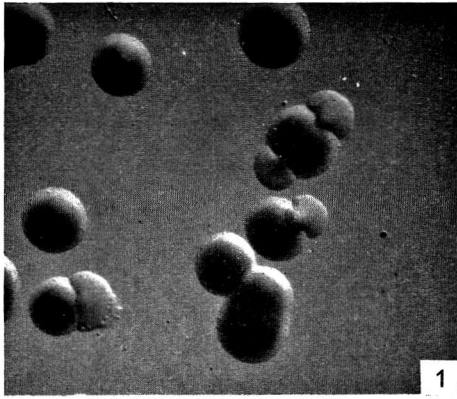
Fig. 4. 6-day colony of strain 316 showing numerous smooth papillae and one early and one fully developed fan-shaped outgrowth. × 40.

Fig. 5. 6-day colony of strain C_9 showing sharp demarcation between adjacent fan-shaped outgrowths. × 25.

Fig. 6. 2-day colonies of the wild-type and F variant of strain 316. F variant colonies are clearly distinguishable by their larger diameter and greater density. × 25.

Fig. 7. A fan-shaped outgrowth on a 6-day colony of strain C_9 showing the development of an R outgrowth from its margin. × 40.

Fig. 8. 8-day colony of strain 300 with a fan-shaped and R outgrowth on its margin. Numerous papillae are present on the surface. × 40.

Studies on the Utilization of Nitrate by *Micrococcus denitrificans*

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SUMMARY

Micrococcus denitrificans is capable of carrying out assimilatory and dissimilatory nitrate reduction, though only the assimilatory process occurs under both aerobic and anaerobic conditions. Aeration affects the dissimilatory activity (reduction of nitrate to nitrogen) of a growing culture in at least three ways: (*a*) it prevents the adaptive formation of the system, (*b*) it partially represses any further synthesis if the system is already present, and (*c*) it inhibits the activity of the preformed system completely. To some extent these effects of oxygen are reflected in the control which it exerts upon the organism's content of nitrate reductase (the enzyme responsible for the initial reduction of nitrate to nitrite) and upon its activity during growth.

Ammonium ions partially inhibit the transformation of nitrate into cell nitrogen but have no detectable effect on the nitrate reductase activity of crude extracts of this organism.

INTRODUCTION

Several attempts have been made to categorize the various routes whereby nitrate ions may be reduced by micro-organisms. Some have been concerned with the nature of the actual components of the reaction sequences, e.g. the criterion of participation of cytochromes suggested by Taniguchi, Sato & Egami (1956). However, until the actual metabolic routes have been more fully characterized it would appear to be advisable to use a classification based on physiological function. Verhoeven (1956) thus distinguished three types of nitrate reduction: (1) Assimilatory; where the nitrate is utilized by the growing organism solely as a source of nitrogen. (2) True dissimilatory; where the nitrate acts as terminal hydrogen acceptor in anaerobic growth of the organism. (3) Incidental dissimilatory; in which nitrate is used as a non-essential hydrogen acceptor with nitrite as the usual product.

That any real distinction may be drawn between 'true' and 'incidental' dissimilation is queried by some, who would group both types under the one heading of 'nitrate dissimilation or respiration' (Fewson & Nicholas, 1961*a*).

Oxygen is known to prevent the development of the true nitrate dissimilatory pathway with its attendant nitrogen production, in several bacteria, e.g. *Pseudomonas denitrificans* (Sacks & Barker, 1949), *P. stutzeri* (Allen & van Niel, 1952), *Micrococcus denitrificans* (Kluyver & Verhoeven, 1954), and *P. aeruginosa* (Collins,

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1955). An inhibitory effect of oxygen on nitrate reduction by washed suspensions of organisms previously adapted to nitrate utilization has also been observed (Sacks & Barker, 1949; Skerman & MacRae, 1957 a, b). On the other hand, there have been reports of loss of nitrate in the form of gaseous nitrogenous products from aerobically growing cultures (Meiklejohn, 1940; Marshall, Dishburger, Mac-Vicar & Hallmark, 1953; Verhoeven, 1956). This seeming contradiction of results has been largely resolved by the studies of Skerman and his colleagues (Skerman, Lack & Millis, 1951; Skerman & MacRae, 1957 a, b; 1961) which related the prevention of nitrate dissimilation to the actual oxygen tension in solution.

With nitrate as sole source of nitrogen for growth, Verhoeven (1956) obtained evidence for assimilatory reduction of nitrate during aerobic growth of *Micrococcus denitrificans*, though in anaerobic growth on a medium containing an alternative source of nitrogen all the nitrate consumed was accounted for as nitrogen or nitrous oxide.

We found that assimilatory reduction of nitrate could be effected by *Micrococcus denitrificans* during anaerobic, as readily as during aerobic growth, i.e. for this organism nitrate is a sufficient if not optimal source of nitrogen for growth. The effect of oxygen on the energy-yielding dissimilatory pathway and of ammonium ions on the assimilatory pathway has been examined.

METHODS

Organism. The strain of *Micrococcus denitrificans* obtained from Dr June Lascelles had been kindly supplied by Dr W. Verhoeven. It was maintained on peptone nitrate slopes containing the following (g./litre): peptone (Evans Medical Supplies Ltd., Liverpool), 10; K_2HPO_4, 1; $MgSO_4.7H_2O$, 0·5; glycerol, 10; agar, 20; the pH was adjusted to 7·0. Inocula for liquid media were taken from such slopes after 20 to 30 hr. incubation at 30°.

Medium. The defined liquid medium consisted of the following (g./litre): sodium succinate, $6H_2O$, 13·5; K_2HPO_4, 6; KH_2PO_4, 4; $MgSO_4.7H_2O$, 0·2; $CaCl_2$, 0·04; sodium molybdate, 0·15; $MnSO_4.4H_2O$, 0·001; and an iron citrate solution, 5 ml. (containing in g./litre, $FeSO_4.7H_2O$, 1·1; citric acid.H_2O, 1·05) with NH_4Cl, 1·6; and KNO_3, 10·1 being added as required.

Growth conditions and preparation of suspensions. Anaerobic cultures were incubated in glass-stoppered bottles completely filled with medium. Aerobic cultures were incubated either in 100 ml. volumes per 1 l. Erlenmeyer flask or in 200 ml. volumes per 2 l. Erlenmeyer flask, shaken at 160 to 200 rev./min. in a gyrotary shaker (New Brunswick Scientific Co., New Brunswick, N.J., U.S.A.). All incubation was at 30°. After harvesting in the exponential phase of growth, all cells were washed in a 0·1 M-phosphate salts buffer, pH 6·8 (0·1 M-phosphate pH 6·8 containing the same concentrations of $MgSO_4$, $CaCl_2$, $MnSO_4$, sodium molybdate and iron citrate as the growth medium).

In those growth experiments where a comparatively large inoculum of adapted organisms was required, cultures were harvested during exponential growth and stored overnight at 2° as a thick suspension in 0·1 M-phosphate salts buffer. On the following day these organisms were used to inoculate large quantities of fresh medium previously warmed to 30°; growth then began almost immediately.

Assessment of growth. The extent of growth was measured with an EEL photo-

electric colorimeter (Evans Electroselenium Ltd., Halstead, Essex) with a neutral density filter and 15·8 mm. sample tubes; the uninoculated medium was used to give zero setting. The relation between 'EEL reading' and dry weight was determined and found linear up to a reading of 35–40 with a progressive departure from linearity thereafter. For the linear part of the curve 10 'EEL units' was equivalent to 0·4 mg. dry weight.

Preparation of cell-free extracts. Exponentially growing organisms were harvested, washed, suspended in 0·04 M-Tris (tris-hydroxymethylaminomethane) buffer pH 7·4, and disrupted by ultrasonic vibration for 5 min. at 25 kc./sec. in a Mullard ultrasonic generator type E 7590B (Mullard Ltd., London, W.C. 1). Unbroken cells and debris were removed by centrifuging at 17,400 *g* for 10 min. at 0°.

Enzyme assays. Nitrate dissimilatory activity (referred to as nitratase) was estimated by manometric measurement of N_2 evolution from nitrate. The main compartment of each manometer vessel contained washed organisms (2·7–3·6 mg. dry wt./ml.); 140 μmole phosphate salts buffer, pH 6·8; 600 μmole sodium succinate; water to 2·5 ml. The centre well contained 0·2 ml. 20% (w/v) KOH. The reaction was started by addition of 100 μmole KNO_3 from the side arm. Incubation was at 30° in an atmosphere of oxygen-free nitrogen (British Oxygen Gases Ltd.).

Nitrate reductase activity was assayed in cell-free extracts. The reaction mixture consisted of 100 μmole Tris buffer, pH 7·9; 100 μmole sodium succinate; 0·1 ml. crude extract (about 1 mg. protein); water to 1·9 ml. The reaction was started by the addition of 20 μmole $NaNO_3$. After 15 min. incubation at 30° protein was precipitated with 0·1 ml. of 2M zinc acetate followed by 1·9 ml. 95% (v/v) ethanol (Fewson & Nicholas, 1961*b*). The precipitate was removed by centrifuging and samples of the supernatant assayed for nitrite.

Succinic dehydrogenase activity of crude extracts was determined spectrophotometrically using 2:6-dichlorophenol-indophenol and phenazine methosulphate (Redfearn & Dixon, 1961).

Protein estimation. This was performed by the method of Lowry, Rosebrough, Farr & Randall (1951) using crystalline bovine plasma albumin (Armour Laboratories Hampden Park, Eastbourne, Sussex) as standard.

Chemical estimations. Nitrite was determined colorimetrically by a method modified from that of Rider & Mellon (1946) as described by Lascelles (1956).

Nitrate was assayed as nitrite after chemical reduction by a method developed by Dr D. J. D. Nicholas (personal communication to Dr June Lascelles). Samples containing up to 2·5 μmole of nitrate were diluted to 2·5 ml. with distilled water, 2·5 ml. of 2N-NaOH was added to bring the pH to between 11 and 11·6, and the mixtures shaken vigorously for 5 min. with about 0·05 g. zinc powder in stoppered test tubes held in a microid shaker. After immediate centrifuging the nitrite content of the supernatant was determined colorimetrically.

Ammonia was estimated by the phenate-hypochlorite method (Conway, 1950), and cell nitrogen similarly after conversion to ammonium sulphate by the Kjeldahl method (Conway, 1950).

RESULTS

Existence of both assimilatory and dissimilatory routes of nitrate utilization

Micrococcus denitrificans grew well with nitrate as sole source of nitrogen under both aerobic and anaerobic conditions (Fig. 1 *b*). The lag period was shorter in the presence of ammonium ions. In a medium containing both nitrate and ammonium ions the lag period was longer and the rate of growth slower in anaerobic than in aerobic conditions (Fig. 1 *a*).
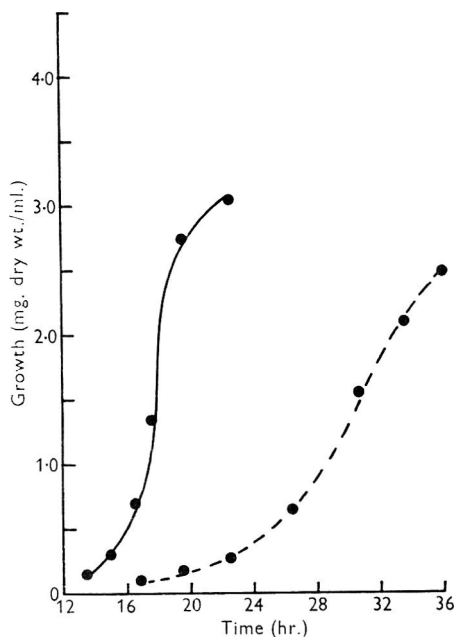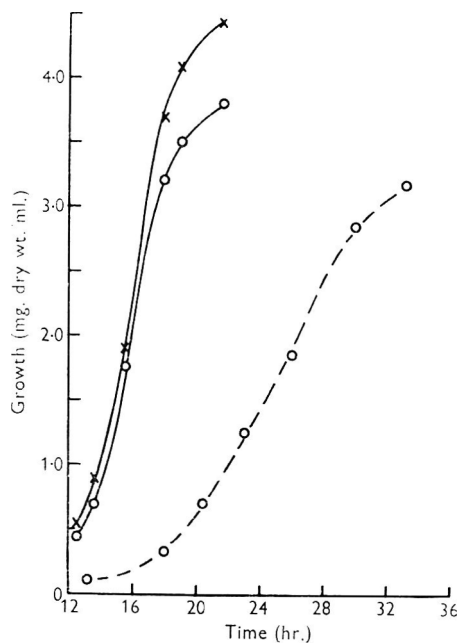
Fig. 1 *a*   Fig. 1 *b*

Fig. 1 *a*. Growth of *Micrococcus denitrificans* on nitrate as sole source of nitrogen. —, aerobic and – – – anaerobic.

Fig. 1 *b*. Growth of *Micrococcus denitrificans* on media containing ammonium ions. —, aerobic; – – –, anaerobic; ×, ammonium chloride only, ○, nitrate plus ammonium ions.

The effect of aeration on the formation of enzymes concerned with nitrate utilization

Two enzyme systems were studied. (1) Nitratase—the sequence of enzymes effecting the reduction of nitrate to nitrogen gas. (2) Nitrate reductase—the enzyme system reducing nitrate to nitrite.

Nitratase. Organisms grown aerobically in the presence of nitrate could not reduce nitrate to nitrogen in contrast to those grown anaerobically with nitrate as hydrogen acceptor, which showed considerable nitratase activity (Table 1).

The effect of aeration on nitratase synthesis was examined in growing cultures. Part of a culture growing anaerobically on nitrate plus ammonia was transferred early in exponential growth to aerobic conditions, the remainder being allowed to continue anaerobically. Nitratase activity and growth were measured at intervals.
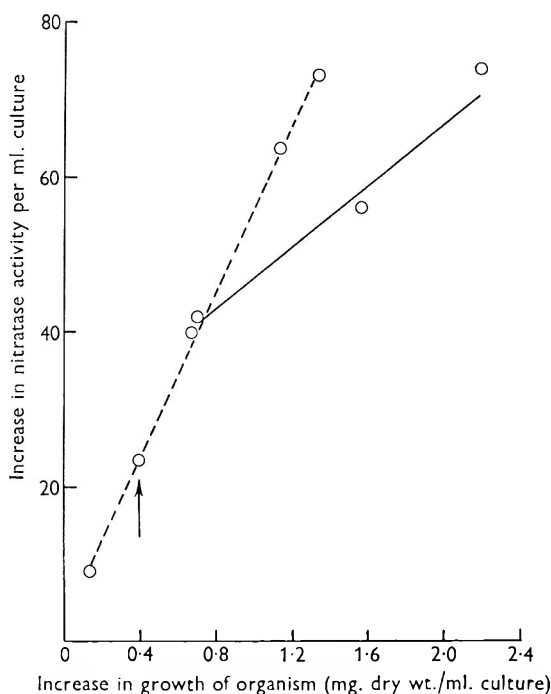
Fig. 2. The effect of aeration on the synthesis of nitratase in cultures of *Micrococcus denitrificans* growing on nitrate plus ammonium ions. Nitratase activity was measured as μl. N_2 produced/mg./hr. ——, aerobic; – – –, anaerobic.

Table 1. *Nitratase activity of washed suspensions of* Micrococcus denitrificans

Nitratase activity was calculated from nitrogen evolution during the first 30 min. of assay.

Nitrogen source in medium	Growth conditions	Nitratase activity μl. N_2 formed/mg. dry wt./hr.
Ammonia	Aerobic	0
Ammonia plus nitrate	Aerobic	0
Ammonia plus nitrate	Anaerobic	54

The results show that there was partial repression of nitratase synthesis after about 1·5 hr. exposure to air introduced at ↑ in Fig. 2.

Nitrate reductase. Crude sonic extracts were tested for nitrate reductase activity under conditions where they showed no detectable nitrite reductase activity (Methods). Preliminary experiments showed that at the pH optimum of 7·9, activity was linear with both protein concentration (over the range 0·15–1·5 mg. protein per ml.) and with time (up to 25 min.). Using the standard assay procedure described, nitrate reductase levels were measured in extracts of organisms grown under conditions in which the supply of oxygen and of nitrate was varied (Table 2).

Extracts of aerobically grown organisms showed no nitrate reductase activity if nitrate had been omitted from their growth medium and only low activity after

growth in its presence. High activity was found only in extracts of anaerobically grown organisms. These results could not be attributed to destruction or irreversible inhibition of the enzyme by oxygen since organisms harvested during exponential growth on ammonium plus nitrate ions under anaerobic conditions showed no loss of activity after 60 min. aeration. It was concluded therefore that oxygen at atmospheric pressure greatly diminishes the synthesis of nitrate reductase.

Since succinate was used as hydrogen donor in the nitrate reductase assay, succinic dehydrogenase activity was measured in each extract. Though there was some variation in the activity of this enzyme under the various conditions of growth, these differences were not of the same order of magnitude as those found in nitrate reductase activity, and in all cases the rate of succinate oxidation was considerably greater than the rate of nitrate reduction (Table 2).

Table 2. *Nitrate reductase and succinic dehydrogenase levels in cell-free extracts of* Micrococcus denitrificans

Nitrate reductase activity expressed as μmole nitrite formed/mg. protein/15 min. Succinic dehydrogenase activity expressed as μmole succinate reduced/mg. protein/ 15 min.

Nitrogen source in growth medium	Growth conditions	Nitrate reductase activity	Succinic dehydrogenase activity
Ammonia	Aerobic	0	9·8
Ammonia plus nitrate	Aerobic	0·03	8·6
Nitrate	Aerobic	0·01	6·1
Ammonia plus nitrate	Anaerobic	1·27	27·6
Nitrate	Anaerobic	1·96	22·4

Table 3. *The effect of aeration on nitrate utilization by growing cultures of* Micrococcus denitrificans

A culture growing anaerobically on nitrate as sole N-source was divided into two portions. One was allowed to continue anaerobically, the other was transferred to aerobic conditions and incubation of both was continued for 9·25 hr. The values listed represent changes occurring from the point of transfer, (−) indicating disappearance and (+) increase in medium component or in organisms. The concentration of all nitrogenous compounds is expressed as μmole N per ml.

	Growth conditions	
	Anaerobic	Aerobic
Growth (mg. dry wt./ml.)	+1·38	+2·74
Nitrate utilized	−41·0	−19·3
Nitrite formed	+26·6	+6·9
Ammonia formed	0	0
Cell nitrogen formed	+3·0	+12·8
Nitrogen balance	−11·4	+0·4

The effect of aeration on nitrate utilization by growing cultures

Aeration partially represses further nitratase synthesis in cultures that had previously been growing anaerobically (Fig. 2). However, it was not known whether the residual enzyme (of considerable activity if assayed under favourable anaerobic conditions), was at all functional under the adverse aerobic conditions. This was examined in balance experiments with growing cultures when the con-

centrations of nitrate, nitrite and ammonia in the medium, together with cell nitrogen content, were measured. Imbalance between supply and recovery of nitrogenous compounds was ascribed to loss of gaseous products, particularly of nitrogen. In these studies *Micrococcus denitrificans* was incubated anaerobically on nitrate as sole nitrogen source and early in the exponential phase of growth a portion was transferred to aerobic conditions. Both cultures were then sampled at intervals, the final balance only being given in Table 3. Anaerobically, as was to be expected, there was considerable loss of nitrogen. Aerobically, the nitrate utilized was completely accounted for as nitrite and cell nitrogen. Thus nitratase is completely inactive under aerobic conditions of growth, which in turn suggests that one at least of the enzymes reducing nitrite to nitrogen is more susceptible to inhibition and possibly repression than is nitrate reductase itself.

The effect of ammonium ions on the aerobic utilization of nitrate by growing cultures

It was thought that ammonium ions which are at the same oxidation level as cell nitrogen and are a better source than nitrate or nitrogen for growth, might repress or inhibit (or both) nitrate assimilation. Ammonium chloride (0·65 g./l.) was added during exponential, aerobic growth of organisms provided with nitrate as sole nitrogen source. Both nitrate uptake and nitrite formation were inhibited (Table 4).

Table 4. *The effect of ammonium ions on the aerobic utilization of nitrate by growing cultures of* Micrococcus denitrificans

A culture growing aerobically on nitrate as sole N-source was divided into two portions. NH_4Cl (650 mg./l.) was added to one, no addition was made to the other and incubation of both was continued for 4 hr. The values listed represent changes occurring from the point of addition of NH_4Cl, the concentration of all nitrogenous compounds being expressed as μmole N per ml.

	Nitrogen source during growth	
	Nitrate	Nitrate plus NH_4Cl
Growth (mg. dry wt./ml.)	+1·0	+1·21
Nitrate utilized	−7·8	−4·4
Ammonia utilized	0	−6·9
Nitrite formed	+1·4	0
Cell nitrogen formed	+5·0	+7·0
Nitrogen balance	−1·4	−4·3

The overall nitrogen imbalance in this experiment could not be due to loss of nitrogen as gas since similarly grown organisms possessed no nitratase activity. In the early stages of aerobic growth on nitrate as sole nitrogen source only part of the nitrate removed from the medium could be recovered while at the end of the exponential phase recovery was complete (Table 5). Nitrate present in the cells but not converted to cell nitrogen would not be quantitatively converted to ammonia by Kjeldahl digestion due to loss as nitric acid vapour (Vogel, 1951). Consequently the early apparent loss of nitrogen may be due to nitrate being taken into the organisms during exponential growth at a rate greater than that at which it is reduced, i.e. being accumulated within the cells.

The effect of ammonium ions on nitrate reductase activity

Since nitrite formation was abolished by the addition of ammonia (Table 4), it was thought possible that ammonium ions might inhibit nitrate reductase activity. However, ammonium chloride (30 mM) had no adverse effect on nitrate reductase activity in extracts of organisms grown aerobically on nitrate as sole source of nitrogen.

Table 5. *Recovery of nitrogen during aerobic growth of* Micrococcus denitrificans
on nitrate as sole source of nitrogen

Growth (mg. dry wt./ml.)	Nitrogen recovered as nitrite, ammonia and cell nitrogen / Decrease in nitrate nitrogen in medium × 100
0·66	77
1·16	62
2·26	82
3·53*	97

* End of exponential growth.

DISCUSSION

Cultures of *Micrococcus denitrificans* grew more rapidly aerobically than anaerobically in a medium containing both nitrate and ammonium ions (Fig. 1b). This could be due to electron transfer to oxygen being more efficient than comparable transfer to nitrate as a source of high energy phosphate. Phosphorylation linked to incidental nitrate reduction has been reported in *Escherichia coli* (Takahashi, Taniguchi & Egami, 1956) and to true dissimilation in *Pseudomonas denitrificans* (Ohnishi & Mori, 1960). However, a comparative study of the energetic yields of all these processes in the same organism has yet to be carried out.

In the control of nitrate dissimilation by oxygen in this organism inhibition of nitratase activity is of primary importance in the short term (Table 3). The function of repression of the development of nitratase by oxygen in the long term is difficult to assess since the rate of synthesis of nitratase in organisms previously grown anaerobically is only partially reduced by aeration (Fig. 2), while cultures grown wholly aerobically under apparently the same oxygen tension contain no detectable nitratase, even when the assay for its activity is performed anaerobically (Table 1).

Ammonium ions apparently inhibit nitrate reduction and its conversion into cell nitrogen. From the results obtained (Table 4), it is evident that the figures for aerobic nitrate disappearance agree well with those for concurrent increase in cell nitrogen. In the presence of ammonium ions, even if all the nitrate utilized aerobically were converted into cell nitrogen, it would be insufficient to account for the total increase in cell nitrogen. Using $N^{15}H_4$ and $N^{15}O_3$, Marshall *et al.* (1953) have shown that ammonium ions largely, if not completely, prevent the incorporation of nitrate into cell protein in *Pseudomonas fluorescens* and *P. denitrificans*. This would seem also to be the situation in *Micrococcus denitrificans*.

An attempt was made to demonstrate a correlation between hydroxylamine

metabolism by washed suspensions of *Micrococcus denitrificans*, and the ability of these organisms to assimilate nitrate, with the prospect that ammonium ions might act in an aerobically growing culture by feed-back inhibition either of an enzyme producing hydroxylamine or of a specific hydroxylamine reductase. In fact, there was no significant difference between organisms grown aerobically on nitrate and those grown on ammonium ions as sole source of nitrogen with respect to hydroxylamine utilization. It is, of course, possible that a basal hydroxylamine reductase activity associated with some other function, such as was reported in the case of sulphite reduction by Mager (1960), could mask a specific 'assimilatory' hydroxylamine reductase activity.

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Some Metabolic Differences Between *Thiobacillus thioparus, T. denitrificans* and *T. thiocyanoxidans*

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SUMMARY

Differences in behaviour of *Thiobacillus thioparus, T. denitrificans* and *T. thiocyanoxidans* were found in growth experiments carried out in thiosulphate or thiocyanate containing media and using aerobic or anaerobic conditions. These differences were: *T. denitrificans* and *T. thioparus* grew anaerobically in thiosulphate + nitrate and thiocyanate + nitrate media; *T. thiocyanoxidans* did not. During growth of *T. thioparus* in thiocyanate + nitrate medium, nitrite accumulated but thiocyanate was not completely oxidized; no nitrite accumulated during growth of *T. denitrificans* under similar conditions. After aerobic, serial subculture in thiosulphate medium *T. denitrificans* no longer grew anaerobically in thiosulphate + nitrate or thiocyanate + nitrate media whereas *T. thioparus* did. *T. thioparus* produced copious growth on thiosulphate agar slopes under aerobic conditions; *T. denitrificans* proved difficult to culture under such conditions, i.e. there was a failure to guarantee growth on each occasion, and where growth occurred this was poor. *T. denitrificans* did not produce the mucoid slime associated with *T. thioparus* cultures. *T. thioparus* accumulated and subsequently used up tetrathionate during aerobic growth in thiosulphate medium; *T. denitrificans* did not.

INTRODUCTION

De Kruyff, Van der Walt & Schwartz (1957) claimed, as a result of growth experiments, that under anaerobic conditions in thiosulphate + nitrate or in thiocyanate + nitrate media *Thiobacillus denitrificans* reduced nitrate to nitrogen but that *T. thioparus* and *T. thiocyanoxidans* reduced nitrate only to nitrite. They suggested, moreover, that the two latter organisms were metabolically identical. Happold, Jones & Pratt (1958) agreed that *T. thioparus* and *T. thiocyanoxidans* grew similarly at the expense of thiocyanate under aerobic conditions. Pratt (1958) showed that *T. thioparus* cultures accumulated polythionates at an earlier stage of growth than was the case with *T. thiocyanoxidans* cultures, grown aerobically in thiosulphate medium. Jones & Happold (1961), in confirming Pratt's results, showed that oxidation of the polythionates was potassium ion dependent. We have consequently attempted to define further the similarities and dissimilarities between these organisms. Vishniac & Santer (1957) stated that *T. denitrificans* 'differed from *T. thioparus* only in ability to grow under anaerobic conditions with nitrate as terminal respiratory electron acceptor, and that *T. denitrificans* could grow aerobically in absence of nitrate in thiosulphate medium but rapidly lost denitrifying ability on aerobic subculture, thus becoming indistinguishable from *T. thioparus*'. The experiments reported in the present paper were designed to resolve the apparent

difference in the views of the above authors, namely, that de Kruyff *et al.* (1957) could grow *T. thioparus* anaerobically but that Vishniac & Santer (1957) could not, and to obtain information which might be used in differentiating between the three species of Thiobacillus used here.

METHODS

Organisms. The initial culture of *Thiobacillus denitrificans* was obtained from Dr K. S. Baalsrud, and was subcultured weekly in screw-capped bottles completely filled with the liquid thiosulphate + nitrate medium of Baalsrud & Baalsrud (1954).

The culture of *Thiobacillus thiocyanoxidans* used was developed from a single cell isolated from a crude culture which oxidized thiocyanate.

The initial culture of *Thiobacillus thioparus* was obtained from the National Collection of Industrial Bacteria (NCIB 8370) described as 'Starkey's original non-motile strain'. From this culture a single colony on a thiosulphate agar plate was picked, grown and used for subculture.

The *T. thiocyanoxidans* and *T. thioparus* cultures were subcultured weekly on thiosulphate agar slopes.

Media. For anaerobic cultures the thiosulphate + nitrate medium of Baalsrud & Baalsrud (1954) and thiocyanate + nitrate medium of de Kruyff *et al.* (1957) were used. For aerobic cultures the thiosulphate medium described by Jones & Happold (1961) was used; thiosulphate agar was prepared according to these authors.

Estimations. Thiosulphate was estimated by titration with iodine according to Vogel (1951); polythionate by titration with iodine after treatment with KOH according to Starkey (1934b); thiocyanate spectrophotometrically by a modification of the method described by Bowler (1944); nitrite spectrophotometrically by the method of Rider & Mellon (1946). To estimate nitrate in presence of nitrate advantage was taken of the observation of Skerman, Lack & Millis (1951) that nitrate and nitrite yielded the same colour when determined spectrophotometrically by the brucine method of Noll (1945), and that with mixtures of nitrate and nitrite the colour densities were additive. Thus it was possible to estimate the nitrate concentration in a mixture of nitrate + nitrite when the concentration of nitrite had been determined by the method of Rider & Mellon (1946). None of the other ions of the concentrations found in the media used affected any of these estimations.

Chromatography. The methods of Jones & Happold (1961) for detecting polythionates were used. Potassium pentathionate prepared by the method of Goehring & Feldmann (1948) was used as an additional marker.

RESULTS

Anaerobic cultures

Thiobacillus denitrificans and *T. thioparus* grew anaerobically in both nitrate-containing media; *T. thiocyanoxidans* did not. These results contrast with those of de Kruyff *et al.* (1957) who reported growth of *T. thiocyanoxidans* anaerobically in both nitrate-containing media, and with the statement of Vishniac & Santer (1957) that '*T. thioparus* was a strictly aerobic bacterium'. Figures 1a and 1b show the thiocyanate, nitrate and nitrite concentrations measured during growth of *T. denitrificans* and *T. thioparus* in screw-capped bottles completely filled with thio-

cyanate + nitrate medium. With *T. thioparus* nitrite accumulated and thiocyanate was not completely used up. These facts may be associated, for the rate of thiocyanate utilization decreased when the nitrite concentration reached 150 μg./ml. and ceased when the nitrite concentration was 200 μg./ml. The decrease in rate and subsequent cessation of thiocyanate utilization by *Thiobacillus thioparus* in thiocyanate + nitrate medium may have been related to nitrite concentration; inhibitory concentrations of nitrite for growth of thiobacilli have been shown as follows: 1×10^{-2} M-NO_2 and $1 \cdot 4 \times 10^{-2}$ M-NO_2 for anaerobic growth of *T. denitrificans* in thiosulphate + nitrate and thiocyanate + nitrate media respectively (Baalsrud & Baalsrud, 1954; Woolley, 1961); $2 \cdot 1 \times 10^{-2}$ M-NO_2 for aerobic growth of *T. thioparus*
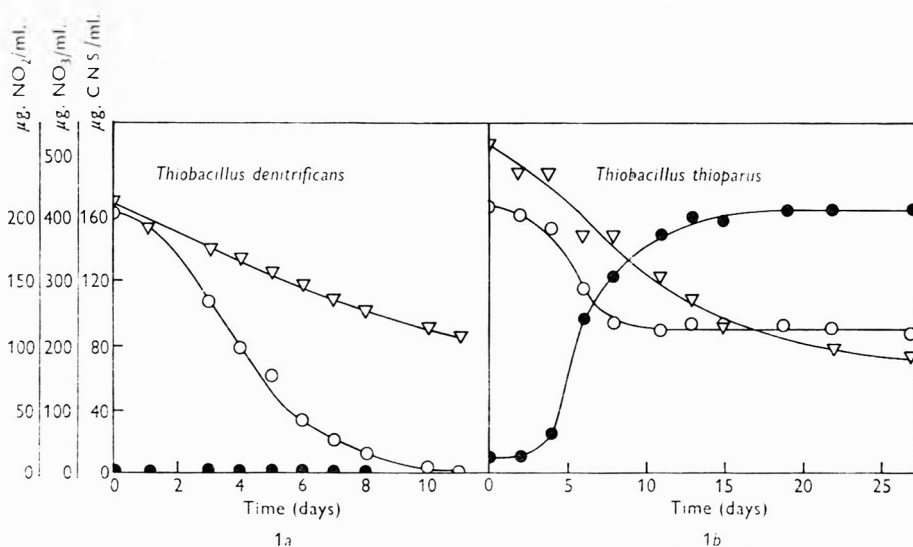
Fig. 1 *a*. Utilization of thiocyanate $\bigcirc$, and nitrate $\triangledown$ and production of nitrite $\bullet$ by a culture of *Thiobacillus denitrificans*.

Fig. 1 *b*. Utilization of thiocyanate $\bigcirc$, and nitrate $\triangledown$ and production of nitrite $\bullet$ by a culture of *Thiobacillus thioparus*.

in thiosulphate medium (Starkey, 1934*a*); $1 \cdot 4 \times 10^{-3}$ M-NO_2 for aerobic growth of *T. thiocyanoxidans* in thiocyanate medium (Youatt, 1953). No quantitative comparisons were made of the anaerobic growth of *T. denitrificans* and *T. thioparus* in thiosulphate + nitrate medium because of difficulties associated with the estimations of thiosulphate and nitrite in the presence of each other.

Aerobic cultures

After aerobic, serial subculture in the thiosulphate medium of Jones & Happold (1961), *T. denitrificans* no longer grew anaerobically when inoculated into thiosulphate + nitrate or thiocyanate + nitrate media, whereas *T. thioparus* which before such subculture has been maintained aerobically, grew immediately under anaerobic conditions in nitrate-containing media.

Whereas *Thiobacillus thioparus* needed 24–48 hr. to produce copious growth on thiosulphate agar slopes under aerobic conditions, *T. denitrificans* proved extremely

difficult to grow on thiosulphate agar under aerobic or anaerobic conditions; minute white colonies took between nine and seventeen days to appear under these conditions. *T. denitrificans* did not produce the mucoid slime associated with *T. thioparus* cultures; cultures of the latter organism took appreciably longer to filter because they were mucoid.

Figures 2*a* and 2*b* show pH values and thiosulphate and polythionate concentrations in 50 ml. cultures of the two organisms grown in 250 ml. conical flasks containing thiosulphate medium (Jones & Happold, 1961). Differences between the organisms
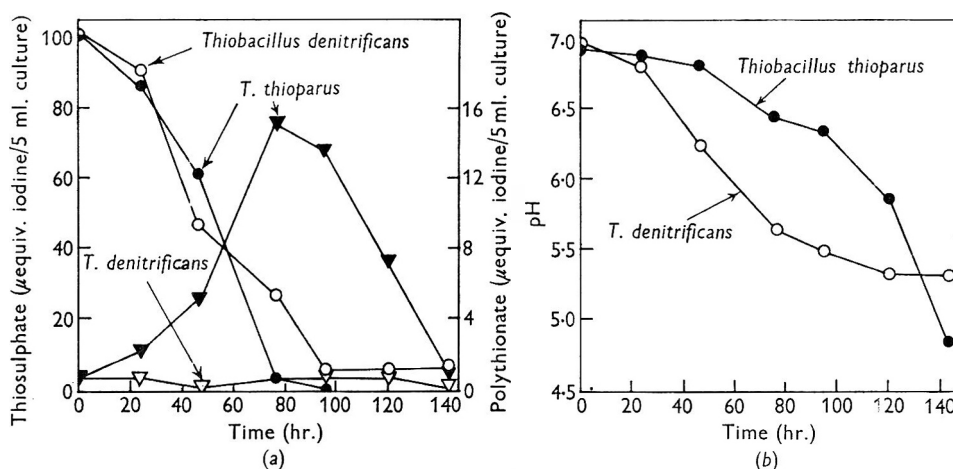
Fig. 2*a*. Utilization of thiosulphate by *Thiobacillus denitrificans* ○ and by *T. thioparus* ● ; production of polythionate by *Thiobacillus denitrificans* ▽ and by *T. thioparus* ▼.

Fig. 2*b*. Acid production during culture of *Thiobacillus denitrificans* ○ and *T. thioparus* ●.

Table 1. *Utilization of thiosulphate and production of polythionate by Thiobacillus thioparus and T. denitrificans*

Time (days)	Medium a				Medium b			
	T. thioparus		T. denitrificans		T. thioparus		T. denitrificans	
	S_2O_3	P	S_2O_3	P	S_2O_3	P	S_2O_3	P
0	187·5	2·5	190	3·0	93·5	1·5	91·5	0·5
1	186·5	0·5	192·5	2·3	88·5	3·0	91	0·5
2	180	3·5	189·5	6·0	83	—	83	2·5
3	176	6·0	182·5	1·5	76	7·0	72	2·0
4	169·5	9·5	175·5	2·5	70·5	8·0	66	0·5
5	164	13·5	171·5	1·5	59·5	15	59·5	1·0
7	150	26·0	156·5	1·5	40	24·5	44·5	0
9	133·5	33·0	143	4·0	24·5	30·5	32·5	0
11	114·5	41·0	130	1·0	5·5	39	18·5	0
14	76·0	59·0	108	17·5	0	21·5	16	0
15	—	—	—	—	0	20	—	—
21	0	30·0	49·5	36·5	0	19	13·5	0·5

Medium *a*: thiosulphate (S_2O_3) of Jones & Happold (1961).
Medium *b*: thiosulphate of Baalsrud & Baalsrud (1954), omitting nitrate.
P = polythionate.
Concentrations expressed as μ equiv. I_2/5 ml. culture.

can be seen (Fig. 2) in respect of polythionate production and rate of acid production. Table 1 shows differences in the amounts of titratable polythionate produced by the two organisms during growth of 500 ml. cultures in 1 l. flasks. The media used were: *a* that described by Jones & Happold (1961); *b* that described by Baalsrud & Baalsrud (1954) but without nitrate. During the latter experiment concentrates of culture filtrates were chromatographed on paper to detect polythionates; Table 2 shows some differences between the two organisms with respect to the polythionates which accumulated.

Table 2. *Chromatographic detection of polythionates in culture filtrate concentrates from cultures of* Thiobacillus denitrificans *and* T. thioparus

Time (days)	Medium	Organism	Polythionates		
			S_3O_6	S_4O_6	S_5O_6
14	*a*, S_2O_3 (Jones & Happold, 1961)	*T. thioparus*	+	−	+
		T. denitrificans	+	−	+
	b, S_2O_3: no NO_3 (Baalsrud & Baalsrud, 1954)	*T. thioparus*	+	−	+
		T. denitrificans	−	−	−
21	*a*	*T. thioparus*	+	+	?
		T. denitrificans	+	+	+
	b	*T. thioparus*	?	+	+
		T. denitrificans	−	?	−

DISCUSSION

The strains of *Thiobacillus denitrificans* and *T. thioparus* used here were capable of anaerobic growth in the presence of nitrate at the expense of thiosulphate or thiocyanate. In contrast to the report of de Kruyff *et al.* (1957), our *T. thiocyan-oxidans* did not grow anaerobically in thiosulphate + nitrate or thiocyanate + nitrate media. In contrast to the statements of Vishniac & Santer (1957) our cultures of *T. denitrificans* and *T. thioparus* were easily distinguished when grown aerobically (as indicated above). Of the differences described, we attach most weight to the loss of capacity for anaerobic growth by *T. denitrificans* in thiosulphate + nitrate or thiocyanate + nitrate media after aerobic subculture, and to the production of slime by the non-motile strain of *T. thioparus*. The differences observed with respect to rate of acid production and polythionate production may have been of degree only for, as Jones & Happold (1961) showed, the type of polythionate which accumulated in cultures of *T. thioparus* was altered by changing the sodium:potassium ion ratio of the growth medium. Furthermore, we found manometrically (Woolley, 1961) that the oxidation of potassium tetrathionate by washed whole cell suspensions of *T. denitrificans* proceeded at different rates according to the sodium: potassium ion ratio of the buffers used to suspend the organisms.

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Vulnerability of Nucleic Acids in Mutant Staphylococci with Impaired Respiration

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SUMMARY

Mutant staphylococci with impaired respiration, induced by ultraviolet radiation, were used for testing various inhibitors with already known mechanisms of action. Inhibitors of protein synthesis (the tetracyclins) affected the growth of parent and mutant cultures to the same degree. To the inhibitors of cell-wall synthesis (the penicillins) mutant staphylococci were more resistant than the parents; this was related to the altered amino acid composition of cell-wall material in the mutants. On the other hand, the mutants were much more vulnerable than parents to the action of inhibitors which affected the nucleic acids—namely, mitomycin C ($\times 50$ times), actinomycin C ($\times 50$), trypaflavine ($\times 300$), nitrogen mustards ($\times 10$–20). A specific thymine synthetase inhibitor (5-fluorodeoxyuridine) strongly inhibited the growth of parent staphylococci, but did not at all inhibit the growth of mutants. This points to some deficiency in the enzymic mechanism of synthesis of thymine in the DNA of mutants. Experiments with 5-bromodeoxyuridine suggest that the mutant staphylococci lacked the enzymic mechanism necessary for effective incorporation of halogenated pyrimidines into the DNA precursor pool.

INTRODUCTION

Mutant yeast cells with impaired respiration, enhanced glycolysis, a distorted cytochrome system, and which formed 'small colonies' (Ephrussi, Hottinguer & Chimenes, 1949), have attracted the attention of numerous investigators (e.g. Lindegren, 1959). The search for mutants with impaired respiration in other micro-organisms has led to detection of characteristic mutant staphylococci, with 'small colonies', respiration deficiency, and altered cytochromes (Gause, Kochetkova & Vladimirova, 1957; Gause, 1960). Mutant staphylococci with impaired respiration are selectively inhibited by substances that primarily affect the synthesis of cell nucleic acids. Some new observations along these lines are described here.

METHODS

Organisms. *Staphylococcus aureus* strain 209 (National Collection of Industrial Bacteria, NCIB, 9308) was used. Mutants with impaired respiration uv-2 (NCIB 9309) and uv-3 (NCIB 9310) were induced in this strain by ultraviolet (u.v.) irradiation, as described previously (Gause *et al.* 1957).

Medium. The organisms were grown in nutrient broth, containing (per litre of medium) tryptic digest of meat, 30 ml.; peptone, 5 g.; sodium chloride, 5 g.; glucose, 10 g. Tryptic digest of meat was a commercially available Hottinguer boullion; for its preparation meat was digested by Pancreatinum Siccum 48 hr. at 37°, filtered, and adjusted to a concentration of 700 mg % NH_2-N.

Estimation of bacteriostatic concentrations. Bacteriostatic action of various inhibitors was estimated in test tubes containing 2 ml. of nutritive broth, at 37°, by serial dilution method. For inoculation, cultures of bacteria were grown overnight in nutritive broth at 37°, adjusted turbidimetrically to a population density of 5×10^8/ml., and added to each tube in the amount of 0·05 ml. After 18 hr. of incubation at 37° the maximal dilutions of preparations were recorded in which the growth of bacteria was absent and the liquid remained transparent. Each experiment was repeated ten times, and the average data are presented in the Tables.

Ultraviolet irradiation. Organisms were treated with u.v. radiation at 2540 Å, with a radiation intensity of 11·53 erg/sec. transmitted to each mm.[2].

RESULTS

Effects of inhibitors of protein synthesis

Gale & Folkes (1953) observed that chlortetracycline and oxytetracycline stopped the growth of staphylococci in nutrient broth by selective inhibition of protein synthesis; nucleic acid synthesis, however, continued in the presence of bacteriostatic concentrations of these antibiotics. Our results showed that tetracycline and chlortetracycline inhibited the growth of the parent and mutant staphylococci to the same degree (Table 1).

Table 1. *Bacteriostatic action of various inhibitors upon the growth of parent and mutant cultures of* Staphylococcus aureus

	Parents	Mutants (uv-2, uv-3)
(1) *Inhibitors of protein synthesis*		
Tetracycline	0·09–0·10 μg./ml.*	0·09–0·15 μg./ml.
Chlortetracycline	0·15–0·18 μg./ml.	0·20 μg./ml.
(2) *Inhibitors of cell-wall synthesis*		
Penicillin G	0·008 units/ml.	0·06–0·07 units/ml.
Phenoxymethylpenicillin	0·007 units/ml.	0·06 units/ml.
(3) *Inhibitors of nucleic acid synthesis*		
Mitomycin C	0·0750 μg./ml.	0·0015 μg./ml.
Actinomycin C	0·250 μg./ml.	0·005 μg./ml.
Trypaflavine	3 μg./ml.	0·01 μg./ml.
N-mustards (Degranol)	2 mg./ml.	0·2 mg./ml.
(4) *Other inhibitors*		
Chloramphenicol	6–8 μg./ml.	2–3 μg./ml.

* Minimum inhibitory concentration estimated as described in Methods.

Effects of inhibitors of cell-wall synthesis

Bacteriostatic concentrations of penicillin which selectively inhibit biosynthesis of cell wall do not disturb the synthesis of proteins and nucleic acids in staphylococci (Strominger, Park & Thompson, 1959; Rogers & Perkins, 1959). It is clear from Table 1 that the mutant staphylococci, as compared with the parents, were about eight times more resistant to the inhibitory action of the penicillins in nutrient broth. It may therefore be suggested that mutants differ from the parent organism in the biosynthesis of the cell-wall material, which is selectively affected by penicillin. Cell-wall material in the mutants appeared to be more resistant to the disruptive action of penicillin. This is in accord with chemical studies which have

shown that the amino acid composition of the cell-wall material in parent staphylococci is different from that of their mutants (Gause, Kochetkova & Vladimirova, 1961).

Effects of inhibitors of nucleic acid formation

Mitomycin C. Shiba, Terawaki, Taguchi & Kawamata (1959) observed that mitomycin C in dilute solution selectively inhibited the formation of deoxyribonucleic acid (DNA) in bacteria, while synthesis of protein and ribonucleic acid (RNA) remained unaffected. Our results showed that mutant staphylococci were fifty times more vulnerable than the parent culture to the action of mitomycin C.

Actinomycin C. Reich, Franklin, Shatkin & Tatum (1961) reported the selective action of actinomycin C on RNA synthesis in bacteria. We observed that the mutants were fifty times more vulnerable than parents to the inhibitory action of actinomycin C.

Trypaflavine. Trypaflavine (3:6-diamino-10-methylacridine chloride) is a mitotic poison which selectively inhibits DNA synthesis (Morthland, De Bruyn & Smith, 1954). We observed that with this compound the mutants were 300 times more vulnerable than the parents.

Table 2. *Concentrations of 5-fluorouracil required to cause inhibition of growth of parent staphylococci and of their mutants, in the presence of various concentrations of uracil**

Micro-organism	Uracil (μg./ml.)	5-Fluorouracil (μg./ml.)
Parent staphylococci	0	125
	10	200
	100	500
	1000	750
Mutant strain uv-3	0	2
	10	6
	100	25
	1000	75

* See footnote to Table 1.

Nitrogen mustards. The nitrogen mustards as a group are potent inhibitors of DNA synthesis. By carefully grading the doses, a value can be chosen where synthesis of DNA is blocked completely, while synthesis of protein and RNA continue (Shepherd, 1958). Degranol [1,6-bis-(β-chloroethylamino)-1,6-deoxy-D-mannitol, manufactured by Chinoin Ltd, Budapest] was used as a representative of this group of compounds; it is a combination of nitrogen mustard with mannitol and is soluble in water. The mutants were ten times more vulnerable than the parents to the action of degranol. The selective inhibition of growth of mutant staphylococci has been recorded for other nitrogen mustards (Gause, 1960). Triethylene melamine, which specifically affects pyrimidines in the synthesis of bacterial DNA (Szybalski, 1960a), also selectively inhibits the growth of mutant staphylococci (Gause *et al.* 1961).

Halogenated pyrimidines. This group of compounds is of particular interest for the study of the vulnerability of nucleic acids in mutant staphylococci with impaired respiration. It is known that 5-fluorouracil acts as a specific inhibitor of RNA

synthesis; it can be incorporated into bacterial RNA, where it may replace, in part, the uracil normally present. The synthesis of DNA, however, is not affected (Horowitz & Chargaff, 1959). We observed that 5-fluorouracil at 125 μg./ml. inhibited the growth of the parent staphylococcus in broth. Addition of thymine (100 μg./ml.) to the nutrient medium produced no effect, but addition of uracil annulled the inhibitory action of 5-fluorouracil. The annulment of the inhibitory action of 5-fluorouracil in the presence of uracil was competitive; see data presented in Table 2.

For the mutant staphylococci (uv-2, uv-3) the inhibitory concentration of 5-fluorouracil was 2 μg./ml.; this inhibitory action was not affected by thymine (100 μg./ml.), but with uracil it was competitively annulled (Table 2). It may be concluded that in the parent and mutant staphylococci 5-fluorouracil specifically inhibited RNA synthesis, but did not affect DNA synthesis. It appears therefore that the synthesis of RNA in mutants was about sixty times more vulnerable to the action of 5-fluorouracil than it was in the parents.

It is of interest that 5-fluorodeoxyuridine (FUDR), in experiments with various bacteria, specifically inhibited synthesis of DNA (Cohen et al. 1958). We observed that FUDR at 25 μg./ml. inhibited the growth of the staphylococcus in broth. Addition of uracil (100 μg./ml.) produced no effect, but addition of thymine competitively annulled the inhibitory action of FUDR on the parent staphylococcus (Table 3). By inhibiting the synthesis of thymine, FUDR apparently interfered with the formation of DNA in the parent staphylococci. It is remarkable, therefore, that FUDR did not inhibit the growth of the mutant staphylococci with impaired respiration (uv-2, uv-3, and all other available mutants of this type), up to the maximal concentration tested (1000 μg./ml.). If FUDR is a specific thymine synthetase inhibitor, the absence of inhibitory action on the mutant staphylococci points to some deficiency in the enzymic mechanism of synthesis of thymine in these mutants.

Table 3. *Concentrations of FUDR required to cause inhibition of growth of parent staphylococci in the presence of various concentrations of thymine**

Thymine (μg./ml.)	FUDR (μg./ml.)
0	25
10	60
100	100
1000	200

* See footnote to Table 1.

We also observed that certain brominated pyrimidines (e.g. 5-bromouracil, 5-bromodeoxyuridine, BUDR), did not inhibit the growth of either parent or mutant staphylococci in nutrient broth, up to the maximal concentration tested (500 μg. ml.). Lorkiewicz & Szybalski (1960) observed that in the presence of FUDR thymine synthetase was inhibited and a state of thymine deficiency was produced in the cells, and the incorporation of BUDR, the thymidine analogue, into the bacterial DNA can be observed. The substitution of thymidine in the DNA by its brominated analogue (BUDR) renders the cells highly sensitive to the killing action of u.v. radiation (Szybalski, 1960b).

We were able to reproduce this phenomenon with the parent staphylococcus used here. The cocci were grown for 18 hr. in nutrient broth containing FUDR (10 μg./ml.), BUDR (10 μg./ml.) or a mixture of FUDR + BUDR. Separately, as well as in admixture, these substances did not inhibit the growth of the staphylococci in the concentrations tested. Then the suspensions of staphylococci were adjusted turbidimetrically to a population density of 10^8/ml. of organisms, 0·05 ml. of dilution poured on to the surface of nutrient agar plates, and the plates u.v. irradiated for different times. The results obtained are shown in Table 4.

It is clear that with the parent staphylococcus grown in the presence of FUDR + BUDR the sensitivity to u.v. radiation was markedly increased. According to Szybalski (1960b) this sensitization directly follows the incorporation of BUDR into the bacterial DNA as a result of substitution of the halogenated analogue in place of thymidine. It is remarkable, therefore, that this phenomenon was not observed in the mutant staphylococci.

Table 4. *Ultraviolet light sensitivity of parent and mutant staphylococci grown in the presence of FUDR, BUDR, or both*

Irradiation time	Mean number of colonies per plate*			
	Control	FUDR	BUDR	FUDR + BUDR
	Parent culture			
15 sec	> 5000	4100	> 5000	540
30	4400	1500	3000	14
45	1700	340	1200	2
60	220	20	30	0
	Mutants (uv-3)			
1 min	> 5000	> 5000	> 5000	> 5000
2	660	650	600	550
3	80	55	80	75
4	35	35	25	37
5	17	12	20	16

* Counted after 72 hr. at 37°.

The data presented in Table 4 show that the mutants were more resistant than the parent to the killing action of u.v. radiation. The interesting point, however, is that in mutants grown in the presence of FUDR + BUDR no sensitization was observed. This suggests that in the mutants the incorporation of halogenated thymidine analogues into DNA did not take place. It can be supposed that the mutant staphylococci lacked the enzymic mechanism necessary for effective incorporation of halogenated pyrimidines into the DNA precursor pool.

Effects of other inhibitors

Chloramphenicol. Chloramphenicol is an inhibitor of protein synthesis; more recent study has shown that this compound interferes with the synthesis of both protein and nucleic acids. It is possible that chloramphenicol blocks some stage in the mechanism of nucleoprotein synthesis, and that the sensitive stage lies somewhere between the activation of amino acids, their fixation by small 'nucleic acid'

components, and the polymerization of these components into macromolecular structures (Gale, 1958). We observed that the mutants were about two to three times more sensitive than the parent to this compound (Table 1). These figures are similar to those for the tetracyclines, which inhibited the growth of the parent staphylococcus and of the mutants to the same degree. It might be supposed that the mechanism of the inhibitory action of chloramphenicol is similar to but not identical with that of the tetracyclines.

Table 5. *Sensitivity to heat of normal staphylococci and mutants with impaired respiration*

	Mean number of colonies per plate*	
Heating time (min.)	Normal staphylococci	Mutant uv-3
5	> 5000	> 5000
10	> 5000	3500
15	> 5000	1600
20	> 5000	35
30	3600	30
60	400	—
90	150	—
120	69	—

* Counted after 72 hr. at 37°.

Effect of heating. Suspensions of staphylococci (10^8/ml. of organisms) were heated in a water bath at 55° for different periods of time, and then poured on to nutrient agar (0·05 ml./ plate). Table 5 shows that the mutant staphylococci were much more sensitive to heat than was the parent. It is difficult to ascribe a reason for this phenomenon, but it is interesting to note that defects in nucleic acids make bacteria more sensitive to the lethal effect of elevated temperatures (Lorkiewicz & Szybalski, 1960).

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Comparative Nutritional Studies of *Pythium* spp.

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SUMMARY

Nutritional experiments were carried out dealing with: (1) the utilization of sulphur compounds; (2) the action of different mineral salts and their interaction. The results showed that the species of Pythium used grew well at 28° buffered with Sorensen's salt to give an initial pH of 6·5 and incubated for 13 days for *Pythium afertile* and 10 days for the remaining species. Acid conditions below pH 5·0 and alkaline conditions above pH 8·0 were not tolerated. The best carbon and nitrogen sources are given. The best sulphur sources were Na_2S, $Na_2S_2O_5$, $Na_2SO_4 . 10 H_2O$, $Na_2SO_3 . 7H_2O$, $Na_2S_2O_5 . 5H_2O$, $K_2S_2O_8$, molecular sulphur, DL-methionine, thioacetamide, L-cysteine, sodium thioglycolate, and DL-cystine Factorial experiments were carried out in which the fungi were incubated at 28° for 13–14 days. Statistical analysis of the results showed that under the given conditions optimal growth measured as mg. dry wt. was obtained in liquid media containing glucose, 10·0 g./l.; KCl, 0·5 g./l.; with varying amounts of K_2HPO_4, KNO_3 and $MgSO_4 . 7H_2O$ for the different species specified. There was a balance between K_2HPO_4 and $MgSO_4 . 7H_2O$ for *P. debaryanum* and there were significant interactions between the salts above taken two at a time for *P. debaryanum* and *P. ultimum* but the interactions for the remaining fungi were more variable. There was interaction between all three salts together for two of the species. *P. debaryanum* is more exacting in its nutritional requirements than the other species.

Some species of Pythium are purely saprophytic, others are normally parasitic but may be saprophytic. Thus Pythium would seem to be a good genus for comparative nutritional studies. Previous work at Newcastle upon Tyne (see Fothergill *et al.*) indicated that for some parasitic fungi the balance of the major inorganic salts in a culture medium was more important for the good growth of the mycelium than the concentration of individual salts, but in the case of some saprophytic species, the balance of salts was much less important. Results, however, over a range of fungi varied. The present experiments are a continuation of work designed to investigate these points. Excluding work done on Pythium as a plant pathogen, a few physiological investigations have been reported mainly dealing with the presence and function of pectinase in these fungi (Chona, 1932; Menon, 1934; Fernando, 1937; Ashour, 1954; Damle, 1952; Gupta, 1956; Wood & Gupta, 1958). The carbon, nitrogen, sulphur and vitamin requirements were investigated by Saksena (1942), Saksena & Mehrota (1949), and Saksena, Jain & Jaffri (1952), but Cantino (1955)

strongly criticized these works because of the lack of pH control during the experiments. No comprehensive nutritional investigation has yet been carried out on Pythium.

METHODS

The following organisms were used. *Pythium debaryanum* Hesse originally isolated from *Lepidium* sp., *P. ultimum* Trow from *Pisum sativum* L., *P. afertile* Kanouse & Humphrey and *P. torulosum* Coker & Patterson. All organisms were obtained from the Centraal Bureau voor Schimmelculture, Baarn. The first two species are parasites causing damping-off disease of seedlings, while the last two species are normally saprophytic soil fungi but they have been reported as growing on Gramineae (Middleton, 1943). Throughout this paper the following abbreviations are used: *P. debaryanum* is designated D; *P. ultimum*, U; *P. afertile*, A and *P. toru-losum*, T.

Initially single tip isolates were used as inocula for each species on Difco potato-extract–glucose agar medium. Stock cultures were kept on slopes of this medium in 6 in. × ¾ in. test tubes and the cultures were stored at room temperature because Church & Scandiffio (1928) showed that cultures of Pythium live longer at room than at lower temperatures. Subcultures were made as required. No alteration of morphological and cultural characteristics of these fungi occurred during the period of the experiments. In all experiments cultures were grown in 30 ml. aliquots in 150 ml. Erlenmeyer flasks. After the growth period the mycelia were filtered on to tared Whatman no. 5 filter-papers and washed with distilled water. The mycelium with the filter-papers was then dried for 24 hr. at 85°, cooled in a desiccator and weighed; results are expressed as mg. dry wt. mycelium/flask average of 4 or 5 replicates. It was not found practicable to separate mycelium from filter-paper after filtration and as the filter-papers used lose 5% of their original weight on drying under the above conditions, appropriate allowance was made for this. For preliminary experiments the following basal defined liquid medium A was used: glucose, 10 g.; NH_4NO_3, 1·0 g.; $MgSO_4.7H_2O$, 0·5 g.; KCl, 0·5 g.; water to 1 l. This medium was buffered with Sorensen's phosphate buffer ($KH_2PO_4 + NaHPO_4$) to give an initial pH of 6·5. Experiments showed that all the *Pythium* sp. grew well at this pH value. The parasitic species tolerated acid conditions, even at pH 5·0, better than the saprophytic species which gave very low mycelial yields at this pH level. None of the species would grow at pH 8·0.

Experiments were carried out to determine the best method of inoculation and it was found that agar disks gave the most consistent mycelial dry weights provided the disks were between 3 and 8 mm. in diameter. Disks of 4 mm. diameter cut from the edge of 48 hr. old cultures were used in all later experiments. Experiments also showed that 4 mm. disks did not significantly affect the nutritional results, that is, the nutritional effect of the Difco agar could be discounted and this was true also for the vitamins which may have been present in the agar. This latter point was tested for the presence of thiamin in the agar. *Pythium vexans* needs an exogenous supply of thiamin for growth (see Leonian & Lilly, 1945). This fungus was tested in (a) the basal medium alone, and in (b) the basal medium plus thiamin using agar inocula. The average weight of 5 replicates showed that in (a) growth was slight, but in (b) 71 mg. mycelium/flask were obtained. Further, the successful use of the agar

disk method in the determination of vitamin requirements of other *Pythium* spp. was shown by Leonian & Lilly (1939) and Saksena (1942). Addition or omission of the usual trace elements, Fe, Mn and Zn made no difference to mycelial dry weights and these may be present in sufficient quantities in the Analar reagents used throughout. Tests also showed that none of the fungi needed an exogenous supply of biotin, nicotinic acid, folic acid, sodium pantothenate, pyridoxine, riboflavin, *para*-aminobenzoic acid or ascorbic acid. Neither thiamin nor its moieties affected growth, which supports the findings of Verma (1944). Robbins & Kavanagh (1938), however, showed that *P. butleri* needed an external supply of this vitamin. The optimum temperature for mycelial growth was 28° but organism T had a rather slower growth rate than the other species. Optimum growth was reached after 13 days incubation for organism A and after 10 days for organisms D, U and T on basal medium A, and it was noted that in these preliminary experiments the mycelial yields of the parasites D and U were about twice those of the saprophytes A and T. Experiments on the effect on mycelial growth of autoclaving the medium were carried out in which the composition of basal medium A was varied by substituting $(NH_4)_2SO_4$ and asparagine respectively for NH_4NO_3 using U as the test organism. Autoclaving these media at 10 lb. pressure for 5 min. had no effect on the dry weight yields of this fungus but for periods above 15 min. dry weight was seriously reduced. Hence all media were subsequently autoclaved at 10 lb. pressure for 5 min.

RESULTS

Sulphur requirements

Preliminary experiments showed that all four isolates grew only slightly without sulphur in medium A. The sulphur requirement was then investigated by varying the sulphur source in this medium to give 0·5 g./l. sulphur and in which $MgSO_4$. $7H_2O$ was omitted and KCl was replaced by 0·5 g./l. $MgCl_2$. The final pH after autoclaving was between 6·5 and 6·6. The media were inoculated and then incubated at 28°. Dry weights of 4 replicate cultures were determined in all cases after 6, 9 and 15 days incubation. The final pH of the media were also determined. The results are shown in Table 1 which records the highest percentage yields expressed in terms of the yield from $Na_2SO_4.10H_2O$ as 100 % for the inorganic sulphur sources and from DL-methionine as 100 % for the organic sulphur sources. With $Na_2SO_4.10H_2O$ the actual highest yields were 88 mg. for organism D, 92 mg. for U, 78 mg. for A and 61 mg. for T, all after 9 days incubation, while with DL-methionine the corresponding highest yields were 78 (after 6 days), 87, 73 and 49 mg. The last three were all after 9 days incubation. In all cases, except two, with inorganic sources of sulphur the highest yields were obtained after 9 days incubation but with organic sulphur sources the highest dry weights varied with the organism and the incubation period.

For the inorganic sulphur sources all the compounds used, except sodium dithionate and ammonium sulphamate, gave good or satisfactory growth with all the fungi, but organism A only gave a moderate yield with molecular sulphur. These *Pythium* species are, therefore, euthiotropic (Volkonsky, 1933). Increasing the oxidation level of the sulphur atom had no significant effect on mycelial growth. Thus molecular sulphur (S), a thiosulphate ($S_2O_3^{2-}$). a metabisulphite ($S_2O_5^{2-}$),

a sulphite (SO_3^{2-}) and a sulphate (SO_4^{2-}) were all good sulphur sources for most of the species. Results using molecular sulphur, however, should be treated with caution. The results for the organic sulphur sources were more variable but, in general, the highest yields were obtained with DL-methionine, thioacetamide, L-cysteine (except for organism U), DL-cystine, sodium thioglycolate (except for organisms A and T). The remaining compounds used gave only moderate or poor growth responses. Sulphanilic acid, DL-ethionine, thiocyanide, thioacetanilide and thiohydantoin were only utilized to a small extent.

Table 1. *The utilization of inorganic and organic sulphur compounds*
by Pythium *spp.*

Highest yields expressed as percentage of yields from A, $Na_2SO_4.H_2O$, and B, DL-methionine taken as 100. Final pH 6·2–6·6. (Mg. dry wt. mycelium/flask average of 4 replicates. 6 and 15 days incubation in brackets.)

Sulphur source	Species			
	D	U	A	T
A. $Na_2SO_4.10H_2O$ as 100 %, 9 days incubation				
Sodium sulphate	100	100	100	100
Sodium sulphide	102	90	79	67
Sodium metabisulphite	102	76	101	—
Sodium sulphite	98	84	103	92
Sodium thiosulphate	97	86	87	94
Sulphur	93	86	40 (6)	72
Potassium persulphate*	82	89	91	100
Sodium dithionate	31	35	8	18
Ammonium sulphamate	28 (6)	21	8	11
B. DL-Methionine as 100 %, 9 days incubation				
DL-Methionine	100 (6)	100 (9)	100 (9)	100 (15)
Thioacetamide	100 (15)	100	57	63 (15)
L-Cysteine	96	47	106	118 (15)
Sodium thioglycolate	90 (15)	67	47	36
DL-Cystine	85 (6)	98	97	84 (15)
Thiourea	56	40 (6)	25	34 (6)
Thiamin	58 (15)	54 (15)	28	38
Djenkolic acid	56 (15)	48 (15)	31 (15)	19
Taurine	29 (15)	21 (6)	47 (15)	104 (15)
Sulphanilic acid	22 (6)	24 (6)	11 (6)	10
DL-Ethionine	17	8	10	8
Thiocyanide	14	11	10	9
Thioacetanilide	14 (15)	33	26 (15)	13 (15)
Thiohydantoin	19	28	12	18 (15)
None	4	5	2	4

* Persulphate breaks down easily to sulphate.
— No growth.

The amino acids DL-methionine, DL-cystine, L-cysteine were very good sources for the species and in most cases these compounds were utilized more rapidly than any of the remaining compounds used. It may be that these compounds are absorbed and assimilated as intact molecules (see Steinberg, 1941). Djenkolic acid, also an amino acid, gave fair yields, but only after 15 days' incubation and it is worth noting that this compound is made up of two cysteine residues linked to a CH_2 group through

the sulphur atoms. Possibly djenkolic acid may have to be converted to cysteine before it is utilized. The poor utilization of thioacetanilide and sulphanilic acid supports Steinberg's conclusion (1941) that compounds containing sulphur attached to a benzene ring are unsuitable for use in assimilation by *Aspergillus niger*. Likewise thiohydantoin gave poor yields and it consists of a five-membered ring. DL-Ethionine was an extremely poor sulphur source for Pythium. Margolis & Block (1957) also showed that five species of yeast could not utilize this compound, while Schlenk & Tillotsen (1959) and Harris & Cohn (1959) showed that ethionine is an antimetabolite of methionine.

Factorial experiments

The object of these experiments was to determine the effect of varying the concentration of the mineral salts in the basal medium. The factorial design and statistical analysis of the results should indicate whether or not a balance between the salts is necessary for high mycelial yields under the given experimental conditions. The direct effect of the individual salts in the medium and the interaction between them is also determined. Previous experiments showed that basal medium A was a satisfactory medium for the four *Pythium* spp., but as it contained NH_4NO_3 as the nitrogen source it required buffering with Sorensen's salt. Haskins & Weston (1950), using *Karlingia rosea*, showed that when KNO_3 was used as a nitrogen source the pH of the culture medium remained approximately constant at pH 6·5 to 7·0. In the following experiments KNO_3 was substituted for NH_4NO_3 and after preliminary experiments basal medium B was evolved containing glucose 10 g.; KNO_3, 2·0 g.; K_2HPO_4, 0·5 g.; $MgSO_4.7H_2O$, 0·5 g.; KCl, 0·5 g.; distilled water to 1 l. The pH of this solution was adjusted to 6·6 with 0·1 N-HCl. With this medium the pH value in the case of organisms D, U and T never fell below 5·35, while with A it never fell below 6·25. The cultures were incubated at 28° for 14 days with organisms D and T and for 13 days with organisms A and U. The concentrations of the salts were fixed on the basis of halving and doubling those in basal medium B. Thus KNO_3, $MgSO_4.7H_2O$ and K_2HPO_4 were each used at three concentrations and all possible combinations of them were set up giving a total of 27 variations. Each combination was done in quadruplicate; thus with any one species of fungus 108 culture flasks were incubated. Each 150 ml. Erlenmeyer flask contained 30 ml. medium. The results are the average mg. dry wt./mycelium of 4 replicates and the final pH values were determined in each case. The grouped results are shown in Table 2 and the grouped analyses of variance are given in Table 3. For abbreviations of the salt concentrations used in the text below see Table 2.

From the single salt analysis it is evident that there was no consistent result for all the organisms. This analysis showed that the differences between mycelial dry weights with KNO_3 were significant for all concentrations of the salt for organisms A and D. The highest mean weight for organism A occurred at the N_3 concentration and for organism D at the N_1 level of KNO_3. For organism T significance between the N_1 and N_3 levels occurred with the highest mean dry weight at the N_1 concentration of salt. For organism U a significant difference occurred between the N_1 and N_2 levels with the highest dry weight at the N_2 concentration. At the N_3 level of KNO_3 with this organism there was a large decrease in mycelial dry wt. With K_2HPO_4

a significant difference was found between the P_1 and P_2 levels for organism A with the highest dry weight at the P_2 level and between the P_1 and P_3 levels for organism T with the highest dry weight at the P_2 level. For organisms U and D no significant differences in dry weights were found between any of the concentrations of K_2HPO_4 used, but for both organisms the highest dry weights occurred at the P_3 level of this salt. With $MgSO_4.7H_2O$ the only significant difference in mycelial dry weight was

Table 2. *Growth responses of four species of Pythium to varying conditions of* K_2HPO_4, $MgSO_4.7H_2O$ *and* KNO_3

Concentration of K_2HPO_4: P_1, P_2, P_3 = 0·25, 0·5, 1·0 g./l.; of $MgSO_4.7H_2O$: Mg_1, Mg_2, Mg_3 = 0·25, 0·5, 1·0 g./l.; of KNO_3: N_1, N_2, N_3 = 1, 2, 4 g./l. (Difference required between means for significance at odds of 99:1 for KNO_3, K_2HPO_4 and $MgSO_4.7H_2O$ = 5·3 mg. for A and T, 6·2 mg. for U and 6·8 mg. for D)

A. *P. debaryanum* (=D) *and* *P. ultimum* (=U)

	P_1						P_2						P_3					
	Mg_1		Mg_2		Mg_3		Mg_1		Mg_2		Mg_3		Mg_1		Mg_2		Mg_3	
	D	U	D	U	D	U	D	U	D	U	D	U	D	U	D	U	D	U

(Mean dry wt. mycelium mg./flask)

N_1	167	168	162	175	160	163	168	172	171	175	163	170	153	163	181	169	172	175
N_2	131	181	146	185	133	182	133	181	142	182	161	158	136	180	136	185	161	172
N_3	149	154	133	151	127	149	139	159	129	146	116	148	127	170	123	151	139	176

B. *P. afertile* (=A) *and* *P. torulosum* (=T)

	P_1						P_2						P_3					
	Mg_1		Mg_2		Mg_3		Mg_1		Mg_2		Mg_3		Mg_1		Mg_2		Mg_3	
	A	T	A	T	A	T	A	T	A	T	A	T	A	T	A	T	A	T

(Mean dry wt. mycelium mg./flask)

N_1	114	122	122	120	104	100	116	104	114	112	105	105	110	101	109	97	105	93
N_2	119	105	127	107	120	93	144	104	147	107	143	105	144	92	144	112	150	106
N_3	134	92	141	97	136	91	156	101	156	112	162	105	152	105	156	87	163	95

Table 3. *Analysis of variance (grouped) for* Pythium *spp. A, T, U, D*

Required 'F' and 't' values taken from Snedecor's tables (1934). Sums of squares and mean squares are omitted from table.

		F required odds					
		Found organism					
Variance	D.F.	A	T	U	D	99:1	19:1
Total	107	—	—	—	—	—	—
KNO_3	2	211·93	7·81	489·64	105·29	4·88	3·11
K_2HPO_4	2	32·54	7·46	3·03	0·42	4·88	3·11
$MgSO_4.7H_2O$	2	1·69	5·53	1·84	1·00	4·88	3·11
$KNO_3 \times K_2HPO_4$	4	12·69	7·18	4·45	2·58	3·56	2·49
$KNO_3 \times MgSO_4$	4	3·10	2·45	5·51	7·48	3·56	2·49
$K_2HPO_4 \times MgSO_4$	4	2·16	3·11	3·20	5·25	3·56	2·49
$KNO_3 \times MgSO_4 \times K_2HPO_4$	8	0·1631	2·46	1·32	2·76	2·74	2·06
Residual error	81	—	—	—	—	—	—

found for organism T between the Mg_2 and Mg_3 levels with the highest dry weight at the Mg_2 concentration of the salt. For organisms A, U and D there were no significant differences between any of the levels of the salt. But for A the highest mean dry weight of mycelium was obtained at the Mg_2 level, for U at the Mg_1 level, and for D at the Mg_3 level of concentration of $MgSO_4.7H_2O$.

Balance between K_2HPO_4 and $MgSO_4.7H_2O$

The grouped analyses of variance showed that there was a significant interaction between K_2HPO_4 and $MgSO_4.7H_2O$ only for the parasitic organism D. This result also indicated that there was a physiological balance between these salts under the conditions of the experiments. Talley & Blank (1941) and Fothergill & Ashcroft (1955) have also shown that a physiological balance exists between these two salts and is important for the good growth of *Phymatotrichum omnivorum* and *Venturia inaequalis*. To confirm this result another experiment was designed to test the change in the amount of growth which might result when the concentrations of the salts were varied while still maintaining the balance between them. Thus the basic concentration of K_2HPO_4 and $MgSO_4.7H_2O$ was 0·5 g./l. and 0·25 g./l. respectively; these concentrations were decreased to half in one set of solutions and increased twice and then 4 times in other solutions. This gave four treatments, each one having the same balance between the salts but the ratio of their concentrations was 0·5:1:2:4. Each of these treatments was tested singly with 0·5, 1·0, 2·0 and 4·0 g./l. of KNO_3. The remaining ingredients of basal medium B remained constant. The initial pH of the media was adjusted to 6·5 prior to autoclaving and the cultures were incubated in 5 replicates at 28° for 14 days. The results expressed as mg. mean dry wt./mycelium are shown in Table 4, where treatments are numbered 1 to 16 in brackets.

Table 4. *Mean dry weights and growth responses of* Pythium debaryanum *after 14 days incubation at 28° on media with the same balance but with different concentrations of certain components*

Concentrations of salts as in Table 2 with following additions: K_2HPO_4: $P_4 = 2\cdot0$ g./l.; $MgSO_4.7H_2O$: $Mg_0 = 0\cdot125$ g./l.; KNO_3: $N_0 = 0\cdot5$ g./l. (The sixteen different solutions numbered in parentheses are referred to in the text as treatments 1 to 16).

Mean dry wt. mycelium (mg./flask)

	P_1 Mg_0	P_2 Mg_1	P_3 Mg_2	P_4 Mg_3	Mean N
N_0	122 (1)	110 (2)	108 (3)	106 (4)	111·5
N_1	140 (5)	147 (6)	162 (7)	143 (8)	148
N_2	158 (9)	182 (10)	164 (11)	172 (12)	169
N_3	158 (13)	163 (14)	164 (15)	157 (16)	160·5

General mean = 147 mg. dry wt. mycelium/flask.

The results showed that the mycelial yields for KNO_3 at N_0 and N_1 levels were significantly different from each other and from the P_2 and N_3 levels but these latter two levels were not different. To show the interaction of K_2HPO_4 and $MgSO_4.7H_2O$ 't' tests were performed. There were no significant differences between: (*a*) treatments 2, 3 and 4 at the N_0 level of KNO_3; (*b*) treatments 5, 6, 7 and 8 at the N_1 level

of KNO_3; (c) treatments 10, 11 and 12 at the N_2 level; and (d) treatments 13, 14, 15 and 16 at the N_3 level. While there were significant differences between treatments 1 and 2 at the N_0 level of KNO_3 and treatments 9 and 10 at the N_2 level, this lack of significant differences at the respective levels of N confirms the conclusion that a balance exists between K_2HPO_4 and $MgSO_4.7H_2O$ and that the concentration of either of these salts can be varied over a wide range without affecting mycelial yields providing that the concentrations of the other salts are varied in proportion. The significance obtained with treatments 1 and 9 at the lowest concentration of salts may indicate that the balance necessary for good growth only comes into operation above a certain minimum concentration of salts. A similar effect with *Venturia inaequalis* was noticed by Fothergill & Ashcroft (1955).

CONCLUSION

The analyses of variance also showed the first- and second-order interactions between the salts in the medium, and indicated some of the nutritional differences between the fungi investigated. For the fungi D and U all the first-order inter-actions (i.e. the interactions of all the salts taken two at a time) were significant at either the 99:1 or 19:1 level of odds. For the fungi A and T the results were more varied. For organism A the interaction of $KNO_3 \times K_2HPO_4$ and $KNO_3 \times MgSO_4.7H_2O$ were significant but not that between $K_2HPO_4 \times MgSO_4.7H_2O$, while for organism T the interactions $KNO_3 \times K_2HPO_4$ and $K_2HPO_4 \times MgSO_4.7H_2O$ were significant but not that between $KNO_3 \times MgSO_4.7H_2O$. In the second-order interactions only those for organisms D and T were significant.

In general the results of the factorial experiments showed that there was a considerable difference between the nutritional requirements of *Pythium debaryanum* (D) and *P. ultimum* (U). With the individual salts the greatest effect was produced by varying the concentration of KNO_3. An absolute balance between all the nutrients was required by *P. debaryanum* (D) for good mycelial growth, but was not so necessary for *P. ultimum* (U). With the species, *P. afertile* (A) and *P. torulosum* (T) variation in the concentration of most of the individual salts produced large effects on mycelial growth and at the highest level of probability the interaction with KNO_3 was significant. Kitchell (1954), Fothergill & Yeoman (1957), and Fothergill & Jones (1958) also found that the concentration of the nitrogen sources and the interaction between these and the phosphorus source had a significant effect on the growth of *Mucor* spp., *Rhizopus stolonifera* and *Zygorhynchus* spp., all saprophytes. These results also support the findings of Fothergill & Jones (1958) who showed that there was a variation of balance in salt concentrations for different *Zygorhynchus* spp. Although each *Pythium* spp. has its own nutritional requirements D and U in general needed a more exact balance of mineral salts and showed less variation in their response to individual salts than did A and T here investigated. Differences in nutritional requirements between these fungi are statistically significant but they are not very large.

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Relationship of Colominic Acid (Poly *N*-Acetylneuraminic Acid) to Bacteria which Contain Neuraminic Acid

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SUMMARY

The bacteriocinic strain of *Escherichia coli* known as $K235L^+OC^+$ which elaborates colominic acid (poly *N*-acetylneuraminic acid) possesses a K antigen and has the serotype *E. coli* O1:K1:HNM in accordance with the Kauffmann–White classification scheme of the enterobacteriaceae. Strains of *E. coli* with O1:K1:HNM or O2:K1:HNM serotype elaborate a similarly constituted colominic acid. Chemical analysis has revealed that all *E. coli* with a K1 serotype contain neuraminic acid, but that strains which have other K serotypes do not contain significant amounts of this substance. The nature of the O serotype and the ability to produce haemolysins or bacteriocines are unrelated to the presence of neuraminic acid in the various strains analysed. Thus, the close association between a K1 serotype and colominic acid in *E. coli* suggests that all strains of this serotype produce colominic acid. *Salmonella dahlem, S. djkarta, E. coli* O10:K5:HNM, Group C *Neisseria meningitidis* 1908 and 1935, and *Citrobacter freundii* O5:H3O (*E. coli* 5396/38) contain neuraminic acid as shown by chemical tests. However, the neuraminic acid is firmly bound and polysaccharides containing this material are not readily elaborated. As no serological relationship exists between these strains and strains of *E. coli* having a K1 serotype, it is concluded that they are non-producers of colominic acid. Thus, bacteria of various genera which contain neuraminic acid have this substance in different chemical combinations.

INTRODUCTION

Numerous investigators have been concerned with the problem of isolation and purification of large molecular weight substances derived from bacteria. These materials, constituted of protein, polysaccharide and lipid, often are shown to be homogeneous macromolecules upon application of physical and chemical criteria. Little is known, however, concerning the specific nature of the chemical binding of protein to carbohydrate in these 'lipoprotein carbohydrate' complexes. With the discovery in recent years of the presence of the acidic amino sugars, muramic acid (3-0-α-carboxyethylglucosamine) (Strange & Powell, 1954) and neuraminic acid (Barry, 1958) in products of bacterial origin, the possibility is presented that these monosaccharides which are hybrids between simple sugars and amino acids may play an essential role in the linking of proteins to carbohydrates. Evidence indicates that muramic acid is linked to other monosaccharides and to amino acids in bacterial cell walls of a large variety of Gram-positive micro-organisms (Cummins & Harris, 1956). However, the demonstration that neuraminic acid also plays an

important role in the composition of bacterial cell walls or of other components of bacteria has yet to be established.

Up to the present time few micro-organisms have been shown to contain derivatives of neuraminic acid. Colominic acid, a unique polymer constituted primarily, if not solely, of units of N-acetylneuraminic acid, is the first polysaccharide obtained from bacterial origin shown to contain a neuraminic acid derivative (Barry, 1957, 1958). This polysaccharide was isolated from an *Escherichia coli* culture filtrate. The isolation of a second polysaccharide, also primarily constituted of units of N-acetylneuraminic acid, was subsequently reported from strains of Group C *Neisseria meningitidis* (Watson, Marinetti & Scherp, 1958). It was first noted in this laboratory (Barry, 1959; Barry, Tsai & Chen, 1960) that neuraminic acid forms a considerable portion of the chemical composition of a strain of *Citrobacter freundii*. The detection of neuraminic acid in two related strains, *Salmonella dahlem* and *S. djkarta*, has also been reported (Westphal, Kauffmann, Luderitz & Stierlin, 1960).

As the distribution of the neuraminic acids among the various species of bacteria is largely unknown, it was the purpose of the present investigation to extend our knowledge concerning the presence of these acids in other micro-organisms. Detection of neuraminic acid or its derivatives in several species of bacteria would materially aid in the establishment of these substances as essential structural components of cells and aid in the determination of their function in micro-organisms.

METHODS

Organisms. In the following experiments several strains of micro-organisms were employed. A variant strain of *Escherichia coli* designated as K235L$^+$OC$^+$ was derived from an *E. coli* known as K235 obtained from Dr Pierre Fredericq of the University of Liège, Belgium. A strain known as *E. coli* 5396/38 obtained from the Walter Reed Army Medical School, Washington, D.C. was kindly provided by Dr Maurice Landy and Dr Marion Webster. The latter also provided transfers of *Salmonella typhi* Ty 2 and Ballerup strain 7851/39. A strain of *S. enteritidis* and two strains of *S. paratyphi* were provided by Dr Frank Holtman, University of Tennessee, Knoxville, Tennessee. Most of the other strains of *E. coli* and Salmonella were kindly sent us by Dr W. H. Ewing, United States Public Health Service, Chamblee, Georgia. Two strains of Group C *Neisseria meningitidis* known as 1908 and 1935 were obtained from Dr H. Scherp, National Institutes of Health, Bethesda, Maryland, and from Dr Glen Watson, Bowman Gray Medical School, Winston-Salem, North Carolina. The strains of Phase I and Phase II *Shigella sonnei* originated from the laboratory of Dr Sarah Branham, Walter Reed Army Medical School, Washington, D.C. and the *Bacillus megaterium* strains KM and 899a from the laboratory of Dr John H. Northrup in California. Several miscellaneous strains of Salmonella, *Bacillus cereus*, *Proteus vulgaris*, *Aerobacter aerogenes*, *Serratia marcescens*, *Rhodopseudomonas palustris*, *Escherichia coli* and *Haemophilus influenzae* Type B and D were sent to this laboratory by different investigators for determination of neuraminic acid content. These are, for the most part, incompletely characterized strains of unknown origin.

Antisera. Antisera were obtained from rabbits which had received multiple

intravenous injections of formalized bacteria followed by inoculations with viable cells in accordance with the procedures described by Edwards (1951) and Edwards & Ewing (1955) for enteric micro-organisms. Antisera against Group C *Neisseria meningitidis* were prepared by the procedure of Rake (1933).

Media and cultivation of organisms. Strains of Group C *Neisseria meningitidis* were maintained on blood agar slopes, while large numbers of organisms were harvested from blood agar plates. Both slopes and plates were incubated for 6 hr. or 18 hr. at 37° in a candle jar at low oxygen tension.

Strains of enteric bacteria were maintained on nutrient agar slopes incubated for 16 hr. at 37°; larger numbers of cells were cultivated at 37° at pH 7·0 in a 1 % (w/v) dialysed casamino acid medium (Difco) enriched with dialysed yeast extract, 0·5 % (w/v) (Difco), and glucose 1·7 % (w/v) contained in 2 litre flasks or in 5-gallon pyrex glass bottles (Barry, 1958).

Strains of *Haemophilus influenzae* were cultivated with aeration at 37° for 6 hr. in a 3·7 % (w/v) brain heart infusion broth (Bacto) enriched by addition of supplement B yeast extract (Bacto, 1 ml./100 ml.) (Neter, 1947).

Rhodopseudomonas palustris was cultivated without aeration at 22° for 48 hr. in a dialysed 1 % (w/v) casamino acid medium. A 200 W. tungsten lamp placed 6 in. away from the flask illuminated the culture during the growth period.

Biological preparations. The Group C *Neisseria meningitidis* hapten employed, generously supplied by Dr Glen Watson, contained 70 % of N-acetylneuraminic acid by our analysis.

Chemical analyses. Protein analyses were made by a modified procedure of Folin and Ciocalteu (Kunkel & Tiselius, 1951) and carbohydrate by a modified anthrone method (Goebel & Barry, 1958). Hexosamine was determined by the procedure of Sorensen (1938); hexuronic acids and pentoses were determined by the methods of Dische (1947) and Dische, Shettles & Osnos (1949).

A modified Ehrlich procedure employed for the detection and estimation of neuraminic acid was carried out as follows. To a ml. sample of an aqueous solution of substance containing 0·05–0·50 mg. of neuraminic acid were added 2 ml. of 30 % (w/v) $Al_2(SO_4)_3$.$18H_2O$ solution and 1 ml. of Ehrlich reagent (5 g. p-dimethyl-aminobenzaldehyde/50 ml. concentrated hydrochloric acid + 50 ml. water (w/v)). A control tube (1 ml. of water + reagents) was also prepared. The covered tubes were heated at 100° for 30 min. in a boiling-water bath. The tubes were rapidly cooled and optical density readings taken in a Beckman spectrophotometer at 530 mμ against the control tube contents. The % neuraminic acid was calculated using a reference curve in which N-acetylneuraminic acid (ovine sialic acid) served as a standard. The advantage of this modified Ehrlich procedure over that originally reported by Werner & Odin (1952) is that a more stable colour complex is formed in the presence of Al^{3+} cation, and the sensitivity of the method is increased by approximately twofold. It should also be noted that the absorption maximum is shifted from 565 to 530 mμ by this change in procedure. In addition to the above procedure, a modified orcinol method (Barry, 1958) was used for the detection of the neuraminic acids.

Biological analyses. Bacteria were tested for their ability to produce bacteriocines as follows. A stab culture of the organism grown on an agar plate for 48 hr. was killed by the addition of 0·5 ml. of chloroform to the cover of the Petri dish. The

dish was incubated for 1 hr. at 37° to evaporate the chloroform. An overlay of 3 ml. of nutrient soft agar seeded with one drop of a 3 ml. slant wash of the indicator strain (*Escherichia coli* strain B, Phase 1 *Shigella sonnei* or *Salmonella enteritidis*) was poured over the stab culture and the plates incubated overnight at 37° after which readings were made. A clear area around the killed stab culture was taken as a positive test.

Agglutination tests of enteric organisms were conducted in the ordinary manner employing cells obtained from 18 hr. nutrient broth cultures. The bacteria were centrifuged, washed in isotonic saline, and resuspended in isotonic saline to a concentration of 2×10^8/ml. The titres were read after incubation at 37° for 2 hr. and after standing overnight at 4°. In all instances, control experiments utilizing similar dilutions of the corresponding normal sera were included in the tests.

Agglutination tests using Group C *Neisseria meningitidis* were conducted by the procedure described by Watson & Scherp (1958).

Univalent anti K antisera was prepared by incubation of whole *Escherichia coli* antisera at 37° with heat-killed cells. This procedure was repeated until no agglutination of heated *E. coli* cells was detectable by agglutination tests. Usually three cycles of absorption were sufficient to remove O agglutinins.

Precipitin tests were conducted as follows. To a series of small tubes (5 mm. × 50 mm.) was added 0·15 ml. of undiluted serum followed by an overlay of 0·15 ml. of serial 10 fold dilutions of test substance dissolved in isotonic saline. After standing for 2–4 hr. at room temperature (22°–25°), the tubes were read for the presence of a precipitate ring at the interface of the two solutions. In all tests, control tubes were included in each series employing normal serum with isotonic saline and normal serum with an isotonic saline solution of the material to be tested.

Haemagglutination tests were conducted by the procedure of Landy & Lamb (1953) employing human O erythrocytes obtained from the blood bank of the University of Tennessee Memorial Hospital.

Haemagglutination blocking tests were conducted as follows. To each of a series of tubes containing 0·5 ml. of an isotonic solution of test substance of 20, 4, 0·5 and 0·2 mg. concentration was added 0·5 ml. antiserum. The tubes were incubated at 37° for 2 hr. A control tube containing 0·5 ml. of antiserum and 0·5 ml. isotonic saline was also incubated. After incubation, the contents of each tube were diluted 1/5 by addition of 4 ml. of isotonic saline. The antiserum in each tube was now equivalent to a 1/10 dilution. Serial two fold dilutions were made in isotonic saline from the control tube and from each of the four tubes containing the test substance and diluted antiserum mixture. To five series of tubes containing 0·2 ml. of each dilution of antiserum was added 0·2 ml. of sensitized human type O erythrocytes. All tubes were incubated at 37° for 2 hr. after which readings were taken.

Bacterial counts were made by the poured plate assay technique on nutrient agar using 0·1 ml. aliquots of a 10^{-5} and 10^{-6} broth dilution of the culture. The plates were incubated overnight at 37° after which readings were made (Adams, 1950).

Haemolysins were detected by streaking the organism on nutrient blood agar plates which contained fresh sheep cells in a concentration of 1·0 % (w/v). The plates were incubated for 16 hr. at 37° after which readings were made. The appearance of a clear area or a greenish zone along the streak or surrounding individual colonies indicated a positive test.

Technique for detection and estimation of neuraminic acid in bacteria

Detection in K antigens of Escherichia coli. To a litre of nutrient medium containing 1% (w/v) of dialysed casamino acids, (5·0 g. dialysed yeast extract in 10 ml. water and 20 ml. 50%, w/v, dextrose) adjusted to pH 7·0 with 0·1 N-phosphate buffer was seeded 0·2 ml. of a 3 ml. slope wash of micro-organism. The culture was grown with aeration for 5 hr. or 18 hr. at 37°. Five ml. of chloroform was added to kill the culture and aeration continued for an additional 15 min. Bacteria were collected by centrifugation, washed in 10 ml. distilled water, collected as before, and resuspended in 10 ml. distilled water. The wash was set aside for analysis. Two ml. of the bacterial suspension was heated in a sealed tube for $2\frac{1}{2}$ hr. at 100° in a water bath, and 2 ml. was maintained at room temperature (22°–25°) to serve as a control. Both the heated and unheated samples were centrifuged at 10,000 g for 30 min. and the clear supernatants removed by pipette. Analyses for neuraminic acid were performed on 1 ml. samples of the heated supernatant employing the modified procedure of Ehrlich. The unheated supernatant served as a control in the analysis. If the solutions were turbid after heating with the analytical reagents, they were rapidly filtered through Whatman no. 1 paper before readings were made at 530 mμ in the Beckman spectrophotometer. In cases where a quantitative evaluation of the amount of neuraminic acid was made, the analytical values obtained for content of neuraminic acid of the wash and supernatant from the heated bacteria were combined and recorded as a single value.

Estimation of neuraminic acid in dried bacteria. When whole bacteria were analysed for content of neuraminic acid, the cells obtained from a litre of culture were lyophilized after washing. Yields of 300–400 mg. of dried cells were usually obtained. The analytical values obtained for content of neuraminic acid of the dried cells and of the cell wash were combined and recorded as a single value.

RESULTS

Agglutination properties of Escherichia coli $K235L^+OC^+$

As the bacteriocinic strain of *Escherichia coli* $K235L^+OC^+$ was the first micro-organism described which produces a substance containing a derivative of neuraminic acid, namely colominic acid, it was of interest to learn more about the properties of this bacterium. It was soon apparent that viable *E. coli* $K235L^+OC^+$ was not readily agglutinated in homologous antiserum. According to Kauffmann (1943), such non-agglutinable strains of *E. coli* contain a capsule which surrounds the micro-organism and prevents O agglutination. Heating such organisms removes the so-called anti O agglutination inhibitor; and following this treatment, O agglutination readily occurs in homologous antisera. The agglutination inhibitor is termed a K antigen (Kauffmann, 1943).

Agglutination experiments of *Escherichia coli* $K235L^+OC^+$ in whole *E. coli* $K235L^+OC^+$ antibacterial antisera and in antisera absorbed with heat killed cells were used to establish the presence of a K antigen.

The results of all agglutination tests are shown in Table 1 where it is seen that the unheated viable cells of *Escherichia coli* $K235L^+OC^+$ are strongly agglutinated to a titre of 1/64 in unabsorbed antibacterial antiserum; whereas, the heated cells

show an agglut.nation titre greater than 1/4096. It is to be noted that viɛble cells show strong agglutination to a titre of 1/32 in antiserum from which the O ɛgglutinins were removed by absorption with heated cells. These results clearly demonstrate that *E. coli* K235⁺OC⁺ contains a heat labile agglutination inhibitor commonly termed a K antigen (Kauffmann, 1943).

Confirmation of the presence of a K antigen in *Escherichia coli* K235L⁺OC⁺ was obtained after submission of the organism for classification of serotype which was found to be *E. coli* O1:K1:HNM, in accordance with the Kauffmann–White classification scheme for identification of enterobacteriaceae.

Table 1. *Agglutination of* Escherichia coli *K235L⁺OC⁺ in homologous antiserum and in antiserum absorbed with heated* E. coli *K235L⁺OC⁺*

Bacteria	Antibacterial antiserum	Dilution of antiserum									
		1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1024	1/2048	1/4096
Viable	Absorbed	4	4	4	3	2	0	0	0	0	0
Heat killed	Absorbed	0	0	0	0	0	0	0	0	0	0
Viable	Unabsorbed	4	4	4	4	3	0	0	0	0	0
Heat killed	Unabsorbed	4	4	4	4	4	4	4	4	4	1

4, Complete agglutination; 0, no agglutination.

Detection of neuraminic acid, bacteriocine and haemolysin in Escherichia coli *of known serotype.* As nothing was known concerning the biological function of neuraminic acid or of its polymer, colominic acid, in *E. coli* K235L⁺OC⁺, attempts were made to correlate the presence of neuraminic acid with other known properties of the bacterium. The presence of a K antigen in *E. coli* K235L⁺OC⁺ suggested that other inagglutinable strains of *E. coli* which have a K antigen might also elaborate colominic acid-like substances. Accordingly, a series of *E. coli* strains of known O and K serotypes was analysed for the presence of neuraminic acid, bacteriocine and haemolysin.

Heat treatment of inagglutinable strains of *Escherichia coli* permits O agglutination to occur in homologous antibacterial antisera. It is apparent that the anti-O agglutination factor, presumably the K antigen, which surrounds the microorganism, is removed from the bacterial surface by the heating procedure. Therefore, chemical analysis of supernatants obtained after centrifugation of heated cells can be used to ascertain if neuraminic acid forms an appreciable part of the chemical composition of the K antigen of the organism.

The results of the analyses for neuraminic acid, bacteriocine and haemolysin in various strains of *Escherichia coli* are recorded in Table 2. Detection of neuraminic acid was made in only two of the nineteen strains analysed, *E. coli* O1:K1:HNM and *E. coli* O1O:K5:HNM. Haemolysins were produced by three strains and bacteriocines were elaborated by seven strains.

It must be concluded, therefore, that neuraminic acid does not form an appreciable portion of the chemical composition of K antigens of *Escherichia coli*. However, the limitations of the chemical procedure do not exclude the presence of trace amounts of the neuraminic acids in K antigens of *E. coli* where they may play an important role in the linkage of carbohydrates to proteins or peptides in these

materials. In addition, it may be noted that no correlation exists between the presence of neuraminic acid and the haemolytic or bacteriocinic properties of the micro-organisms. The bacteriocinic and haemolytic properties are similarly unrelated to each other.

Table 2. Escherichia coli *analysed for neuraminic acid haemolysin and bacteriocine*

Organism tested	Neuraminic acid	Haemolysin	Test organism for bacteriocine		
			E. coli 'B'	*S. enteritidis* PhI.	*Sh.sonnei*
E. coli O1:K1:HNM	+	−	+	−	−
E. coli O6:K53:HNM	−	+	+	−	−
E. coli O20:K17:HNM	−	−	−	−	−
E. coli O10:K5:HNM	+	−	−	−	−
E. coli O4:K12:HNM	−	+	+	−	−
E. coli O21:K20:HNM	−	−	−	−	−
E. coli O11:K10:HNM	−	−	−	−	−
E. coli O8:K44:HNM	−	−	+	−	−
E. coli O9:K29:HNM	−	−	+	−	−
E. coli O9:K55:HNM	−	−	−	−	−
E. coli O9:K35:HNM	−	−	−	−	−
E. coli O9:K38:HNM	−	−	−	−	−
E. coli O9:K33:HNM	−	−	−	−	−
E. coli O26:K60:HNM	−	+	−	−	−
E. coli O127a:K63:HNM	−	−	−	−	−
E. coli O86a:K61:HNM	−	−	−	+	−
E. coli O111ab:K58:HNM	−	−	+	−	−
E. coli O112ac:K66:HNM	−	−	−	−	−
E. coli O55:K59:HNM	−	−	−	−	−

Detection of neuraminic acid, bacteriocine and haemolysin in various Escherichia coli *which have a K1 and various O serotypes.* As shown previously, the serotype of *Escherichia coli* K235L$^+$OC$^+$ was determined as *E. coli* O1:K1:HNM. If the K serotype of the organism is related to the presence of neuraminic acid, then different strains of *E. coli* which possess in common a K1 combined with different O serotypes should contain neuraminic acid. Accordingly, five strains of *E. coli*, all containing a K1 and different O serotypes, were analysed for neuraminic acid, bacteriocine and haemolysin. The results are given in Table 3; it may be noted that all five strains tested contain neuraminic acid. Three of the strains tested show haemolytic properties, and four elaborate bacteriocine.

It is concluded that all *Escherichia coli* which possess a K1 serotype contain a derivative of neuraminic acid and that the haemolytic or bacteriocinic properties which may be associated with the bacteria are unrelated to the presence or absence of this substance.

Neuraminic acid content of various Escherichia coli *as percentage of dry weight.* Although neuraminic acid was shown by qualitative tests to form part of the chemical composition of strains of *Escherichia coli* with a K1 serotype, it was of interest to ascertain whether these different strains contain equivalent amounts of neuraminic acid. Bacteria harvested from cultures during the logarithmic phase and the final phase of growth were dried and analysed for content of neuraminic acid. The data recorded in Table 4 show typical analytical values obtained for content of neuraminic acid determined as percentage of dry weight. It is evident that all strains of *E. coli* analysed contain approximately equivalent amounts of

neuraminic acid if cultivated and harvested under similar conditions. However, bacteria obtained during the logarithmic phase contain a higher percentage of neuraminic acid than do mature bacteria isolated in the final phase of growth. Thus, it is apparent that neuraminic acid itself or substances containing neuraminic acid are elaborated into the culture medium during growth of these strains.

Table 3. Escherichia coli *with a K1 and various O serotypes analysed for neuraminic acid, haemolysin and bacteriocine*

			Test organism for bacteriocine		
Organism tested	Neuraminic acid	Haemolysin	E. coli 'B'	S. enteritidis PhI.	Sh.sonnei
E. coli O1:K1:HNM	+	+	+	−	−
E. coli O2:K1:HNM	+	+	+	−	−
E. coli O7:K1:HNM	+	+	+	−	−
E. coli O16:K1:HNM	+	−	−	−	−
E. coli O25:K1:HNM	+	−	−	−	+

Table 4. *Neuraminic acid content of* Escherichia coli *with a K1 and various O serotypes grown at 37° and harvested after 5 hr. or 18 hr.*

	Neuraminic acid in percentage dry weight	
Organism tested	5 hr.	18 hr.
E. coli O1:K1:HNM	0·97	0·27
E. coli O2:K1:HNM	1·15	0·46
E. coli O7:K1:HNM	1·82	0·23
E. coli O16:K1:HNM	1·69	0·19
E. coli O25:K1:HNM	1·23	0·11

Neuraminic acid content per bacterium of various Escherichia coli. Experiments were next performed to determine the content of neuraminic acid per bacterium in each of the different *E. coli* of K1 serotype isolated during the logarithmic phase of growth. In Table 5 are recorded typical values for content of neuraminic acid per bacterium for five different *E. coli* strains. The results clearly reveal that no gross differences exist in content of neuraminic acid per bacterium in the various strains analysed.

Isolation of colominic acid from Escherichia coli *O1:K1:HNM and O2:K1:HNM.* Although strains of *E. coli* which have a K1 serotype contain neuraminic acid, it was not known if the same derivative of this acid is present or whether the neuraminic acid derivative is in a chemical combination similar to that found in colominic acid obtained from *E. coli* O1:K1:HNM. Thus, a polysaccharide containing neuraminic acid was isolated from culture supernatants seeded with *E. coli* O2:K1:HNM. The physical and chemical properties of this polysaccharide were compared with those of the polysaccharide, colominic acid, obtained from culture supernatants seeded with *E. coli* O1:K1:HNM.

Fifteen litres of culture medium (Barry, 1958) were seeded with 5×10^4 cells of *Escherichia coli* O1:K1:HNM or *E. coli* O2:K1:HNM growing in the logarithmic phase. The culture was maintained at 37° and aerated at the rate of 4 litres per min. for 18 hr. The pH of the culture was held at 7·0 by an electronic device (Goebel,

Barry & Shedlovsky, 1956). The culture was killed by addition of 100 ml. of chloroform and aeration continued for an additional 30 min. The suspension was clarified in a Sharples Super centrifuge, refrigerated at 0°–4° by a cooling coil located within the bowl of the apparatus. The clear supernatant was concentrated at 20° *in vacuo* to 1·5 l. in a glass circulating evaporator maintained at 1–2 mm. by means of a Kinney vacuum pump Model KC8. The fluid was fed into the evaporator with an electronic device (Barry & Pierce, 1959) which maintained the liquid in the bowl of the evaporator at a constant level. The concentrated medium was dialysed by employing 5 ft. lengths of Nojax sausage casing (Visking Corp.) size 18/32, against distilled water for 16 hr. at 4°. The bag contents were concentrated *in vacuo* to 500 ml., dialysed, concentrated *in vacuo* to 100 ml. and redialysed. The solution was further concentrated to 100 ml. *in vacuo*, filtered and lyophilized. Yields of 5–5·5 g. of non-dialysable polysaccharide containing 20–30 % neuraminic acid were obtained.

Table 5. *Neuraminic acid content of various* Escherichia coli *grown for 5 hr. at 37°*

Organism tested	Titre bacteria per ml. × 10⁸	Volume culture (ml.)	Total number of bacteria (× 10¹¹)	Total amount neuraminic acid recovered (mg.)	Calculated amount neuraminic acid per bacterium (μg. × 10⁻⁹)
E. coli O1:K1:HNM	3·3	482	1·59	1·94	12·2
E. coli O2:K1:HNM	6·2	431	3·54	1·68	4·75
E. coli O7:K1:HNM	7·2	433	3·11	2·17	6·96
E. coli O16:K1:HNM	10·0	483	4·83	3·52	7·30
E. coli O25:K1:HNM	4·4	505	2·22	2·80	12·6

A 3 % (w/v) solution of the substance in 0·02 M-sodium acetate buffer was adjusted to pH 4·5 and chilled to 4°. Absolute ethanol at − 10° was added to a concentration of 75 % (v/v) and the solution stored for 2 hr. in the deep freeze at − 25°. The solution was centrifuged at 10,000 *g* at − 10° in a Lourdes centrifuge and the supernatant decanted. The precipitate was dissolved in 100 ml. distilled water and the solution dialysed against 15 l. of water at 4° for 16 hr. The solution was concentrated to 150 ml. *in vacuo* and filtered. The solution was stirred at room temperature and solid ammonium sulphate (77·7 g.) was added to 75 % saturation. After standing for 1 hr., the precipitate was removed by centrifugation and to the clear supernatant was added with stirring ammonium sulphate to 90 % saturation (21·2 g.). One hr. later the solution was centrifuged. To the supernatant was added with stirring concentrated hydrochloric acid until incipient turbidity (approx. 0·15 ml.) and the solution filtered through a sintered glass filter of medium porosity. To the stirred filtrate was added additional concentrated hydrochloric acid until no more precipitate formed (approx. 0·9 ml.) and the solution kept at 4° for 2 hr. The solution was filtered through a sintered glass filter of medium porosity and the sticky precipitate dissolved in 50 ml. distilled water. Two ml. of 1 N-hydrochloric acid was added and the solution dialysed until free of chloride and sulphate ions. The solution was concentrated to 50 ml. and traces of salt removed by electrodialysis. After concentrating the solution to 40 ml. *in vacuo*, it was filtered and lyophilized. Yields of 0·5–1·7 g. of purified polysaccharide were obtained.

Comparison of the properties of colominic acid obtained from Escherichia coli
*O1:K1:HNM with those of the polysaccharide containing neuraminic acid
obtained from* E. coli *O2:K1:HNM*

Purified colominic acid isolated from the *E. coli* O1:K1:HNM culture super-
natant and the purified polysaccharide, which contains neuraminic acid obtained
from *E. coli* O2:K1:HNM, are white amorphous powders soluble in water. Aqueous
solutions are markedly acidic (pH 3·7) and not viscous. Humin is formed upon
heating either substance in dilute mineral acid or by boiling in aqueous solution.
Only feeble anthrone and Folin–Ciocalteu tests are given by either material. Hexos-
amines, pentoses and hexuronic acids are absent. Both substances give reddish
purple colours when heated with Bial's orcinol or with Ehrlich's reagent. On the
basis of the modified Ehrlich procedure, a typical analysis of colominic acid from
E. coli O1:K1:HNM shows 99 % N-acetylneuraminic acid. A typical analysis of the
polysaccharide containing neuraminic acid obtained from *E. coli* O2:K1:HNM
shows 98·5% *N*-acetylneuraminic acid. Neither substance precipitates in anti-
serum of rabbits immunized with the micro-organisms from which they are derived.

Table 6. *Chemical analysis of polysaccharides containing neuraminic acid
obtained from* Escherichia coli *O1:K1:HNM and O2:K1:HNM*

		Substance analysed				
Source	Material analysed	C (%)	H (%)	N (%)	CH_3CO (%)	Empirical formula
E. coli O1:K1:HNM	Colominic acid	42·83	6·46	4·79	14·45	$(C_{11}H_{19}NO_9)_n$
E. coli O2:K1:HNM	Acidic polysaccharide	42·73	6·36	4·71	13·88	$(C_{11}H_{19}NO_9)_n$
Sheep salivary mucin	*N*-Acetylneuraminic acid	42·72	6·19	4·53	13·92	$(C_{11}H_{19}NO_9)$

Chemical analyses. Analyses for carbon, hydrogen, nitrogen and acetyl were
performed on several preparations of colominic acid obtained from *Escherichia coli*
O1:K1:HNM and on the polysaccharide containing neuraminic acid obtained from
E. coli O2:K1:HNM. The materials were dried to constant weight at 80°. The results
of typical analyses are given in Table 6. From the analytical data, the formula
$C_{11}H_{19}NO_9$ can be calculated as representing the monomer unit of both colominic
acid and the acidic polysaccharide containing neuraminic acid obtained from
E. coli O2:K1:HNM. It should be mentioned that the empirical formula $(C_{11}H_{19}NO_9)_n$
given for each of the polysaccharides includes a mole of bound water per $C_{11}H_{19}NO_9$
unit.

The optical rotation of a 5 % (w/v) aqueous solution of colominic acid obtained
from *Escherichia coli* O1:K1:HNM when measured in a 2 dm. tube, gave the value
$[\alpha]_D^{20°} = -51° \pm 2°$. The optical rotation of the acidic polysaccharide obtained
from *E. coli* O2:K1:HNM measured under identical conditions gave the value
$[\alpha]_D^{20°} = -50° \pm 2°$.

Paper chromatographic analysis of the hydrolysate of either polysaccharide
showed *N*-acetylneuraminic acid to be the major component (Barry, 1958).

An infra red absorption spectrum of colominic acid obtained from *Escherichi acoli*
O1:K1:HNM and of the acidic polysaccharide obtained from *E. coli* O2:K1:HNM

was taken in a potassium bromide pellet using 1·4 mg of substance mixed with 350 mg. of the salt. The spectra are recorded in Fig. 1.

It is apparent from the elemental analytical data recorded in Table 6 and from the infrared absorption spectra shown in Fig. 1 that colominic acid isolated from the *Escherichia coli* O1:K1:HNM culture supernatants is similar in all respects to the polysaccharide containing neuraminic acid obtained from the *E. coli* O2:K1:HNM culture supernatants. Thus, it can be concluded that other strains of *E. coli* which possess a K1 serotype and which contain neuraminic acid also elaborate colominic acid.
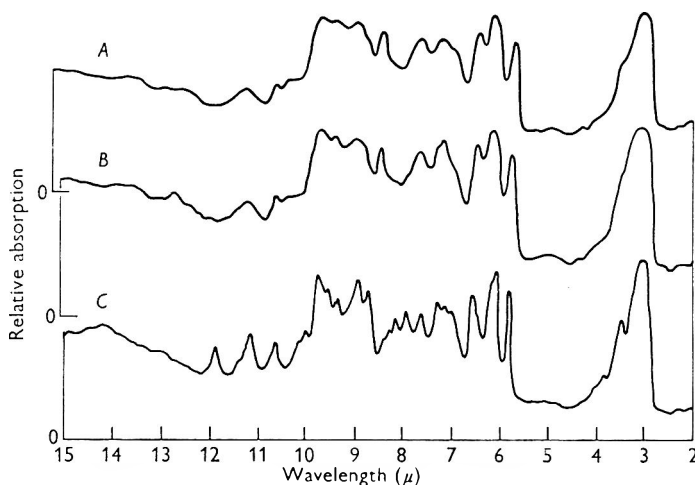

Fig. 1. Infrared spectrograms taken on 1·4 mg. of substance in 350 mg. of potassium bromide pellet. Curve *A*, colominic acid isolated from culture supernatant of *Escherichia coli* O1:K1:HNM; Curve *B*, colominic acid isolated from culture supernatant of *Escherichia coli* O2:K1:HNM; curve *C*, *N*-acetylneuraminic acid.

Chemical analysis of other species of bacteria for content of neuraminic acid. As derivatives of neuraminic acid have been found in only a few closely related enteric micro-organisms, it was of interest to determine if these substances were present in other enterobacteriaceae and in unrelated species of bacteria. As derivatives of neuraminic acid were first found in *Escherichia coli*, a genus of the enterobacteriaceae, it appeared that other genera of this family should be examined for the presence of these substances. Thus, 25 Salmonella, 7 Shigella, 2 *Aerobacter aerogenes*, a *Citrobacter freundii* and a strain of *Klebsiella pneumoniae* were analysed. Of these, only three strains, *S. dahlem*, *S. djkarta* and *C. freundii*, contain a neuraminic acid derivative in readily detectable quantities.

Negative tests for neuraminic acid were obtained for 3 strains of *Bacillus megaterium*, 2 of *Bacillus cereus*, 2 of *Haemophilus influenzae* type B and D and a strain of *Rhodopseudomonas palustris*. Two strains of Group C *Neisseria meningitidis*, 1908 and 1935, were analysed for content of neuraminic acid. These strains were reported to contain a derivative of neuraminic acid (Watson *et al.* 1958).

In Table 7 are recorded typical values found for content of neuraminic acid in various bacteria expressed as percentage of dry weight. The neuraminic acid content varies over a 20-fold range in concentraton. It is of interest to mention that the

detection of neuraminic acid in the clarified and concentrated culture supernatants in which *Salmonella dahlem, S. djkarta, Escherichia coli* O1O:K5:HNM or *Citrobacter freundii* were grown was difficult and usually unsuccessful. Thus, it is concluded that these organisms do not readily elaborate polysaccharides which contain neuraminic acid into the culture medium as do strains of *E. coli* of K1 serotype.

Table 7. *Neuraminic acid content of various bacteria grown at 37° for 18 hr.*

Organism tested	Neuraminic acid % dry weight
C. freundii O5:H30	1·92
S. dahlem	1·19
S. djkarta	0·51
N. meningitidis Group C 1908	0·64
N. meningitidis Group C 1935	0·26
E. coli O1O:K5:HNM	0·10

Serological relationship of bacteria which contain neuraminic acid

As the neuraminic acid content of *Salmonella dahlem, S. djkarta, Citrobacter freundii* and Group C *Neisseria meningitidis* is of the same order of magnitude as is found in *Escherichia coli* of K1 serotype, it appeared that a colominic acid-like substance may be produced by these strains. In this connexion, it should be mentioned that the Group C hapten isolated from Group C *N. meningitidis* 1935 was reported to be 85 % constituted of units of *N*-acetylneuraminic acid and to contain glucosamine (Watson *et al.* 1958). It was of interest, therefore, to determine by agglutination tests whether the various bacteria known to produce polysaccharides that contain neuraminic acid are related to each other.

The results of the agglutination experiments are recorded in Table 8. It is apparent that *Escherichia coli* O1:K1:HNM and O2:K1:HNM, which elaborate colominic acid, are unrelated to any of the other bacteria which contain neuraminic acid by cross-agglutination tests. However, *E. coli* O1:K1:HNM and O2:K1:HNM are related to each other. The strains of Group C *Neisseria meningitidis* are related to each other but to no other strain listed in Table 8. *Salmonella dahlem, S. djkarta* and *Citrobacter freundii* show an inter-relationship one to the other but are unrelated to either strain of *E. coli* or to the strains of Group C *N. meningitidis*. Thus, on the basis of the results of the agglutination experiments in homologous and heterologous antisera the strains examined can be divided into three separate and unrelated groups.

Precipitation of colominic acid and of Group C Neisseria meningitidis *hapten in homologous and heterologous antibacterial antisera.* Despite the foregoing observations that no relationship was demonstrable by cross-agglutination tests between strains of *Escherichia coli* which elaborate colominic acid and strains of Group C *Neisseria meningitidis* which contain a polysaccharide constituted primarily of *N*-acetylneuramic acid, experiments were performed to ascertain whether purified polysaccharides containing neuraminic acid obtained from these bacteria would precipitate in *E. coli* and Group C *N. meningitidis* antibacterial antisera. The results of the precipitin tests are recorded in Table 9. It is apparent from the data that colominic acid does not precipitate in any of the antibacterial antisera, whereas the

Group C *N. meningitidis* hapten readily precipitates in Group C *N. meningitidis* antibacterial antisera, but not in *E. coli* O1:K1:HNM or O2:K1:HNM antibacterial antisera.

Haemagglutination of human O erythrocytes sensitized with adsorbed Group C Neisseria meningitidis *hapten or with colominic acid in homologous and heterologous antibacterial antisera.* The incubation of erythrocytes in saline solutions of poly-

Table 8. *Agglutination of bacteria which contain neuraminic acid in homologous and heterologous antisera*

Organism tested	Antibacterial antiserum	$\frac{1}{5}$	$\frac{1}{10}$	$\frac{1}{20}$	$\frac{1}{40}$	$\frac{1}{80}$	$\frac{1}{160}$	$\frac{1}{320}$	$\frac{1}{640}$	$\frac{1}{1280}$	$\frac{1}{2560}$	$\frac{1}{5120}$	$\frac{1}{10240}$
S. djkarta	*S. djkarta*	4	4	4	4	4	4	4	4	4	1	±	0
S. dahlem		4	4	4	4	4	4	2	1	±	0	0	0
C. freundii		4	4	4	4	3	2	0	0	0	0	0	0
E. coli O1:K1:HNM		0	0	0	0	0	0	0	0	0	0	0	0
E. coli O2:K1:HNM		0	0	0	0	0	0	0	0	0	0	0	0
N. meningitidis 1908		0	0	0	0	0	0	0	0	0	0	0	0
N. meningitidis 1935		0	0	0	0	0	0	0	0	0	0	0	0
S. djkarta	*S. dahlem*	4	4	4	4	4	4	4	3	2	0	0	0
S. dahlem		4	4	4	4	4	4	4	4	4	3	2	1
C. freundii		4	4	4	4	4	2	±	0	0	0	0	0
E. coli O1:K1:HNM		0	0	0	0	0	0	0	0	0	0	0	0
E. coli O2:K1:HNM		0	0	0	0	0	0	0	0	0	0	0	0
N. meningitidis 1908		0	0	0	0	0	0	0	0	0	0	0	0
N. meningitidis 1935		0	0	0	0	0	0	0	0	0	0	0	0
S. djkarta	*C. freundii*	4	4	4	4	4	4	4	4	4	1	0	0
S. dahlem		4	4	4	4	4	4	4	4	4	1	0	0
C. freundii		4	4	4	4	4	4	4	4	4	4	4	2
E. coli O1:K1:HNM		0	0	0	0	0	0	0	0	0	0	0	0
E. coli O2:K1:HNM		0	0	0	0	0	0	0	0	0	0	0	0
N. meningitidis 1908		0	0	0	0	0	0	0	0	0	0	0	0
N. meningitidis 1935		0	0	0	0	0	0	0	0	0	0	0	0
S. djkarta	*E. coli* O1:K1:HNM	0	0	0	0	0	0	0	0	0	0	0	0
S. dahlem		0	0	0	0	0	0	0	0	0	0	0	0
C. freundii		0	0	0	0	0	0	0	0	0	0	0	0
E. coli O1:K1:HNM		4	4	4	4	3	2	±	0	0	0	0	0
E. coli O2:K1:HNM		4	4	4	4	2	1	0	0	0	0	0	0
N. meningitidis 1908		0	0	0	0	0	0	0	0	0	0	0	0
N. meningitidis 1935		0	0	0	0	0	0	0	0	0	0	0	0
S. djkarta	*E. coli* O2:K1:HNM	0	0	0	0	0	0	0	0	0	0	0	0
S. dahlem		0	0	0	0	0	0	0	0	0	0	0	0
C. freundii		0	0	0	0	0	0	0	0	0	0	0	0
E. coli O1:K1:HNM		4	4	4	4	4	2	±	0	0	0	0	0
E. coli O2:K1:HNM		4	4	4	4	4	4	2	±	0	0	0	0
N. meningitidis 1903		0	0	0	0	0	0	0	0	0	0	0	0
N. meningitidis 1935		0	0	0	0	0	0	0	0	0	0	0	0
S. djkarta	*N. meningitidis* Group C 1908	0	0	0	0	0	0	0	0	0	0	0	0
S. dahlem		0	0	0	0	0	0	0	0	0	0	0	0
C. freundii		0	0	0	0	0	0	0	0	0	0	0	0
E. coli O1:K1:HNM		0	0	0	0	0	0	0	0	0	0	0	0
E. coli O2:K1:HNM		0	0	0	0	0	0	0	0	0	0	0	0
N. meningitidis 1908		4	4	4	4	4	4	2	2	1	0	0	0
N. meningitidis 1935		4	4	4	4	4	4	2	2	±	0	0	0
S. djkarta	*N. meningitidis* Group C 1935	0	0	0	0	0	0	0	0	0	0	0	0
S. dahlem		0	0	0	0	0	0	0	0	0	0	0	0
C. freundii		0	0	0	0	0	0	0	0	0	0	0	0
E. coli O1:K1:HNM		0	0	0	0	0	0	0	0	0	0	0	0
E. coli O2:K1:HNM		0	0	0	0	0	0	0	0	0	0	0	0
N. meningitidis 1908		4	4	4	4	4	4	2	2	1	0	0	0
N. meningitidis 1935		4	4	4	4	4	4	3	2	1	0	0	0

4, complete agglutination. 0, no agglutination. ±, trace

saccharides frequently leads to the adsorption of these substances to the cell surface. Addition of antiserum prepared against the polysaccharide to a suspension of the sensitized erythrocytes results in their haemagglutination. This sensitive

Table 9. *Precipitin tests of colominic acid and* Neisseria meningitidis *Group C hapten in* E. coli *O1:K1:HNM, O2:K1:HNM and* N. meningitidis *Group C 1908 and 1935 antisera.*

Substance tested	Antiserum	Concentration of substance (μg./ml)					
		1000	100	10	1	0·1	0·01
Colominic acid	E. coli O1:K1:HNM	0	0	0	0	0	0
	E. coli O2:K1:HNM	0	0	0	0	0	0
	N. meningitidis 1908	0	0	0	0	0	0
	N. meningitidis 1935	0	0	0	0	0	0
N. meningitidis	E. coli O1:K1:HNM	0	0	0	0	0	0
Group C 1908	E. coli O2:K1:HNM	0	0	0	0	0	0
hapten	N. meningitidis 1908	4	3	2	1	0	0
	N. meningitidis 1935	4	3	2	1	0	0

4, heavy precipitation. 0, no precipitation

Table 10. *Haemagglutination of type O erythrocytes sensitized with* Neisseria meningitidis *Group C hapten or colominic acid in* N. meningitidis *Group C 1908, 1935,* Escherichia coli *O1:K1:HNM and O2:K1:HNM antisera*

Sensitizing agent	Antiserum	Dilution of antiserum											
		$\frac{1}{20}$	$\frac{1}{40}$	$\frac{1}{80}$	$\frac{1}{160}$	$\frac{1}{320}$	$\frac{1}{640}$	$\frac{1}{1280}$	$\frac{1}{2560}$	$\frac{1}{5120}$	$\frac{1}{10,240}$	$\frac{1}{20,480}$	$\frac{1}{40,960}$
N. meningitidis	N. meningitidis												
Group C. hapten	Group C 1908	4	4	4	4	4	4	3	3	1	1	0	0
	1935	4	4	4	4	4	3	2	2	1	0	0	0
	E. coli												
	O1:K1:HNM	0	0	0	0	0	0	0	0	0	0	0	0
	O2:K1:HNM	0	0	0	0	0	0	0	0	0	0	0	0
Colominic acid	N. meningitidis												
	Group C 1908	0	0	0	0	0	0	0	0	0	0	0	0
	1935	0	0	0	0	0	0	0	0	0	0	0	0
	E. coli												
	O1:K1:HNM	0	0	0	0	0	0	0	0	0	0	0	0
	O2:K1:HNM	0	0	0	0	0	0	0	0	0	0	0	0

4, complete haemagglutination. 0, no haemagglutination.

test is employed for the demonstration of materials which have weak serological properties. Thus, fresh human O erythrocytes were incubated with saline solutions of purified colominic acid or with Group C *Neisseria meningitidis* 1908 hapten. Sensitized cells were then added to *Escherichia coli* O1:K1:HNM, *E. coli* O2:K1:HNM, Group C *N. meningitidis* 1908 and 1935 antibacterial antisera. The results are given in Table 10 where it is to be noted that erythrocytes sensitized with the Group C *N. meningitidis* hapten are readily agglutinated in *N. meningitidis* antibacterial antiserum prepared against either the 1908 or 1935 strains. However, these cells are not agglutinated in antisera prepared against *E. coli* O1:K1:HNM

or *E. coli* O2:K1:HNM. In the experiments where cells sensitized with colominic acid were used, no haemagglutination occurred in any of the four antisera. Thus, no serological relationship was demonstrable between colominic acid and the Group C *N. meningitidis* hapten.

Table 11. *Inhibition of haemagglutination by colominic acid of type O erythrocytes sensitized with* Neisseria meningitidis *Group C* 1908 *hapten in* N. meningitidis *Group C* 1908 *antiserum*

Inhibitor	Inhibitor concentration (μg./ml.)	Dilution of antiserum								
		1/20	1/40	1/80	1/100	1/320	1/640	1/1280	1/2560	1/5120
Colominic acid	10,000	4	4	4	4	4	4	3	2	0
	2,000	4	4	4	4	4	4	3	1	0
	400	4	4	4	4	4	4	2	1	0
	100	4	4	4	4	4	4	3	1	0
	0	4	4	4	4	4	3	3	1	0

4, complete haemagglutination. 0, no haemagglutination

Inhibition of haemagglutination of human type O erythrocytes sensitized with Group C Neisseria meningitidis 1908 *hapten by colominic acid.* Despite the observation that human type O erythrocytes sensitized with Group C *N. meningitidis* 1908 hapten failed to haemagglutinate in *Escherichia coli* O1:K1:HNM or *E. coli* O2:K1:HNM antisera, it appeared that a relationship between colominic acid and the Group C *N. meningitidis* hapten might be demonstrable by employing a haemagglutination blocking method where *N. meningitidis* antiserum is incubated with colominic acid prior to the addition of erythrocytes sensitized with Group C *N. meningitidis* 1908 hapten. The results are shown in Table 11, where it is seen that no inhibition of the haemagglutination of the erythrocytes sensitized with Group C *N. meningitidis* hapten occurs in any of the incubated antisera containing colominic acid. Therefore, it is concluded that colominic acid does not combine with antibody present in Group C *N. meningitidis* antibacterial antiserum which precipitates Group C *N. meningitidis* 1908 hapten.

DISCUSSION

The discovery of the neuraminic acids in materials derived from mammalian origin (Blix, 1936) laid the foundation for a new field of biochemistry. Many animal tissues contain derivatives of neuraminic acid in combination with carbohydrates, lipids and proteins. The wide distribution of neuramino-mucoproteins in animal secretions and excretions suggests that these materials have a protective function in cells. The epithelial surfaces of the respiratory, digestive and urogenital tracts are covered by mucins rich in neuramino-proteins. Certain bacteria and viruses which inhabit or invade these tracts possess neuraminidases which can split neuraminic acid from neuramino-proteins and thus a possible mechanism of invasion of underlying cells by these agents is indicated. The discovery of substances containing neuraminic acid in materials derived from bacteria opened up a new area in the biochemistry of bacteria. However, the distribution and biological significance of neuraminic acid in bacteria has remained largely unknown.

The work presented here has shown that few strains of Gram-negative bacteria contain readily detectable amounts of neuraminic acid. Among the 70 different Gram-negative micro-organisms analysed, only 11 contain neuraminic acid. Of these, 6 are *Escherichia coli*, 2 are Salmonella, 2 are Group C *Neisseria meningitidis* and one is a *Citrobacter freundii*. It is of interest to note that the strains which contain neuraminic acid were isolated from pathological material obtained from mammalian sources. The incorporation of neuraminic acid derivatives in complex bacterial products, approaching the host neuramino-mucoproteins' composition, may be the microbe's method of establishing itself in various tissues. However the virulence of strains of *E. coli* containing neuraminic acid was shown by Forbes & Kunk (1961) to be no greater than that of strains which do not contain this material.

The development of a systematic classification scheme for the enterobacteriaceae, based on the antigenic composition of the micro-organisms, has made it possible to identify enterobacteriaceae isolated from different sources. The determination of the serotype of *Escherichia coli* K235L$^+$OC$^+$ as O1:K1:HNM was of considerable value in the finding of other *E. coli* which produce colominic acid (poly *N*-acetyl-neuraminic acid) and to a determination of the relationship of colominic acid to other properties of these bacteria. Thus, the isolation of colominic acid from culture supernatants of different *E. coli* which possess a K1 but different O serotypes, namely *E. coli* O1:K1:HNM and *E. coli* O2:K1:HNM, strongly suggests that other *E. coli* which contain neuraminic acid and have a K1 serotype also produce colominic acid (Barry *et al.* 1960). This view is further supported by the observation that 5 strains of *E. coli* with a K1 but different O serotypes contain equivalent amounts of neuraminic acid. Furthermore, the absence of neuraminic acid in *E. coli* which have other K serotypes demonstrates that this material is not a common component of strains which possess a K serotype. The association of the K1 antigen of *E. coli* with colominic acid is evident and it suggests that the genes which control the biosynthesis of these materials are closely linked. Finally, it was shown that other properties of *E. coli*, such as ability to produce haemolysins or bacteriocines, cannot be directly associated with the production of colominic acid or the presence of neuraminic acid. The earlier observations (Barry, 1958) and those reported here concerning the close association of neuraminic acid with a K1 serotype in various strains of *E. coli* were recently confirmed (De Witt & Rowe, 1961; De Witt & Zell, 1961).

The detection of neuraminic acid in species of bacteria other than *Escherichia coli* suggested that colominic acid may be produced by these micro-organisms. The cross-agglutination experiments clearly revealed that no serological relationship exists between *E. coli* which elaborate colominic acid and other micro-organisms which contain neuraminic acid. The strain of *Citrobacter freundii* which is constituted of 1·9 % neuraminic acid does not possess a K antigen but has O and H antigens which are related to the O5 and H30 antigens present in bacteria of the Arizona group of enterobacteriaceae. Although it is possible to detect the presence of neuraminic acid in complex materials isolated from the culture supernatants of *C. freundii* O5:H30, it is apparent that the greater portion of the neuraminic acid is firmly bound. On the other hand, strains of *E. coli* which produce colominic acid readily elaborate this material into the culture medium. These results indicate a different chemical binding of the neuraminic acid in *C. freundii* and in the related strains *Salmonella dahlem* and *S. djkarta*.

In 1958, subsequent to the discovery of colominic acid, Watson & Scherp (1958) described the isolation and characterization of the group C hapten obtained from Group C *Neisseria meningitidis*. This material was reported to be constituted of units of *N*-acetylneuraminic acid to the extent of 70–85 % and to contain glucosamine (Watson *et al.* 1958). The Group C hapten is serologically active, whereas colominic acid is not. All experiments reported here have failed to show any serological relationship between colominic acid and the Group C *N. meningitidis* hapten. It must be concluded, therefore, that these two substances have either a different arrangement of the *N*-acetylneuraminic acid units within the macro-molecule or that the serological properties of the Group C *N. meningitidis* hapten are attributable to factors other than the neuraminic acid portion of the polysaccharide.

Neuraminic acid materials are present in three genera of the enterobacteriaceae family; however, the detection of these substances has been made in only a few strains. The detection of neuraminic acid in a genus of the neisseriaceae has shown that complex substances which contain neuraminic acid also exist in bacteria of other families.

It is not yet possible to assess fully the biological importance or function of neuraminic acid in bacteria. Furthermore, little can be said about the biosynthesis or metabolism of neuraminic acid in cells. It is apparent, therefore, that information concerning the distribution and nature of the chemical binding of neuraminic acid in micro-organisms is desirable, for this will provide the means for further investigations.

From the data which have been discussed, it is concluded that colominic acid (poly *N*-acetylneuraminic acid) is produced by *Escherichia coli* strains which possess a K1 serotype. Other bacteria which contain neuraminic acid have this substance in a chemical combination which is different from that of colominic acid.

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The Catabolism of Cystathionine by *Escherichia coli*

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SUMMARY

L-Cystathionine is rapidly degraded to homocysteine, pyruvate and ammonia by cell-free enzyme preparations of an auxotrophic strain of *Escherichia coli* which requires pyridoxin, glycine or serine for growth. One molecule of each of the products was formed from each molecule of cystathionine throughout the course of the reaction. The preparation did not form pyruvate from L-serine and L-alanine (which were possible intermediates); it is concluded that the cleavage is a single step reaction. After precipitation with ammonium sulphate and dialysis the enzyme required both pyridoxal phosphate and magnesium ions for full activity, but no dependence on magnesium was found with preparations from another strain. The reaction was inhibited totally by cyanide and cupric ions and partially by isonicotinic acid hydrazide and sulphydryl compounds (cysteine, homocysteine, glutathione). An auxotrophic strain of *E. coli* which grew with methionine or homocysteine, but not with cystathionine, did not contain the enzyme.

INTRODUCTION

The nutritional requirements of certain induced mutants of *Escherichia coli* (Lampen, Roepke & Jones, 1947; Gots & Koh, 1950) suggest that cysteine is converted to methionine, as in Neurospora (Horowitz, 1947), through the successive intermediate formation of cystathionine (Fig. 3) and homocysteine. The conversion of homocysteine to methionine by *E. coli* has been studied extensively in this laboratory, with suspensions of intact organisms and with cell-free enzyme preparations (Gibson & Woods, 1960; Guest & Woods, 1962). There is little detailed information, however, about the bacterial metabolism of cystathionine, the probable immediate precursor of homocysteine. Binkley & Hudgins (1953) stated briefly that cell-free preparations of *Proteus morganii* degraded cystathionine both to homocysteine and to cysteine, the former pathway being predominant with concurrent formation of pyruvate and ammonia; pyridoxal phosphate was required. The object of the present work, which forms part of a wider study of methionine synthesis by *E. coli*, was to determine whether this organism has the necessary enzymic mechanisms to convert cystathionine to homocysteine and to investigate the nature and course of the reaction. An auxotrophic strain (B 166) which requires pyridoxin for growth was used initially in the hope of facilitating the study of any requirement for this vitamin in its coenzyme form (pyridoxal phosphate). A brief report of part of this work has been given (Wijesundera & Woods, 1953).

In mammalian liver cystathionine is degraded mainly to cysteine and α-keto-

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butyrate and not to homocysteine and pyruvate (Binkley, Anslow & du Vigneaud, 1942; Carroll, Stacey & du Vigneaud, 1949). This enzyme has been highly purified (Matsuo & Greenberg, 1958a) and shown also to require pyridoxal phosphate (Binkley, Christensen & Jensen, 1952; Matsuo & Greenberg, 1958b, 1959a). The work of Fischer (1957) with cell-free preparations of *Neurospora crassa* suggests that this organism can degrade cystathionine by both pathways, that leading to homocysteine being the major one.

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

Organisms. *Escherichia coli* B 166, an auxotroph requiring either pyridoxin, serine or glycine for growth in a medium containing autoclaved glucose (Morris & Woods, 1959) was the main organism used; it was obtained originally from Dr J. S. Gots. *E. coli* 122/33 and 26/18 (both from Dr B. D. Davis) were methionine auxotrophs responding respectively to cystathionine or homocysteine (122/33) and homocysteine only (26/18). *E. coli* F was a prototrophic laboratory strain. Stock cultures of all the organisms were maintained on tryptic digest of meat agar slopes, subcultured monthly (18 hr. at 37°) and stored at 4°.

Media and growth. Medium L (Morris & Woods, 1959), containing lactate as carbon source, was used for strain F. Medium A, used for the auxotrophic strains, was the glucose + salts medium of Davis & Mingioli (1950) except that the glucose (and any necessary growth factor) was added before autoclaving. With strain B 166 it was desired to obtain organisms grown without added pyridoxin; although either DL-serine or glycine alone will support growth under these conditions, best yields were obtained with a mixture of DL-serine, glycine and DL-alanine (each mM). The supplement used for strains 122/33 and 26/18 was DL-methionine (0·5 mM).

The appropriate medium (500 ml. in 1 l. Roux bottles) was autoclaved at 115° for 10 min. and inoculated with 0·5 ml. of a 24 hr. culture in the same medium. The Roux bottles were incubated at 37° for 16–20 hr. in a sloped position (20° to the horizontal) to increase aeration. Organisms from 20–30 bottles were harvested in a continuous centrifuge, washed twice on an angle centrifuge with 100 ml. volumes of phosphate buffer (67 mM, pH 7·4) and stored (when necessary) as a thick paste at 4°.

Cell-free extracts. Organisms (equiv. 1 g. dry wt.) in 8 ml. phosphate buffer (67 mM, pH 7·4) were placed in each cup of a tissue disintegrator (Mickle, 1948) with 8 g. glass balls (Ballotini no. 13; Chance Bros., Smethwick, Staffs.) and tributyl citrate (0·1 ml.). The cups were vibrated at maximum amplitude for 1 hr. at 2° and the crushed organisms washed out with about 25 ml. phosphate buffer (67 mM, pH 7·4) at 0°. The clear yellowish supernatant fluid obtained by centrifuging for 15 min. at 18,000 g contained about 85% of the total enzyme activity of the crushed material. It was stored at 0° after adding a few drops of chloroform and retained full activity for at least two weeks.

General procedure in experiments with enzymes. The reaction mixture contained enzyme preparation (0·2–1·0 ml.), pyridoxal phosphate (10 μm-moles), magnesium sulphate (1 μmole), substrate (10 μmoles of L- or 20 μmoles of DL-) and phosphate buffer (pH 7·4) to a final volume of 2 ml. and final molarity of 0·067. The substrate solution (at 37°) was added after incubating the remaining constituents for 10 min. at 37°; the final incubation was normally for 30 min. at 37° in tubes (125 mm. × 16 mm.) shaken gently in air. When keto acids were to be assayed or identified the

reaction was stopped (and proteins precipitated) by the addition of 25% (w/v) trichloroacetic acid (0·5 ml.). Protein-free filtrates for the estimation of ammonia and homocysteine were obtained by warming the reaction mixture with an equal volume of N-HCl.

Chemical estimations. A modification of the method of Friedeman & Haugen (1943) was used for pyruvic acid. Samples were diluted to 2 ml. and mixed with 1 ml. of 2% (w/v) 2,4-dinitrophenylhydrazine in 2N-HCl; after 15 min. at 25° an equal volume of 2·5N-NaOH was added. The absorption of the resulting 2,4-dinitrophenylhydrazone was measured at 450 mμ after the colour had fully developed (15 min.). Authentic sodium pyruvate was used as standard and absorption was linear from 0 to 0·35 μmole; test samples outside this range were suitably diluted with 1·25N-NaOH.

Ammonia was distilled from samples (2·5–3·0 ml.), using the apparatus described by Markham (1942), into 0·5% (w/v) boric acid in 20% (v/v) ethanol in water containing a mixed indicator (Conway & O'Malley, 1942); 0·01N-HCl was used for the titration.

Assay of homocysteine. A microbiological method based on the growth requirement of *Escherichia coli* 26/18 was used. This strain responds to homocysteine or methionine but not to cystathionine or cysteine. The materials under test were free from methionine as judged by lack of activity when tested (*a*) in the methionine assay of Gibson & Woods (1960) with *Streptococcus equinus* P 60 and (*b*) with a methionine-requiring auxotroph of *E. coli* (M 200) which does not respond to homocysteine.

The lactones of L- and D-homocysteine were equally active in supporting the growth of the test organism; DL-homocysteine, which was more readily available, was therefore used as standard. The following other possible degradation products of L-cystathionine were inactive when tested at 0·1 and 0·5 mM, either alone or in the presence of pyridoxal phosphate (2·5 μM): cysteine (tested as L), homoserine, serine, alanine, α-aminobutyric acid (all tested as DL).

Medium A (final volume 4 ml. in 150 mm. × 19 mm. tubes) was supplemented with test samples or DL-homocysteine (0·1–0·8 μmole) and autoclaved at 115° for 7 min. The inoculum was about 2×10^3 organisms harvested from a fresh 24 hr culture on tryptic digest of meat agar and incubation was for 40 hr. at 37°. Extent of growth was assessed in an EEL photoelectric colorimeter (Evans Electroselenium Ltd., Halstead, Essex) with a neutral density filter. The dose-response curve (Fig. 1) was linear in the range 0·1–0·3 μmole DL-homocysteine; the curve for a typical experimental sample could be superimposed on the standard curve throughout the range tested (Fig. 1) thus confirming the authenticity of the assay. The mutant showed a tendency to revert to wild type at low frequency and occasional assays failed because of random full growth in tubes containing sub-optimal quantities of homocysteine.

Chromatography. Amino acids were identified by the use of descending chromatograms developed on Whatman no. 1 paper either with butanol + acetic acid (the upper layer of a mixture of *n*-butanol, glacial acetic acid and water in the proportions (v/v) of 4 + 1 + 5) or with phenol + water (160 g. phenol + 40 ml. water). The detecting agent was ninhydrin, and also, in the case of the sulphur-containing amino acids, the platinic iodide reagent of Toennies & Kolb (1951).

Keto acids were identified by a modification of the method of Metzler & Snell (1952 a). The sample (2 ml.) of deproteinized reaction products was mixed with 0·1 ml. of 0·2 % (w/v) 2,4-dinitrophenylhydrazine in 2 N-HCl. After 10 min. the resulting hydrazone was extracted into ethyl acetate and samples placed on Whatman no. 1 paper on spots previously treated with 0·01 ml. phosphate buffer (67 mM, pH 7·4). The buffer neutralized any acid carried over and decreased spreading of the spots. The chromatograms were developed with a mixture of *n*-butanol, water and ethanol (5 + 4 + 1 by vol.) and the hydrazones detected directly as yellow spots; faint spots were seen more easily in ultraviolet radiation.

Chemicals. L-Cystathionine was isolated from the mycelium of *Neurospora crassa* H 98 A essentially as described by Horowitz (1947) who found it to be identical with the synthetic compound, i.e. S-(L-β-amino-β-carboxylethyl) L-homocysteine (Anslow, Simmonds & du Vigneaud, 1946). Our material darkened with decomposition at 280° and gave the same R_f value as a specimen of synthetic L-cystathionine on chromatograms developed with butanol + acetic acid and phenol + water. It also gave an identical dose-response curve to authentic material when tested with an auxotroph of *N. crassa* (38706) which requires cystathionine, homocysteine or methionine for growth. All other amino acids used were obtained commercially.

RESULTS

Nature of the reaction

Identification of products. Homocystine was detected chromatographically (R_f 0·12 in butanol + acetic acid) in the products of a reaction catalysed by cell-free extracts of *Escherichia coli* strain B 166; the R_f value of the original cystathionine was 0·04. The material also supported the growth of *E. coli* strain 26/18, an auxotroph requiring homocysteine or methionine, and there was excellent correspondence between the response curve given by increasing amounts of the sample and of authentic homocysteine (Fig. 1). Such samples were free from methionine as judged by the tests described in the Methods section.

Cysteine was also a possible product of cystathionine metabolism. It was not, however, detected on chromatograms developed with phenol + water nor did samples of the reaction products support the growth of *Streptococcus equinus* P 60 on a medium devoid of cysteine (Barton Wright, 1952); in the latter test it was necessary first to confirm chromatographically that all the cystathionine had been removed since it partially replaces cysteine for the growth of the organism.

The presence of a keto acid or acids in the reaction products was indicated by production of a red colour after treatment of deproteinized samples with 2,4-dinitrophenylhydrazine reagent and making alkaline. Chromatography of the hydrazones (see Methods) showed that the keto acid was pyruvic acid (Table 1); the appearance of two spots for each hydrazone is probably due to the different rates of travel of the *syn*- and *anti*-isomers of the hydrazones (Metzler & Snell, 1952 a). No spots corresponding with the hydrazone of α-ketobutyric acid (another possible product of cystathionine metabolism) were detected. The absorption spectrum (400–600 mμ). given in alkaline solution by the hydrazone formed from the test sample was identical within experimental error with that of the hydrazone of authentic pyruvic

acid (peak 450 mμ) and differed from that of the α-ketobutyric acid derivative (peak 436 mμ).

A volatile base giving the characteristic reaction with Nessler's reagent was also formed during the action of the cell-free enzyme preparation on cystathionine; it was assumed to be ammonia.

Course of the reaction. The rate of production of homocysteine, pyruvate and ammonia was linear and, within experimental error, equimolar throughout the course of the reaction (Fig. 2). There was thus no indication of the accumulation of an intermediate. The final values for homocysteine and ammonia corresponded to 1 mole per mole of cystathionine. The value for pyruvate was slightly lower (0·9 mole) and decreased a little on further incubation suggesting the presence in the preparation of enzymes metabolizing pyruvate slowly. For these experiments a
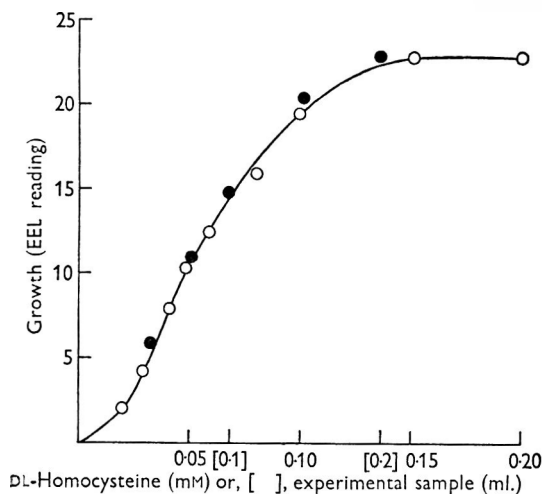
Fig. 1
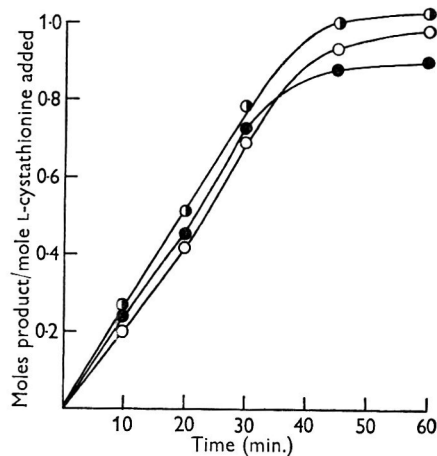
Fig. 2

Fig. 1. Response of *Escherichia coli* strain 26/18 to DL-homocysteine (○) and to a sample of degradation products of L-cystathionine (●); the sample was obtained after incubating for 30 min. the standard reaction mixture containing L-cystathionine (20 μmoles) and an enzyme preparation of *E. coli* strain B 166 (0·5 ml., equiv. 30 mg. dry wt. organism).

Fig. 2. Rate of production of homocysteine (○—○), pyruvate (●—●) and ammonia (◑—◑). The standard reaction mixture (20 ml.) contained L-cystathionine (200 μmoles) and an enzyme preparation of *Escherichia coli* strain B 166 (8 ml., equiv. 400 mg. dry wt. organism).

Table 1. *Chromatography of the 2,4-dinitrophenylhydrazones of keto-acids*

L-Cystathionine (10 μmoles) was incubated for 10 min. in the standard reaction mixture with enzyme derived from *Escherichia coli* strain B 166 (1 ml., equiv. 50 mg. dry wt. organism). Samples of the products and of authentic pyruvate and α-ketobutyrate (each 10 μmoles) were converted to 2,4-dinitrophenylhydrazones and chromatographed as described in the Methods section.

Material	R_f values
Experimental sample	0·52, 0·71
Pyruvate	0·53, 0·74
α-Ketobutyrate	0·67, 0·78

reaction mixture of total volume 20 ml. (in a 100 ml. conical flask) was used and samples (2 ml.) withdrawn at intervals. Since the microbiological assay of homocysteine was time-consuming and occasionally unreliable (see Methods) the production of pyruvate was used henceforward as a routine for estimating the activity of the enzyme; the method is both convenient and sufficiently sensitive. Endogenous control values were less than with ammonia.
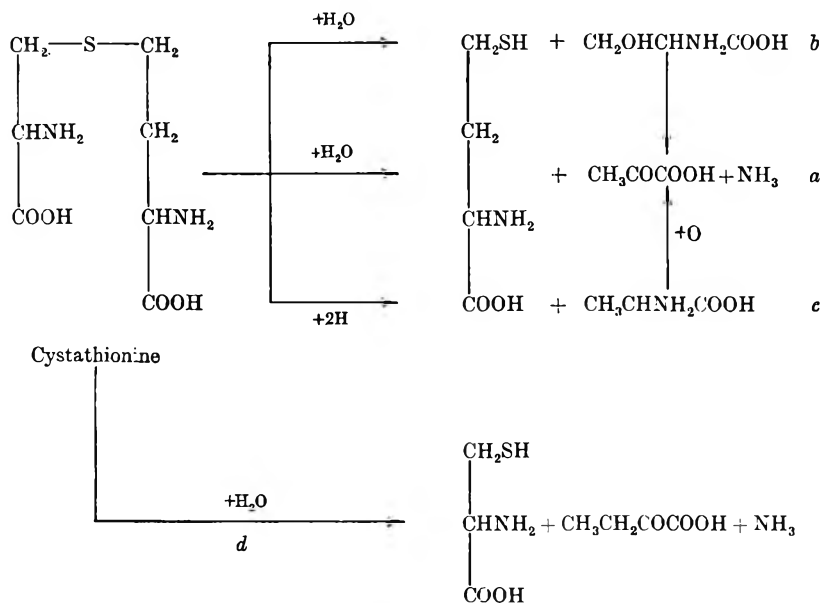

Fig. 3. Possible pathways for the degradation of cystathionine; (a), (b) and (c) refer to the bacterial enzyme while (d) is the pathway probable in animal tissues.

Table 2. *Comparative activity of the enzyme with cystathionine, serine and alanine*

Substrate (10 μmoles of L- or 20 μmoles of DL-) was incubated for 30 min. in the standard reaction mixture with enzyme preparation from *Escherichia coli* strain B 166 (0·5 ml., equiv. 25 mg. dry wt. organism).

	Pyruvate (μmoles) formed in	
Substrate	Expt. 1	Expt. 2
None	0·00	0·00
L-Cystathionine	7·10	4·36
DL-Serine	7·33	8·80
L-Serine	—	< 0·02
D-Serine	—	6·45
DL-Alanine	0·07	—

Overall mechanism of the reaction. There are three possible types of reaction (Fig. 3) by which cystathionine could by the addition of the elements of water give rise to homocysteine, pyruvate and ammonia: (a) Simultaneous fission of the bond linking the S atom to the C atom of the three-carbon chain and deamination of the three-carbon fragment; this would be analogous to the degradation of tryptophan to indole, pyruvate and ammonia by the tryptophanase enzyme of *Escherichia coli*

(Wood, Gunsalus & Umbreit, 1947). (*b*) Initial hydrolytic cleavage of the above S—C bond with formation of homocysteine and serine, the latter being deaminated to pyruvate and ammonia; serine deaminase is known to be present in *E. coli* (Gale & Stephenson, 1938; Wood & Gunsalus, 1949; Metzler & Snell, 1952*b*). (*c*) Initial reductive fission of the above S—C bond yielding homocysteine and alanine, the latter in turn giving pyruvate and ammonia by oxidative deamination; an oxidative deaminase attacking DL-alanine is also present in *E. coli* (Stephenson & Gale, 1937).

Little or no pyruvate was formed from DL-alanine (Table 2) but the production of pyruvate from DL-serine somewhat exceeded that from L-cystathionine. Experiments with the individual amino acid isomers however showed that only D-serine was attacked (Table 2); the fact that D-serine yielded less pyruvate than the equivalent amount of the DL-compound is accounted for by the known impurity of the
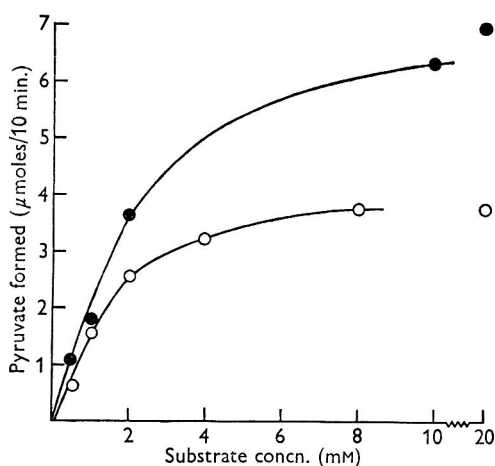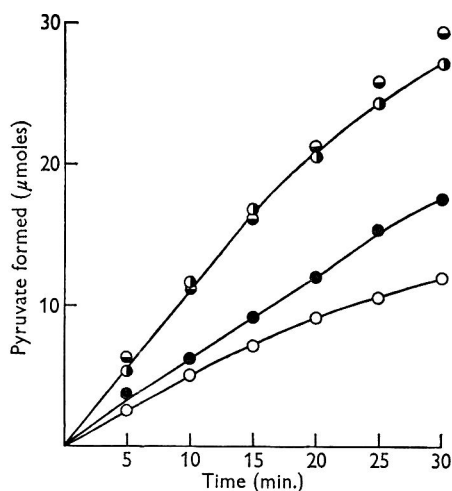

Fig. 4. Effect of the concentration of L-cystathionine (O—O) and DL-serine (●—●) on the initial rate of formation of pyruvate. Cystathionine was omitted from the standard reaction mixture when serine was present: the enzyme preparation (0·4 ml., equiv. 25 mg. dry wt. organism) was obtained from *Escherichia coli* strain B 166 and had been stored at 0° for 4 days.

Fig. 5. Rate of production of pyruvate from L-cystathionine (O—O), DL-serine (●—●) and from a mixture of the two (◐—◐). The points (◓) represent the sum of the individual values for the two substrates. The standard reaction mixture contained 40 μmoles of each or both substrates and an enzyme preparation (0·4 ml., equiv. 25 mg. dry wt. organism) from *Escherichia coli* strain B 166.

specimen of D-serine used. L-Cystathionine would be expected to yield L-serine or L-alanine; since neither was attacked by an enzyme preparation which formed pyruvate actively from L-cystathionine, it is clear that neither can be a free intermediate in the reaction. This would exclude mechanisms (*b*) and (*c*) (Fig. 3) and direct formation of homocysteine, pyruvate and ammonia (*a*) is therefore probable.

The relation between substrate concentration and initial velocity of the reaction was measured (Fig. 4) both for L-cystathionine and for D-serine (supplied as the DL-compound); the K_m values were 1·2 and 1·0 mM, respectively. The rate of for-

mation of pyruvate was linear from each substrate but, when both were present, each at a concentration (20 mM) sufficient fully to saturate the enzyme, the rate was clearly additive (Fig. 5). It is likely, therefore, that cystathionine and D-serine deaminase activities are not separate functions of the same enzyme. This conclusion is supported by the fact that the ratio of the two activities was different with different enzyme preparations (e.g. Table 2) and that, on prolonged storage at 0°, cystathionase activity was lost more rapidly than that of D-serine deaminase; both enzymes, however, were stable for 2 weeks. Almost complete separation of the two activities was finally achieved by maintaining the preparation at 55° in phosphate buffer (pH 7·4, 67 mM). After 12 min. 35 % of the D-serine deaminase was lost but cystathionase was unaffected; the former enzyme was entirely lost after 50 min. while about 25 % of the cystathionase activity remained.

Table 3. *Effect of pyridoxal phosphate and magnesium ions on the degradation of cystathionine*

The standard reaction mixture contained L-cystathionine (10 μmoles) and (where indicated) pyridoxal phosphate or pyridoxal (20 μmoles), ATP (2 μmoles) and metal ions (100 μmoles). The enzyme (1 ml., equiv. 40 mg. dry wt. organism) was a preparation of *Escherichia coli* strain B 166 which had been resolved as described in text. Incubation was at 37° for 30 min.

Additions		Pyruvate formed
'Coenzyme'	Metal ion	(μmoles)
—	—	0·01
Pyridoxal phosphate	—	0·30
—	Mg^{2+}	0·18
Pyridoxal phosphate	Mg^{2+}	2·10
Pyridoxal phosphate	Mn^{2+}	0·52
Pyridoxal phosphate	Zn^{2+}	0·37
Pyridoxal + ATP	Mg^{2+}	0·18

Effect of pyridoxal phosphate. Initial experiments were carried out with enzyme preparations of *Escherichia coli* strain B 166 harvested after growth in absence of vitamin B_6 derivatives (see Methods) and therefore presumably deficient in this vitamin. Suspensions of the organism grown similarly have been shown to require vitamin B_6 absolutely for the conversion of homocysteine to methionine, with serine as source of the one-carbon residue (Wijesundera, Cross & Woods, 1960). In the present case pyruvate formation from cystathionine by the cell-free enzyme was increased 50–100 % by pyridoxal phosphate (5 μM). Complete resolution of the enzyme was achieved by storing a preparation for five days at 0° followed by exposure in phosphate buffer (pH 7·4, 67 mM) at 0° to bright sunlight for 5 hr. over a period of 2 days. The enzyme was then precipitated by saturation with ammonium sulphate and the precipitate redissolved in water and dialysed for 20 hr. at 4° against running water. The preparation now had negligible activity but was activated by a mixture of pyridoxal phosphate and magnesium ions (Table 3); alone each had only a small effect. Pyridoxal plus adenosine triphosphate did not replace pyridoxal phosphate. Manganous and zinc ions replaced magnesium only partially. It was then found that complete resolution with regard to pyridoxal phosphate could be obtained with enzyme preparations of *Escherichia coli* F simply by dialysis after storage for 5 days at 0°; the requirement for magnesium ions was not shown. With

such preparations cystathionase activity increased almost linearly with pyridoxal phosphate concentration from 0 to 10 μM (Fig. 6); the K_m was about 8 μM.

Temperature and pH. The optimum temperature for the cystathionase reaction in phosphate buffer at pH 7·4 was 42°; activity decreased to 26 % at 30° but only to 86 % at 50°. The effect of pH was studied in three buffers (Fig. 7); the optimum pH in tris buffer (8·6) was rather higher than in phosphate and borate (8·2).

Inhibitors. Sulphydryl compounds (cysteine, homocysteine, reduced glutathione) markedly inhibited cystathionase activity though sulphide itself had only a small effect (Table 4); homocysteine is also of course one of the reaction products. Isonicotinic acid hydrazide, which is sometimes regarded as an antimetabolite of the vitamin B_6 group, was also strongly inhibitory; cyanide and cupric ions caused total inhibition (Table 4).
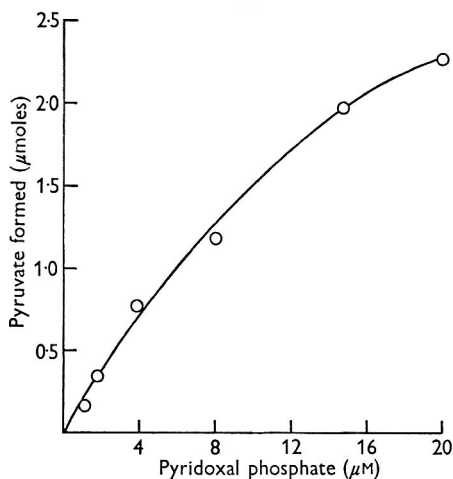

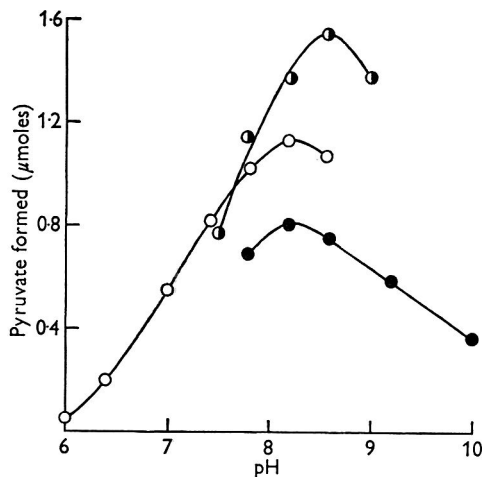Fig. 6   Fig. 7

Fig. 6. Effect of the concentration of pyridoxal phosphate on cystathionase activity. The standard reaction mixture was modified by increasing the pH value to 8·2 and the amount of Mg^{2+} to 0·1 m-mole. The enzyme preparation (0·5 ml.; equiv. 25 mg. dry wt. organism) was obtained from *Escherichia coli* strain F and had been dialysed after storage for 5 days at 0°. Pyridoxal phosphate was incubated for 10 min. with the reaction mixture before adding L-cystathionine (10 μmoles) and incubating at 37° for a further 30 min.

Fig. 7. Effect of pH value on the activity of cystathionase in phosphate (O—O), borate (●—●) and tris (◐—◐) buffers. The phosphate in the standard reaction mixture was replaced as required by borate or tris of equivalent concentration. The enzyme preparation (0·5 ml. from equiv. 25 mg. dry wt. organism, of *Escherichia coli* strain F) had been dialysed to remove phosphate. Time of incubation (37°) 15 min.

Cystathionase in other strains of Escherichia coli

Escherichia coli strain 122/33 is a methionine auxotroph which also grows when given homocysteine or cystathionine; as expected, it contained the cystathionase enzyme though this was rather less active (or less easily extracted) than that of strains B 166 and F (Table 5). The main interest, however, centred on methionine auxotrophs which respond to homocysteine but not to cystathionine. Such organisms (e.g. strain 26/18) would be expected not to contain the enzyme; this

proved to be the case (Table 5). Many cases are known, however, in which the genetic block is incomplete or in which the lesion results in an enzyme with changed properties (Mitchell, 1953). The properties of strain 26/18 were therefore re-examined under a variety of conditions. Cystathionine did not support growth when glucose (medium A) was replaced by lactate (medium L) as sole carbon source or when incubation was at 30° instead of 37° on either medium. Furthermore, no cysta-thionase activity was detected in cell-free extracts at any pH value between 6 and

Table 4. *Inhibition of cystathionine degradation*

The standard reaction mixture contained L-cystathionine (10 μmoles) and enzyme derived from *Escherichia coli* strain B 166 (0·25 ml., equiv. 12 mg. dry wt. organism) and was incubated for 15 min. in the presence or absence of the substances indicated. Unless otherwise stated, sodium salts were used. Results are expressed as % decrease in pyruvate formed compared with controls containing no inhibitor.

Addition	Concn. (mM)	Inhibition (%)
Cyanide	1	100
Copper sulphate	1	100
Isonicotinic acid hydrazide	1	87
Glutathionine (reduced)	5	56
DL-Homocysteine	10	65
L-Cysteine	10	45
Azide	10	20
Sulphide	10	11
Fluoride	10	0
Arsenate	10	0

Table 5. *Cystathionase activity of extracts of strains of* Escherichia coli

The standard reaction mixture contained 0·25 ml. (equiv. 13 mg. dry wt. organism) of cell-free extracts derived from the *E. coli* strains indicated and was incubated at 37° for 15 min. The results given are the mean values from at least three experiments.

Strain of *E. coli*	Growth requirement	Cystathionase activity (μmoles pyruvate/mg. dry wt. organism/hr.)
F	None; prototrophic	1·63
B 166	Pryridoxal, serine or glycine	1·15
122/33	Methionine, homocysteine or cystathionine	0·85
26/18	Methionine or homocysteine	0·00*

* No activity was again observed when the amount of enzyme preparation and the time of incubation were both increased fourfold.

10, in phosphate or borate buffer as appropriate. Possible lack of an additional cofactor was tested by supplementing the reaction mixture with adenosine-5'-phosphate, glutathione, biotin and yeast extract (either singly or as a mixture) or with a heated extract of a known active strain (122/33) of *Escherichia coli*; in no case did any cystathionase activity result. Adenosine-5'-phosphate and glutathione have been found to reactivate certain amino acid deaminases (Wood & Gunsalus, 1949). Apparent lack of the enzyme might also be due to the presence of an inhibitor. One

batch of the cell-free extract was exhaustively dialysed whilst another was fractionated with 50 % ammonium sulphate and both fractions tested after dialysis. All three dialysates were inactive even on addition of pyridoxal phosphate and magnesium ions. Cell-free extracts of the inactive strain (26/18) did not affect the formation of pyruvate from cystathionine by cell-free extracts of an active strain (122/33). This experiment shows not only that strain 26/18 does not contain a dissociable inhibitor but also that it does not contain some additional enzyme which destroys cystathionase itself. Although the possibility cannot be excluded that strain 26/18 contains cystathionase inactivated by an inhibitor which does not dissociate, it is more likely that the enzyme is not formed by this auxotroph.

DISCUSSION

The properties of methionine auxotrophs of *Escherichia coli* suggest strongly that cystathionine is an intermediate in the biosynthesis of methionine by this organism (see Introduction). The experiments of Bolton, Cowie & Sands (1952), who used an isotope tracer technique, however, cast doubt on this view; the addition of unlabelled cystathionine to cultures growing with [^{35}S]sulphate as sole source of sulphur did not, as would be expected, decrease the extent of incorporation of ^{35}S into methionine. The present work shows that *E. coli* has active enzymic mechanisms for the conversion of cystathionine to homocysteine which is the undoubted immediate precursor of methionine. Other work in this laboratory (Rowbury, 1961, 1962) shows that *E. coli* also has enzymes for the synthesis of cystathionine from precursors (homoserine + cysteine) indicated by nutritional studies with mutants of *E. coli* and other micro-organisms. There remains little doubt therefore that cystathionine is an intermediate and that the results of the isotope experiments must be explained in some other way; it is possible, for example, that exogenous cystathionine may not equilibrate with the cystathionine formed within the organism and perhaps remains tightly bound to the enzyme systems which catalyse its synthesis and further metabolism. The cystathionase was, as expected, not found in a methionine auxotroph of *Escherichia coli* which responded to homocysteine but not to cystathionine; it was also absent from a similar auxotroph of *Neurospora crassa* (Fischer, 1957).

Cystathionine could be degraded by the cleavage of the bond which unites the S atom to: either (1) the three-carbon chain with formation of homocysteine, pyruvate and ammonia (Fig. 3, *a*); or (2) the four-carbon chain, with formation of cysteine, α-ketobutyrate and ammonia (Fig. 3, *d*). Mechanism (2) operates in animal tissues while both mechanisms appear to occur in *Neurospora crassa* and *Proteus morganii* although (1) predominates (see Introduction). In the present work no evidence for mechanism (2), even as a minor pathway, was found with cell-free extracts of *Escherichia coli*. It remains possible that such an enzyme is present, but is destroyed during the preparation of the cell-free extract. There is, however, a stable and highly active cystathionase which catalyses mechanism (1).

The cystathionase of *Escherichia coli* was obtained free from enzymes which form pyruvate from possible initial intermediates (L-serine, L-alanine) formed by hydrolytic or reductive fission of the S—C bond (Fig. 3*b*, *c*). Direct formation of homocysteine, pyruvate and ammonia is therefore postulated (Fig. 3*a*), though it

remains possible that the unstable α-aminoacrylic acid, $CH_2:C(NH_2)COOH$, might be formed transiently; there is isotopic evidence that this compound is an intermediate during the formation of tryptophan from serine and indole by preparations of *Neurospora crassa* (Tatum & Shemin, 1954). Matsuo & Greenberg (1959 b), who, using a highly purified crystalline cystathionase from animal tissues and cystathionine labelled with ^{14}C at the x-position in the four-carbon chain, obtained no evidence for free homoserine as an intermediate in the formation of α-ketobutyric acid, which is the product in this case (Fig. 3 d); these authors also postulated an unsaturated amino acid, vinyl glycine $(CH_2:CH.CH(NH_2)COOH)$, as a transient intermediate.

The cystathionase of *Escherichia coli*, like that of the similar enzyme of *Proteus morganii* (Binkley & Hudgins, 1953) and the other type of cystathionase in mammalian liver (Matsuo & Greenberg, 1959 a), required pyridoxal phosphate as coenzyme. Preparations from one strain of *E. coli* (B 166) also required magnesium ions, but this was not demonstrated with either strain F in the present work or with other strains (R. J. Rowbury, unpublished observations). It is possible that the extracts of *E. coli* strain B 166 contained traces of inhibitory metal ions whose toxicity was overcome by magnesium; Binkley (1950) found that magnesium abolished the inhibitory effect of cupric and ferric ions on a purified thionase enzyme. A non-enzymic cleavage of L-cystathionine to homocysteine, pyruvate and ammonia at 37° and catalysed by pyridoxal was observed by Binkley (1955); the other conditions necessary were a pH value of 9 and the presence of cupric ions and a chelating agent.

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Complex Flagellar Phases in Salmonella

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SUMMARY

Salmonella cultures in which loss of a major antigenic component resulted in the formation of stable serotypes of simpler constitution, previously were thought to have lost a third flagellar phase. Further study of cultures of *Salmonella hamilton. S. worthington*, and *S. meleagridis of* complex constitution indicated that loss of a third phase was not involved but that instead the organisms lost a major antigenic constituent present in both phases of diphasic types and in the single flagellar phase of monophasic types. Attention was drawn to the similarity of their behaviour to that of *S. salinatis*, *S. montgomery*, and certain other salmonellae of complex constitution.

INTRODUCTION

Flagellar variation in Salmonellas has been the subject of numerous studies since Andrewes (1922) first described specific and non-specific phases. Until recently, natural, reversible phase variation involved only serotypes possessing two alternative flagellar (H) antigens. This diphasic nature has been seen in numerous Salmonella serotypes as well as in the Arizona group. Taylor, Lee, Edwards & Ramsey (1960) mentioned the presence of three flagellar phases in a culture of *Salmonella worthington* with the antigenic formula 1, 13, 23, 37:z:1, w:z_{43}, about the same time that Le Minor & Edwards (1960) found that *S. hamilton* (3, 15:z_{27}) of Moran & Edwards (1958) also possessed the H antigens *e, h* and 1, 2 of *S. georlitz.* These Salmonella cultures were isolated in their 'third' phases, z_{43} and z_{27} respectively, but unlike readily reversible types, were changed 'irreversibly' to well known diphasic serotypes by passage through semisolid medium containing serum active on the third phase. In the ensuing discussion the terms 'irreversible variation' and 'permanent loss variation' are used to characterize the loss of an antigenic component which subsequently could not be recovered or could be recovered only in rare instances and with some difficulty.

A form of phase variation has been encountered occasionally in Salmonella cultures in which a common major antigenic component is present in each phase of a diphasic organism. This common component can be permanently lost, either spontaneously or through cultivation in semisolid medium containing agglutinins for that factor. *Salmonella salinatis* (4, 12:d, e, h:d, e, n, z_{15}) of Edwards & Bruner (1942) and *S. montgomery* (11:d, a:d, e, n, z_{15}) of Edwards, Kauffmann & Huey (1957) possess the major flagellar antigen d in both phases and on irreversible loss variation yield typical strains of *S. san diego* (4, 12:e, h:e, n, z_{15}) and *S. luciana* (11:a:e, n, z_{15}), respectively. Similar variations involving d as the common H

antigen were described in sucrose fermenting Salmonella-like organisms by Edwards, Moran & Bruner (1948) and Edwards (1950). A culture having a hitherto unrecognized flagellar antigen as the major component of both phases was reported recently (Edwards, McWhorter & Douglas, 1962). This serotype (6, 7:z_{49}, r:z_{49}, 1, 5) was changed irreversibly by passage through semisolid agar containing z_{49} serum to typical $S.$ $infantis$ (6, 7:r:1, 5). The present paper is a re-evaluation of the reportedly triphasic $S.$ $worthington$ and $S.$ $hamilton$ cultures, and presents findings of similar characteristics in a recently studied culture of $S.$ $meleagridis$ having three naturally occurring flagellar antigens.

METHODS

The cultures studied consisted of the stock strains of $Salmonella$ $hamilton$ (CDC 1833–57) isolated from sick turkey poults at the Texas Agricultural Experiment Station; $S.$ $worthington$ (CDC 787-60) isolated from bone meal at the Salmonella Reference Laboratory, Colindale; and $S.$ $meleagridis$ (CDC 4946–61) isolated and identified by Dr S. Hofmann at the Robert Koch Institute, Berlin. Biochemical and serologic studies were performed as described by Edwards & Ewing (1962). All H antigen determinations were done on single colony isolations. Changes in flagellar phases were accomplished by the inoculation of tubes of semisolid medium containing appropriate antisera with single colony growth. When a change in phase occurred, the culture was plated and isolated single colonies selected for further study.

RESULTS

$Salmonella$ $hamilton$ (CDC 1833–57). The biochemical characteristics and antigenic constituents (3, 15; e, h;1, 2; and z_{27}) of this culture were reported by LeMinor & Edwards (1960) and confirmed in this study. After plating, over 100 single colonies were examined by slide agglutination with suitable dilutions of e, h; 1, 2;

Table 1. *Agglutination reactions of H antigens of* Salmonella hamilton, 1833-57

	Sera		
Antigens	*S. reading* phase 1 (e, h)	*S. paratyphi B* phase 2 (1, 2)	*S. simsbury* (z_{27})
1833–57 phase 1	< 50	< 50	6,400
1833–57 phase 2	< 50	6,400	3,200
1833–57 e, h variant	6,400	< 50	< 50
1833–57 1, 2 variant	< 50	12,800	< 50

Figures indicate highest dilution in which agglutination was observed.

and z_{27} sera. All were agglutinated well by z_{27} ($S.$ $simsbury$) serum at a dilution eliminating any O reaction. In addition, a large number of the same colonies also were agglutinated by 1, 2 ($S.$ $paratyphi$ B, phase 2) serum. None was agglutinated by e, h ($S.$ $reading$, phase 1) serum. Similar numbers of colonies reacting only with z_{27} serum and of those reacting both with z_{27} and 1, 2 sera were picked and H antigens prepared from each. These antigens were titrated with e, h, 1, 2 and z_{27} sera, the results obtained with a representative colony of each type being given in Table 1.

A portion of each colony used for tube-agglutination tests also was placed in

semisolid medium containing z_{27} serum. Migration through the serum occurred slowly in the colonies agglutinating only with z_{27} serum, over one-half of such colonies requiring two or three serial passages before another antigen migrated well through the medium. Sixteen colonies gave rise to antigens agglutinated to titre by e, h serum, but unaffected by 1, 2 and z_{27} sera. Six colonies yielded antigens which reacted to the titre of *S. paratyphi B*, phase 2 serum (1, 2), but which were unaffected by e, h or z_{27} serum. One colony had not migrated through z_{27} serum after three passages. On the other hand, of 25 colonies which reacted both with 1, 2 and z_{27} sera, practically all passed through the semisolid medium in 24–48 hr., giving rise to 1, 2 variants in 22 cases and e, h variants in three. The reactions of typical e, h, and 1, 2 forms isolated after passage through z_{27} serum are shown in Table 1.

It was apparent that the original culture of *Salmonella hamilton* had two distinct H phases instead of three and that each phase possessed a common major antigenic component, z_{27}. Phase 1 was agglutinated by z_{27} serum in high titre, and, although not agglutinated by e, h serum, tended primarily to produce e, h forms as its loss variant. Phase 2 was agglutinated actively by z_{27} and 1, 2 sera and yielded 1, 2 forms as its loss variant. Spontaneous phase variation had occurred in the majority of the original z_{27} and z_{27}, 1, 2 colonies when they were held on agar slants for 6 weeks after isolation. The e, h and 1, 2 variants isolated after passage through z_{27} serum readily were reversible one to the other on cultivation in appropriate sera, but such phases were immoblilized in semisolid medium containing both e, h and 1, 2 sera. Serial passage over a period of a month failed to produce antigenic change. This suggested that from a diphasic organism of complex form having the antigenic formula 3, 15:z_{27}, (e, h):z_{27}, 1, 2, a typical *S. goerlitz* (3, 15:e, h:1, 2) had been obtained, and that this did not readily revert to the original form.

Table 2. *Agglutination reactions of H antigens of* Salmonella worthington, 787-60

Antigens	Sera		
	S. poona, phase 1 (z)	*S. worthington*, phase 2 (1, w)	*S. senftenberg*, 'phase 2' (z_{43})
787–phase 1	< 50	< 50	12,800
787–60 phase 2	< 50	12,800	6,400
787–60 z variant	12,800	< 50	< 50
787–60 1, w variant	< 50	12,800	< 50

Figures indicate highest dilution in which agglutination was observed.

Salmonella worthington (CDC 787-60). Aside from failure to produce H_2S, this culture was biochemically identical with usual cultures of the serotype. The antigenic constituents (1, 13, 23, 37; z; 1, w; and z_{43}) were as described by Taylor *et al.* (1960). An investigative procedure similar to that used with *S. hamilton* was followed. Using appropriately absorbed and diluted z (*S. poona*, phase 1), 1, w (*S. worthington*, phase 2), and z_{43} (*S. senftenberg*, 'phase 2') sera, slide-agglutination tests revealed two antigenically different types of colonies and these were confirmed by expanded tube tests. All colonies were agglutinated by z_{43} serum to high titres, and approximately 50 % were agglutinated strongly by 1, w serum as well. No colonies were agglutinated by absorbed z serum. Results with representative colonies are shown in Table 2.

In contrast to *Salmonella hamilton*, loss variation in this culture occurred spontaneously and without difficulty. Passage through one tube of plain semisolid medium without serum resulted in the loss of the z_{43} component. Twenty-one colonies of each type were inoculated directly into tall tubes of plain semisolid medium. All 21 colonies which were agglutinable only by z_{43} serum gave rise to a form which reacted to titre in z serum, but which was unaffected by either z_{43} or 1, w serum. All colonies with z_{43} and 1, w antigens yielded forms which were agglutinated to the titre of 1, w serum but which were not agglutinated by z_{43} or z serum (Table 2). The resulting z and 1, w phases were mutually reversible when cultivated in appropriate sera and, in fact, exhibited spontaneous variation on several occasions. Inoculation into semisolid medium containing z + 1, w sera immobilized these phases during serial passages over a 1-month period. Spontaneous phase variation, $z_{43}(z) < \ldots \ldots > z_{43}$, 1, w, was demonstrated in a number of original single colonies 6–16 weeks after isolation.

It seemed apparent from these results that the previously termed 'triphasic' *Salmonella worthington* possessed two flagella phases containing a common major antigen, had the formula 1, 13, 23, 37:z_{43}, (z): z_{43}, 1, w and gave rise to a loss variant, 1, 13, 23, 37:z:1, w from which the z_{43} antigen was not recovered.

Salmonella meleagridis (CDC 4946-61). The culture possessed the usual biochemical properties of the type. It was correctly typed as *S. meleagridis* with an additional H antigen, z_{43} (3, 10; e, h; 1, w; and z_{45}) by Dr Hofmann and confirmed by Dr Kauffmann and this laboratory. Serologic examination of over 100 single colonies

Table 3. *Agglutination reactions of H. antigens of* Salmonella meleagridis,
4946-61

	Sera		
Antigens	S. reading, phase 1 (e, h)	S. worthington phase 2 (1, w)	S. senftenberg, 'phase 2' (z_{45})
4946–61 phase 1	200	< 50	12,800
4946–61 phase 2	< 50	12,800	6,400
4946–61 e, h variant	6,400	< 50	< 50
4946–61 1, w variant	< 50	12,800	< 50

Figures indicate highest dilution in which agglutination was observed.

by slide agglutination with appropriately absorbed and diluted e, h; 1, w; and z_{45} (*S. senftenberg*, 'phase 2') sera, and expanded tube tests with selected colonies, yielded results similar to those obtained with the two aforementioned cultures. All colonies were agglutinated to high titres by z_{45} serum. Approximately two-thirds of the colonies also were agglutinated in low dilutions of e, h serum. The remainder were agglutinated by high dilutions of 1, w serum. The former (z_{45}, e, h) was designated phase 1 and the latter (z_{45}, 1, w), phase 2. Results of typical agglutinations of the two phases are shown in Table 3.

Similar numbers of colonies of each phase were inoculated into semisolid medium which contained z_{45} serum. General spreading in the medium was observed within 24–48 hr. Of the 31 phase 1 colonies, 27 gave rise to typical e, h variants, two yielded both e, h and 1, w colonies, and two gave rise to 1, w forms. Of 28 phase 2 colonies, 21 gave rise to typical 1, w variants, five gave a mixture of e, h and 1, w colonies

and only one yielded e, h forms. The reactions of typical e, h and l, w variants are shown in Table 3. Spontaneous phase variation (z_{45}, e, h $< \ldots \ldots > z_{45}$, l, w) was noted in practically all single colonies eight weeks after isolation. Loss variants produced by passage through z_{45} serum, readily were reversible between the e, h and l, w phases. Spontaneous loss of the z_{45} component was found only in three colonies after passage through plain semisolid medium. As with the other two cultures, the common major antigen was not recovered once it was lost. Both e, h and l, w phases were immobilized when placed in semisolid medium containing e, h + l, w sera, even after several passages over a six-week period. CDC 4946-61 was represented by the antigenic formula 3, 10:z_{45}, e, h:z_{45}, l, w and gave rise to loss variants identical with typical *Salmonella meleagridis* (3, 10:e, h:l, w).

<center>DISCUSSION</center>

Using CDC 4946-61 as an example, Fig. 1 shows the type of antigenic variation found in the three cultures studied. In all respects, the behaviour of these cultures resembled that of *Salmonella salinatis*, *S. montgomery*, and the complex form of *S. infantis* mentioned above and, therefore, they should not be considered as triphasic serotypes. All possessed three flagellar antigens, but behaved as diphasic organisms. One antigen was common to, and prominent in, both phases, and once lost it was not recovered. In all instances phase 2 of these complex organisms contained the component which was present in the simpler loss variant in easily demon-
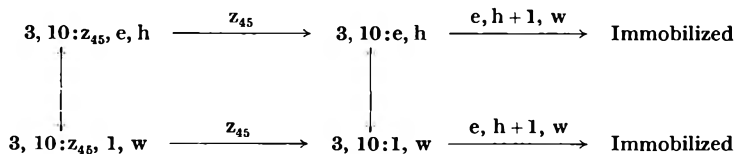
Arrows indicate direction of changes in phase. Symbols on arrows indicate antisera added to produce change. No symbol indicates change occurs spontaneously.

<center>Figure 1. Phase variation in *Salmonella melagridis*, 4946–61.</center>

strable form. On the contrary, the component of phase 1 which emerged in the loss variants was present in small amount or in an inapparent form, and became a major antigen only after passage through appropriate serum. Of all the complex cultures studied to date, only in *S. hamilton* and *S. worthington* was the minor antigen of phase 1 not demonstrable in the original colonies at serum dilutions of 1/50. However, some single colonies of the other strains also were found which were not agglutinated by serum for the minor phase 1 component at 1/50. Because this component appeared readily after serum passage, even when not initially evident by titration, it was felt that the antigenic symbol should be included as a phase 1 constituent.

The question arises as to the nature of the flagellar variation described by Taylor *et al.* (1960) in which the flagellar antigens of a 'diphasic' Salmonella varied only in one direction. Cultures usually found in monophasic g, s, t forms, as *Salmonella kingston*, *S. senftenberg*, *S. westhampton*, and *S. halmstad*, were isolated with 'phase 2' antigens, z_{43}, z_{45}, or z_{46}. On transformation to the g, s, t phase these cultures could not be reverted to the originally isolated phase, but behaved as

typical g, s, t cultures. Similar 'one-way' variation in usually monophasic strains has been noted in *S. simsbury* containing z_{27} antigen (Edwards, Moran & Bruner, 1947), *S. wichita* with z_{37}, *S. cubana* with z_{37}, *S. alachua* with z_{37} or z_{45}, and *S. california* with z_{45} (unpublished data). It is quite probable that this type of flagellar variation in typically monophasic serotypes is analogous to the complex antigenic structure of *S. hamilton*, *S. salinatis*, etc. Thus, a 'diphasic' z_{43} form of *S. senftenberg*, for example, should be considered monophasic, i.e. 1, 3, 19:z_{43}, g, s, t: rather than 1, 3, 19: g, s, t:z_{43}, possessing a complex phase with z_{43} as the major irreversible component and g, s, t as the minor antigen. Attempts have been made to demonstrate g, s, t antigens in such a complex but the appearance of spontaneous loss variants in the strains examined rendered it difficult to differentiate minor antigenic components of a complex phase from small proportions of loss variants. The problem might be solved by transduction of complex phases *in toto* to diphasic serotypes which possessed other H antigens. This was attempted by using high titred temperate phages cultivated on $z_{43}(z)$ forms of *S. worthington* and on z_{43}, g, s, t; z_{45}, g, s, t; and z_{46}, g, s, t forms of *S. senftenberg*. Numerous attempts to transduce these antigens failed.

A discussion of the possible origin and significance of such antigenically complex forms has been given in a recent report by Edwards, McWhorter & Douglas (1962) and will not be considered here.

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