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The Mucopeptides of Bacterial Cell Walls. A Review

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The cell walls are rigid insoluble structures of bacteria. Attempts at isolating walls were first made last century (Vincenzi, 1887), and there was much speculation about their chemical nature—chiefly as to whether they consisted of chitin or cellulose, materials known to provide rigid structures in other organisms. Even 10 years ago practically nothing could be said on the subject except that the walls were not chitin or cellulose. Since then we have achieved at least the beginning of an answer (see Reviews, Salton, 1956*a*; Work, 1957; Zilliken, 1959); but it will be long before we have the full story for all types of bacteria. The first information about the chemical constituents of bacterial walls was in a report by Holdsworth (1951) that phenol-insoluble residues from *Corynebacterium diphtheriae* contained nearly all the glucosamine and α,ϵ-diaminopimelic acid of the organism, and that these compounds were associated with a polysaccharide. The perfection of techniques for preparation of walls (Salton & Horne, 1951), by mechanical disintegration and differential centrifugation, enabled apparently homogeneous samples to be examined. Walls of streptococci were shown simultaneously by Salton (1952*a*) and McCarty (1952) to contain about 70 % of polysaccharides and hexosamines and a further component consisting of only 10 amino acids, of which lysine, alanine and glutamic acid preponderated. Rhamnose was the main polysaccharide; in fact, over 90 % of the total rhamnose of the cell was in the wall. Further work by Salton showed that walls of other species also had an unusual composition, which was not then understood.

The problem was attacked systematically by Cummins & Harris (1956*a*, *b*, 1958; Cummins, 1956) who examined walls of over a hundred Gram-positive bacteria. The separated walls were treated with trypsin and ribonuclease, and were thus freed from wall proteins and cytoplasmic constituents. A resistant residue was left; its main components, identified by paper chromatography of acid-hydrolysates, were invariably the two hexosamines glucosamine and muramic acid, and three amino acids, glutamic acid, alanine and either lysine or diaminopimelic acid; in some cases there were also up to five sugars, one or two other amino acids, or galactosamine. A recurring type of 'basal unit' in Gram-positive cell walls was soon recognized (Work, 1957). This 'basal unit' can be termed a 'mucopeptide'; evidence for its existence will be presented in this review. It contains the aforementioned hexosamines, glucosamine and muramic acid, and three amino acids bound in peptide linkage. In addition, each bacterial genus and even each species often has a characteristic pattern of amino acids, amino sugars and sugars superimposed on the basal mucopeptide unit. This pattern may prove of value in bacterial classification.

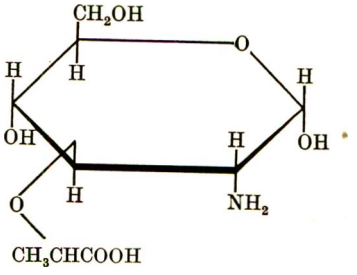
The basal unit was apparent from the results of Cummins & Harris, because they had, so to speak, stripped the meat off the walls with enzymes, leaving only the rigid framework. However, these results give an over-simplified idea of cell-wall structure, and it should be realized that freshly separated walls of Gram-positive organisms may contain up to 40 % of their weight of protein and other compounds. For example, the teichoic acids, which are polyribityl phosphates, were found by Armstrong *et al.* (1958) to make up as much as 30 % of the walls of certain Gram-positive organisms. The walls or 'coats' of bacterial spores also contain mucopeptide units with considerable proportions of proteins and other unidentified substances (Strange & Powell, 1954; Strange & Dark, 1956; Salton & Marshall, 1959).

The walls of Gram-negative bacteria are more complex than those of Gram-positive bacteria; in addition to polysaccharide, the trypsin-treated walls contain a high proportion of lipid and most of the amino acids found in proteins (Salton, 1956*a*). The specific mucopeptide constituents, glucosamine, muramic acid and diaminopimelic acid, are also present, but their overall concentrations are lower than in the walls of Gram-positive organisms. The walls of *Escherichia coli* appear to have three layers visible with the electron microscope (Kellenberger & Ryter, 1958). Chemically, they can be separated into an outer pliable lipoprotein coat very soluble in phenol, and an inner insoluble rigid layer containing mucopeptide constituents; a further less soluble constituent is a lipopolysaccharide which is probably the site of the O-antigen (endotoxin) (Weidel & Primosigh, 1958; Weidel, Frank & Martin, 1960; Westphal, 1960). The mucopeptide components are different from those of walls of Gram-positive bacteria in that they contain both diaminopimelic acid and lysine.

The rigidity and insolubility of at least part of the walls of nearly all types of bacteria suggest that the mucopeptide is highly polymerized and perhaps highly cross-linked. Owing to this insolubility, the chemical homogeneity of the rigid portions of wall preparations cannot be assessed, nor can chemical information on their undegraded structures be obtained directly. However, it is possible to purify soluble subunits of the rigid portions, and their analysis has produced evidence for the mucopeptide structures.

Structure and metabolism of some specific constituents of mucopeptides

Several constituents of bacterial mucopeptides have not been found elsewhere in nature, and may be specific to these structures. Muramic acid was isolated by Strange (1956) from a product obtained from the exudates of germinating spores of *Bacillus megaterium*; it is glucosamine in ether linkage through its 3-position with lactic acid (Strange & Kent, 1959):



Muramic acid

Muramic acid is a component of walls of all bacteria, but has not yet been identified in other types of organisms. It probably originates from glucosamine (Zillikin, 1959; Richmond & Perkins, 1960*c*).

Whenever the optical configuration of the amino acids in Gram-positive cell walls has been examined, almost all the glutamic acid and at least 50 % of the alanine have been identified as the so-called 'unnatural' D-isomers (Ikawa & Snell, 1960; Salton, 1957*b*). D-aspartic acid has also been found (Toennies, Bakay & Shockman, 1959), but never D-lysine. Rydon (1948) stated that D-amino acids are certainly present in bacterial extracellular products such as antibiotics or capsular polyglutamic acids, but that they had not been encountered in the intracellular components of micro-organisms. Now, only 12 years later, we know that certain D-amino acids are major components of many bacterial walls, which themselves make up 25 % or more of the total dry weight of the organisms. The origin of these D-amino acids can be traced partly to the action of alanine racemase, a widely distributed bacterial enzyme. In *Bacillus* species, D-glutamic acid originates from L-glutamic by a series of reactions. A transamination between L-glutamic and pyruvic acids gives L-alanine, which is then racemized to the racemic mixture; of this, the D-alanine transaminates with α-oxoglutarate to form D-glutamic acid and pyruvic acid (Thorne, Gomez & Housewright, 1955; Thorne, 1956). The transaminase responsible for the last reaction is stereospecific, and will only carry out the reverse step with D-glutamic or D-aspartic acids, the L-isomers being inactive. A glutamic acid racemase has recently been isolated from *Lactobacillus arabinosus* (Glaser, 1960), but its significance in wall metabolism is unknown.

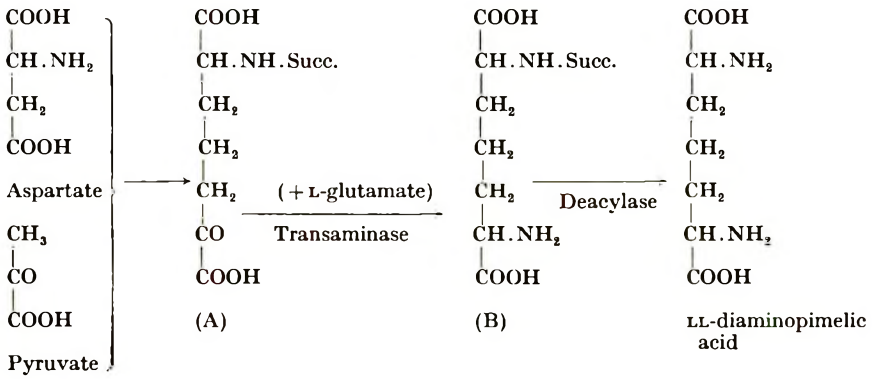
α,ϵ-Diaminopimelic acid is another compound specific to bacteria (see Review by Rhuland, 1960). It is not found in any other micro-organisms with the exception of the blue-green algae (Myxophyceae) which are closely related to bacteria. It is present in all bacteria, with the exception of most Gram-positive cocci and various lactobacilli (Work, 1951; Work & Dewey, 1953; Hoare & Work, 1957). Chemically diaminopimelic acid $\begin{matrix}\mathrm{COOH}\\ \mathrm{NH_2}\end{matrix}\!\!>\!\mathrm{CH.CH_2.CH_2.CH_2.CH}\!<\!\begin{matrix}\mathrm{COOH}\\ \mathrm{NH_2}\end{matrix}$ is unlike most other naturally occurring amino acids in that it has 2 amino and 2 carboxyl groups. It has 3 stereoisomers, LL, DD, and one *meso* (DL) form, the molecule being symmetrical about the γ-carbon atom. The form which was originally isolated from *Corynebacterium diphtheriae* was optically inactive and was proved to be the *meso* isomer (Work *et al.* 1955). A solvent which will separate the *meso* isomer from the other two on paper chromatograms has enabled the isomer present in any organism to be determined (Rhuland *et al.* 1955; Hoare & Work, 1955). The *meso* form is that most commonly found, but in certain families, notably Propionibacteria, and many Streptomyces, the LL isomer is present; a few organisms contain both *meso* and LL forms (Hoare & Work, 1955, 1957). The findings of Cummins & Harris and of Salton on distribution and isomeric form of diaminopimelic acid in cell walls of various species are identical with our results on whole bacteria. In fact, the majority of the cellular diaminopimelic acid is located in the wall, although small amounts are sometimes found in soluble fractions.

Diaminopimelic acid undergoes the general types of metabolic reactions which occur with other amino acids. The *meso* and LL isomers are attacked by certain L-amino acid oxidases (Work, 1955); some more specific bacterial enzymes decarboxylate, racemize, oxidize or transaminate one or other isomer (Dewey & Work,

1952; Dewey, Hoare & Work, 1954; Antia, Hoare & Work, 1957; Meadow & Work, 1958; Antia & Work, 1961). The racemase and decarboxylase have been studied in detail.

Diaminopimelic racemase converts either the *meso* or LL isomer to an equilibrium mixture of the two, while the decarboxylase converts *meso*-diaminopimelic acid to L-lysine with evolution of 1 molecule of CO_2. Thus, *meso*-diaminopimelic acid is attacked by these two enzymes at the D 'end'; this is the only known enzymic decarboxylation of a carboxyl group in the D-configuration. Since the DD isomer is not attacked by either enzyme, the L-configuration at one end of the molecule is evidently essential for fixation to the enzymes.

The bisosynthesis of diaminopimelic acid with cell-free preparations of various *Escherichia coli* mutants has been partly worked out (Gilvarg, 1957, 1958, 1959; Kindler & Gilvarg, 1960; Peterkofsky & Gilvarg, 1959).



Some of these preparations can synthesize the amino acid from aspartic acid and pyruvic acid in the presence of glutamate, ATP and various cofactors. A monoketo-monoamino acid (compound A) was identified as an intermediate: the amino group was derived directly from that of aspartate and was acylated with a succinyl radical. The acylation protects the molecule from cyclization, which would undoubtedly occur if the amino group were free to react with the keto group. A transamination with L-glutamate as specific amino donor results in the formation of *N*-succinyl-L-diaminopimelic acid (compound B), which is converted to LL-diaminopimelate by a specific deacylase. Since *E. coli* contains *meso*-diaminopimelic acid, the next step is probably racemization of the LL isomer. There are several lines of evidence that this step occurs in the whole organism (Hoare & Work, 1955; Meadow, Hoare & Work, 1957), and a metabolic function for diaminopimelic racemase has been suggested.

The function of diaminopimelic decarboxylase is the formation of lysine. This was first established when it was found that certain mutants of *Escherichia coli* which require lysine lacked the decarboxylase (Dewey & Work, 1952). Formal proof of *in vivo* conversion of diaminopimelic to lysine was obtained by examining the

incorporation of radioactive diaminopimelic acid into the amino acids of growing cultures of *E. coli* (Meadow & Work, 1959). It was possible to trace the radioactivity from diaminopimelic acid to lysine, as excellent recovery of exogenous radioactive lysine was obtained in the cellular lysine. Also, radioautography showed that radioactive diaminopimelic acid was converted only into radioactive lysine, and that neither amino acid interchanged its carbon chain with other amino acids. To determine the proportion of lysine originating from diaminopimelic acid, two mutants of *E. coli* were studied: one (173–25), had a relative requirement for lysine and an absolute requirement for diaminopimelic acid (Davis, 1952); the other (D), was derived from mutant 173-25 by 'training' it to dispense with lysine, when it required to be given only diaminopimelic acid (Meadow *et al.* 1957). The mutants

Table 1. *Summary of* ^{14}C*-incorporation experiments with diaminopimelic acid (DAP)-requiring mutants of* Escherichia coli

The organisms were grown in minimal media containing glucose (0·5%), lysine (0·1 mM) and DAP (0·1 mM). In each experiment one of these sources of carbon was radioactive (totally labelled). Phenol-insoluble fractions of walls were hydrolysed and the radioactivity of amino acids determined (Meadow & Work, 1959).

Mutant 173–25 requires to be given lysine and DAP; mutant D requires only DAP.

Expt.	^{14}C source	Mutant	Radioactivity of wall amino acids			Radioactive lysine as % of total lysine of fraction
			DAP	Lysine	Others	
1	DAP	173-25	+	+	0	80
		D	+	+	0	55
2	Lysine	173-25	0	+	0	10
		D	0	+	0	10
3	Glucose	173-25	0	+	+	10
		D	0	+	+	20

were grown in a basal salts and glucose medium with lysine and diaminopimelic acid, but in each experiment either the glucose, lysine or diaminopimelic acid was totally labelled with ^{14}C. The cell walls were fractionated with phenol, and the insoluble portions, containing most of the mucopeptide, were hydrolysed and the proportion of the lysine originating from each carbon source was calculated (Table 1). Expt. 1 showed that labelled diaminopimelic acid was converted to lysine, but that it accounted for 80 % of the total wall lysine in mutant 173-25 which required to be given lysine for full growth, and only 55 % in mutant D. This difference between the two mutants was not accounted for by differences in their utilization of exogenous lysine, since they incorporated identical amounts of radioactivity from ^{14}C-lysine (Expt. 2). When labelled glucose was used (Expt. 3), all the amino acids except diaminopimelic were labelled, and the mutant with no lysine requirement incorporated twice as much radioactivity into lysine as the other mutant. Expt. 3 proved that part of the lysine was derived from a route other than through diaminopimelic acid, and that there were at least two biosynthetic paths to lysine in *E. coli*. The major route is through diaminopimelic acid, while the other utilizes some unknown carbon source. The latter route was evidently strengthened by 'training' the mutant to dispense with added lysine, since the diaminopimelic decarboxylase activity was not changed by the process (Meadow *et al.* 1957).

Bacteriolytic enzymes

Although the mucopeptides of bacterial cell walls are resistant to proteolytic enzymes (perhaps because of the presence of D-amino acids), they are often very susceptible to attack by bacteriolytic enzymes. The best known is lysozyme, first shown by Fleming (1922) to lyse living cultures of *Micrococcus lysodeikticus*, and later found to attack certain other living Gram-positive bacteria. Salton (1952*b*) showed that the substrate for lysozyme is the cell wall itself; wall preparations from susceptible species are completely solubilized by lysozyme treatment, with a simultaneous decrease in optical density and the liberation of substances giving reactions for reducing sugars and *N*-acetylhexosamines (Reviews, Salton, 1957*a*, 1958).

A so-called disaccharide has been identified in some lysozyme digests (Salton, 1956*b*). It is probably a $\beta(1 \rightarrow 6)$ glycoside of *N*-acetylglucosamine and *N*-acetylmuramic acid, and is responsible for the reducing action of the digests. The reducing group belongs to muramic acid (Salton & Ghuysen, 1959; Perkins, 1960*a*) so that the 1-carbon of glucosamine may be linked to the 6-carbon of muramic acid. Small amounts of a lysozyme-sensitive tetrasaccharide also occur in the digests; this may be a dimer of the disaccharide joined by $\beta(1 \rightarrow 4)$ linkages. Since free *N*-acetylglucosamine or *N*-acetylmuramic acid have not been found in digests, it seems possible that at least part of the mucopeptide molecule contains these compounds bound in pairs by lysozyme-sensitive $\beta(1 \rightarrow 4)$ linkages alternating with lysozyme-resistant $\beta(1 \rightarrow 6)$ linkages; they might, in fact, form part of a backbone to which are linked peptides and polysaccharides. If this theory is correct, the $\beta(1 \rightarrow 4)$ glycosidic linkages in the backbone are the substrate for lysozyme. The susceptible links may be unevenly distributed along the backbone, since the fragments are of very different sizes and composition, some even have molecular weights up to 15,000, and contain sugars, amino acids, muramic acid and glucosamine. There are great differences in susceptibility to lysozyme among various bacterial species. Little is known about the exact structure in walls which determines the extent of lysozyme sensitivity; no correlation has been found between overall composition and this sensitivity. All we know is that certain lysozyme-resistant organisms contain more O-acetyl groups in their walls than do lysozyme-sensitive strains of the same organisms, and that the removal of these groups with mild alkali often renders the cells more sensitive (Brumfitt, 1959).

Most living Gram-negative bacteria are resistant to lysozyme, but they can be rendered susceptible by subjection to extremes of temperature or pH or by exposure to surface-active agents such as detergents, bile salts, chloroform, polymyxin or ethylene diamine tetra-acetic acid (EDTA) (see Review, Salton, 1958). These treatments probably all cause damage to the outer lipoprotein coat, and thus allow access of lysozyme to the inner mucopeptide substrate. Wall suspensions from Gram-negative bacteria when treated with lysozyme, show no appreciable changes in optical density, probably owing to their high content of non-mucopeptide constituents. Soluble constituents are, however, liberated from the walls; some contain diaminopimelic acid, alanine, glutamic acid, glucosamine and muramic acid (Salton, 1958; Work & Lecadet, 1960).

Bacteriolytic enzymes are also produced by some bacteria. Many of these enzymes resemble lysozyme in their substrate specificity and in the nature of their

digestion products (Richmond, 1959*a*, *b*); but there are often small differences of specificity and point of attack between the different enzymes, which are not yet understood. The bacteriolytic enzymes which do not resemble lysozyme were reviewed by Work (1957) and by Strange (1959). These enzymes have been found in the culture filtrates of certain Streptomyces species or associated with spores and vegetative cells of aerobic sporulating species of the genus *Bacillus*. The enzymes from spores are not entirely species-specific, although they are often more active towards walls of their own species; they will even attack walls of Gram-negative bacteria after treatments similar to those used for sensitization to lysozyme (Work, 1959). It has been suggested (Strange, 1959) that the lytic enzymes of species of *Bacillus* are involved in the sporulation cycle, since they are most active in the sporulating and germinating phases, when they release soluble mucopeptides from the sporangial wall or spore coat (Powell & Strange, 1956).

The main mucopeptide released from germinating spores of *Bacillus* species has been isolated (Powell & Strange, 1953; Strange & Powell, 1954). It has a molecular weight of about 15,000 and consists mostly of 1 molecular proportion of D-glutamic acid, 3 of alanine (D and L isomers), and 1 of *meso*-diaminopimelic acid, and a mixture of 8 molecular proportions of acetylglucosamine and acetylmuramic acid (Strange, 1959). This mucopeptide served as the original source of muramic acid. The mucopeptide is the only high-molecular-weight degradation product of wall mucopeptides to have been purified and analysed; the sequence of amino acids in it has not yet been established. It is further degraded by lysozyme and by β-glucosaminidase. Both glucosamine and muramic acid are present as the *N*-acetyl derivatives. This is the case with all the enzymic degradation products of walls so far examined (Salton, 1956*b*; Salton & Ghuysen, 1959; Perkins, 1960*a*, *b*; Ghuysen & Salton, 1960) and it may be assumed that the amino groups of these hexosamines are always acetylated.

Other lytic enzymes of bacteria are associated with the cell walls of vegetative organisms (Mitchell & Moyle, 1957), and may be responsible for the rapid autolysis which sometimes occurs in dense suspensions of these organisms. Autolytic activity, if present, is stronger in rapidly growing cultures, and may play an active part in cell division, since localized breakdown of rigid cell-wall material might well occur immediately before division, followed by immediate resynthesis.

Lysis by bacteriophages. Lytic enzymes which attack cell walls are also associated with bacteriophages (Panijel & Huppert, 1957; Koch & Weidel, 1956; Maxted, 1957; Murphy, 1957; Koch & Jordan, 1957; Brown & Kozloff, 1957; Ralston, Lieberman, Baer & Krueger, 1957; Krause, 1958; Koch & Dreyer, 1958; Weidel & Primosigh, 1957, 1958). Probably these enzymes are localized in the phage tails and are responsible both for the penetration of the phage infective principle through the bacterial rigid wall layer, and for the subsequent lysis of infected cultures (see review by Weidel, 1958). The lysates themselves often also show lytic activity. A defective lysogenic mutant (P_{32}) of *Escherichia coli* $K_{12}(\lambda)$ produces a lysate which contains a lytic enzyme (Jacob & Fuerst, 1958). Some of the properties of this enzyme (known as λ-endolysin) have been investigated after partial purification from a lysate induced by nitrogen mustard (Work, 1960). λ-Endolysin does not attack viable bacteria, even the host strain of *E. coli*, except after certain preliminary treatments, e.g. with chloroform, EDTA, acetone-drying. The bacteria

are only susceptible during the exponential phase of growth. Many Gram-negative species are attacked, and even some Gram-positive species (*Bacillus megaterium*, *Staphylococcus aureus*). *Micrococcus lysodeikticus*, the substrate *par excellence* for lysozyme is not susceptible to λ-endolysin. Cell walls of *B. megaterium* are completely lysed by λ-endolysin, and have been used as substrate to examine its properties. The optimum pH value is about 7·0; some activation is produced by NaCl or KCl (10^{-2}M). Phosphates and certain heavy-metal ions are strongly inhibitory, the amounts which give 50% inhibition (in presence of NaCl) being KH_2PO_4, 10^{-3}M; Zn, 5×10^{-4}M; Fe^{+++}, 10^{-5}M; Co^{++} and Mn^{++}, 10^{-3}M no inhibitions, Co^{++} stimulates slightly. In the complete absence of heavy metals, EDTA is without effect but was usually added to walls before treatment with the endolysin as a protection against inhibitory metal ions. λ-Endolysin resembles the lytic enzyme of disrupted mature phage λ (Fisher, 1959), but it differs from lysozyme in its sensitivity to metals and phosphates and in its specificity. Thus, λ-endolysin is distinct from the lytic enzyme of T_2 coli phage or of lysates produced by the phage; this enzyme has been reported to resemble lysozyme (Koch & Dreyer, 1958).

To provide further grounds for the comparison of λ-endolysin with lysozyme, the products of digestion by these enzymes of walls of *Escherichia coli* B and *Bacillus megaterium* were examined (Work & Lecadet, 1960). The soluble fragments were separated by dialysis into two fractions, each of which was treated with 1-fluoro-2:4-dinitrobenzene and then subjected to paper chromatography or paper electrophoresis. The resulting yellow spots were eluted, hydrolysed and examined qualitatively for free and dinitrophenylated amino acids. The hexosamines appeared to be partially destroyed during the hydrolysis of the dinitrophenylated fragments, so that no conclusions could be drawn about their presence. However, in nearly all cases, glucosamine and muramic acid (one or both) were detected in the hydrolysates, as well as a limited number of amino acids, so most of the yellow fragments were probably mucopeptide in nature.

The fragments produced by λ-endolysin from *Bacillus megaterium* were indistinguishable from those produced by lysozyme. Five spots were obtained by paper chromatography (butanol-acetic acid solvent) from each of the dialysable digests (Table 2). The slower-moving spots contained mainly alanine and glutamic acid and had no identifiable end-amino groups; the more mobile spots were larger and also contained dinitrophenyl-alanine and mono-dinitrophenyl-diaminopimelic acid. In the non-dialysable fractions, which were separated by paper electrophoresis at pH 3·6, similar distributions of amino acids occurred in the various spots, except that here diaminopimelic acid was present with both amino groups bound as well as with one attached to dinitrophenol.

λ-Endolysin and lysozyme did not produce identical fragments from *Escherichia coli* walls (Table 3). The digests contained a variety of amino acids, including both lysine and diaminopimelic acid, also glycine, serine and aspartic acid in addition to alanine and glutamic acid. In the dialysable fraction, only glutamic acid was invariably present in all spots; in fact in one spot from the endolysin digest, it was the only major amino acid detected. None of the less mobile spots contained alanine; diaminopimelic acid and lysine were fairly randomly distributed, but only occurred together in spots which were obviously inhomogeneous (e.g. the immobile or most mobile ones). One spot from the endolysin digest contained very large amounts of

alanine, while a neighbouring spot from the lysozyme digest contained nearly all the usual amino acids of a protein but no diaminopimelic acid. A colourless dialysable fragment in digests from both organisms was identified on paper chromatograms by the acetylhexosamine reaction; it was probably the dimer of *N*-acetylglucosamine and *N*-acetylmuramic acid studied by Salton (1956*b*) in lysozyme digests from Gram-positive organisms. In the non-dialysable fractions of *E. coli*, the differences between the actions of the enzymes were not so marked as in the dialysable fractions, but they were apparent. Several spots contained both diaminopimelic acid and lysine, with either both or one of their amino groups bound, or with both free. Further purification of the material from these spots has not yet been done.

Table 2. *Amino acid contents of some peptides in digests of walls of* Bacillus megaterium, *produced by λ-endolysin (of* Escherichia coli $K_{12}(\lambda)$ *lysogenic mutant* P_{32}*) or lysozyme*

Identical samples of walls (2 mg.) were digested with each enzyme under optimal conditions. The digests were separated by dialysis, and each fraction was dinitrophenylated with fluorodinitrobenzene. The dialysable fractions were spread on Arche 310 paper and irrigated with butanol + acetic acid + water (4:1:1) solvent for 2 days; the non-dialysable fractions were subjected to paper electrophoresis on Whatman 3MM paper at pH 3·6, 500 V for 9 hr. After drying the papers, the yellow spots were each cut out, eluted, hydrolysed with 6N-HCl and examined qualitatively by paper chromatography for free and dinitrophenylated amino acids. Controls without walls were treated identically; they showed small amounts of a few amino acids (originating from the filter paper) which have been allowed for in presenting the results.

The figures in the columns represent the distances (cm.) travelled by each spot. The two enzymes produced fragments identical in mobility and amino acid contents.

Dialysable fraction		Non-dialysable fraction	
cm.	Amino acids	cm.	Amino acids
2 → 5·5	Glu, Ala	−1 → −8	Glu, Ala
6 → 12	Glu, Ala	0 → +11	Dap, Glu, Ala, Gly, DNP-Dap, DNP-Glu
13 → 18	Glu, Ala	+15 → +22	Dap, Glu, Ala, DNP-Dap, DNP-Ala
20 → 27	Glu, Ala, DNP-Dap, DNP-Ala		
		+24 → +26	Glu, Ala
30 → 42	Glu, Ala, DNP-Dap, DNP-Ala		

Dap = diaminopimelic acid. DNP = dinitrophenyl group. Glu = glutamic acid. Ala = alanine. Gly = glycine.

Certain conclusions can be drawn from the results. Not every peptide fragment in the digests contained all the amino acids of the whole walls. This is contrary to results so far reported about the products of action of various bacteriolytic enzymes. where the non-dialysable peptides, produced by enzymic digestion of fresh walls or those already treated with fluoro-dinitrobenzene have been reported to contain all the constituents of wall mucopeptides (Salton, 1956*b*; Ingram & Salton, 1957; Koch & Dreyer, 1958). Digestion products from the action of λ-endolysin or lysozyme on dinitrophenylated walls of *Escherichia coli* and *Bacillus megaterium* were different from those produced from untreated walls (Work & Lecadet, 1960); the former therefore cannot be regarded as normal reaction products. The marked differences between the reaction products produced by λ-endolysin and lysozyme

Table 3. *Amino acid contents of some peptides in soluble fractions of λ-endolysin (from* Escherichia coli $K_{12}(\lambda)$ *lysogenic mutant* P_{32}*) or lysozyme digests of walls of* E. coli *B.*

Samples (40 mg.) of walls were exhaustively digested with two successive portions of either enzyme in the presence of chloroform. After removal of insoluble products by centrifugation, digests were treated as in Table 2. The spots in the two digests were not identical, and are listed separately. The figures in the columns represent the distances (cm.) travelled by each spot.

λ-Endolysin products, dialysable		Lysozyme products, dialysable	
cm.	Amino acids	cm.	Amino acids
0	Dap, Lys, Asp, Glu, Ser, Gly		
4–5·5	Dap, Glu, Gly	3–5	Dap, Lys, Asp, Glu, Ser, Gly
6–7	Glu	6·5–7·5	Lys, Asp, Glu, Ser, Gly
8–10	Dap, Glu, Ser, Gly, Ala,* DNP-Dap	9–11	Lys, Asp, Glu, Ser, Gly, Thr, Ala, Val, Leu, Arg, Cys
16–18	Lys, Asp, Glu, Ser, Gly, Val, DNP-Lys, DNP-Ala		
21–22	Glu, DNP-Dap	20–25	Lys, Asp, Glu, Ser, Gly, Thr, Ala, Val, Leu
30–36	Lys, Asp, Glu, Ser, Gly, Ala, Val, Leu, Arg, DNP-Dap, DNP-Lys, DNP-Glu, DNP-Ser, DNP-Ala	30–36	Lys, Asp, Glu, Ser, Gly, Ala, Val, Leu, Arg, DNP-Dap, DNP-Lys, DNP-Glu, DNP-Ser, DNP-Ala
36–42 (solvent front)	Lys, Asp, Glu, Gly, Thr, Ala, Val, Leu, DNP-Dap, DNP-Lys, Di-DNP-Lys, DNP-Glu, DNP-Ser, DNP-Ala	36–42 (solvent front)	Asp, Glu, Ser, Gly, Ala, DNP-Dap, Di-DNP-Lys, DNP-Glu, DNP-Ser, DNP-Ala

* Very large amount

non-dialysable		non-dialysable	
−2 → +2	Lys, Asp, Glu, Ser, Gly, DNP-Dap	−2 → +2	Lys, Asp, Glu, Ser, Gly, DNP-Dap
+ 8 → +13	Dap, Asp, Glu, Ser, Gly, Ala, DNP-Dap	+ 8 → +13	Dap, Glu, DNP-Dap,
+17 → +21	Dap, Lys, Glu, Ser, Gly, Ala, DNP-Dap, Di-DNP-Dap, Di-DNP-Lys, DNP-Glu, DNP-Ser	+17 → +21	Dap, Lys, Glu, Ala, DNP-Dap, Di-DNP-Lys, DNP-Glu, DNP-Ser
+21 → +26	Dap, Glu, Ser, Gly, Ala, DNP-Dap, Di-DNP-Dap, DNP-Lys, Di-DNP-Lys, DNP-Glu, DNP-Ser, DNP-Ala	+21 → +26	Dap, Lys, Glu, Ser, Ala, DNP-Dap, Di-DNP-Dap, DNP-Glu, DNP-Ser, DNP-Ala
+26 → +28	Lys, Asp, Glu, Ser, Gly, DNP-Dap	+26 → +28	Lys, Glu, Ser, Gly, DNP-Dap

Ala = alanine. Arg = arginine. Asp = aspartic acid. Cys = cystine. Dap = diaminopimelic acid. Glu = glutamic acid. Gly = glycine. Leu = leucine. Lys = lysine. Ser = serine. Thr = threonine. Val = valine. DNP = dinitrophenyl group.

from *E. coli* walls show that the enzymes are not identical in their action on this material, although they appeared to act identically on walls from *B. megaterium*. The reason for this is not known; it may be connected with the fact that walls of *E. coli* are the natural substrate for λ-endolysin. The lytic enzyme from T_2 coli phage was reported by Koch & Dreyer (1958) to resemble lysozyme in the fragments

which were produced from dinitrophenylated walls of *E. coli* B. Also, walls of *E. coli* already digested by T_2 coli phage enzyme were not further attacked by lysozyme, and *vice versa*. With λ-endolysin, on the contrary, the insoluble residues of dinitrophenylated walls of *E. coli* were further digested by lysozyme, while the lysozyme-resistant residues were also attacked by λ-endolysin. It is thus apparent that the lytic enzyme of λ-phage is different from that of T_2-phage. The results also indicated that *E. coli* walls are more complicated than was originally suggested by Weidel & Primosigh (1958). Each of the so-called 'layers' contained mucopeptide components which are partially, but not entirely, solubilized by lysozyme or λ-endolysin; lipids were also solubilized and were even found in dialysable fractions. Apparently many structures are held together in these walls by non-covalent links which are broken as a secondary result of solubilization of the mucopeptides. Under the influence of lytic enzymes the walls disintegrate into many types of fragments which differ according to whether lysozyme or λ-endolysin is acting.

The function of cell walls

When whole susceptible organisms are treated with lysozyme or other bacteriolytic enzymes the rigid mucopeptide component of the cell wall is attacked, and the immediate cause of lysis is the bursting of the mechanically unprotected cytoplasmic membrane by the internal osmotic pressure. When the digestion is carried out in isotonic solution, e.g. 10 % (w/v) sucrose, the cytoplasmic membrane remains intact and spherical forms are produced (see review by Weibull, 1958). With Gram-positive organisms the resulting bodies are known as protoplasts; chemical and immunological analyses have shown them to be free from wall components, including specific proteins and mucopeptides (Freimer, Krause & McCarty, 1959; Vennes & Gerhardt, 1959). Similar spherical bodies are produced from Gram-negative bacteria after appropriate preliminary sensitizing treatment, but cannot strictly be called protoplasts: not only do they still retain the wall lipoproteins and lipopolysaccharides, but they also contain mucopeptide components such as diaminopimelic acid and muramic acid (Salton, 1958); their membranes also react positively with cell-wall antibodies (Holme, Malmborg & Cota-Robles, 1960). The term spheroplast is now usually applied to the spherical osmotically sensitive forms from Gram-negative bacteria (Brenner *et al.* 1958). Protoplasts and spheroplasts are reasonably stable in media of sufficiently high osmotic pressure; they burst when transferred to water, leaving a residue of membranous ghosts, similar in size to the original body. The composition of the ghosts (cytoplasmic membranes) from Gram-positive bacteria is quite different from that of cell walls; they contain considerable quantities of lipids, but no hexosamines, wall sugars or diaminopimelic acid (Gilby, Few & McQuillen, 1958; Weibull & Bergstrom, 1958). The permeability properties of protoplasts or spheroplasts are the same as those of the bacteria from which they were derived, showing that their membranes are the osmotic barrier of the intact cell. Protoplasts under appropriate conditions can synthesize protein, can grow, divide and produce bacteriophage after infection or induction (McQuillen, 1955; Borek & Ryan, 1959). Thus they contain the main essential biosynthetic mechanisms of the cell, for which the wall mucopeptides are apparently not required when protection from osmotic effects is provided. Cell walls are permeable to dextrans of molecular weight up to 100,000 and to proteins excreted by the organisms,

suggesting that they have a sieve- or sponge-like structure. Their main function would appear to be that of a rigid porous envelope which protects the delicate cytoplasmic membrane within. Gerhardt (1959) and Butler, Crathorn & Hunter (1958) obtained evidence which suggests that certain amino acids may be stored in the walls; so walls may have the further function of a reservoir for certain metabolites.

Since the cell walls of all bacteria contain this specific mucopeptide structure, one ought to be able to prevent wall synthesis by growing exacting organisms in the absence of one of the mucopeptide components necessary for growth. This has been done with diaminopimelic acid (Meadow *et al.* 1957; Rhuland, 1957), lysine (Toennies & Shockman, 1958) and glucosamine (Zilliken, 1959). Meadow *et al.* (1957) used the two mutants of *Escherichia coli* (173–25 and D, see p. 171) which require to be given diaminopimelic acid for growth. When grown in glucose salts media supplemented with a constant (optimal) amount of lysine, and different amounts of diaminopimelic acid, cultures of mutant 173–25 had initial growth rates which were independent of the diaminopimelic acid concentration. Organisms grown in media with low concentrations of diaminopimelic acid lysed when the diaminopimelic acid was exhausted from the medium. Mutant D, grown in a similar series of media but without lysine, did not lyse, but just stopped growing when the diaminopimelic acid was used up. When lysine was present, lysis occurred on cessation of growth as with the cultures of mutant 173–25. This lysis can be explained on the basis that, provided some diaminopimelic acid is present, the organisms grow normally and form walls; as soon as the diaminopimelic acid is exhausted, new walls cannot be formed and the organisms burst, probably at the next division. The reason for the obligatory presence of lysine for lysis to occur is not known: it is not due to the fact that lysine allows cytoplasmic protein synthesis to continue so that the cell contents outgrow their walls (suggested by McQuillen, 1958*a*), since lysine can be replaced by α-*N*-acetyllysine as a growth factor, and in this case lysis does not occur.

When these diaminopimelic-requiring mutants are grown in the presence of sucrose and low concentrations of diaminopimelic acid, spheroplasts are formed (Meadow *et al.* 1957; Bauman & Davis, 1957; McQuillen, 1958*b*). These spheroplasts are able to grow, and to synthesize inducible enzymes in the presence of all amino acids except diaminopimelic, showing that this latter amino acid is not essential for protein synthesis.

Lysis through lysine deprivation occurs with *Streptococcus faecalis*, which contains no diaminopimelic acid, but has lysine instead in its wall mucopeptides (Toennies & Gallant, 1949; Toennies & Shockman, 1958). This organism requires to be given eight amino acids, including lysine, for normal growth; the effect of deprivation of each of these amino acids in turn was investigated. With suboptimal concentrations of lysine, the growth curves were very similar to those of the exacting *Escherichia coli* deprived of diaminopimelic acid, and the organisms lysed after exhaustion of the lysine present. A different phenomenon occurred with organisms depleted of valine or threonine, which are not wall amino acids (Shockman, Kolb & Toennies, 1958; Toennies *et al.* 1959; Shockman, 1959*a*). Exponential growth ceased when valine or threonine was exhausted from the medium, but the cultures continued to increase slowly in optical density and mass up to 40 hr. This so-called 'post-exponential growth' was particularly marked in the case of threonine-depleted organisms and was largely due to increase in wall mucopeptide with no concomitant

protein synthesis. Comparison of the molar ratios of the amino acids in the walls at various stages of growth indicated that the mucopeptides had the same composition at all stages. But when the amino acids were expressed as percentage of wall weight, it was evident that the overall composition of the wall had changed during post-exponential growth, possibly owing to variation of a non-mucopeptide component.

There is a tendency among workers in the field under consideration to examine only the molar ratios of specific wall constituents, not their absolute amounts in terms of percentage composition. This can be misleading, as it does not give a true picture of wall composition. More work of the kind carried out by Shockman and colleagues might lead to a better understanding of various interesting problems connected with cell walls, such as why bacteria harvested during exponential growth are more susceptible than older ones to attack by lytic enzymes (see Douglas & Parker, 1958). We do not know whether this is simply due to the presence of thicker walls in older organisms, or whether the older walls contain proportionately more enzyme-resistant structures or less enzyme-sensitive groups.

The biosynthesis of mucopeptide

The experiments just discussed show that synthesis of cell wall and cytoplasmic protein can continue independently in nutritionally-exacting organisms grown under certain conditions; this type of phenomenon has been termed 'unbalanced growth' (McQuillen, 1958*a*). Another way of dissociating the two types of synthesis is to suspend washed organisms in buffered glucose containing certain amino acids. This approach was used with *Staphylococcus aureus* by Mandelstam & Rogers (1958) and Hancock & Park (1958). Study of the incorporation of radioactive amino acids into walls and proteins of suspensions of *S. aureus* showed that when the amino acids of the suspending media were limited to those of the wall mucopeptides, radioactivity was incorporated selectively into the walls; but when 18 amino acids present in proteins were given, both walls and cytoplasmic proteins took up radioactivity. Added chloramphenicol inhibited 94 % of the uptake of amino acids into protein but had no effect on uptake into walls. Penicillin had the opposite effect and selectively inhibited the uptake of amino acids into the wall fraction. Aureomycin was quite unselective; it inhibited both wall and protein synthesis. Mandelstam & Rogers (1959) showed that the presence of penicillin or bacitracin prevented the usual increase in weight of mucopeptide observed during incubation, and therefore resulted in a genuine inhibition of mucopeptide synthesis.

As might be expected, since penicillin selectively inhibits the synthesis of a component of cell walls, the final result of penicillin action on bacteria growing in normal media is lysis. In fact, the growth curve of *Escherichia coli* in the presence of penicillin is similar to that produced by diaminopimelic acid deprivation (Meadow *et al.* 1957). Penicillin lysis has long been known, but the cause of it was not realized until spheroplasts of *E. coli* were produced by growth in penicillin broth containing 10 % sucrose (Lederberg, 1956; Liebermeister & Kellenberger, 1956; Hahn & Ciak, 1957). Bizarre forms of various Gram-negative bacteria growing on solid media in the presence of penicillin are well known; these may be regarded as spheroplasts which are stabilised mechanically by the solid medium in which they grow (Lederberg & St Clair, 1958). Analytical data on L-forms or penicillin-induced

spheroplasts show that the cells are deficient, but not always completely lacking in diaminopimelic acid and muramic acid (Kandler & Zehender, 1957; Weibull, 1958; Salton & Shafa, 1958). Their lipid and polysaccharide components are normal.

Before the site of penicillin action was known Park (1952) observed that when *Staphylococcus aureus* was grown in the presence of penicillin, certain uridine nucleotides accumulated in the cells. The main nucleotide was uridine diphosphate (UDP) linked through *N*-acetylmuramic acid to a peptide containing lysine, glutamic acid and alanine. At that time, the connexion of this structure with cell walls was not appreciated, nor was muramic acid identified. After the work of Strange & Powell (1954), Cummins & Harris, (1956*a*, *b*, 1958) and Lederberg (1956), a connexion with wall composition was seen (Park & Strominger, 1957); the nucleotide contained all the components of staphylococcal walls except glucosamine and glycine. The composition of the peptide has now been partly deduced (Fig. 1), although there is as yet no formal proof of the amino acid sequence (Strominger, 1959*a*, *b*; Strominger & Threnn, 1959).

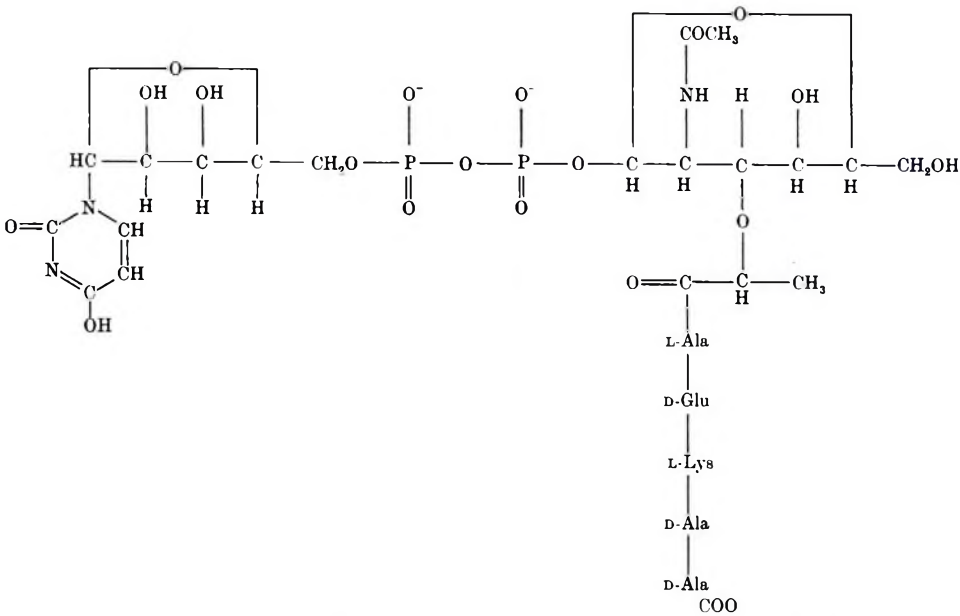


Fig. 1. The structure of a uridine nucleotide which accumulates in penicillin-inhibited *Staphylococcus aureus*.

The accumulation of other uridine nucleotides in *Staphylococcus aureus* grown under various conditions has led Strominger to propose a scheme for the biosynthesis of mucopeptide units (summarized by Strominger, 1960). This involves a 'UDP cycle' (Fig. 2) in which uridine nucleotides serve as catalytic carriers. For the purposes of this review, UDP-acetylmuramic acid can be taken as the starting point. This compound was assumed to originate from UDP-acetylglucosamine through UDP-acetylglucosamine-pyruvate (Strominger, 1958). UDP-acetylmuramic acid was one of several nucleotides accumulating in cells grown in the presence of gentian violet; it was suggested that gentian violet acts by inhibiting the attachment of alanine to the muramyl carboxyl group. After depriving *S. aureus* of lysine,

UDP-acetylmuramyl-L-alanyl-D-glutamate was isolated. An enzyme which catalyses the attachment of lysine to this peptide was partially purified, and found to be specific for L-lysine. D-Cycloserine (oxamycin), another antibiotic known to produce spheroplasts from *Escherichia coli* (Ciak & Hahn, 1959), caused accumulation of the uridine peptide which lacked only the two terminal D-alanine molecules (Strominger, Threnn & Scott, 1959). All the effects of D-cycloserine in different species were competitively reversed by adding D-alanine (see also Shockman, 1959*b*), but they were not produced by the enantiomorph, L-cycloserine. It seems that this antibiotic acts directly as an analogue of D-alanine. Finally, the last suggested step in the synthesis of wall mucopeptide is a transglycosylation of the UDP-acetylmuramyl-peptide with an acceptor, so far unidentified. It is this last step which is supposed by Park & Strominger to be inhibited by penicillin, but there is as yet no positive evidence for this assumption.

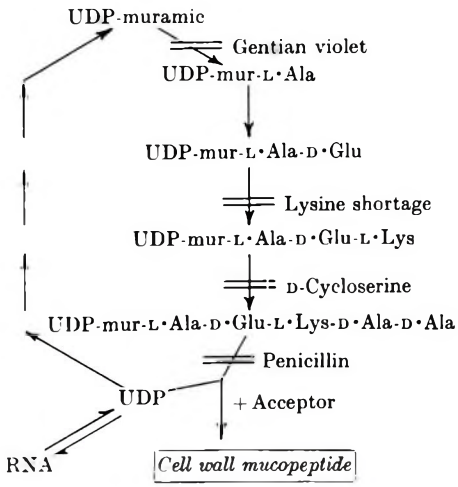


Fig. 2. Scheme for the biosynthesis of part of the cell wall of *Staphylococcus aureus*.

Other possible sites for penicillin action are inhibition of synthesis of the acceptor, or of transfer of one or more low-molecular weight precursors through the semi-permeable cytoplasmic membrane to the site of wall synthesis outside the membrane. Although UDP-peptide accumulation occurs very rapidly after addition of penicillin to growing or resting cultures (Strominger, 1957), this is not the only immediate effect of penicillin. There is also a very rapid release of ultraviolet-absorbing material into the culture fluid with *Escherichia coli*, which suggests that the osmotic barrier has been disturbed (Prestidge & Pardee, 1957; Meadow, 1960). Penicillin also interferes with the synthesis of ribonucleic acids in a way quantitatively related to total uridine nucleotide synthesis. This can be explained by the UDP-cycle, since the product of transglycosylation of the UDP-muramyl-peptide would be UDP, a direct precursor of ribonucleic acid. In the presence of penicillin, UDP would be irreversibly trapped in the accumulated uridine peptides and so would be unavailable for synthesis of ribonucleic acids; the inhibition of this synthesis is therefore a secondary result of penicillin action.

A transglycosylation step in mucopeptide synthesis is certainly an attractive

hypothesis; it is based on the knowledge that similar steps occur in the biosynthesis of low and high molecular-weight polysaccharides, including hyaluronic acid, glucan, cellulose and glycogen; here 'active' sugar fragments are transferred from uridine nucleotides to acceptors (Strominger, 1960). It is possible that this uridine peptide is indeed a precursor of wall mucopeptides, especially because of its content of D-amino acids, but it is not the only UDP-peptide found in *Staphylococcus aureus* treated with penicillin; similar peptides also contain the other staphylococcal wall amino acids, glycine and aspartic acid (Ito, Ishimoto & Saito, 1959). Direct evidence for the incorporation of any of these peptides as complete units into cell walls has yet to be produced as proof that the uridine peptides are, in fact, immediate cell-wall precursors. The method of insertion of glucosamine into the muramyl peptides is not yet known, but is an important step still to be revealed in mucopeptide biosynthesis.

Bacterial wall structure

Until we know more about the homogeneity of individual units of wall mucopeptides, it is perhaps not very useful to speculate about their possible precursors. The molar ratios of wall constituents are seldom exactly one, and their digestion products are of great variety (Rogers & Perkins, 1959; Perkins, 1960*b*). This suggests that whole walls may contain either a mixture of different highly polymerized mucopeptides, or one polymer made from various UDP peptides (monomers), or both. There may well be some favoured monomers which occur in many different organisms; these may be the 'basal units' of walls where there are also various other peptides derived from monomers which are more type-specific. The only uridine peptides which have been isolated from Gram-negative species have been found in a diaminopimelic acid-requiring mutant of *Escherichia coli* grown under unspecified conditions (Strominger, Scott & Threnn, 1959). One peptide had the same amino acid sequence as in the nucleotide peptide of penicillin-grown *Staphylococcus aureus* (Fig. 1) except that *meso*-diaminopimelic acid took the place of lysine. Under conditions of diaminopimelic deprivation, the peptide contained only L-alanyl-D-glutamic acid. Possibly the sequence acetylmuramyl–L-alanine-D-glutamic—(diaminopimelic or lysine)—D-alanine—D-alanine may be a favoured monomer which provides the 'basal unit' of walls of both Gram-positive and Gram-negative bacteria.

Perhaps a part at least of the mucopeptide structure can be envisaged as a backbone of *N*-acetylglucosamine—*N*-acetylmuramate, possibly in alternating (1 → 4), (1 → 6) β-glycosidic linkages, bound by peptide links through the carboxyl groups of muramic acid to various amino acids. This peptide link is often, but not invariably, through alanine: exceptions are the muramyl-glycyl sequence identified by Perkins & Rogers (1959) in partial acid-hydrolysates of walls of *Micrococcus lysodeikticus*, and the alanine-free peptides which were found in enzymic digests of *Escherichia coli* walls by Work & Lecadet (1960). The sugars of walls are also linked to the hexosamines and often are present as high molecular-weight lipopolysaccharides which are responsible for the group-specific antigenic properties of cell walls. Little is known about the sequences or compositions of the peptide chains except in the case of the uridine-peptides already described. There are few free amino groups in Gram-positive walls, usually that of alanine and one amino group of lysine or diaminopimelic acid (Ingram & Salton, 1957). Many of the pairs of amino groups of

lysine and diaminopimelic acid of *E. coli* are not free to react with fluorodinitrobenzene (Salton, 1957*b*; Work & Lecadet, 1960). This suggests that these amino groups act as cross-linking agents between adjacent chains, or form cyclic peptides. The cross-linking of chains is known to be one of the best ways of producing rigid structures, and this may be the function of the two amino acids lysine and diaminopimelic acid, which are the only long-chain amino acids in the mucopeptide structure. Lysine and diaminopimelic acid have identical chains—the latter compound having just one more carboxyl group; they appear to be mutually exclusive in the walls of Gram-positive organisms. Glutamic acid is invariably present in mucopeptides, but whether in α- or γ-linkages is not known. As a whole, the amino acids of mucopeptides present plenty of opportunity for cross-linking or cyclization; if not doubly linked they could tend to form strong non-covalent links either with adjacent mucopeptides or with other charged molecules such as the teichoic acids.

Table 4. *Amino acid components of walls of certain species of bacteria*

Glutamic acid and alanine were always present and are not included.

Genus	No. of species examined	Lysine	Diaminopimelic acid		Glycine
			Meso	LL	
Staphylococcus	9	+	−	−	+
Micrococcus	2*	−	+	−	+
Propionibacterium	4	−	−	+	+
Propionibacterium	1	−	+	−	−
Bacillus	3	−	+	−	−
Clostridium	1	−	−	+	+
Nocardia	4	−	+	−	−
Nocardia	1	−	−	+	+
Micromonospora	5	−	+	+	+

* Unstable species, diaminopimelic acid not always present.
+ = present; − = absent.

The D-configuration of the glutamic acid and alanine in walls is of great interest; it may account both for the stability of the mucopeptides against attack by proteolytic enzymes, and for the obviously different mechanisms of peptide biosynthesis in walls and in cytoplasmic proteins. The most common form of diaminopimelic acid is the *meso*-isomer with a D-configuration at one end and an L the other. In the *Escherichia coli* uridine-peptide, *meso*-diaminopimelic acid occurs between D-alanine and D-glutamic acid and might be linked in peptide bonds through its D 'end'. The unreacted L-end of diaminopimelic acid might either cross-link with L-amino acids in other peptides, or might retain the biological reactivity of the peptide for further biosynthesis by its capacity to react with enzymes in the usual L-configuration. This biological reactivity occurs in the decarboxylation and racemization of *meso*-diaminopimelic acid (p. 170), where the L 'end' of the molecule is thought to react with the enzymes. An interesting point arising from a study of the data about cell-wall composition is that where walls contain LL-diaminopimelic acid, glycine is invariably present, but when *meso*-diaminopimelic occurs, glycine is seldom found (Table 4). Walls of the species which contain LL-diaminopimelic acid have not yet been examined in detail, but if one assumes that they contain the D-isomers of

glutamic acid and alanine, it may be suggested that glycine, with its freely rotating amino and carboxyl groups might be able to align LL-diaminopimelic acid in a peptide chain containing D-amino acids.

CONCLUSION

In dealing with a structure—the cell wall—found in so many different types of organism, it is obvious that the work of a few years described here cannot give more than a preliminary sketch of the subject. This review, is therefore, not an attempt to cover the complete field, but deals only with certain topics in the hope that it may serve as a pointer for future work. One obvious need is for detailed analyses of mucopeptide components from a wide variety of bacterial species. Without this, no valid generalizations can be made.

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The Action of Acriflavine on Brewers' Yeasts

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SUMMARY

The absorption of acriflavine by strains of *Saccharomyces carlsbergensis* and *S. cerevisiae* occurred at approximately the same rate and extent and was greatest in the range pH 4–5. In this range, the proportion of respiration-deficient cells produced by acriflavine treatment was also maximal. Furthermore, under more alkaline conditions, the acriflavine exerted a pronounced toxic effect. At pH 4–5, the proportion of mutant cells produced was about 50 times greater in strains of *S. cerevisiae* than in those of *S. carlsbergensis*, while fewer cells were killed. With *S. cerevisiae* the production of mutants was rapid and in certain strains exposure for 1 hr. to 50 μg. acriflavine/ml. led to about one-third of the cells mutating, while after 4 hr., all the cells were respiration-deficient. In contrast, exposure of strains of *S. carlsbergensis* for 24 hr. at this concentration of acriflavine induced only about one-tenth of the cells to mutate. The mutants took up no measurable amount of oxygen when examined in Warburg respirometers. As compared with the parent cultures, there was no difference in the ability of the cells to utilize carbohydrates and to undergo flocculation.

INTRODUCTION

Ephrussi and co-workers studied respiration-deficient mutants of bakers' yeast which they called 'la mutation petite colonie' (summary, Ephrussi & Hottinguer, 1951), while Lindegren and his associates refined the techniques for detecting such mutants (Ogur, St John & Nagai, 1957). Furthermore, the latter, by the use of selective media, demonstrated the effectiveness of a number of agencies in inducing this mutation (Ogur, Lindegren & Lindegren, 1954; Ogur & St John, 1956; Nagai & Nagai, 1958). With this background, the present work was concerned with the mode of action of acriflavine (euflavine), probably the most potent of the relevant agents. From a study of the absorption of acriflavine, its toxicity, and its ability to induce respiration-deficiency in a range of brewing yeasts, quantitative differences in its effect on *Saccharomyces cerevisiae* and *S. carlsbergensis* were observed. An attempt has been made to define the optimal conditions for such effects.

METHODS

Organisms. The following yeasts, obtained from the British National Collection of Yeast Cultures (NCYC), were used:

Saccharomyces cerevisiae NCYC, nos. 239, 1062, 1063, 1108 and 1109.
Saccharomyces carlsbergensis NCYC, nos. 396, 397, 398, 399 and 400.

These strains were selected to cover a range of behaviour, certain aspects of which are important under brewery fermentation conditions, namely ability to form flocs, yeast heads, and chains of cells (Eddy, 1958).

Media. The yeasts were maintained as stock cultures in a liquid malt extract + yeast extract + glucose + peptone medium (MYGP; Wickerham, 1951). Before testing, active cultures were prepared by transferring a small inoculum (0·25 ml.) to fresh medium (100 ml.) and incubating at 25° with gentle shaking for 18 hr. Investigations were mostly conducted in a defined medium (A) with a normal pH of 4·5 described by Kilkenny & Hinshelwood (1951). Where precise conditions of pH stability were required equal volumes of M/15 citrate + phosphate buffer and double strength medium A were mixed.

Stock solutions of acriflavine (B.P.C. quality) in distilled water at concentrations of 100 and 1000 μg./ml. were kept in the dark. They were sterilized by steaming on three successive days, and the concentrations adjusted by measurement of optical density at 4400 A., followed by appropriate dilution with sterile distilled water.

Detection of respiration-deficient colonies. After suitable treatment, cell populations were diluted and plated on MYGP agar, and incubated for 48 hr. at 25°. The colonies were then overlaid with a solution of triphenyl tetrazolium chloride (TTC) solidified with agar, and buffered to pH 7 (Ogur *et al.*, 1957). Glucose (0·5 %, w/v) was added to the overlay mixture for improved resolution of mutant (white) and normal (red) colonies (Nagai, 1959).

Estimation of viability and growth rate in solid media. Suspensions of yeast in media containing gelatin were prepared and examined microscopically over a period of 4–5 hr. at 18° in a haemocytometer (Gilliland, 1959). Suspensions were prepared in the appropriate medium, with or without acriflavine, containing 6 % (w/v) gelatin and held at 25° to preserve the liquid state. While still liquid, one drop of a suspension was transferred to a haemocytometer and the edges of the cover-glass sealed with vaseline. After a period to allow the gelatin to set, the preparation was examined microscopically and the position of each cell noted.

Absorption of acriflavine. Suspensions of yeast cells (10^8/ml.) were prepared, in M/15 citrate + phosphate buffer of appropriate pH, together with glucose (2 %, w/v) when required. The suspensions were kept agitated with a wrist action shaker. At zero time a solution of acriflavine was added to 50 μg./ml. Samples were removed at once, and at suitable intervals subsequently, and centrifuged. A measured sample of the clear supernatant liquid was diluted appropriately and the optical density (OD) determined, to provide a measure of acriflavine concentration. At 4400 A. there was a linear relationship between OD and acriflavine concentration within the range 0–10 μg./ml.

Measurement of carbon dioxide evolution and oxygen consumption. These were carried out in a conventional Warburg apparatus, at 25°. Yeasts were grown in MYGP medium for 18 hr. at 25° in gently shaken culture, centrifuged, and re-suspended in M/15 KH_2PO_4 solution (pH 4·5). Cell material corresponding to 2 mg. dry wt. was used in each vessel. Respiratory substrates (2 %, w/v) were prepared in a similar KH_2PO_4 solution.

Growth rate of respiration-deficient strains in liquid media. This was followed by turbidimetric estimation of suitably diluted samples.

RESULTS

Production of respiration-deficient strains of Saccharomyces cerevisiae *and* S. carlsbergensis

Strains of the yeasts were exposed to acriflavine at concentrations of 10, 50 and 100 μg./ml. in medium A at pH 4·6 for 4 hr. A cell concentration of 10^6/ml. was used. The typical results shown in Table 1 demonstrate that acriflavine had a pronounced mutagenic effect on the strains of *Saccharomyces cerevisiae* used, but only a small one on *S. carlsbergensis*. These studies were repeated on two strains of

Table 1. *Production of mutants of* Saccharomyces cerevisiae *and* S. carlsbergensis *by acriflavine*

Cultures were treated with different concentrations of acriflavine for 4 hr. at 25° C. After dilution and plating, the resultant colonies were overlaid with a mixture of agar and tetrazolium salt. 'Sectored' colonies are those derived from juxtaposed mutant and normal cells.

		Acriflavine (μg./ml.)					
		10		50		100	
	Yeast strain	% mutant colonies	% sectored colonies	% mutant colonies	% sectored colonies	% mutant colonies	% sectored colonies
S. cerevisiae	239	9	22	86	12	100	0
	1026	0	9	98	2	—	—
	1108	90	9	77	16	94	6
S. carlsbergensis	396	1	25	0	28	1	5
	398	6	7	6	12	15	19
	399	0	0	0	0	0	0

S. carlsbergensis with the exposure time lengthened to 24 hr., but respiration-deficient cells still made up only a small proportion of the final population (Table 2). 'Sectored' colonies where they occurred could be accounted for by the tendency of certain strains to form either chains of cells, or flocs, resulting in the juxtaposition of unchanged and mutant cells. This inevitably makes it difficult to draw quantitative conclusions where sectoring was common; therefore, in the majority of trials, the strains *S. carlsbergensis* 396 and *S. cerevisiae* 239, with reasonably unclustered growth habits, were used.

Table 2. *Production of mutants of* Saccharomyces carlsbergensis *by acriflavine*

Experimental procedure was as in Table 1, except that the cells were exposed to acriflavine for 24 hr.

		Acriflavine (μg./ml.)					
		10		50		100	
	Yeast strain	% mutant colonies	% sectored colonies	% mutant colonies	% sectored colonies	% mutant colonies	% sectored colonies
S. carlsbergensis	396	9	2	40	6	42	7
	399	0	29	1	96	—	—

Characteristics of respiration-deficient strains

Cultures obtained by transfer of 'white' colonies after TTC overlay were maintained on slopes of MYGP agar after two transfers in MYGP broth with checking by TTC overlay at each transfer. Their stability was demonstrated by regular testing during periods of up to one year using the TTC overlaying technique and manometric measurements of gas exchange. These tests revealed consistent inability either to reduce TTC or absorb oxygen.

Colony size could not be measured objectively, but diameters of the order of one-fifth that of the parent strains were regularly observed after a 48 hr. incubation period.

Spectroscopic examination of respiration-deficient strains, grown in MYGP under aerobic conditions at 25°, revealed the existence of a strong absorption band at 5500 A., corresponding to reduced cytochrome *c*; bands at 6000 and 5620 A. corresponding to the *a* and *b* components were not visible. All the bands were evident when the parent strain was examined.

It was therefore concluded that the cytochrome complement of the respiration-deficient cultures is similar to that of the 'petite colonie' strains described by Slonimski (see Ephrussi, 1953).

Further evidence of the similarity to the 'petite' strains was provided by the inability of the respiration-deficient strains to grow in media with acetate or lactate as carbon sources.

Demonstration of the direct mutagenic action of acriflavine

The presence of a high proportion of respiration-deficient cytoplasmic mutants in a cell population that had been exposed to acriflavine might be due to: (*a*) accelerated growth of pre-existing mutants; (*b*) direct mutagenic action; (*c*) accelerated growth of induced mutants. Nagai & Nagai (1958) suggested that the first alternative is unlikely and the following investigation shows that only the second alternative is applicable.

Saccharomyces cerevisiae 239 and *S. carlsbergensis* 396 and respiration-deficient mutants derived from them, 239A and 396A, were examined by means of the slide-culture technique after it had been established that substantially all the cells were viable. The relative number of dead cells was taken as the proportion of cells staining after suspension in 0·01 % (w/v) methylene blue, buffered at pH 5·0. The increase in numbers of normal and mutant cells with and without acriflavine present was followed for a period equivalent to that used in the preparation of mutants (Table 3). Clearly, in the presence of acriflavine the respiration-deficient cells were inferior in their rate of cell division to the corresponding normal cells and, far from being selected, are placed at a severe disadvantage. Their presence must therefore have been a direct effect of the acriflavine. Further, it will be recalled that Table 1 shows, for example, 86 % of respiration-deficient cells in the entire population of *S. cerevisiae* 239 after treatment with acriflavine (50 μg./ml.) for 4 hr. Table 3 shows that in this period cell numbers have increased by 76 %. Hence an original population of 100 cells has increased to 176 cells and 86 % of these (151 cells) are respiration-deficient. It follows, therefore, that at least 51 of the original population had mutated.

Table 3. *Growth of yeasts whilst exposed to acriflavine*

Haemocytometer slides were set up containing yeast suspensions in gelatin-solidified medium A, together with appropriate amounts of acriflavine. The cultures were incubated at 18° for 4 hr., and examined microscopically at the beginning and end of this period. Buds having a diameter less than one-fifth of the parent cell were not counted.

Yeast strain		Acriflavine (μg./ml.) 0	10	50	100
		Increase in cell number as % original no.			
Saccharomyces cerevisiae	239	140	96	76	68
	239A	100	68	20	5
S. carlsbergensis	396	128	66	23	11
	396A	28	12	0	0

The optimal conditions for the production of respiration-deficient cytoplasmic mutants

Effect of pH *on mutagenic action.* *Saccharomyces cerevisiae* 239, and *S. carlsbergensis* 396 were incubated in medium A at a range of concentrations of acriflavine, and at pH values between 4 and 8. The incidence of respiration-deficient colonies in the suspension was estimated by the overlay technique (Tables 4 and 5). Clearly mutation was most pronouced at low pH values, but the effect of increasing alkalinity was not only to decrease mutagenic action but also to increase toxicity (Albert, 1951).

Table 4. *The effect of* pH *on the mutagenic action of acriflavine on* Saccharomyces cerevisiae 239

Tubes were set up containing yeast suspension in medium A buffered appropriately and acriflavine at 50 or 100 μg./ml.; incubation for 4 hr. at 25°. After incubation, the suspensions were diluted and plated on MYGP agar, incubated at 25° for 48 hr. and overlaid with TTC agar. The results given are the means of six replicates.

Acriflavine (μg./ml.)	pH value of medium 4 Colonies per plate	4 % mutants	6 Colonies per plate	6 % mutants	8 Colonies per plate	8 % mutants
0	465	0	357	0	262	0
50	353	84	151	32·5	96	22
100	262	97	131	42	68	54

The original suspension gave 188 colonies/plate, all normal.

Effect of pH *value on the absorption of acriflavine.* A study of absorption of acriflavine over a range from pH 3 to 8 revealed no quantitative or qualitative differences between strains *Saccharomyces cerevisiae* 239 and *S. carlsbergensis* 396; the results for the former strain are shown in Fig. 1. The optimal pH value for absorption, pH 5, was also similar in the two strains. This was close to the optimal pH value for mutagenic action. On the other hand, a considerable absorption took place at pH 8, when the acriflavine was more toxic and little mutation occurred.

Table 5. *The effect of* pH *on the mutagenic action of acriflavine on* Saccharomyces carlsbergensis 396

For experimental details, see Table 4. Period of incubation, 24 hr.

	pH of medium							
	4		5		6		8	
Acriflavine (μg./ml.)	Total no. per plate	% mutants	Total no. per plate	% mutants	Total no. per plate	% mutants	Total no. per plate	% mutants
0	230	0	190	0	370	0	150	0
10	90	13	30	15	No growth	—	No growth	—
50	33	9	2	0	No growth	—	No growth	—
100	17	5	3	3	No growth	—	No growth	—

Glucose had no effect on the rate or extent of the absorption. The mutagenic action at pH value of 3 and less was not examined since little absorption took place.

Viability after exposure to acriflavine. Since *Saccharomyces cerevisiae* and *S. carlsbergensis* differed in their susceptibility to the mutagenic effect of acriflavine, it seemed possible that they might differ also in sensitivity to the toxic action of the mutagen. Estimates of the toxic effect were obtained by using the slide-culture technique. Yeasts were exposed to acriflavine whilst in medium A, and were washed three times in saline solution before microscopic examination. The results (Table 6) show that *S. cerevisiae* was resistant to the toxic effect of acriflavine whilst being susceptible to the mutagenic effect, the reverse applying with *S. carlsbergensis*.

Table 6. *Viability of yeast after exposure to acriflavine for* 4 *hr.*

Yeast strain	Acriflavine (μg./ml.)	Cells observed initially	Non-budding cells observed after 5 hr.	Viability %
Saccharomyces cerevisiae 239	0	82	3	96
	10	106	2	98
	50	89	10	89
	100	80	5	94
S. carlsbergensis 396	0	85	0	100
	10	110	10	91
	50	94	34	64
	100	103	44	58

The rate of production of respiration-deficient cells. To follow the pattern of appearance of mutants with time at different initial acriflavine concentrations, *Saccharomyces cerevisiae* 239 was used since it is particularly susceptible to the mutagenic effects over short periods during which the toxic effects are negligible (Fig. 2). The considerable incidence of mutants after 1 hr. at an acriflavine concentration of 50 μg./ml. is of interest because less than 12% of the cells have cleaved during that period in the presence of acriflavine (Table 7). A second feature is the limited mutagenic effect of acriflavine at 10 μg./ml.; at the cell density used such an initial concentration never led to the whole population mutating.

On increasing the concentration of the yeast suspension at constant acriflavine concentration, the production of mutants was progressively decreased.

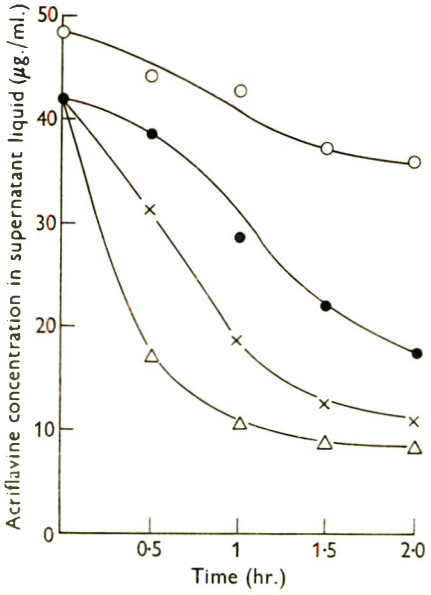

Fig. 1

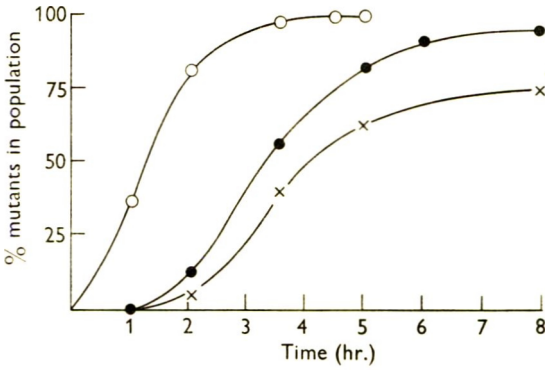

Fig. 2

Fig. 1. The absorption of acriflavine by *Saccharomyces cerevisiae* 239. The system comprised: 100 ml. yeast (10^8 cells/ml.) suspended in M/15 citrate + phosphate buffer. Acriflavine solution was added at zero time, and a sample removed for assay. Suspensions were shaken at 20°. ○ = pH 3; △ = pH 5·0; × = pH 7·0; ● = pH 8·0.

Fig. 2. The production of respiration-deficient mutants with time. The system comprised: *Saccharomyces cerevisiae* 239, suspended in medium A + acriflavine. Cell concn. 10^6/ml. Total volume 10 ml. Samples (0·5 ml.) withdrawn at times shown. Suspensions shaken gently at 25°. Acriflavine (μg./ml.): ○ = 50; ● = 20; × = 10.

Selected physiological characteristics of mutant strains

Estimates of the endogenous gas exchange were made over 30 min. in the Warburg apparatus. The quotients $Q^{air}_{O_2}$, $Q^{air}_{CO_2}$, and $Q^{N_2}_{CO_2}$ were then measured over the period 10–40 min. after addition of glucose. No measurable endogenous gas exchange was observed with the ten strains tested. The $Q^{air}_{CO_2}$ and $Q^{N_2}_{CO_2}$ values for any one strain were the same, and for all strains were within the range 150–200 μl. CO_2/mg. dry wt./hr. No oxygen uptake was ever observed. However, Slonimski (1958) quoted a diminution of oxygen consumption of 98%, and a residual uptake

Table 7. *Growth of yeast in liquid medium + acriflavine*

System comprised *Saccharomyces cerevisiae* 239 suspended in medium A + acriflavine (50 μg./ml.). Cell concn. 10^6/ml. Suspension shaken gently at 25°. At intervals, samples were removed aseptically, diluted, and plated on MYGP agar. Colonies scored after incubation for 48 hr. at 25°. Figures are means of six replicates.

Time	Colonies
0	102
1	114
2	125
4	180

of 2% (about 4 μl./vessel/hr.) would not be measured with certainty in a conventional Warburg respirometer.

Fermentation characteristics. Four mutant strains were grown on brewers' wort (containing principally fructose, sucrose, glucose, maltose, maltotriose and maltotetraose), and their ability to utilize these constituents compared with that of the equivalent normal strain. No differences were detected.

Flocculation. Eight strains of respiration-deficient mutants showed no change in this characteristic when compared with the equivalent parent strain.

DISCUSSION

Ephrussi & Hottinguer (1950) contended that when a population of yeast cells is exposed to acriflavine, respiration deficiency is only manifested in those daughter cells which are produced during the period of exposure or shortly afterwards. The parent cells appear not to be affected by the mutagen and retain their normal respiratory abilities. However, in the present work there is some evidence for direct mutagenic action on the parent cells. Thus from Fig. 2 it is evident that a high proportion of mutants appeared in a population of *Saccharomyces cerevisiae* which was exposed to acriflavine at 50 μg./ml. for 1–2 hr. under conditions where little cell division had occurred (Table 7) and the viability was over 94% (Table 6).

Differences between *Saccharomyces carlsbergensis* and *S. cerevisiae* with regard to the mutagenic and toxic actions of acriflavine are not associated with the rates of adsorption of the dye. Perhaps the organisms differ instead in the rate at which acriflavine penetrates the cytoplasmic membranes. However, since in *S. carlsbergensis* relatively few mutants were produced after long exposure, this explanation is not entirely satisfactory. It would seem much more likely that there is a difference between the two species in the materials responsible for synthesis of, for example, cytochrome components. A further explanation can be suggested, namely that equal numbers of mutants are initially produced by the two species but that many of these newly formed mutants of *S. carlsbergensis* are incapable of division. It is, however, unlikely that direct evidence for this explanation can be obtained.

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The Function of Glycerol, Cholesterol and Long-Chain Fatty Acids in the Nutrition of *Mycoplasma mycoides*

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SUMMARY

Mycoplasma mycoides var. *mycoides* requires for growth a number of preformed lipid precursors. Media containing glycerol, cholesterol, a saturated and an unsaturated fatty acid, defatted bovine serum albumin and an additional defatted serum protein fraction (Fraction C) can supply these requirements. Albumin is believed to function by binding fatty acids, and Fraction C by binding cholesterol. The requirement for a saturated fatty acid can be satisfied by myristic, palmitic, stearic or margaric acid, lauric acid being less effective. The requirement for an unsaturated fatty acid can be satisfied by oleic acid, linoleic and linolenic acids being less effective. Organisms incubated in a medium deficient in either glycerol, the Fraction C + cholesterol system, or oleate, but adequate with respect to all other nutrients, died rapidly. Death was accompanied by lysis. Death due to a deficiency of glycerol or of cholesterol was prevented either by the omission of uracil (an essential nutrient) or by addition of chloramphenicol. Death due to oleate deficiency was not prevented by the omission of uracil. Morphological changes which resulted from each of these deficiencies are illustrated by electron micrographs. The hypothesis is advanced that glycerol, cholesterol and long-chain fatty acids are all needed for the synthesis of an undetermined cell component which is necessary for the structural integrity of the cell, and that the synthesis of this is more sensitive to a deficiency of these nutrients than is the synthesis of cytoplasm.

INTRODUCTION

A medium of partly defined composition which supported good growth of *Mycoplasma mycoides* was described by Rodwell (1960). It contains a heat-stable defatted serum protein fraction (Fraction C), cholesterol, an unsaturated fatty acid (or 'Tween 80'), serum albumin, glycerol, high concentrations of DL- or L-lactate and glucose. It enabled requirements for adenine, guanine, uracil, thymine, riboflavin, thiamine, nicotinic acid, α-lipoic acid, pantothenic acid and biotin to be recognized. The amino acid requirements were not defined. The function of the medium components which serve as lipid precursors has now been investigated.

The concentration of glycerol required for maximum growth increased from about

5 μg./ml. in static cultures to about 50 μg./ml. in cultures rotated to give a moderate degree of aeration during incubation (Rodwell, 1960). Glycerol is rapidly oxidized to acetate and CO_2 by suspensions of *Mycoplasma mycoides* grown aerobically. Evidence for a flavoprotein-catalysed oxidation of glycerophosphate was reported by Rodwell & Rodwell (1954). If this pathway were irreversible, glycerophosphate could not be formed from hexose, and an exogenous source of glycerol would be needed for lipid synthesis. Experiments have shown that (^{14}C)-labelled glycerol is incorporated, probably without dilution from hexose, into lipids by growing cultures of *M. mycoides* (Plackett, 1961). The quantitatively greater requirement for glycerol under aerobic growth conditions is due to the greater rate of oxidation and consequent loss of glycerophosphate for synthetic reactions. The observation that cultures of *M. mycoides* undergo rapid lysis when growing under conditions of glycerol deficiency, but not when some other nutrients are growth-limiting, suggested that glycerol deficiency might cause the phenomenon known as unbalanced growth.

The function of sterols in the growth of mycoplasma organisms is of considerable interest, since other bacteria are not known to require or to synthesize them, except possibly in trace amounts by some species (Dauchy, Kayser & Villoutreix, 1956; Fiertel & Klein, 1959). Smith (1959, 1960) reported both cholesterol esterase (ester-synthesizing) and lipase (ester-splitting) activity in a strain of human origin, and Lynn & Smith (1960) studied the distribution of esterified and free cholesterol between the soluble and insoluble fractions of suspensions of disrupted organisms from several strains. Their results indicated that both free and esterified cholesterol were present in both fractions. Sterol was not detected in two strains of *Mycoplasma laidlawii* which did not require sterol for growth. It was suggested previously that cholesterol exercised a protective function during the growth of *M. mycoides*. From the experimental data it could not be determined whether cholesterol is required as a nutrient in the strict sense (i.e. whether it is incorporated into the cellular structure) in addition to the postulated protective function (Rodwell, 1956). A detoxifying function for cholesterol was also suggested to explain its growth-promoting effect for *M. laidlawii* (Butler & Knight, 1960; Razin & Knight, 1960).

A requirement for an unsaturated fatty acid for the growth of *Mycoplasma mycoides* was also postulated previously (Rodwell, 1956). The partly defined medium (Rodwell, 1960) contains bovine serum albumin and 'Tween 80'. The fatty acid requirements and the functions of serum albumin and of fatty acids have now been investigated.

METHODS

Organism. The strain used was the V5 strain of *Mycoplasma mycoides*, isolated from a case of bovine pleuropneumonia in 1936.

Medium A. The composition of a partly defined medium was previously reported (Rodwell, 1960). Details for its preparation are now described. It contains: $Na_2HPO_4 + KH_2PO_4$ (pH 7·8), 0·04 M; Na DL-lactate, 0·14 M; cholesterol, 10 μM; and (per l.) glucose, 5·0 g.; bovine serum Fraction C, 0·5 g.; bovine serum albumin Fraction V (Armour), 2·0 g.; Tween 80, 5·0 mg.; glycerol, 0·05 g.; acid-hydrolysed casein (Difco, vitamin-free), 2·5 g.; tryptic digest of casein, $\equiv$ 2·0 g. casein; L-cystine, 2·5 mg.; L-tryptophan, 5·0 mg.; $MgSO_4.7H_2O$, 50 mg.; $FeSO_4.7H_2O$, 0·25 mg.; $MnSO_4.4H_2O$, 0·25 mg.; adenine, 5·0 mg.; guanine, 5·0 mg.; uracil,

5·0 mg.; thymine, 2·5 mg.; Ca pantothenate, 0·25 mg.; riboflavin, 0·25 mg.; pyridoxal-HCl, 0·05 mg.; nicotinamide, 0·5 mg.; thiamine, 0·5 mg.; biotin, 0·01 mg.; DL-α-lipoic acid, 0·01 mg. The medium was distributed in 20 mm. diameter optically matched test tubes covered with aluminium caps and sterilized by autoclaving at 10 lb./sq.in. for 10 min. Bovine serum Fraction C was added to the medium before autoclaving. Bovine serum albumin Fraction V and glucose were added from sterile (filtered) solutions after autoclaving the bulk of the medium. The final volume after these additions was 5 ml./tube. The pH value of the medium was 7·5.

Medium B. As a result of the studies on the fatty acid requirements to be described medium A was modified. Bovine serum albumin Fraction V was replaced by Fraction V extracted by *iso*-octane + glacial acetic acid mixture (19 + 1; Goodman. 1957) at the decreased concentration of 1·0 g./l. Tween 80 was replaced by Na palmitate + Na oleate each at 20 μmole/l. Inositol (1 mg./l.), choline chloride (1 mg./l.) and leucovorin (folinic acid) (0·25 mg./l.) were also added.

Preparation of medium constituents. Na DL-lactate was prepared from CP or AR grade lactic acid. The Zn salt was made, twice recrystallized, decomposed with sodium carbonate, and the remaining traces of zinc removed by successive treatments with 8-hydroxyquinoline in chloroform (Waring & Werkman, 1942). Excess carbonate was then decomposed by boiling with additions of HCl until the pH value remained at about 7·0. Alternatively, the Zn salt was suspended in water and shaken with a small excess of ion-exchange resin (Amberlite IR.120, H^+ form). The resin was drained, washed, the supernatant fluid and washings passed through a small column of fresh resin, and the acid titrated to pH 7·0 with NaOH. Solutions prepared by either method had equal activity. Several samples of CP and AR grade lactic acid when tested without purification inhibited growth at higher concentrations.

Bovine serum Fraction C was made from ox serum. Serum was diluted with an equal volume of water, heated to 70°, cooled, adjusted to pH 5·6 with 5 % (v/v) acetic acid, heated to 80°, cooled, and filtered on a Büchner funnel. The filtrate was saturated with ammonium sulphate. The orange-coloured precipitate which rose to the surface was collected by gravity filtration through folded Whatman no. 2 paper. It was suspended in a small volume of water and the suspension dialysed thoroughly against four to six changes of distilled water. The dialysed solution containing some insoluble material was adjusted to pH 4·8–5·0, whereupon about half the protein was precipitated. The suspension was chilled to 0–2°, and 10 vol. cold ethanol added slowly. The precipitate was washed twice with the same volume of ethanol at −5° on the centrifuge, then successively with ethanol, ethanol + ether mixture, and ether on a Büchner funnel. The yield of cream-coloured powder was 1·5–2·0 g./l. serum taken. The product was stable indefinitely. For use in the medium, it was ground in a mortar with water, 0·05N-NaOH added to pH 7·4 (about 7·0 ml./g. powder), and the mixture stirred for about 30 min. The concentration was adjusted to 1 % (w/v) with water, and a small amount of insoluble material centrifuged off and discarded.

Cholesterol was added to the medium as a colloidal dispersion in water. One volume of a 20 μM solution in ethanol at 60° was injected rapidly into 19 vol. of stirred water at 60°. Cholesterol dispersions were stored in evacuated Thunberg tubes in a refrigerator. To avoid precipitation it was necessary to add the cholesterol dispersion to the medium after the bovine serum Fraction C had been added.

Tween 80 was purified as described by Davis (1947).

Oleic acid was purified from commercial redistilled oleic acid by precipitation of the long chain saturated fatty acids from acetone at $-20°$, followed by five recrystallizations of the unsaturated acid from acetone at $-60°$ (Brown & Shinowara, 1937).

Linoleic and linolenic acids were prepared by saponification followed by bromination of the fatty acid mixtures, and would therefore be a mixture of the *cis* and *trans* isomers.

Lauric, myristic, palmitic and stearic acids were Eastman-Kodak products and were recrystallized several times from acetone. Margaric acid was a product of L. Light and Co. (Colnbrook, Bucks). Fatty acid impurities were not detected in any of the acids by reversed-phase circular paper chromatography as described by Nowotny, Lüderitz & Westphal (1958). Margaric acid migrated at a speed intermediate between those of palmitic and stearic acids in this solvent system. The fatty acids were added to the medium as the Na salts. Sparingly soluble salts were warmed to dissolve them before adding them to the medium.

Bovine serum albumin Fraction V (Armour) was heated at 56° for 30 min. at pH 7·0 to inactivate lipase (Davis & Dubos, 1946). Bound fatty acids were extracted with *iso*-octane containing glacial acetic acid as described by Goodman (1957). The fatty acid content of the albumin after extraction was not determined.

Tryptic digest of casein was made from 'vitamin-free' casein (Glaxo) and was charcoal-treated as described by Roberts & Snell (1946).

The other reagents were commercial products. Stock solutions were stored in a deep-freeze cabinet and were replaced at monthly intervals.

Preparation of inocula. Organisms for inocula were grown in BVF-OS medium (Turner, Campbell & Dick, 1935). Cultures were centrifuged at 15,000*g* for 15 min., the tubes drained, the deposit washed once with 0·4 M-sucrose solution containing 0·01 M-phosphate buffer (pH 7·0) and resuspended in sucrose + phosphate solution.

Growth tests. Duplicate tubes were seeded with about 2×10^6 viable elements/ml., and the growth estimated turbidimetrieally at 660 mμ at intervals during static incubation at 37°. Streaming birefringence was judged visually by the amount of 'swirl' when the cultures were gently agitated. In many instances the cultures were examined by dark-field microscopy.

Nutritional deficiency experiments. Replicate tubes of partly defined medium with the additions or omissions indicated in the text were seeded with inocula of 5×10^7 to $5{\cdot}5 \times 10^8$ viable elements/ml. and were incubated at 37° in an upright position without shaking. One tube of each replicate set was used for each viable count or for the preparation of specimens for electron microscopy. Two tubes of each replicate set, and also two tubes of uninoculated medium, were reserved for turbidity measurements at 440 mμ.

Counts of viable elements. These were made by a modification of the Miles & Misra (1938) method, as adapted for counting *Mycoplasma mycoides* by Mr G. S. Cottew (personal communication).

Preparation of specimens for electron microscopy. The cultures were chilled to 0°, centrifuged in the cold, the pellets resuspended in sucrose + phosphate solution and the suspensions squirted into 10 vol. of sucrose + phosphate containing 4% (w/v) formaldehyde. After standing for 4–18 hr. at room temperature they were centrifuged and the pellets washed twice and resuspended in distilled water.

RESULTS

Effect of glycerol deficiency

Tubes of (i) Medium A, (ii) Medium A lacking glycerol, (iii) Medium A lacking uracil, and (iv) Medium A lacking both glycerol and uracil, were seeded with $5{\cdot}5 \times 10^8$ viable elements/ml. Viable element counts and turbidity measurements were made at intervals during incubation for 24 hr. During this period, the viable count increased about fourfold in (i), decreased to about 15 % of the number inoculated in (ii), and remained almost stationary in (iii) and (iv) (Fig. 1*b*). That is, a

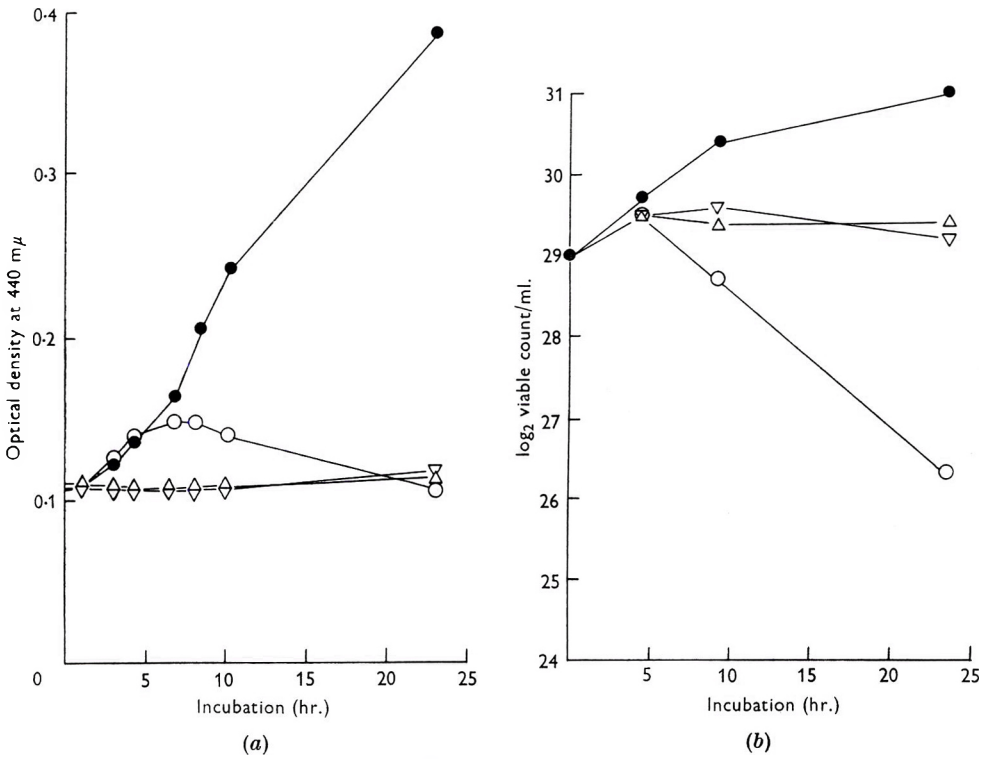

Fig. 1. The effect of the omission of glycerol and uracil on the turbidity and viability of cultures of *Mycoplasma mycoides*. (*a*) Turbidity; (*b*) viable element count. ●, Complete medium; ○, glycerol omitted; △, uracil omitted; ▽, glycerol and uracil omitted.

deficiency of uracil prevented death which would otherwise have occurred through glycerol deficiency, throughout this incubation period. Incubation in (ii) resulted in: an early increase, followed by a decrease, in turbidity (Fig. 1*a*); an early (after about 4 hr. of incubation) loss of streaming birefringence; later, an obvious increase in the viscosity of the culture, suggesting that extensive cellular lysis had occurred. Profound morphological changes culminating in lysis during incubation in the glycerol-deficient medium were evident in electron micrographs. Owing to technical difficulties, specimens for electron microscopy were obtained from a duplicate experiment on which turbidity measurements, but not viable counts, had been made. Electron micrographs prepared from cultures in (iii) (Pl. 1, fig. 3) showed a range of

forms characteristic of the organism grown in (i) (Pl. 1, figs. 1, 2) throughout the 24 hr. incubation period. In (ii) most of the forms were enlarged within 4–8 hr. of incubation; many had shrunken appendages, and a few flattened ghost-like forms were present (Pl. 1, fig. 4). After 24 hr. of incubation there was a great preponderance of ghost-like forms, most of which contained a single, small, electron-dense granule (Pl. 2, figs. 5, 6); there were only a few electron-dense forms which were probably representative of the remaining viable forms (Pl. 2, fig. 5). After 24 hr. incubation in (iv), the forms resembled preparations from (ii) after only 4 hr. incubation (Pl. 2, fig. 7). That is, a deficiency of uracil retarded the onset of the morphological changes caused by glycerol deficiency.

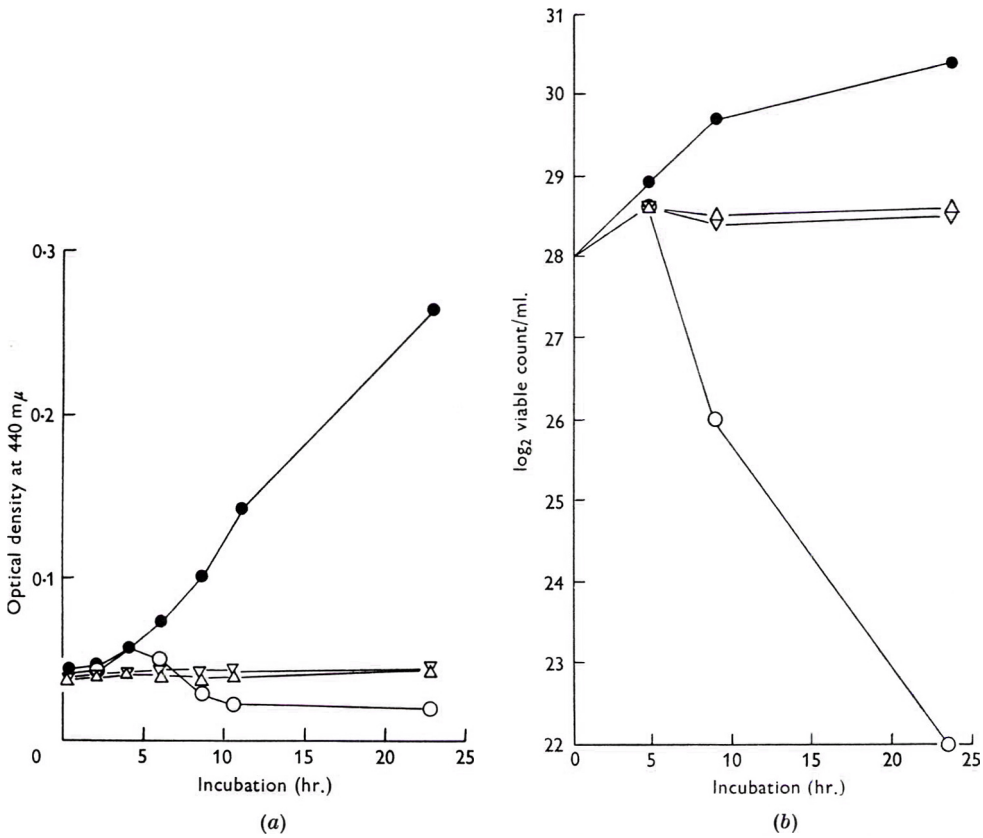

Fig. 2. The effect of the addition of chloramphenicol (CAP) on 'glycerol-less death' of *Mycoplasma mycoides*. (*a*) Turbidity; (*b*) viable element count. ●, Complete medium; ○, glycerol omitted; △, CAP (μg./ml.) added; ▽, glycerol omitted and CAP (20μg./ml.) added.

Like the omission of uracil, the addition of chloramphenicol (CAP) prevented death caused by glycerol deficiency and the associated turbidity changes (Fig. 2*a*, *b*). Viable counts remained stationary during a 24 hr. incubation period in Medium A with the addition of 20μg. CAP/ml., and in glycerol-deficient medium containing 20μg. CAP/ml. Only about 1·5% of the viable elements inoculated survived after 24 hr. of incubation in glycerol-deficient medium. There was a maximum mortality

rate of about 80 % per generation time. The greater death rate found in this experiment as compared with that illustrated in Fig. 1*b*, may have been due to differences in the size of the inocula used in the two experiments.

Effect of cholesterol + bovine serum Fraction C deficiency

Although direct evidence is lacking, it is thought that the function of bovine serum Fraction C is to bind cholesterol in a water-soluble assimilable form. Incubation of mycoplasma organisms in Medium A lacking both cholesterol and Fraction C resulted in an early loss (after about 5 hr. of incubation) of streaming birefringence.

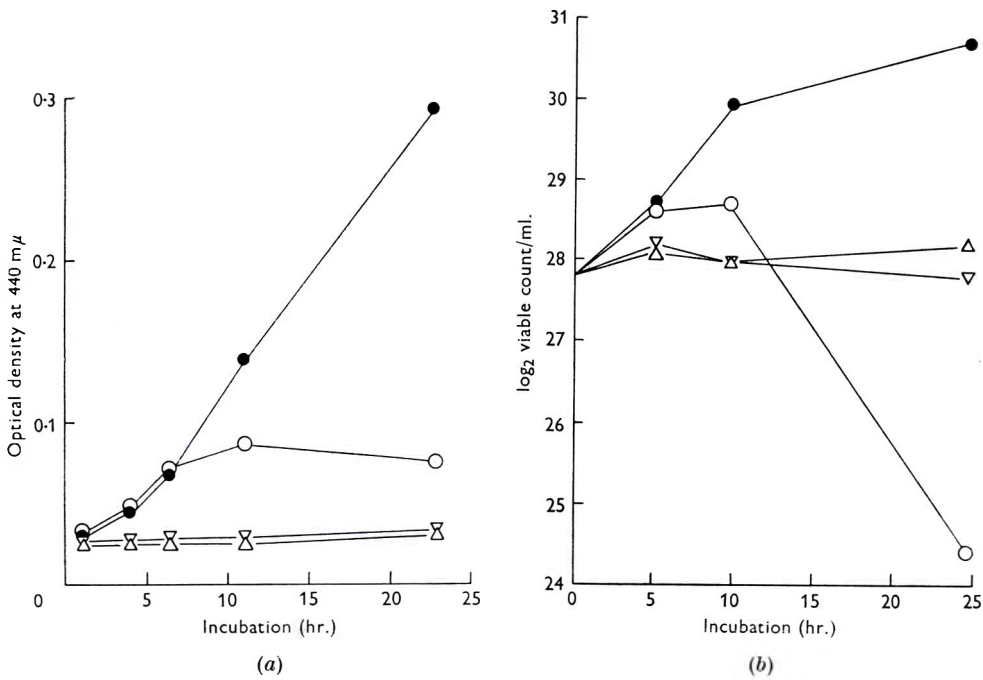

Fig. 3. The effect of the omission of cholesterol, bovine serum, Fraction C and uracil on the turbidity and viability of cultures of *Mycoplasma mycoides*. (*a*) Turbidity; (*b*) viable element count. ●, Complete medium; ○, cholesterol and bovine serum, Fraction C omitted; △, uracil omitted; ▽, cholesterol, bovine serum Fraction C and uracil omitted.

an increase followed by a gradual decrease in turbidity (Fig. 3*a*) and, after about 10 hr. of incubation, a rapid decrease in viability (Fig. 3*b*). As with glycerol deficiency, these changes did not occur when uracil was omitted. After 5 hr. of incubation many of the forms seen were swollen, and some had tenuous outgrowths which appeared to be in the process of becoming pinched off. A tendency for the forms to aggregate when the suspensions were dried on the grids was noticeable (Pl. 2, fig. 8). After longer incubation, the material appeared to be largely aggregated into masses in which little cellular structure could be seen (Pl. 3, figs. 9, 10). It is possible that a deficiency of cholesterol + bovine serum Fraction C caused the forms to become more susceptible to damage during the fixing and drying processes; nevertheless, the morphological changes differed from those caused by glycerol deficiency.

The omission of either bovine serum Fraction C or cholesterol singly caused decreases in viable counts, but the death rate was greater when both components were omitted (Fig. 4*a*, *b*).

Requirements for long-chain fatty acids

Medium A contains bovine serum albumin Fraction V. Tween 80 was also included, although it increased growth only slightly in the presence of Fraction V. Extraction with *iso*-octane + glacial acetic acid almost abolished the growth-promoting activity of Fraction V when Tween 80 was omitted. Its activity was partly restored

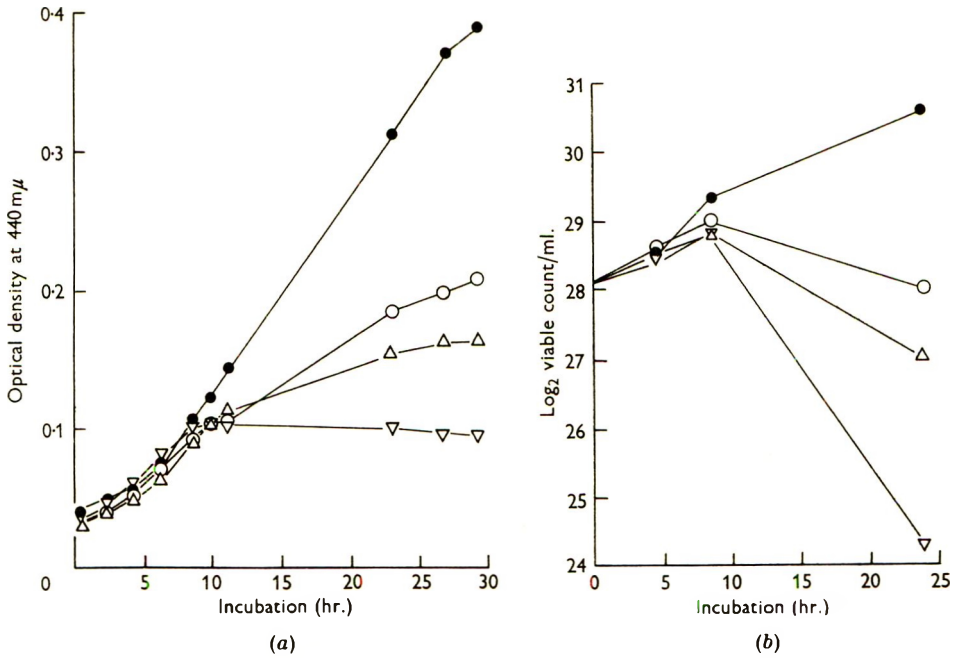

Fig. 4. The effect of the omission of cholesterol and/or of bovine serum Fraction C, on the turbidity and viability of cultures of *Mycoplasma mycoides*. (*a*) Turbidity; (*b*) viable element count. ●, Complete medium; ○, cholesterol omitted; △, bovine serum Fraction C omitted; ▽, cholesterol and bovine serum Fraction C omitted.

by the addition of Tween 80 or of oleate, and fully restored by a mixture of a saturated and an unsaturated fatty acid. Of the even-numbered saturated fatty acids from C_{12} to C_{18}, palmitate and stearate were of about equal activity; laurate was less active when growth tests were performed in the presence of a constant concentration of oleate (Fig. 5). Myristate was as active as palmitate or stearate but the growth rate was slower (Fig. 6). The odd-numbered C_{17} acid, margaric acid, was as active as palmitic or stearic acids (Fig. 7). Oleate, linoleate and linolenate were the only unsaturated fatty acids tested; oleate was the most active, the others being active only over a very narrow concentration range (Fig. 8).

The presence of *iso*-octane-extracted Fraction V was essential. The optimum concentration in the presence of 0·02 μmole each of palmitate and oleate/ml. was about 1·0 mg./ml.; growth was not significantly improved by increasing the concen-

trations of any of these components, or in the presence of more complex fatty acid mixtures.

All of these growth tests were performed in the presence of inositol, choline and leucovorin, although any influence these may have had on growth was not determined. Medium B was formulated as a result of these tests.

Cultures of *Mycoplasma mycoides* strain V5 in medium B showed very marked streaming birefringence, and electron micrographs showed a well developed branched mycelial morphology. It is now realized that Medium A is slightly deficient or disproportionate in its fatty acid composition (compare Pl. 1, fig. 1, and Pl. 3, fig. 11).

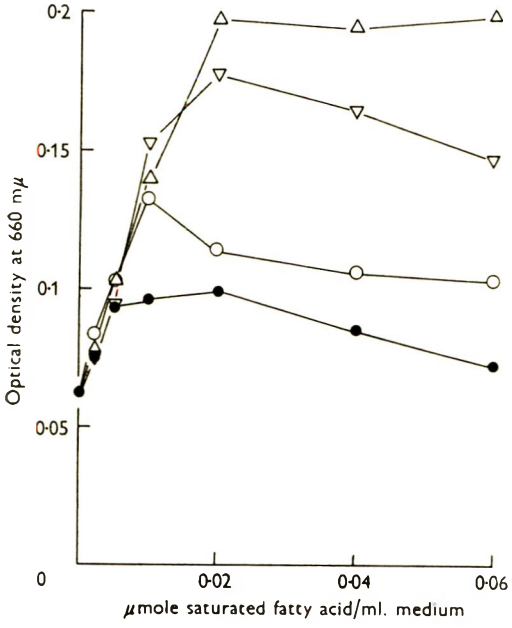

Fig. 5

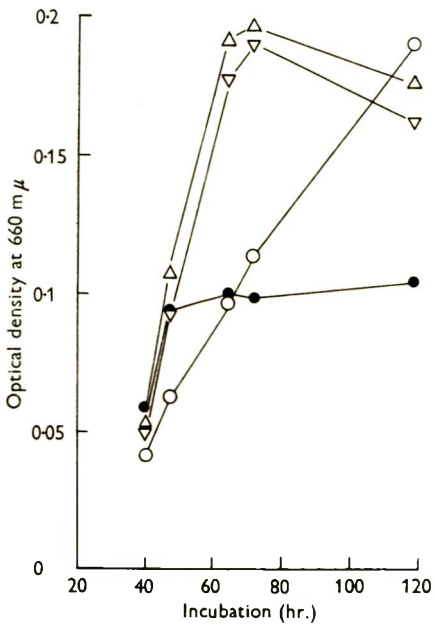

Fig. 6

Fig. 5. The effect of saturated fatty acids on growth of *Mycoplasma mycoides* in the presence of 0·01 μmole oleate/ml. Incubation period 71 hr. ●, Laurate added; ○, myristate added; △, palmitate added; ▽, stearate added.

Fig. 6. The effect of the C_{12} to C_{18} saturated fatty acids, in the presence of oleate, on the growth rate of *Mycoplasma mycoides*. Fatty acid concentration, 0·01 μmole/ml. ●, Laurate; ○, myristate; △, palmitate; ▽, stearate.

Effect of fatty acid deficiencies

Replicate tubes of Medium B with the following omissions—(i) complete; (ii) without palmitate; (iii) without oleate; (iv) without palmitate and oleate; (v) without palmitate and uracil; (vi) without oleate and uracil; and (vii) without palmitate, oleate and uracil—were sown with $5{\cdot}0 \times 10^7$ viable elements/ml. Viable counts and turbidity changes during incubation for 45 hr. are shown (Fig. 9*a*, *b*). The following points may be seen: (*a*) Without added palmitate there was a large increase in turbidity, the optical density after 45 hr. of incubation reaching a value about half of that of the cultures in complete medium; increases in the viable count almost equalled those in complete medium. (*b*) In the absence of oleate, there was an

increase, followed by a slow decrease, in turbidity, and, after about 15 hr. of incubation, a rapid decrease in viability. (*c*) In the absence of palmitate and oleate, the increase in turbidity was greater, and the decrease in viability less, than in the cultures deficient only in oleate. (*d*) The omission of uracil did not entirely prevent an increase in the number of viable elements when incubated in medium without added palmitate, nor did it prevent a decrease in the number of viable elements in media without oleate, or without oleate and palmitate. In two other experiments, however, uracil omission slowed the decrease in the number of viable elements in medium lacking both fatty acids.

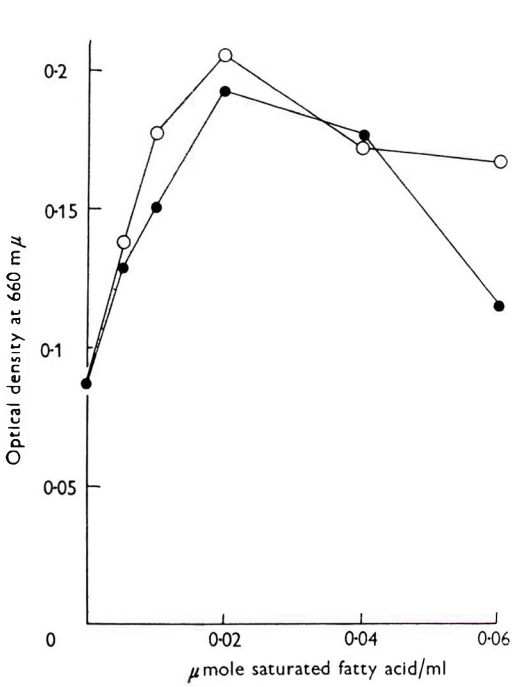

Fig. 7

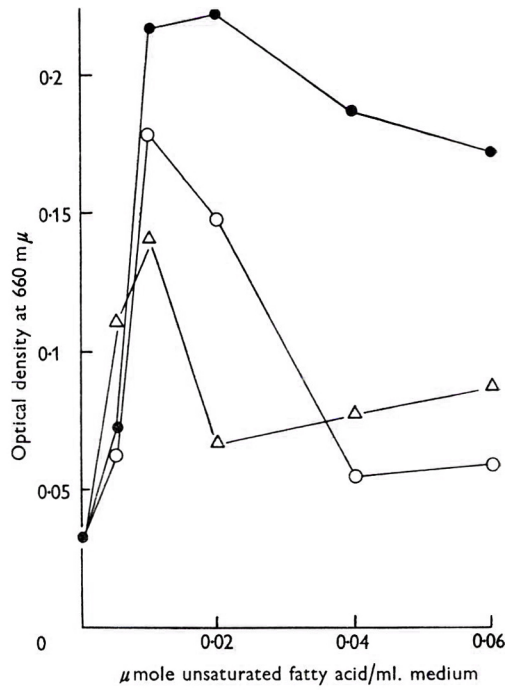

Fig. 8

Fig. 7. The effect of margarate and palmitate on growth of *Mycoplasma mycoides* in the presence of 0·01 μmole oleate/ml. Incubation period 70 hr. ●, Margarate added; ○, palmitate added.

Fig. 8. The effect of unsaturated fatty acids on the growth of *Mycoplasma mycoides* in the presence of 0·01 μmole palmitate/ml. Incubation period 71 hr. ●, Oleate added; ○, linolenate added; △, linoleate added.

Electron micrographs were made from a similar series of cultures, incubated for 20 hr., in which the turbidity changes closely paralleled those shown in Fig. 9*a*. The forms seen in growth in Medium B were highly branched tangled filaments of uniform thickness (Pl. 3, fig. 11). In Medium B from which palmitate had been omitted the forms were short plump pear-shaped or oval, and many had short outgrowths (Pl. 3, fig. 12). The forms in Medium B from which oleate had been omitted were of varied morphology (Pl. 4, fig. 13); flat disks, some with a thickened rim, and some with a number of bead-like structures ranged round the periphery, were all characteristic. The forms with the thickened rim, and those with the bead-like outgrowths,

doubtless correspond to the ring forms and to the asteroids, respectively, which have often been described in the past in morphological studies with the light microscope. An example of an asteroid is shown shadowed in Pl. 4, fig. 14, and after 'staining' with phosphotungstic acid in Pl. 4, fig. 15. The stain has penetrated the flat central portion of the form more readily than the bead-like outgrowths, as also in the ring form shown in Pl. 4, fig. 16. The form illustrated in Pl. 4, fig. 17, has five deeply stained areas (? vacuoles), some clearly defined lightly stained areas round part of the periphery, and a single lightly stained spot.

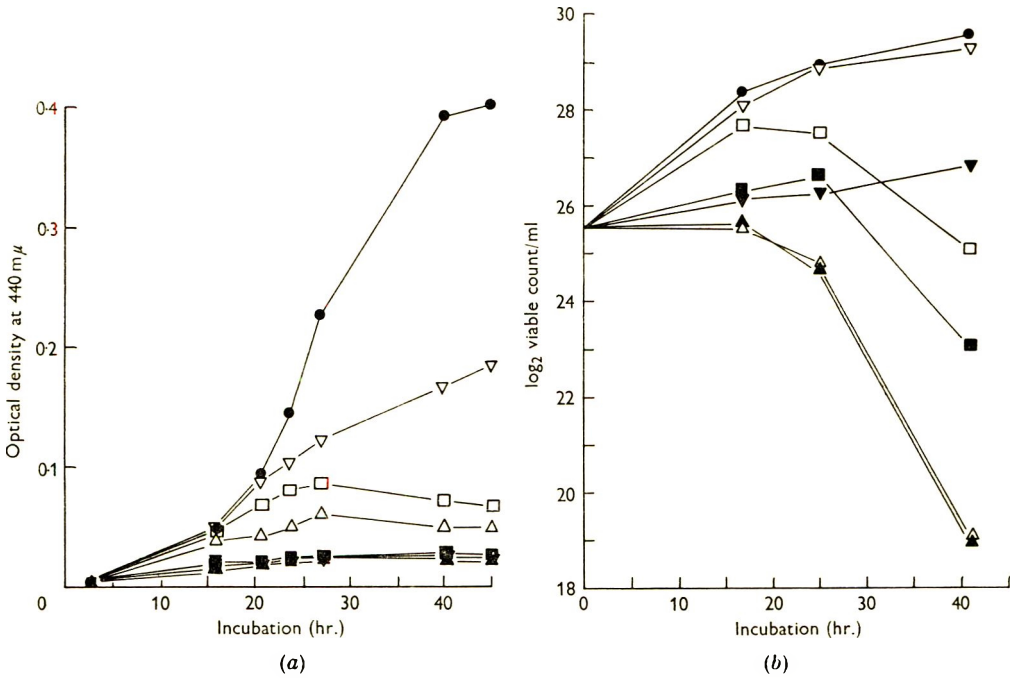

Fig. 9. The effect of the omission of palmitate, oleate and uracil on the turbidity and viability of cultures of *Mycoplasma mycoides*. (*a*) Turbidity; (*b*) viable element count. ●, Complete medium; △, oleate omitted; ▲, oleate and uracil omitted; ▽, palmitate omitted; ▼, palmitate and uracil omitted; □, palmitate and oleate omitted; ■, palmitate, oleate and uracil omitted.

DISCUSSION

The lipid precursor requirements for growth of *Mycoplasma mycoides* are complex. The observations suggest that a deficiency of glycerol, cholesterol, and probably other lipid precursors causes unbalanced growth, leading to the death of viable elements and lysis. When cytoplasmic synthesis was prevented (by uracil deprivation or by the addition of chloramphenicol) the death of viable elements and lysis were also prevented. The hypothesis is advanced that these nutrients are required for the synthesis of an undefined cell component which is necessary for the structural integrity of the cell, and that the synthesis of this is more sensitive to a deficiency of these nutrients than is the synthesis of cytoplasm. It is not excluded that the death of viable elements and lysis are secondary consequences of a primary defect elsewhere in the cell.

The effects of fatty acid deficiency require further investigation. The experiments were complicated by the probability that Medium B from which fatty acids were omitted was contaminated with suboptimal amounts of fatty acids derived from other medium constituents, e.g. from incompletely extracted bovine serum albumin Fraction V, or from the hydrolysed-casein preparations. Fatty acids may also have been carried over in significant amounts with the inoculum, because the fatty acid-dependence was found to vary with the size of the inoculum. It was necessary to diminish the size of the inoculum as compared with that used for the glycerol and cholesterol experiments, and to incubate for longer periods. Under these conditions, a deficiency of oleate caused decreases in counts of viable elements and lysis which were not prevented by uracil omission. Palmitate deficiency in the experiment described did not cause decreases in viable counts, but in other experiments in which incubation was continued still longer, such decreases were beginning to occur and might have been expected to be more rapid under conditions of greater deficiency. The highly branched filaments seen in Medium B may have contained many more 'nuclear equivalents' or *potential* colony-forming units than the observed viable count would indicate. This may explain why the viable count was no greater than in palmitate-deficient medium (Fig. 9*b*). The highly branched filaments would also have a higher surface/volume ratio than the plump elements seen in palmitate deficiency. The latter forms might therefore require less oleate to maintain their viability. The experiments suggest that the ratio of saturated to unsaturated fatty acids is an important factor. Shorb & Lund (1959) reported that a saturated and an unsaturated fatty acid, each inactive alone, were together required for the growth of two species of trichomonads; they also stressed the need for a proper balance of fatty acids. The growth-promoting activity of the C_{17}-acid, margaric acid, for *Mycoplasma mycoides* may indicate that this acid is incorporated into the lipids without alteration of chain length.

Knowledge of the limiting membrane of mycoplasma organisms might help to define their relations with other micro-organisms. Mycoplasma organisms appear to lack a rigid wall structure, and are known to lack the bacterial cell-wall 'muco-complex' (Kandler & Zehender, 1957; Plackett, 1959). It may be suggested that the limiting membrane of *Mycoplasma mycoides* depends for its integrity on lipid components. Whether the galactan, described by Plackett & Buttery (1958) and Buttery & Plackett (1960), which accounts for about 10 % of the dry weight of the organism, and which also contains a lipid component, is associated with this structure, is not known.

The morphological changes which occur during incubation of mycoplasma forms in media deficient in glycerol, cholesterol, oleate and palmitate, appear to differ. An adequate concentration of fatty acids is needed for the development of branched filaments. The appearance of ring forms, asteroids, etc., often seen in cultures incubated for long periods in crude media, may be due to an unsaturated fatty acid deficiency.

The suggestion of a general loss of structure during incubation in medium deficient in the cholesterol + bovine serum Fraction C system is of interest because cholesterol is believed to be interspersed between the phospholipid residues in biological membranes, and to stabilize the structure. Butler & Knight (1960) described growth inhibition of *Mycoplasma laidlawii* by certain steroids, and its annulment by chol-

esterol. It would be of interest to know whether the growth-inhibitory steroids, cause unbalanced growth with subsequent death and lysis.

The nature of the compounds into which glycerol is incorporated is being studied. A substantial part is incorporated into a cardiolipin-like compound (Plackett, 1961). Glycerophosphate polymers of the teichoic acid type described in the cell-walls of certain species of bacteria, where they replace part or all of the ribitol phosphate (Armstrong, Baddiley & Buchanan, 1959), or large polymers of the type described by McCarty (1959), were not found. The glycerophosphate polymers forming part of the protoplast membrane of bacteria (Mitchell & Moyle, 1956; Gilby, Few & McQuillen, 1958) have not been characterized.

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EXPLANATION OF PLATES

Figs. 1–7 illustrate the morphological changes caused by incubation of *Mycoplasma mycoides* in medium deficient in glycerol and uracil; figs. 8–10 those resulting from deficiency in cholesterol and bovine serum Fraction C, and figs. 11–17 those from deficiency in palmitate and oleate. The specimens were photographed at a magnification of ×7200. The final magnification of figs. 1–13 is ×14,400 and that of figs. 15–17 is ×36,000.

Figs. 1–4 and 6–8 were shadowed with gold-palladium; figs. 11–13 with gold-manganin and figs. 15–17 were stained with phosphotungstic acid.

PLATE 1

Fig. 1. After 8 hr. incubation in Medium A.
Fig. 2. After 24 hr. incubation in Medium A.
Fig. 3. After 24 hr. incubation in Medium A, uracil omitted.
Fig. 4. After 8 hr. incubation in Medium A, glycerol omitted.

PLATE 2

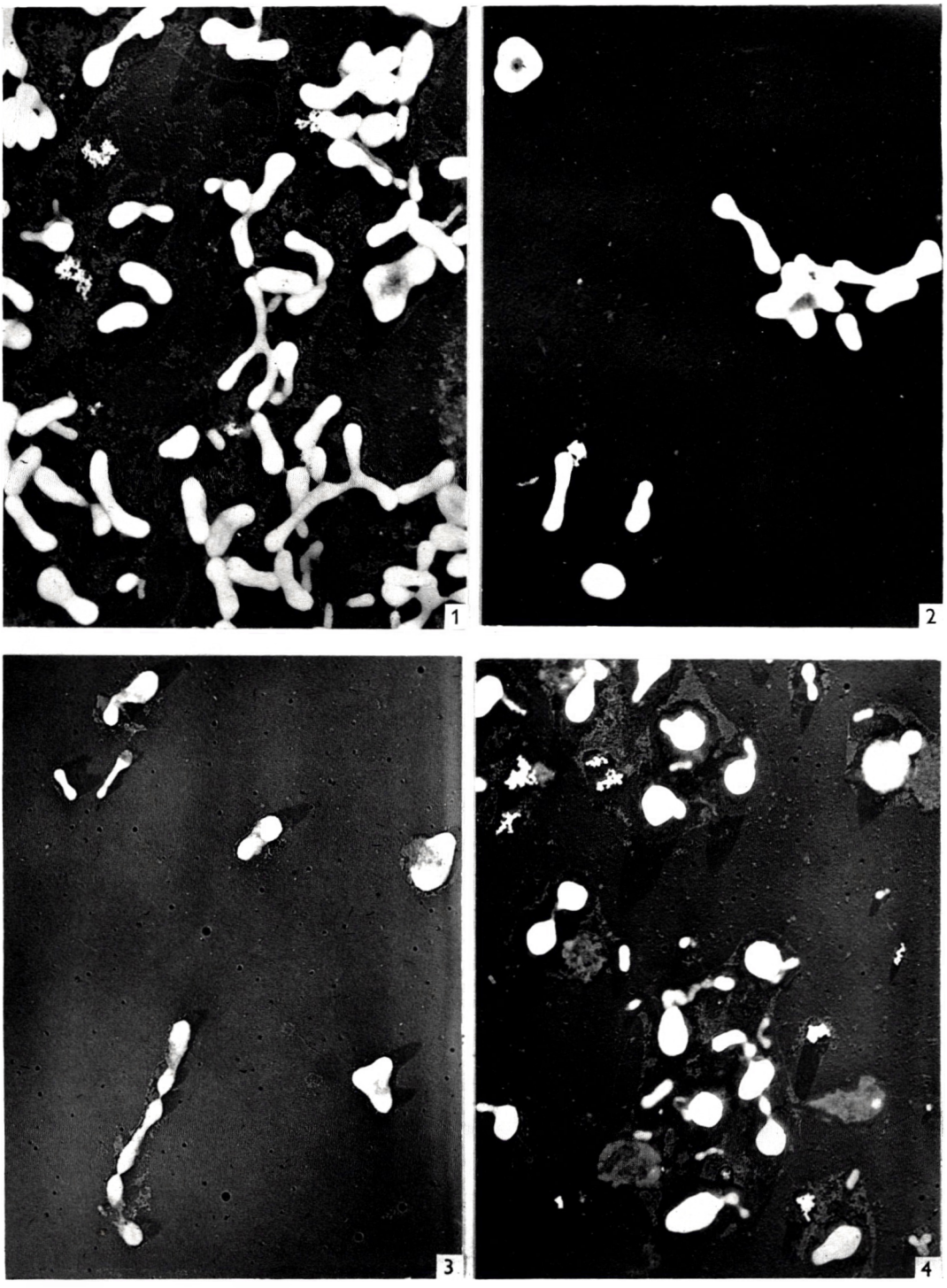
Fig. 5. After 24 hr. incubation in Medium A, glycerol omitted; unshadowed.
Fig. 6. As for fig. 5, shadowed.
Fig. 7. After 24 hr. incubation in Medium A, glycerol and uracil omitted.
Fig. 8. After 5 hr. incubation in Medium A, cholesterol and Fraction C omitted.

PLATE 3

Fig. 9. After 20 hr. incubation in Medium A, cholesterol and Fraction C omitted.
Fig. 10. As for fig. 9, from another experiment.
Fig. 11. After 20 hr. incubation in Medium B.
Fig. 12. After 20 hr. incubation in Medium B, palmitate omitted.

PLATE 4

Fig. 13. After 20 hr. incubation in Medium B, oleate omitted.
Fig. 14. As for fig. 13.
Fig. 15. As for fig. 13.
Fig. 16. As for fig. 13.
Fig. 17. As for fig. 13.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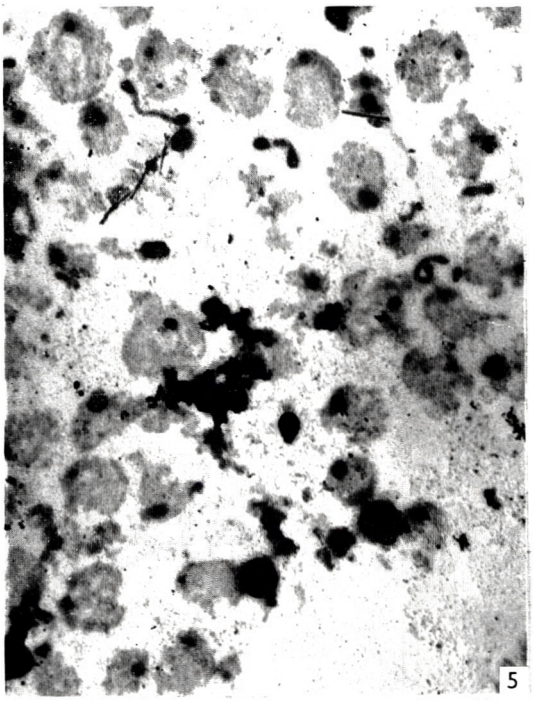
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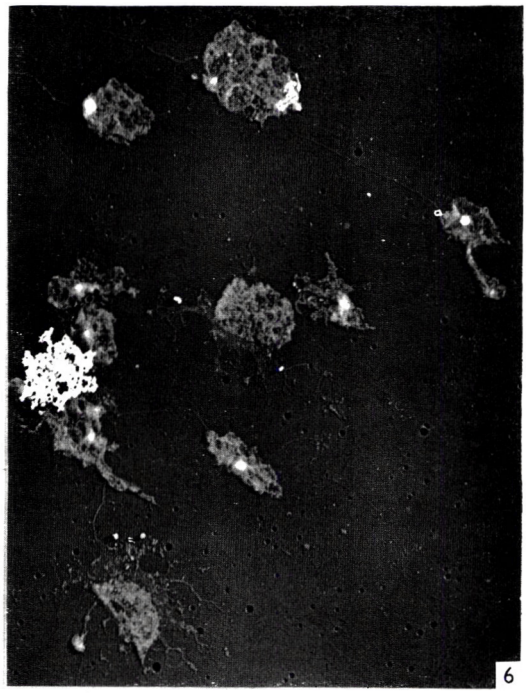
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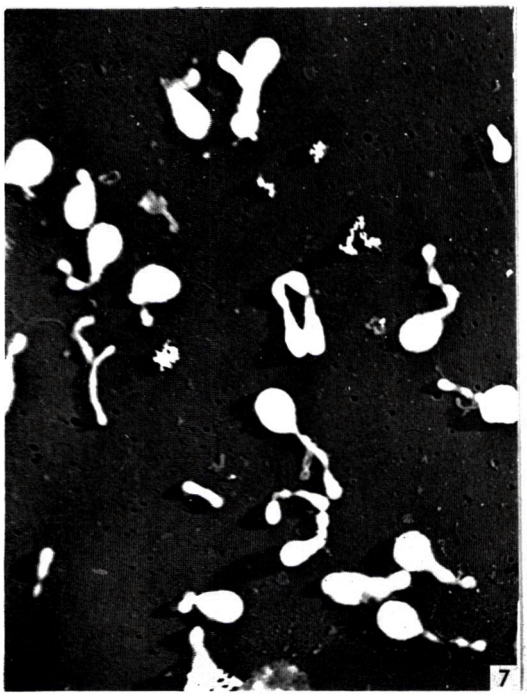
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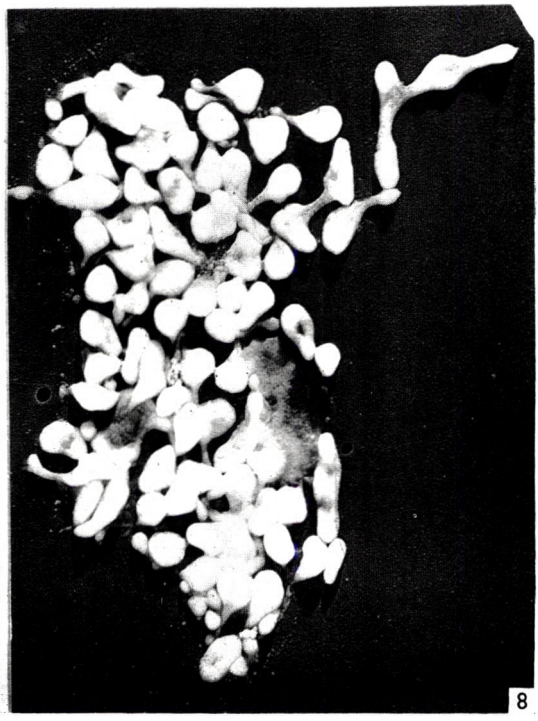
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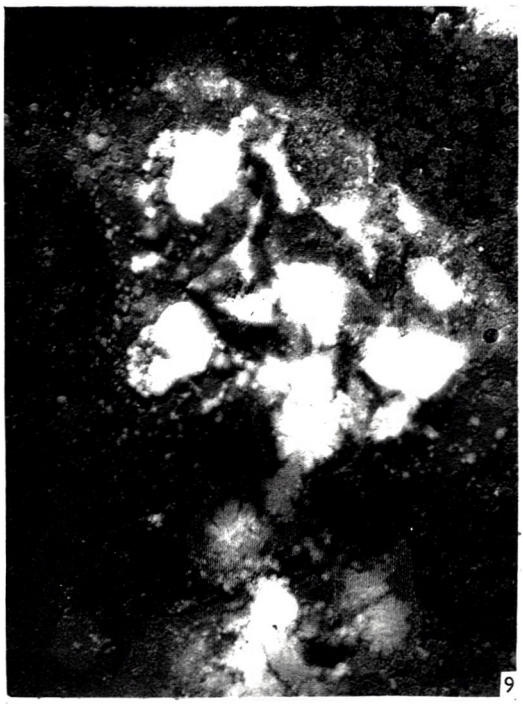
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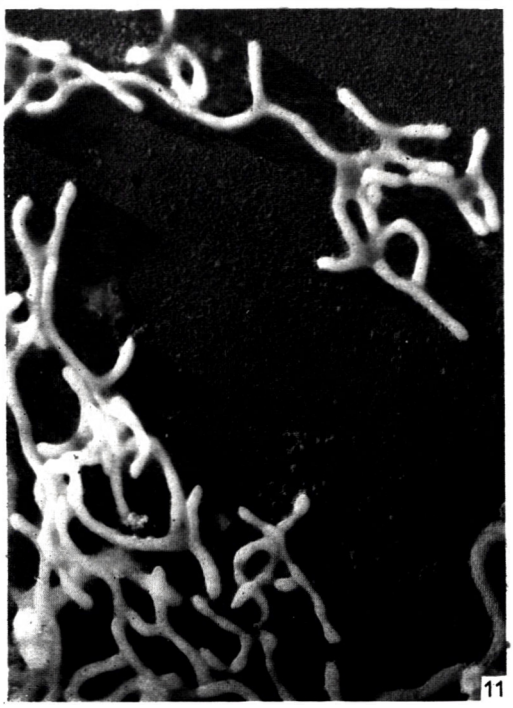
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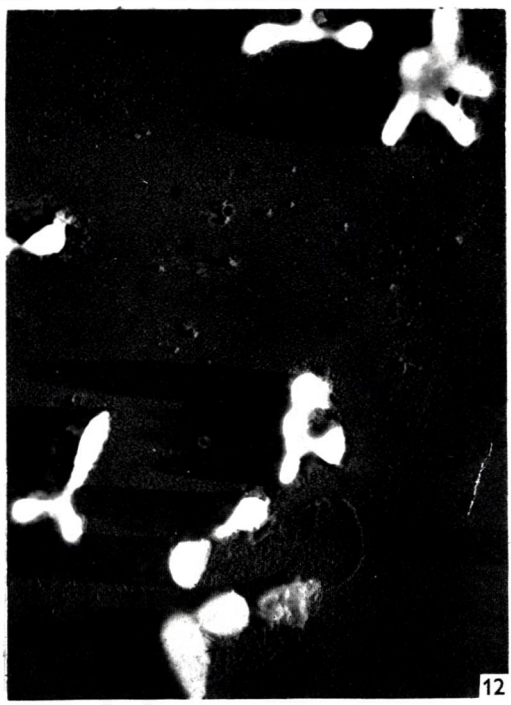
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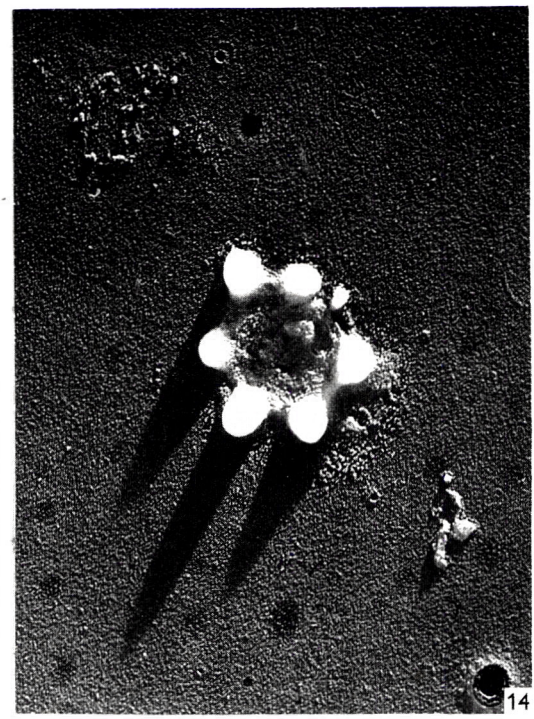
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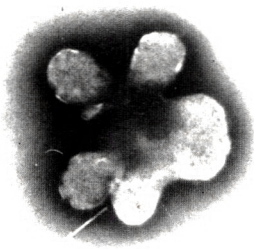
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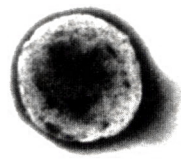
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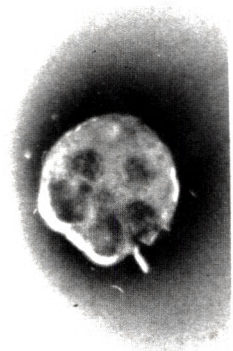
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Glyceride Hydrolysis and Glycerol Fermentation by Sheep Rumen Contents

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SUMMARY

Microbial hydrolysis of triglycerides was observed when these were incubated anaerobically at 37° with sheep rumen contents. The extent of hydrolysis was variable, but was often considerable (> 90 %) when linseed oil was used as substrate. The free fatty acids liberated were analysed by gas chromatography and, as compared with the acids present initially in glyceride combination, they were less unsaturated because of microbial hydrogenation. Linolenic acid was particularly effectively hydrogenated. No synthesis of long-chain fatty acids took place during the incubations and, apart from the possibility that in some experiments a limited conversion of stearic acid to palmitic acid took place, there was no evidence of significant degradation of long-chain acids. Glycerol liberated during the hydrolysis was completely metabolized, in part to volatile fatty acids, largely propionic acid. No mono- or diglycerides were detected as intermediates in the lipolysis of triglycerides. Analysis of the contents of the rumen, abomasum and small intestine of each of two slaughtered sheep, one of which had previously been fed on a diet rich in linseed oil, showed that most of the total higher fatty acids present in each of these three portions of the alimentary tract was in the form of free acids. It is concluded that microbial lipolysis results in the pre-digestion of much of the lipids ingested by the sheep as part of its feed.

INTRODUCTION

Attention was first directed to the influence of the rumen on lipids by Reiser (1951) who found a marked decrease in the linolenic acid content of linseed oil when it was incubated with sheep rumen contents. This effect was attributed to hydrogenation of double bonds by rumen micro-organisms and was substantiated by comparisons of the unsaturation of feed lipids with that of the lipids of rumen contents in sheep (Shorland, Weenink & Johns, 1955) and goats (Reiser & Reddy, 1956) and the detailed examination of the products of incubation of oleic, linoleic and linolenic acids with sheep rumen contents (Shorland, Weenink, Johns & McDonald, 1957). In all the foregoing studies the total lipid of rumen contents was saponified before determination of the unsaturation of the component fatty acids. In the present work, to examine more fully the effect of mixed rumen micro-organisms on esterified unsaturated fatty acids, several triglycerides were incubated with sheep rumen contents, and subsequently the lipids were extracted without previous saponification. These experiments, which resulted in the finding that

lipolysis could be effected by rumen organisms, are reported in the present paper, together with related observations on the nature of the lipids in different parts of the alimentary tract of the sheep. Preliminary accounts of some of these results have already been published (Garton, Hobson & Lough, 1958; Garton, Lough & Vioque, 1959; review by Garton, 1959).

METHODS

In vitro *experiments*

Incubation of glycerides with rumen contents. Samples of rumen contents were obtained from sheep with permanent rumen fistulas. The animals were fed on a mixture of hay and concentrates with free access to water and the samples (100–500 ml. as required) were taken 3–4 hr. after the last feed. After thorough mixing, portions (usually 100 ml.) were transferred to sterile 250 ml. conical flasks fitted with Bunsen valves. Each flask was placed up to its neck in a water bath maintained at 37°, and gassed with CO_2 to remove air. The pH value of the rumen contents was then determined by capillator (British Drug Houses, Ltd., Poole, Dorset) and adjusted when necessary to pH 6·5 by the dropwise addition of a sterile solution of Na_2CO_3 (25%, w/v). A weighed amount of glyceride, contained in a small glass cup, was then put into the rumen contents and the flask shaken; it was then flushed again with CO_2. At hourly intervals thereafter for 8 hr. the flask was shaken and adjusted to pH 6·5. The incubation was continued overnight for a further 16 hr. after which the pH value was recorded and the mixture acidified to pH 2·0 with 10 N-H_2SO_4. Other flasks containing rumen contents only, or rumen contents which had previously been heated at 90° for 1 hr., were incubated at the same time and treated in the same way.

Extraction and fractionation of lipids. At the end of the incubation period 100 ml. ethanol were added to the acidified rumen contents and the mixture boiled under reflux for 10 min. After cooling, the aqueous ethanol was decanted and 200 ml. of a mixture of ethanol and ether (3 + 1, v/v) were added to the solid residue in the flask, which was then heated under reflux for 15 min. The ethanol + ether extract was filtered off and the solids remaining on the filter washed with warm ethanol + ether. The total ethanol + ether extracts were pooled with the aqueous ethanolic extract and this solution, containing the total lipid, was concentrated to 20–30 ml. by distillation on a water bath. To remove volatile fatty acids from the concentrated extract 50 ml. of a phosphate buffer (pH 6; containing 5·68 g. anhydrous Na_2HPO_4 + 49·0 g. anhydrous KH_2PO_4/l. water) were added and the mixture was extracted thrice with 50 ml. ether, the volatile acids remaining in the buffer solution as salts.

The combined ethereal extracts were shaken gently with 3 × 50 ml. 0·5% (w/v) aqueous KOH, followed by one washing of the ether with 50 ml. water; this treatment removed the free higher fatty acids as potassium soaps, leaving neutral lipids in solution in the ether. The solution of soaps was acidified dropwise with 10 N-H_2SO_4 until no further precipitation of fatty acids took place. The acids were extracted thrice with 50 ml. ether and the combined ethereal extracts washed with water until free from mineral acid. The extract was then taken to dryness *in vacuo* in a tared flask to give the weight of free higher fatty acids, which were stored

under N_2 at $+1°$ in the dark. After allowing for the weight of free fatty acids in the rumen contents alone, the extent of hydrolysis of a glyceride was derived from the weight of free fatty acids liberated expressed as % of the calculated weight of acids produced on complete hydrolysis of the glyceride. The slight increase in weight of the free acids resulting from the hydrogenation of unsaturated components was neglected.

In some experiments the alkali-extracted ethereal solution of neutral lipids was fractionated further; the solution was taken to dryness and saponified under reflux with excess 0·5N-ethanolic KOH. The saponified mixture was acidified dropwise with 10 N-H_2SO_4 and the resultant mixture of fatty acids and unsaponifiable matter extracted with 3×50 ml. ether. From these combined extracts the fatty acids were recovered as described above for free fatty acids, leaving the unsaponifiable matter in the ether.

Incubation of glycerol with rumen contents. Following preliminary experiments in which glycerol was determined in rumen contents before and after the incubation with linseed oil, known amounts of free glycerol were incubated with rumen contents under the same conditions as those described for the incubation of glycerides. Glycerol was estimated by the method of Lambert & Neish (1950) following treatment of the rumen contents according to Johns (1953).

Fatty acid analyses. The free fatty acids resulting from lipolysis and, where appropriate, those derived from the residual neutral lipids, were converted to their corresponding methyl esters by refluxing with excess methanol containing 1% (w/w) H_2SO_4. The recovered esters were analysed by gas chromatography at 170° using polymerised ethylene glycol adipate (prepared according to Farquhar *et al.* 1959) as liquid phase and argon as carrier gas. Appropriate model mixtures of fatty acid esters were chromatographed and the fatty acid composition of the samples from the incubations was calculated as described by Farquhar *et al.* (1959). The fatty acid composition of the glycerides used for incubation was determined in a similar way following saponification of the material with alkali and conversion of the acids to methyl esters.

Volatile fatty acids present in rumen contents before and after incubation with a triglyceride or glycerol were determined titrimetrically following acidification and steam-distillation of rumen contents (Friedemann, 1938); the mixtures of sodium salts of fatty acids so obtained were analysed qualitatively by paper chromatography (Elsden & Lewis, 1953).

Alimentary tract contents of slaughtered sheep

Samples of the contents of various parts of the alimentary tracts of two sheep were obtained immediately at slaughter which, for each animal, was 7 hr. after the last feed. One sheep had been fed for several months on a diet consisting largely of hay, linseed meal and maize to which 40 g. of linseed oil were added daily; the other had been fed on a mixture of concentrates including maize meal, groundnut meal, molassine meal, dried grass, oat dust and bruised oats. Each sample of alimentary tract contents was mixed with twice its weight of absolute methanol and filtered. The residue was retained and the filtrate was distilled to dryness on a water bath; the retained solids and the methanol-extracted matter were combined and extracted under reflux with excess chloroform + methanol mixture (2 + 1, v/v) for 15 min. The

mixture was filtered and the solids on the filter washed with hot chloroform + methanol, after which the combined filtrates were distilled to dryness to give the crude lipid extract. This material was dissolved in a convenient volume of ether before proceeding with fractionation into free fatty acids and neutral lipids as described above for incubated rumen contents.

RESULTS

Incubation of glycerides with rumen contents

In the first series of experiments 1·00 g. amounts of linseed oil were incubated with 100 ml. portions of whole rumen contents (in which the oil readily emulsified); after 24 hr. the free higher fatty acids were extracted and their iodine values determined. Hydrolysis usually resulted in the liberation of 60 % to more than 90 % of the esterified fatty acid residues of the original oil; occasionally the extent of hydrolysis was lower, in some experiments as low as 20 %. The results of two experiments showing considerable hydrolysis are given in Table 1. No lipolysis was observed in the flasks containing the rumen contents which had previously been heated (flasks 4 and 8) and the slight difference between the amounts of free acids recovered from flasks 3 and 4 and those from flasks 7 and 8 was due to traces of free acid present initially in the linseed oil. That hydrogenation of unsaturated acids had also taken place was indicated by the low iodine value of the recovered acids compared with the value (189·0) of the acids of the linseed oil itself; calculation gives the iodine value of the free acids derived from the oil as 121·4 (flask 2) and 112·2 (flask 6). Only traces of free glycerol were found in the rumen contents before incubation; following incubation in the presence or absence of linseed oil, none at all was detected.

Table 1. *Lipolytic activity of sheep rumen contents towards linseed oil*

Each flask contained 100 ml. rumen contents and, where indicated, 1·00 g. of linseed oil.

Flask	Treatment before incubation	Addition	Free fatty acids: Weight (mg.)	Free fatty acids: Iodine value
		Experiment 1		
1	None	None	245	22·8
2	None	Linseed oil	859*	97·4
3	Heated at 90° for 1 hr.	None	236	31·6
4	Heated at 90° for 1 hr.	Linseed oil	268	44·7
		Experiment 2		
5	None	None	291	29·2
6	None	Linseed oil	1144†	94·3
7	Heated at 90° for 1 hr.	None	253	41·3
8	Heated at 90° for 1 hr.	Linseed oil	311	50·0

* Representing about 62 % hydrolysis of the esterified fatty acids of the oil.
† Representing about 87 % hydrolysis of the esterified fatty acids of the oil.

In other experiments similar to those outlined in Table 1 linseed oil was incubated: (i) with rumen contents from which almost all the micro-organisms had been

removed by centrifugation; (ii) with mixed sheep saliva (kindly collected for us by our colleague Dr R. N. B. Kay). In neither case was any hydrolysis of the oil observed. It was therefore concluded that lipolysis was probably due to the bacteria and/or protozoa of the rumen contents. When differential centrifugation was used to separate food particles and large protozoa from bacteria and small protozoa, almost all the lipolytic activity was found to be associated with the latter group of mixed organisms, which consisted mainly of bacteria. Attempts were made to prepare from mixed rumen organisms a cell-free extract which possessed lipase activity, but none was successful. The methods used included acetone-powders, grinding, shaking with ballotini beads, and rupture of the organisms with various detergents.

Further incubation experiments were carried out in which the free fatty acids resulting from the hydrolysis of linseed oil, olive oil and cocoa butter by whole rumen contents were analysed by gas chromatography. In addition, neutral lipids were extracted at the conclusion of the incubation period and volatile fatty acids were determined in the rumen contents incubated with and without the addition of triglyceride. Cocoa butter and olive oil did not form such stable emulsions in rumen contents as did linseed oil, and a few small globules of these triglycerides remained on the surface of the rumen contents throughout the incubation period.

The extent to which the esterified fatty acid residues in the glycerides were hydrolysed was about 95%, 68% and 40%, respectively, for linseed oil, olive oil and cocoa butter. The combined weight of free fatty acids and neutral lipid recovered after the incubations showed almost quantitative recovery of the total weight of triglyceride added initially, due allowance being made for the amounts of lipid in the rumen contents *per se* and for the small amounts of glycerol liberated in the hydrolysis. The incubation of the glycerides resulted in a slightly enhanced formation of steam-volatile fatty acids as compared with the amounts found in rumen contents incubated under the same conditions without the addition of glycerides. This was probably due to some fermentation of liberated glycerol (see later). In Table 2 are shown the amounts and composition of the free fatty acids which resulted from hydrolysis of the glycerides, the fatty acid composition of the glycerides used, and the amounts and composition of the free acids present in the rumen contents alone. It is assumed that the free fatty acids extracted from the rumen contents alone were also present in the acids extracted from the glyceride incubations; Table 1 indicates that this is a reasonable assumption since a comparison of the amounts of free fatty acids in heated and unheated rumen contents (flasks 1 and 3 and flasks 5 and 7) showed that only very small amounts of free acids were produced during the incubation from lipids initially present in the rumen contents. Further, an even smaller amount might be expected to arise in this way when triglyceride was also present because of competition for enzyme by the additional substrate. Table 2 shows that, as compared with the composition of the fatty acids present initially in the triglycerides, the acids resulting from hydrolysis were extensively modified. Hydrogenation of the C_{18} unsaturated fatty acids led to the production of enhanced proportions of stearic acid (cf. Shorland *et al.* 1957) and, in addition, palmitic acid appeared in increased proportions, particularly from linseed oil and olive oil. Small amounts of (as yet) unidentified acids were formed from linseed oil and olive oil, but not from cocoa butter. These acids, present in the

Table 2. *Composition and amounts of free fatty acids found after incubating sheep rumen contents with and without triglycerides and, for comparison, the fatty acid composition of the triglycerides*

Rumen contents (100 ml.) with and without 1·00 g. of triglyceride. Dash (—) indicates not detected.

	Expt. 3					Expt. 4		
	Acids from rumen contents alone (177 mg.)	Linseed oil		Olive oil		Acids from rumen contents alone (271 mg.)	Cocoa butter	
		Acids from incubation (940 mg.)*	Acids present in glycerides initially	Acids from incubation (659 mg.)*	Acids present in glycerides initially		Acids from incubation (353 mg.)*	Acids present in glycerides initially
Fatty acid	Composition (weight %)							
Unidentified ('acid A')	4·4	2·6	—	2·0	—	3·7	—	—
Unidentified ('acid B')	5·4	2·4	—	1·7	—	5·8	—	—
C_{16} saturated (palmitic)	17·9	13·8	5·6	27·2	10·3	20·5	27·4	24·5
C_{16} mono-unsaturated	—	—	—	—	0·7	—	—	—
C_{18} saturated (stearic)	57·5	31·7	5·8	26·7	2·9	61·5	46·9	34·1
C_{18} mono-unsaturated	17·8	30·2	21·6	42·4	80·7	8·5	25·7	38·1
C_{18} di-unsaturated	—	14·2	12·5	—	5·4	—	—	3·1
C_{18} tri-unsaturated	—	5·1	54·5	—	—	—	—	—

* Weight after subtraction of contribution of the free fatty acids from rumen contents alone.

free fatty acids of rumen contents *per se*, were not found in the dietary lipids of the sheep. On the gas chromatograms their methyl esters appeared in positions between methyl laurate and methyl tridecanoate, and between the latter and methyl myristate.

Whilst the studies of Shorland *et al.* (1957) showed that unsaturated fatty acids as such were hydrogenated when they were incubated with rumen contents, the experiments so far described here do not preclude the possibility that hydrogenation might take place whilst the acids were still in glyceride combination. Though in most of the experiments with linseed oil hydrolysis was almost complete, in others (as previously mentioned) the extent of hydrolysis was much less. Analysis of the neutral lipids remaining after partial (20 %) hydrolysis of 1·0 g. of linseed oil by 100 ml. rumen contents from one sheep showed that almost no hydrogenation of the esterified unsaturated acids had taken place. However, when rumen contents of a second sheep were used the neutral lipids remaining after 32 % hydrolysis of the oil did show changes in fatty acid composition though, as Table 3 shows, the free fatty acids were more extensively modified. In this particular experiment, in contrast to previous experiments with rumen contents of a different sheep (Table 2), hydrogenation did not result in enhanced amounts of stearic acid being produced (compare Wright, 1960), though much of the linolenic acid was converted to C_{18} mono- and di-unsaturated acids; further, no additional palmitic acid was produced.

Table 3. *Composition of the free fatty acids and the fatty acids of the neutral lipids following partial hydrolysis of linseed oil and, for comparison, that of the fatty acids of the linseed oil glycerides*

Dash (—) indicates not detected.

	Source of acids		
	Free fatty acids*	Neutral lipids after incubation*	Linseed oil glycerides
Fatty acid	Composition (weight %)		
Unidentified	0·7	—	—
C_{16} saturated (palmitic)	5·7	5·3	5·7
C_{18} saturated (stearic)	4·0	5·2	4·2
C_{18} mono-unsaturated	24·4	18·8	15·6
C_{18} di-unsaturated	38·4	21·6	13·2
C_{18} tri-unsaturated	26·8	49·1	61·3

* Due allowance having been made for the contribution of the lipids in the rumen contents *per se*.

Incubation of glycerol with rumen contents

The hydrolysis of glycerides raises the question of the fate of the glycerol which is liberated. As already noted, no glycerol was detected in rumen contents following the almost complete hydrolysis of linseed oil. Similarly, when 0·5 g. amounts of glycerol were incubated anaerobically at 37° for 24 hr. with 100 ml. rumen contents (from a sheep fed on hay and concentrates) no glycerol was detectable after this time. In further experiments, 0·5–1·0 g. glycerol was incubated with 100 ml. rumen contents and small samples (1·0 ml.) were removed for glycerol determination at intervals up to 24 hr. after the start of the incubation. Within 2 hr. less than 80 %

of the initial amount of glycerol was present, within 4 hr. only about 50% remained and the rest gradually disappeared within 24 hr. (Table 4). In no experiment did volatile fatty acids account for more than 50% of the glycerol which was metabolized; the predominant acid produced was propionic acid.

Table 4. *Metabolism of glycerol in sheep rumen contents*

Glycerol (0·580 g.) was added to 100 ml. rumen contents.

Time after adding glycerol (hr.)	Recovery of added glycerol (%)
0	100
2	77·8
4	50·0
6	28·3
9	13·3
23	1·1
24	0

Possible intermediates in glyceride hydrolysis

In incubation experiments in which hydrolysis of linseed oil was less than 80% after 24 hr. the neutral lipids were examined for the presence of possible intermediates of lipolysis, namely, mono- and di-glycerides. The chromatographic procedures of Borgström (1954), Barron & Hanahan (1958) and Hirsch & Ahrens (1958) all failed to reveal the presence of partial glycerides. Similar negative findings also resulted from analyses of the neutral lipids of samples of the incubation mixture taken at several intervals during the course of a 24 hr. incubation of linseed oil with rumen contents, although 'normal' hydrolysis progressively took place as evidenced by the increasing proportion of free fatty acids in the total lipids of the samples.

Table 5. *Free fatty acids in the alimentary tracts of two slaughtered sheep*

For diets of sheep, see Methods.

Source of digesta	Free fatty acids as % total fatty acids
Sheep 1	
Rumen	92
Abomasum	90
Small intestine (upper)	96
Small intestine (lower)	94
Sheep 2	
Rumen	78
Abomasum	94
Small intestine (total)	97

Alimentary tract contents

The proportion of free fatty acids in the total fatty acids obtained from the rumen, abomasum and small intestine of two sheep is given in Table 5. As described under Methods, sheep no. 1 was given the diet containing linseed oil and sheep

no. 2 was fed largely on concentrates. The greater part of the total fatty acids of the alimentary tract contents of both animals was in the form of free higher fatty acids; gas chromatographic analysis of those derived from sheep no. 2 showed that stearic acid, palmitic acid and C_{18} mono-unsaturated acid were the major components of the acids in each of the three parts of the tract which were examined (compare the free fatty acids of rumen contents given in Table 2).

DISCUSSION

Though it has been known for some time that carbohydrates and proteins undergo hydrolytic and fermentative changes in the rumen, little attention has hitherto been paid to the fate of lipids which constitute as much as 5–10% of the dry weight of many common feedingstuffs. As outlined in the Introduction, it has been established within the last decade that centres of unsaturation of fatty acids can be hydrogenated in the rumen, apparently under the influence of the micro-organisms present. The findings of the present work now show that the micro-organisms can effect considerable hydrolysis of fatty acids from glyceride combination and, since the preliminary report of these studies appeared (Garton *et al.* 1958), Dawson (1959) has found that phospholipids too can be hydrolysed to their components (i.e. fatty acids, glycerol and a nitrogenous base) by sheep rumen organisms. Thus ingested feed lipids can be pre-digested in the rumen and most of the higher fatty acids in the digesta leaving the rumen are, as Table 5 shows, in the free state. This is a situation markedly different from that in simple-stomached animals in which ingested lipids are not subject to hydrolysis until they reach the small intestine.

It is concluded that rumen bacteria produce a lipase and, as the following paper (Hobson & Mann, 1961) describes, lipolytic bacteria have been isolated from sheep rumen contents. Since no enzyme activity was detected in rumen contents except in the presence of micro-organisms, the lipolysis must take place either within the bacteria or at the point of contact between bacterium and lipid particle. No mono- or di-glycerides were detected during the lipolysis of triglyceride, indicating that, if formed at all, these possible intermediates have a very transient existence. Indeed, it appears that the enzyme system is non-specific in its activity towards fatty acids occupying the α- and β-positions on the glycerol molecule; in this respect the system seems to resemble lipoprotein lipase rather than pancreatic lipase (compare Korn, 1960).

The hydrogenation of the unsaturated fatty acids of triglycerides apparently takes place more rapidly when they are split off rather than when they are in glyceride combination. It should be mentioned that hydrogenation results in the production of spatial and positional isomers of unsaturated acids (Shorland *et al.* 1957); these are included, where appropriate, in Tables 2 and 3 under the heading 'C_{18} mono-, di- and tri-unsaturated acids'. Linolenic acid forms a very large proportion of the total fatty acids of pasture grasses (Garton, 1960). Our studies have confirmed the observations of Shorland *et al.* (1957) that this acid is particularly effectively hydrogenated. The micro-organisms responsible for hydrogenating double bonds and the source of the necessary hydrogen have not yet been investigated, though Wright (1959, 1960) has shown that, in addition to bacteria, protozoa are probably involved.

In the experiments illustrated in Table 2 some additional palmitic acid was apparently formed in some way at the expense of the C_{18} fatty acids of linseed oil, olive oil and cocoa butter. Though no significantly increased amounts of lower volatile fatty acids were produced during these incubations, it seems possible that the palmitic acid was formed from stearic acid. This question forms part of a current investigation using synthetic glycerides containing ^{14}C-labelled stearic acid. In connexion with the occurrence of free higher fatty acids in rumen lipids, it may be noted that there is no evidence that they can be synthesized in significant amounts *de novo* in the rumen other than in the formation of the lipids within the micro-organisms themselves. Gray, Pilgrim, Rodda & Weller (1952) showed that when ^{14}C-labelled acetate or ^{14}C-labelled propionate were incubated with sheep rumen contents, butyrate arose by condensation of acetate units, and valerate by condensation of acetate and propionate units; no significant synthesis of longer-chain acids was observed.

Johns (1953) showed that the fermentation of free glycerol by sheep rumen contents *in vitro* and *in vivo* led to the formation of propionic acid. That propionic acid is the principal volatile fatty acid produced was confirmed in the present work, though no more than 50 % of the glycerol which was metabolized could be accounted for on a 'carbon balance' basis; the fate of the remainder has still to be investigated. As described in the following paper (Hobson & Mann, 1961), relatively more of the glycerol carbon was accounted for as volatile fatty acids (largely propionic) when glycerol-fermenting organisms from sheep rumen were grown in media containing glycerol or glycerol + acetate.

Triglycerides, though present in the lipids of concentrates made from seeds (e.g. linseed meal, maize meal, groundnut meal), do not form a very high proportion of the lipids of such feeds as grasses and clover in which galactosyl glyceryl esters of fatty acids predominate (Weenink, 1959; Garton, 1960). Though the metabolism of these esters by rumen micro-organisms has not yet been studied in detail, preliminary experiments indicate that they are readily hydrolysed.

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The Isolation of Glycerol-Fermenting and Lipolytic Bacteria from the Rumen of the Sheep

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SUMMARY

Facultatively anaerobic bacteria appear to play little part in glycerol fermentation in the sheep rumen. Amongst the most important members of the glycerol-fermenting flora are strict anaerobes of the group *Selenomonas ruminantium* var. *lactilyticas*. Three isolates of a different group of strictly anaerobic curved Gram-negative rods, which hydrolyse linseed oil and tributyrin as well as fermenting glycerol, were obtained in numbers which suggest they are amongst the more important lipolytic bacteria in the rumen. The main product of fermentation of glycerol by the selenomonads and the lipolytic bacteria is propionic acid, but other acids are also formed. The properties of these bacteria and some others isolated during the experiments are described. All the bacteria appear to be normal inhabitants of the rumen of sheep fed a number of different rations. The numbers of these organisms were not appreciably increased by feeding glycerol or triglycerides to the animals.

INTRODUCTION

Over the last few years a number of papers have been published about the effects of dietary fats and oils on the digestibility of foodstuff components. Also, the hydrogenation of unsaturated fatty acids in the rumen has been investigated by a number of workers (see review by Garton, 1960). During investigations on the nitrogen metabolism of ruminants Dr M. I. Chalmers of this Institute found that the addition of oils or fats to sheep rations had a marked effect on the ruminal ammonia concentrations. The separate components of these triglycerides (fatty acids, glycerol) also affected ammonia concentrations as might be expected, since it has been shown that triglycerides of long-chain fatty acids can be rapidly hydrolysed by mixed rumen organisms (Garton, Hobson & Lough, 1958; Garton, Lough & Vioque, 1961). The work described here was undertaken to see whether some of the bacteria presumed to be responsible for the hydrolysis of dietary glycerides and the fermentation of the glycerol formed could be isolated and studied in more detail. Also to be examined was the question whether such bacteria were an intrinsic part of the normal rumen flora, or whether they increased in numbers (or differed in types) in the rumens of sheep being fed different amounts of glycerides or glycerol. Since the ruminal ammonia concentrations were altered by feeding these substances it was of interest to see whether glycerol-fermenting or lipolytic bacteria utilized or produced any large amount of ammonia during growth. A number of glycerol-containing media were used in an endeavour to isolate not only strictly anaerobic rumen bacteria, but also facultative anaerobes and bacteria which do not need the exacting conditions of

some rumen bacteria (see review by Bryant, 1959). Acetate was included in some of the media in an attempt to isolate bacteria which fermented glycerol and acetate to butyric acid (compare *Clostridium tyrobutyricum*) since the experiments with whole rumen contents suggested that propionic acid was not the only end product of glycerol fermentation in the rumen as suggested by Johns (1953), and acetate is a normal constituent of rumen fluid. Linseed oil was chosen as substrate for the lipolytic bacteria since it was being fed to some of the sheep in linseed cake and as added oil; further, the component fatty acids of the glycerides of linseed oil are similar to those of total grass lipids.

METHODS

Media

Some of the important bacteria isolated from the rumen will not grow under the conditions usually used for growth of 'strict anaerobes' (for example clostridia). Special techniques for the isolation of these bacteria have been devised and these are briefly outlined below. In addition to a low E_h and CO_2 some rumen bacteria need growth factors found in rumen fluid, and this is often included in media for isolation of rumen bacteria.

General methods. The technique used for the preparation of the media specified as 'incubated under CO_2' (no. 4–14) was based on that originated by Hungate (1950). In this method the various solutions making up the media are saturated with CO_2 and mixed under a stream of oxygen-free CO_2, and contain a reducing agent which brings the E_h below that at which resazurin is colourless. The oxidation-reduction potential of the media described in this paper, measured between a calomel and a platinum electrode, was *c.* −350 mV. Small amounts of these media (*c.* 10 ml.) were dispensed into 6 in. × $\frac{5}{8}$ in. tubes under a stream of CO_2 and tightly stoppered with rubber bungs. Larger volumes of media were prepared in rubber-stoppered bottles using a similar technique. All inoculations of this type of medium were carried out under a stream of CO_2. Any media showing a trace of pink oxidized resazurin were discarded as the strictly anaerobic rumen bacteria will not grow under such conditions.

General constituents of media. Mineral solution (*a*) contained (g./l.): KH_2PO_4, 3·0; $(NH_4)_2SO_4$, 6·0; NaCl, 6·0; $MgSO_4$, 0·6; $CaCl_2$, 0·6. Mineral solution (*b*) contained (g./l.): K_2HPO_4, 3·0. Rumen fluid was prepared by straining rumen contents (freshly obtained from a hay-fed sheep) through gauze and centrifuging at 62,000*g* for 10 min. The clear liquid was kept for not more than a few days at 2° before use.

Media used for glycerol-fermenting bacteria

Media 1 to 3 were used for the isolation of rumen bacteria less exacting in requirements for a low E_h and presence of CO_2. *Medium* 1 contained, per 100 ml.: Bactocasitone, 1·5 g.; Difco yeast extract, 0·5 g.; NaCl, 0·5 g.; L-cystine, 0·075 g.; thioglycollic (mercaptoacetic) acid, 0·03 ml.; agar, 0·075 g.; glycerol, 1 ml.; bromocresol purple, 0·1 ml. of 1·6 % (w/v) ethanolic solution; water to 100 ml. All the constituents except the glycerol, which was added as a sterile filtered solution, were autoclaved together at 120° for 15 min. The medium was dispensed in 9 ml. amounts in tubes plugged with cotton wool and tenfold dilutions of rumen contents prepared in this medium. Incubation was in air. *Medium* 2. This medium was like medium 1, but contained agar 2 g., and was incubated in plates in a McIntosh & Fildes anaero-

bic jar under hydrogen. *Medium* 3 contained: mineral solution (*a*), 15 ml.; mineral solution (*b*), 15 ml.; rumen fluid, 20 ml.; yeast extract, 0·25 g.; glycerol, 1 ml.; agar, 2 g.; water, 49 ml.; bromocresol purple, 0·1 ml. of 1·6 % (w/v) ethanolic solution. All the constituents except glycerol, which was added as a filtered solution, were autoclaved together. This medium was incubated in plates in a McIntosh & Fildes anaerobic jar under hydrogen, except where noted.

Media 4 to 14 were used for the isolation or testing of rumen bacteria exacting in requirements for a low E_h and CO_2, and were prepared as described under General Methods and incubated under CO_2. *Medium* 4 contained mineral solution (*a*), 15 ml.; mineral solution (*b*), 15 ml.; rumen fluid, 40 ml.; Difco yeast extract, 0·25 g.; water, 29 ml.; bromocresol purple, 0·1 ml. of 1·6 % (w/v) ethanolic solution; glycerol, 1 ml.; sodium acetate, hydrated, 1·49 g.; $NaHCO_3$, 0·4 g.; cysteine HCl, 0·05 g.; pH 6·8. The mineral solutions, rumen fluid, yeast extract, indicator and water were autoclaved together at 120° for 15 min. Immediately on removing from the autoclave oxygen-free CO_2 was bubbled through the solution and the flask stoppered. The glycerol, acetate, bicarbonate and cysteine were then added as a sterile filtered solution, and the medium dispensed in 9 ml. amounts. Tenfold dilutions of rumen contents were usually made directly into this medium. *Medium* 5. This medium was like medium 4 except that bromocresol purple was omitted and agar (2 %, w/v) and resazurin (0·0001 %, w/v) were added. *Medium* 6 was like medium 5, but without agar. *Medium* 7 was like medium 4, except that sodium acetate was omitted and the glycerol was replaced by the appropriate 'sugar' to 0·5 % or 1 % (w/v). *Medium* 8 contained per 100 ml.: mineral solution (*a*), 15 ml.; mineral solution (*b*), 15 ml.; rumen fluid, 20 ml.; water, 49 ml.; $NaHCO_3$, 0·4 g.; glycerol, 1 ml.; cysteine HCl, 0·05 g.; resazurin, 0·1 ml. of 0·1 % (w/v) solution. *Medium* 9 was like medium 8 but contained in addition yeast extract, 0·25 g. After incubation for 2 days ammonia in inoculated and uninoculated media was determined by the method of Conway (1957). The lipolytic bacteria were also tested in media 8 and 9.

Media used for lipolytic bacteria

These media were prepared and incubated under CO_2 as described under General Methods. To prevent alteration of the linseed oil by heating, the oil was taken from the lower part of a deep bottle kept at 2° and added to media without sterilization. No bacteria were ever found to grow from such samples of oil placed in uninoculated media. Saliva was used as an emulsifying agent for the linseed oil.

Saliva-based medium. *Medium* 10 contained, per 100 ml.: glucose, 0·2 g.; yeast extract, 0·5 g.; NaCl, 0·5 g.; cysteine HCl, 0·1 g.; sheep parotid saliva, 99 ml.; linseed oil, 1 ml.; resazurin, 0·1 ml. of 0·1 % (w/v) solution; K_2HPO_4, 0·087 g.; KH_2PO_4, 1·29 g.; final pH 6·9. The phosphates, yeast, NaCl, cysteine and resazurin were mixed in 95 ml. saliva, gassed with CO_2 and heated in boiling water until the dye was reduced. The bottle was then stoppered tightly under CO_2 and autoclaved at 120° for 15 min. The linseed oil was then added as an emulsion and the glucose as a concentrated filtered solution in amounts sufficient to bring the constitution of the medium to that given above. The complete medium was then mechanically shaken to produce an even emulsion. Medium 10 was dispensed in 9 ml. amounts and inoculated with 1 ml. portions of tenfold dilutions of rumen contents made in a solution of phos-

phates, cysteine and resazurin in saliva in the concentrations used in the medium. The dilution blanks were autoclaved under CO_2 and dilutions made in an apparatus enabling CO_2 to be continuously bubbled through the blanks.

Rumen fluid-based media (11 *to* 13). *Medium* 11 contained, per 100 ml.: mineral solution (*a*), 15 ml.; mineral solution (*b*), 15 ml.; rumen fluid, 40 ml.; water, 29 ml.; resazurin, 0·1 ml. of 0·1 % (w/v) solution; $NaHCO_3$, 0·4 g.; cysteine HCl, 0·05 g.; glucose, 0·1 g.; linseed oil, 1 ml. The minerals, rumen fluid, water and resazurin were autoclaved together at 120° for 15 min. The $NaHCO_3$, cysteine and glucose were added as a sterile filtered aqueous solution, and the linseed oil as a 50 % (v/v) emulsion in sterile rumen fluid. The completed medium no. 11 was shaken under CO_2 to emulsify the oil and then dispensed in 9 ml. amounts. Tenfold dilutions of rumen contents were made under CO_2 in a sterile solution of minerals (*a*) + (*b*), cysteine and resazurin in the percentage proportions used in the medium. Portions (1 ml.) of each dilution were added to 9 ml. medium. *Medium* 12 was like medium 11 except that glucose was omitted. *Medium* 13 was like medium 12 but containing 2 % (w/v) agar. The linseed oil emulsion was added to medium 13 at a temperature of about 70° and it was shaken immediately so that the agar did not set whilst the oil was being emulsified. Medium 13 was dispensed and kept at 50° until inoculated, after which the tubes were rolled under cold water; dilutions of rumen contents were made directly in this medium. Medium 13 + 1 % (v/v) tributyrin in place of oil was used to test for esterase activity.

Casitone + yeast extract-based medium. Medium 14 was like medium 12 except that the rumen fluid was replaced by Bacto-casitone (1·5 g.) and yeast extract (0·25 g.).

Properties of glycerol-fermenting bacteria

Biochemical tests on selenomonads. Hydrogen sulphide was tested for in medium 6 by lead acetate paper, gelatin liquefaction in the same medium with sterile gelatin + charcoal tablet added (Kohn, 1953), and nitrate reduction in this medium with 0·2 % (w/v) KNO_3 added. Indole and Voges-Proskauer tests were made in a medium similar to medium 6 but with 0·5 % (w/v) glucose in place of glycerol, Bacto-casitone added to 1 % (w/v) and the rumen fluid decreased to 10 % (v/v). Products of glycerol fermentation were determined in medium 6 with rumen fluid decreased to 10 % (v/v). Fermentation reactions were tested in medium 7.

The properties of other bacteria. The fermentation reactions of the *Clostridium* and *Aerobacter* spp. were tested in peptone water and other reactions by the methods given in *Manual of Microbiological Methods* (1957). Products of glycerol fermentation by the clostridia were determined in cultures grown in medium 1.

Motility tests were made on selenomonad cultures grown 17 hr. in medium 6 + 0·1 % (w/v) glucose replacing glycerol. Organisms were examined in sealed capillary tubes and stained for flagella by Liefson's method (*Manual of Microbiological Methods*, 1957).

Properties of lipolytic bacteria

Biochemical tests. Fermentation reactions were tested in medium 12 or in medium 14 (without oil) with the 'sugar' at 1 % or 0·5 % (w/v) concentration and bromocresol purple added. Hydrogen sulphide and indole production were tested in medium 14 without oil but with glycerol, nitrate reduction in the same medium with

0·1 % (w/v) KNO_3 added, and gelatin hydrolysis also in this medium with a gelatin + charcoal tablet added.

Motility tests were made on cultures grown 17 hr. in medium 14 + 0·1 % (v/v) glycerol in place of oil. Organisms were examined in wet preparation by dark-ground illumination and stained for flagella by Liefson's method (*Manual of Microbiological Methods*, 1957).

General Methods

Determination of lipolytic activity. The whole culture, or uninoculated medium blank (10 ml.) was brought to pH 5·6–5·8 by addition of 2N-HCl and extracted with 3 × 10 ml. portions of ether. The ether extract was then washed with successive 10 ml. portions of water saturated with ether until the washings were at pH 6·5–7. The ether was removed by distillation (for the last few minutes *in vacuo*) the oily residue dissolved in 10 ml. 95 % (v/v) ethanol in water and titrated with 0·05N-NaOH (phenol red as indicator). In preliminary experiments the results obtained by this method were checked against those obtained by extraction and weighing of the free fatty acids by the methods given in the previous paper (Garton *et al.* 1961).

Determination of fermentation products. Volatile fatty acids were steam-distilled from the acidified medium after removal of cells (Friedemann, 1938) and analysed qualitatively by paper chromatography (Elsden & Lewis, 1953) or quantitatively by chromatography on celite columns (Bueding & Yale, 1951). Lactic acid and formic acid were determined colorimetrically (Barker & Summerson, 1941; Grant, 1947) and succinic acid by a manometric method (Umbreit, Burris & Stauffer, 1957). Glycerol was determined by the method of Lambert & Neish (1950).

Stock cultures. Stock cultures of the clostridia were kept in Robertson's cooked meat medium and the Aerobacter strains on nutrient agar slopes. The selenomonads were kept initially at −20° on slopes of medium 5, and subcultured every 10–14 days. Later slopes stored at 2° were found to keep just as effectively. Cultures were also freeze-dried in 7·5 % (w/v) glucose serum broth and in 10 % whole milk. These dried cultures kept at 2° could be revived after 3 months. The lipolytic organisms were maintained at 2° on slopes of medium 14 with agar + glycerol in place of oil, transferred every 4 weeks. Cultures freeze-dried as previously described did not revive.

Sheep rations and the collection of rumen samples. The basal ration consisted of a mixture of linseed cake meal, casein and a concentrate mixture (ground maize, crushed oats, bran) with hay fed separately. Sheep 8, 8N, 186, 97 and 6 received this ration except when recorded as 'fed oil', which meant that 40 g. linseed oil/day was added to the ration, or 'fed glycerol', which meant that 20 g. glycerol/day was added. Sheep 44 always had the basal diet + glycerol. Sheep 9 received a diet of hay and grass cubes only. Samples were taken via rumen cannulas about 3 hr. after feeding.

RESULTS

Glycerol-fermenting bacteria

Growth in a medium without rumen fluid. Rumen contents from sheep 6 were diluted in medium 1 and incubated for 3 days. Cultures which showed acid formation (up to $1/10^6$ dilution) were subcultured on solid medium 2 and incubated for 2 days. Acid-producing colonies were transferred to a liquid medium (medium 1

without agar). A mixed flora grew in the initial cultures, but the chief bacteria which grew on subculture were large, thick Gram-positive rods from dilutions up to $1/10^4$. From the initial $1/10^5$ and $1/10^6$ dilutions Gram-positive cocci were obtained which gave a weak acid reaction with glycerol; this activity was lost on subculture. The Gram-positive rods were 2μ to $5\mu \times 1\mu$, did not grow aerobically, on blood agar they gave β-haemolysis. Colonies were 2·5 mm. in diameter, smooth, whitish, translucent with slightly raised centre and uneven edge. Glycerol was fermented to give acid, and glucose, maltose, lactose and sucrose gave acid and gas; mannitol and salicin were not fermented. In litmus milk a stormy clot reaction was given and the lecithinase test was positive within 48 hr. The organisms were identified as *Clostridium perfringens* (*Welchii*).

Growth in a medium with rumen fluid incubated under hydrogen. Tenfold dilutions of rumen contents from sheep 6 (fed glycerol) were prepared in sterile saline, and 1 ml. portions of the dilutions mixed with medium 3 in Petri dishes and incubated under hydrogen for 3 days. Acid-forming colonies appeared up to $1/10^3$ dilution. The primary colonies were subcultured into medium 3 without agar, and further subcultures made from those cultures which showed acid formation. From the initial $1/10^3$ dilution some very small Gram-positive rods were obtained which did not survive further subculture. From the $1/10^2$ dilution Gram-negative rods of different sizes were obtained; they were non-motile and straight or slightly curved with some variation in length. Organisms grown aerobically on medium 3 had large capsules. Anaerobically very small capsules were formed and the organisms tended to be short and coccoid. Grown anaerobically and aerobically these bacteria were non-haemolytic. Colonies growing aerobically at 38° on medium 3 containing different amounts of rumen fluid were always opaque, mucoid, raised with entire edge, creamy white and about 3·5 mm. diameter; anaerobically the colonies were 0·5–1·75 mm. diameter and not mucoid. These bacteria fermented dulcitol, inositol, glycerol, mannitol, sorbitol, xylose, arabinose, rhamnose, aesculin, salicin, glucose, galactose, lactose, trehalose, dextrin and starch, giving acid and gas; adonitol and inulin were not fermented. Litmus milk after 17 hr. gave acid without a clot; at 48 hr. a soft clot had formed. Catalase reaction, nitrate reduction, Voges-Proskauer reaction and growth in Koser's citrate medium were all positive. Indole, methyl red and Eijkman tests, gelatin liquefaction and examinations for motility were all negative. The bacteria were classified as *Aerobacter aerogenes*.

Growth in media with rumen fluid incubated under carbon dioxide. Cultures were made of dilutions of rumen contents from sheep 6 (fed glycerol), 8, 9 and 44. Medium 4 was used for all samples except that from sheep 8, in which case the medium base was modified by adding rumen fluid to only 20 % (v/v) and agar to 2 % (w/v) and omitting the indicator. This modified medium 4 was used without glycerol or acetate, or with glycerol alone, acetate alone, or glycerol + acetate in the concentrations given under Methods. These media were inoculated with dilutions of rumen fluid (from sheep 8) prepared in a fluid similar to that of Doetsch, Robinson & Shaw (1952). All four media gave from sheep 8 a growth consisting predominantly of curved Gram-negative rods up to $1/10^9$ dilution. Growth was denser in the media with added glycerol. From the medium + glycerol alone isolations were made of a curved Gram-negative rod (A/10/B), and a very small Gram-negative coccus, the latter not surviving continued subculture.

Cultures from sheep 44 showed a growth predominantly consisting of curved Gram-negative rods up to $1/10^{12}$ dilution. No isolations were made from this experiment.

Cultures from sheep 6 (fed glycerol) made in medium 4 gave good growth and acid formation up to $1/10^{8}$ dilution; again Gram-negative rods predominated. From the $1/10^{7}$ and $1/10^{8}$ dilutions subcultures were made into medium 5. The pure cultures finally obtained and examined in detail were all from the $1/10^{8}$ dilution. There were six isolates (numbered, 3, 6, 7, 8, 9, 12) all strictly anaerobic Gram-negative curved rods with pointed ends, motile, evenly stained in young, vigorously growing cultures, but in older cultures showing granulation and uneven staining. The rods were $1{\cdot}8\mu \times 0{\cdot}4\mu$ to $3\mu \times 0{\cdot}6\mu$, without capsules or iodine-staining polysaccharide. Growth on glycerol slopes (medium 5) gave opaque, light-brown colonies with a creamy periphery. Deep colonies were similar, but the centres were very brown and this colour deepened on prolonged incubation. Suspensions of organisms in saline were sometimes very dark-coloured, almost black.

Table 1. *Reactions of glycerol-fermenting selenomonads*

Substrate	Isolation number and reaction										
	3	6	7	8	9	12	17	18	19	20	21
Xylose	−	−	−	−	−	−	+	+	+	+	+
Starch	+	+	+	+	+	+	−	−	−	−	−
Dextrin	+	+	+	+	+	+	−	−	−	−	−
Sorbitol	+	+	+	+	+	+	−	−	−	−	−
Dulcitol	−	−	+	−	−	−	−	−	−	−	−
Inulin	−	−	−	−	−	−	+*	+*	+*	+*	+*
Melezitose	+	+	+	+	+	+	−	−	−	−	−
Amylose	+	+	+	+	+	+	−	−	−	−	−
Nitrate reduction	+	+	+	+	+	+	−	−	n.t.	−	−

* Inulin fermentation much slower than other reactions.

n.t. = not tested.

All isolates fermented cellobiose, maltose, glucose, sucrose, glycerol, lactose, mannitol, galactose, arabinose, rallinose, salicin, aesculin, melibiose, ribose, fructose, sodium lactate, mannose, glucose-1-phosphate, glucosamine HCl. No isolates fermented trehalose, rhamnose, inositol, adonitol, xylan, cellulose, glycogen, sorbose, sodium acetate, fumarate, citrate, pyruvate, succinate. No isolates liquefied gelatin, formed indole, acetyl methyl-carbinol, or catalase, or grew aerobically. All formed H_2S and were motile. All grew at 30° and 38°, but not at 18° or 50°.

Cultures were also isolated from sheep 9 in the same manner. In the initial cultures growth and acid formation took place up to $1/10^{9}$ dilution. Pure cultures were obtained from this dilution (isolates labelled 17, 18, 19, 20, 21). In morphology and growth the cultures were similar to those from sheep 6 being motile curved Gram-negative rods $1{\cdot}6\mu \times 0{\cdot}6\mu$ to $3\mu \times 0{\cdot}6\mu$. The biochemical reactions of all cultures are given in Table 1. Six isolates tested (numbers 3, 6, 9, 12, 17, 21) showed no lipolytic activity, and two isolates (3, 17) tested did not hydrolyse tributyrin. All isolates grew in medium 4 with 10% or 20% (v/v) rumen fluid or in a casitone+yeast medium without rumen fluid, and growth in a rumen fluid-type medium (similar to 4) was much better when casitone or yeast extract was added. As shown later, acetate was not utilized along with the glycerol, but it appeared to act as a growth factor, as growth in medium 4+acetate was much better than without acetate at 24 hr.; after 48 hr., however, growth was much the same in each medium.

These curved Gram-negative glycerol-fermenting rods appear on morphological grounds (see Fig. 1) to belong to the genus *Selenomonas*. Since they all ferment lactate they may be classified as *S. ruminantium* var. *lactilyticas* (Bryant, 1956).

Lipolytic bacteria

Growth in saliva-based media. Experiments were made in which dilutions of rumen contents were inoculated into medium 10. Although growth was obtained, little lipolysis appeared to be taking place; the following results are typical. Tenfold dilutions of rumen contents from sheep 8N (fed linseed oil) were made in the diluting fluid and portions transferred to tubes of medium 10, medium 10 without glucose, and medium 10 without glucose and yeast extract. The cultures were incubated vertically in tubes in a shaker with a short, rapid travel. The shaker was kept going for about 7 hr. out of every 24 hr. during an 8-day period of incubation. The linseed oil emulsion made all the tubes too optically dense to see whether growth had taken

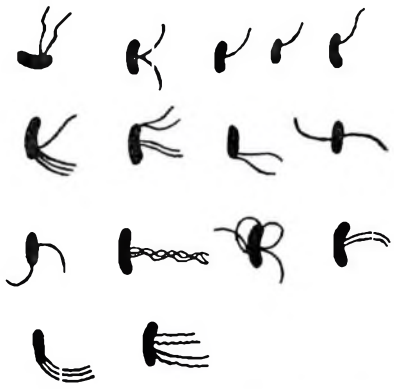

Fig. 1. Arrangement of flagella on glycerol-fermenting selenomonas. Not to scale.

place, but examination of stained films showed that a mixed growth of bacteria with many Gram-positive cocci occurred in medium 10 tubes up to $1/10^6$ dilution. From $1/10^7$ to $1/10^{12}$ dilutions the growth was mainly small indeterminate Gram-negative rods. Tubes of medium 10 minus glucose showed a similar growth, but the Gram-positive cocci were slightly more numerous in the greater dilutions; Gram-negative rods were always present. The tubes of medium 10 without glucose and yeast extract showed a few bacteria in dilutions $1/10^1$ and $1/10^2$, but nothing at greater dilutions. A representative number of tubes were taken for determination of lipolysis; the results are shown in Fig. 2. It will be seen that although there was some extra acid formed in cultures in medium 10, with and without glucose, the extent of lipolysis was similar to that produced by dilution of the rumen fluid in medium 10 without glucose and yeast extract, and no lipolysis due to growth of the bacteria in the greater dilutions was detected.

Growth in rumen-fluid based media. Preliminary tests established the composition of the medium which would give a stable emulsion of linseed oil and allow growth of bacteria. The following are the results of a preliminary experiment with medium 11. Dilutions of rumen fluid from sheep 8N in medium 11 were incubated in a nearly horizontal position on a rocking shaker making 1 rock per minute, as it was thought

that the shaker previously used was too violent. This slow rocking kept the linseed oil emulsion fairly well dispersed, only a slight amount of oil appearing on the surface of the medium. Incubation was continued for 5 days. The 5- to 6-day incubation period was initially used because it appeared to give greatest growth on visual examination of cultures; it was later confirmed that with pure cultures the viable count was higher after incubation for 6 days than for 3 or 10 days. Examination of cultures showed a mixed growth, with many Gram-positive cocci and Gram-negative rods of different shapes and sizes, up to $1/10^9$ dilution, the greatest made, except for dilutions $1/10^7$ and $1/10^8$ where the Gram-negative rods were present in very small numbers. The extent of lipolysis was determined on all tubes and is shown in Fig. 2. The result for the $1/10^5$ dilution may be low because of a mishap in the extraction, but it is evident that more lipolysis was taking place in general in the greater dilutions than could be accounted for by dilution of the rumen fluid; it appeared to be connected with the presence of Gram-negative rods, so more trials of the medium

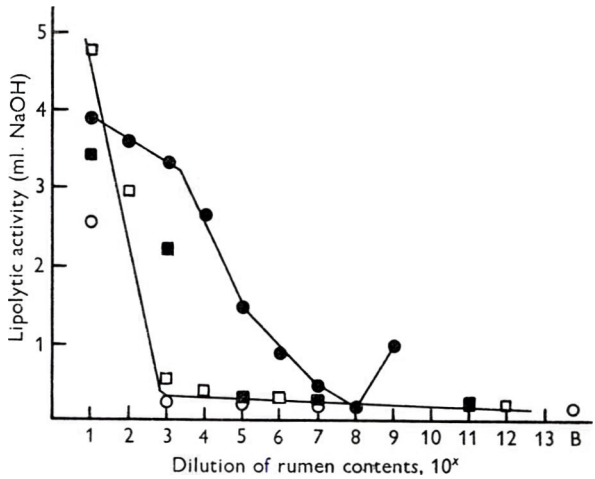

Fig. 2. Lipolytic activity of dilutions of rumen contents cultured in different media. Saliva-based medium: with linseed oil + glucose, □; linseed oil alone, ■; linseed oil without nitrogen source, no bacterial growth, ○. Rumen fluid-based medium: linseed oil + glucose, ●.

were made. For the isolation of lipolytic bacteria dilutions of rumen contents from sheep 8N (fed linseed oil) were made in medium 11 and incubated for 5 days. Stained films showed a mixed flora including Gram-positive cocci up to the $1/10^6$ dilution; from the $1/10^6$ to $1/10^9$ dilutions Gram-negative rods of different morphology were the organisms observed. All the dilutions except $1/10^9$ showed lipolysis and from the original $1/10^7$ and $1/10^8$ dilutions three isolates of lipolytic bacteria were obtained by dilution and subculture on alternate liquid and solid media (12 and 13). These were small Gram-negative rods labelled 7ES, 8ES and 8ER. The lipolytic activity in liquid medium (12) was tested at intervals while the cultures were kept; it did not diminish on subcultivation. Up to about 70 % hydrolysis of the oil occurred in 6 days. In morphology, growth characteristics and biochemical reactions all these isolates were similar, except that isolate 7ES grew much more poorly than the others and did not keep so well in stock cultures. The organisms were strictly anaerobic,

Gram-negative rods variable in size, but generally $1{\cdot}2\mu$ to $1{\cdot}8\mu \times 0{\cdot}4\mu$ in young cultures. They were more or less curved depending on the size of the given organism. Organisms in older cultures became faintly staining and developed a central granule; these organisms were generally larger (up to 3μ to $3{\cdot}6\mu \times 0{\cdot}4\mu$). Later the organisms seemed to disintegrate leaving only a granular mass. The organisms were actively motile with a single polar flagellum. No iodine-staining polysaccharide or definite capsules were seen, but organisms of isolates 8ES and 8ER were buried in masses of slime. Isolate 7ES was not so noticeably slimy. In glycerol agar colonies were lenticular and brownish. Towards and on the agar surface, colonies were round, raised, whitish and mucoid; on continued incubation, the colonies spread to give a very slimy growth all over the agar surface. In linseed oil agar roll tubes colonies were minute or invisible to the naked eye unless incubation was continued for a long time, and growth was usually noticed only by the circular clear areas which developed in the agar. Clear zones, taken to indicate esterase activity, were formed in the tributyrin medium.

These bacteria hydrolysed linseed oil, but did not apparently utilize the resultant long-chain fatty acids and no growth occurred in a medium containing the acids from hydrolysed linseed oil. Fermentation tests were originally carried out in medium 12 + the appropriate sugar in place of oil. Of the substances listed in Table 1 only glycerol, fructose and ribose were fermented, the first more quickly than the others. However, in all cases growth was not luxuriant, so some of the tests were repeated using medium 14. Growth was much better in this medium, but only the same substances were utilized. In medium 12 with glycerol alone the final pH value was 5·6, in medium 14 it was pH 5·0. Hydrogen sulphide was formed, nitrate was not reduced to nitrite, indole was not formed, nor was gelatin hydrolysed. Growth occurred at 38°, but not at 20°, 30° or 50°. In rumen fluid-based media growth was somewhat better when 30 % or 40 % rather than 10 % or 20 % (v/v) rumen fluid was used. These lipolytic bacteria could not be identified with any of the rumen bacteria so far described.

Products of glycerol fermentation

Some of the bacterial isolates were tested for the products of glycerol fermentation. The mixed flora growing from $1/10^3$ and $1/10^4$ dilutions of rumen contents inoculated into medium 1 produced formic, acetic, propionic, butyric, *iso*valeric and caproic acids and four clostridial isolates from this medium all produced formic, acetic and butyric acids. Other products were not tested for. The Gram-negative curved rods were all very similar in fermentation products. Isolate A/10/B, isolated from sheep 8 in medium 4 containing only glycerol (see above), produced mainly propionic acid when grown with glycerol or glycerol + acetate. The six isolates of the selenomonads from sheep 6 all produced mainly propionic acid in a glycerol + acetate medium (6). Selenomonad isolate 3 produced in one experiment (μmole acid/ml. medium): propionic, 50·4; lactic, 8·2; succinic, 14·6; acetic, 4·2; no formic or butyric acids. These products accounted for 111·3 % of the carbon of glycerol utilized. In other experiments very slight growth appeared to take place in the medium without glycerol with the formation of some volatile fatty acids. This might account for this high carbon recovery. Selenomonad isolate 21 in the same medium

produced mainly propionic acid, with some lactic acid (succinic acid not tested for, no carbon balance done). The lipolytic bacteria which utilized glycerol in medium 14 without oil produced mainly propionic acid. In one experiment the products from isolate 7ES were (μmole acid/ml. medium): acetic, 3·4; propionic, 36·2; butyric, 0·6; succinic, 11·0; no formic or lactic acid. These products accounted for 89·1 % of the carbon of the glycerol fermented. Isolates 8ES and 8ER behaved similarly. Isolate 8ES produced in one experiment (μmole acid/ml. medium): acetic, 6·0; propionic, 54·0; butyric, 1·0; succinic 8·0; no formic or lactic acid; carbon recovery 90·0 %. There was no obvious gas formation with glycerol media, but since no quantitative measurements were made, some CO_2 or bacterial slime might account, partially at least, for the low carbon recoveries.

Ammonia utilization or formation

The utilization of ammonia by nine isolates of selenomonads and the three lipolytic isolates when grown in either of the media 8 and 9 was not definitely detected after growth for 2 days. Except in three cases the ammonia concentration in the growing culture was within 0·5 μmole/ml. of that of the control. In the three cases the difference was about 1 μmole/ml., but it was not consistent and was ignored. Some rumen bacteria have been shown to utilize ammonia when growing in media in which this compound was almost the only source of nitrogen, others have been found to need amino acids or peptides as well as ammonia. The two media described here should have provided both conditions. Growth in medium 9 with added yeast extract was much better than in medium 8.

Counts of lipolytic bacteria in rumen contents

Viable counts of lipolytic bacteria in the rumen contents of sheep 9, and of sheep 97 and 186 on the basal ration alone and fed linseed oil were made on medium 13 in roll tubes. The number of clear zones in the medium after incubation for 6–7 days was noted. As it was very difficult to see some of these zones, only approximate counts were made. Clear zones were found in media inoculated from sheep 9 up to $1/10^9$ dilution, from sheep 97 to $1/10^{10}$ and from sheep 186 to $1/10^9$ on each ration. Although colonies of bacteria not visibly utilizing the oil could be seen, in the smaller dilutions especially, the zones of clearing contained colonies that were barely visible to the naked eye, as with the pure cultures of lipolytic bacteria. On prolonged incubation the colonies and the clear zones became larger, but by this time the majority of bacteria appeared to have disintegrated (compare the pure cultures) and no definite morphological structures could be identified.

DISCUSSION

It is evident from the experiments with sheep 8 that the addition of glycerol to a rumen fluid medium did not lead to the growth only of bacteria capable of utilizing this compound; many bacteria seem to have requirements such that they can grow on the substrates provided by rumen fluid + yeast extract. However, the media used here should have permitted the growth of a good selection of bacteria able to utilize glycerol even if others grew also. The results show that whilst bacteria like

those found in other habitats play some part in the rumen fermentation of glycerol, they are present only in small numbers. The results are similar to other reports in which different media have shown similar numbers of bacteria such as *Aerobacter aerogenes* to be present in the rumen. The numbers in which the selenomonads grew show that they must form a major component of the glycerol-utilizing flora. It is also evident that they are a basic part of the rumen flora and are not found solely or even mainly in the rumen of sheep fed glycerol-containing diets. These selenomonads, however, can utilize a large selection of 'sugars' and so would be expected to exist on substrates provided by almost any diet. Although there are obvious differences in fermentation reactions between the isolates of selenomonads from sheep 6 and 9, in general the reactions agree with those of the two bovine strains of lactate-fermenting selenomonads described by Bryant (1956) which also showed some strain differences. Isolates 3 and 21 were also tested qualitatively for volatile fatty acids formed by fermentation of glucose; propionic acid and acetic acid were the only ones detected. In this these bacteria are similar to *Selenomonas ruminantium* var. *lactilyticas*, both strains of which produced mainly propionic and acetic acids from glucose. In general the size of the isolated selenomonads was less than that of the selenomonads seen in the rumen; further experiments are in progress to see whether the bacteria described here are really typical large rumen selenomonads which have become smaller on cultivation *in vitro*. In the absence of acetate, glycerol fermentation *in vitro* was much slower than the fermentation of many other carbohydrates, so that the presence of acetate in the rumen would aid in the rapid utilization of glycerol by the selenomonads. Whilst glycerol-fermenting bacteria other than the selenomonads and lipolytic rods are present in the rumen and would contribute small amounts of mixed volatile fatty acids to its contents (e.g. clostridia), the selenomonads and the lipolytic bacteria described here must constitute a significant part of the flora and their products of fermentation of glycerol should bear some relationship to those found in the rumen. The main fermentation product in all cases was propionic acid, with some lactic and succinic acid. The amounts of volatile fatty acid formed differed in different experiments, but were always less than the theoretical amounts provided by the complete conversion of the glycerol utilized; in some cases the amount was only 50–60 % of the theoretical. These results are similar to those found with whole rumen contents *in vitro* where only a fraction of the glycerol fermented could be accounted for as volatile acids, considered as propionic (cf. Garton *et al.* 1961). If the other products were lactic or succinic acids one might expect them to be rapidly fermented by the whole rumen contents giving, again, propionic acid. Thus, either other so far unidentified products are formed, or the fermentation of lactate and succinate takes place so slowly under the *in vitro* conditions used that these products accumulate. That this could happen is shown by the fact that small amounts of lactic acid were found amongst the products of glycerol fermentation by the selenomonads, although these bacteria will utilize lactate when it is the sole substrate present. The selenomonads and the lipolytic bacteria did not utilize ammonia or produce it under the conditions of the test, so no obvious reason appears for the presence of glycerol having an effect on ammonia levels in the rumen.

Other studies on lipid metabolism by bacteria have been mainly confined to aerobic or facultatively anaerobic bacteria growing on substrates containing glycerides of lower fatty acids. In some cases the hydrolysis of esters of the higher fatty

acids has been demonstrated, again principally with aerobes or facultative anaerobes. In earlier (unpublished) experiments on rumen contents the presence was shown of a small number (about 10^4/ml.) of bacteria of the genus *Bacillus*, which were capable of growing on relatively simple media and of hydrolysing tributyrin. The lipolytic bacteria described here seem to be the only ones so far isolated from the rumen which will hydrolyse triglycerides of long-chain fatty acids. Although morphologically similar to many types of rumen bacteria they differ from all known species in their limited fermentation reactions. It would thus appear that they might be put in a new classification, but it is probably better to leave this until further strains are isolated. These bacteria are probably not the only ones which will hydrolyse glycerides in the rumen, but from the numbers present (about 10^8/ml.) they would appear to have a large part in this action. Again, the bacteria seem to be regular inhabitants of the rumen, and not to be encouraged by feeding linseed oil or glycerol, but all the rations fed to the animals would contain some, if only a very small amount, of lipid. Although these bacteria readily hydrolyse linseed oil they do not apparently metabolize the higher fatty acids formed; this is in accordance with the experiments in which rumen contents were incubated with triglycerides (Garton *et al.* 1961) where the liberated acids accumulated quantitatively. Further experiments will be needed to see whether these lipolytic bacteria are also responsible for the hydrogenation of unsaturated fatty acids in the rumen. Although the lipolytic bacteria described here were tested for hydrolytic activity only towards linseed oil and tributyrin, it is possible that they will also hydrolyse other triglycerides, as do mixed rumen bacteria (Garton *et al.* 1961).

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With 1 *plate*
Printed in Great Britain

The Isolation and Characterization of Bacteriophages from *Listeria monocytogenes*

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SUMMARY

The bacteriophages of *Listeria monocytogenes* have been studied with respect to isolation techniques and their use as diagnostic tools and as aids in epidemiological investigations. The occurrence of lysogeny was investigated in 123 strains isolated from human and animal sources throughout the world. Conventional procedures for isolation of phage were unreliable with Listeria since lysogenic strains did not always, by spontaneous lysis, release a detectable amount of phage. However, after exposure to ultraviolet radiation, such strains were induced to produce up to 10^7 plaque-forming particles/ml. Some strains which did not release phage produced substances after irradiation possibly analogous to colicines. The lytic spectrum of 11 phages against 149 strains of Listeria was studied and a system of classification, with five of these phages, was used to place 127 of these strains in 8 phage types. Nearly all of the untypable strains were rough, undergoing dissociation, or were lysogenic. Phage susceptibility appeared to be closely associated with the serological type of the strain, but showed no relation to the animal source or the geographical origin. These studies indicated that Listeria phages could be used as a means of generic identification and also as a substitute for or an adjunct to serological typing in epidemiological investigations.

INTRODUCTION

The increasing frequency of reports of human and animal listeriosis tend to give this disease a measure of importance not formerly accorded it (Seeliger, 1958; Gray, 1959; Welshimer & Winglewish, 1959). However, the epidemiological picture of listeriosis is incomplete, and neither biochemical nor serological procedures have been completely satisfactory in clarifying this picture. The successful application of bacteriophage typing to other bacterial genera suggests that such a method might be of value in tracing outbreaks of listeriosis or in proving transmission of the organism. Moreover, identification and differentiation of Listeria from other genera of Gram-positive rods still presents a problem in many laboratories. All too frequently, cultures of Listeria are erroneously identified as diphtheroids. Serological procedures for the identification of Listeria are expensive and are not readily adapted to most small laboratories. Bacteriophage filtrates, on the other hand, are

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inexpensive, easily prepared, relatively stable, and can be used by many laboratories not able to maintain a complete stock of reagents and typing sera for diagnosis of the less frequently encountered bacterial pathogens. Therefore, an evaluation of specific phage as an aid to the identification of genus *Listeria* seemed warranted.

Bacteriophage typing was first established as a practical and precise means of demonstrating subtle strain differences by Craigie & Yen (1938) in their classic studies on *Salmonella typhosa*. The use of adapted phage permitted them to detect differences among strains of Vi-positive *S. typhosa* which were undetectable by biochemical and serological means. This method found wide acceptance in tracing typhoid outbreaks and has since been applied to other groups of bacteria including *S. paratyphi B* (Felix & Callow, 1951), *Shigella sonnei* (Hammarström, 1949), and *Pseudomonas pyocyanea* (Warner, 1950). In addition, sensitivity to phage has been found to be a reliable method of identifying members of the genus *Salmonella* (Cherry, Davis, Edwards & Hogan, 1954; Chi, 1956; Pickett & Laughner, 1960), *Malleomyces* (Smith & Cherry, 1957), and for differentiating *Pasteurella pestis* from *P. pseudotuberculosis* (Gunnison, Larson & Lazarus, 1951). Phage typing of *Staphylococcus aureus*, originally developed by Fisk (1942*a*, *b*), and later modified by others (Wilson & Atkinson, 1945; Williams & Rippon, 1952; Hood, 1953; Blair & Carr, 1953, 1960), is now extensively used in epidemiological investigations. *Listeria monocytogenes* phage was described by Schultz (1945) but, except for a preliminary report (Sword & Pickett, 1958), phage typing of this species has not previously been reported.

To establish the reliability of a phage typing scheme, we first evaluated both the extra- and intrageneric specificity of Listeria phages. The former was pertinent in view of reported serological relationships between Listeria and both *Staphylococcus aureus* (Drew, 1946; Seeliger & Sulzbacher, 1956) and enterococci (Seeliger, 1955*a*). Also, phage pools were examined for evidence of interference among heterologous phages; the activity of undiluted stock phages and preparations diluted to routine test doses (RTD; as defined by Williams & Rippon, 1952) were compared; and the relationship between colonial dissociation of the host and its susceptibility to phage was studied.

METHODS

Test strains and their routine culture. The 149 strains of Listeria used in this work were from human and animal sources throughout the world. They were obtained from at least twelve animal species through the courtesy of workers in ten different countries. The identity of all strains was confirmed by observation of motility in semi-solid agar as well as microscopic and colonial morphology. All cultures were maintained on Tryptose agar (Difco), stored at 4°, and transferred at 6-month intervals. Blood agar base (Difco) and heart infusion broth (Difco) were used for propagating phages and for typing procedures.

Serological properties. In many cases information about the serotype was supplied by the source laboratory. However, to confirm this and to identify untyped cultures the procedures for immunization, agglutinin-absorption, and typing as described by Paterson (1939, 1940) and Seeliger (1955*b*) were used. The following strains from the National Collection of Type Cultures (London, England) were used as antigens: 2167, 5105, 5214 and 5348.

Isolation of bacteriophage. Cultures of certain strains (44-24 L, 44-50, 44-51, and 44-67 L) which have been shown by the method of Fisk (1942*a*, *b*) to carry phage, did not always contain enough free phage particles to be readily detectable when spotted on indicator strains. Preliminary use of the ultraviolet induction technique of other workers (Liegeois-Muller & Fredericq, 1952*a*, *b*; Thibaut & Fredericq, 1952) yielded promising results with these strains. A mineralight lamp (Model V41 Ultraviolet Products, Inc. San Gabriel, Calif., U.S.A.) which emitted approximately 52 % ultraviolet radiation with a wavelength of 2537 A was used. The lamp (with filter) at a distance of 45 cm. from the object being irradiated was shown by a General Electric germicidal ultraviolet intensity meter to produce 685 ergs/cm^2/sec. Irradiation for 1–2 min. at a distance of 25 cm., followed by incubation at 37° for 90 min. yielded maximal phage titres, often as high as 10^7 plaque-forming particles/ml. Listeria strains to be irradiated were grown overnight at 37°. Small volumes (2–3 ml.) were spread in a thin layer over the surface of open Petri dishes and irradiated. The dishes were swirled periodically during irradiation to provide maximum exposure, and the procedure was carried out in semi-darkness to avoid photo-reactivation. After irradiation and incubation the strains were spotted at spaced intervals by Pasteur pipettes on plates seeded with indicator strains. The indicator strains were included in a thin layer of semi-solid agar on the surface of blood agar base plates as described by Adams (1950). The spots were allowed to dry and the plates incubated at 37°. In this manner, each strain could serve in turn as the substrate on which the others were spotted and all desired combinations of strains could be achieved. A group of 123 strains was tested for the presence of phage by spotting on 52 strains chosen to include those of as many geographic, host and serological groups as possible.

Purification and preparation of stock phage suspensions. Spotted areas which showed the presence of plaques or confluent lysis were selected for further study. A small amount of the semisolid agar from such areas was removed by a wire loop and shaken in 1 ml. broth to elute the phage; 0·1 ml. of this broth was then incorporated into semi-solid agar overlays along with the appropriate seed organism. In this manner newly isolated phages were transferred two to three times from single plaques to insure purity of the final phage preparation. Stock phage preparations were obtained by harvesting from agar overlay plates by a modification of the method of Swanstrom & Adams (1951). Phage suspension was incorporated into seeded agar overlay on blood agar base plates. After incubation the agar overlay was scraped from the surface of the agar base and suspended in heart infusion broth. Phage was recovered from the agar by elution overnight at 4° followed by centrifugation at 2000 *g* for 30 min. The resultant supernatant fluid was sterilized by adding 0·001 % (w/v) thymol or by passage through a millipore HA filter pad (Millipore Filter Corp., Bedford, Mass., U.S.A.) and stored at 4°. Titres of phage suspensions ranged from 10^8 to 10^{11} plaque-forming particles/ml. Preparations were stable at 4° for at least 6 months and often for as long as 1 year.

Electron microscopy. Phage particles were concentrated from stock preparations by centrifugation at 15,000 *g* for 1 hr. in a Spinco (Model L) ultracentrifuge. The deposits were resuspended to one-tenth the original volume with broth and centrifuged at 2000 *g* for 1 hr. to remove large particles. High speed centrifugation was then repeated on the supernatant fluid, and the final pellet suspended in 1 ml. of

neutral isotonic ammonium benzoate (1·8 %, w/v). This suspension was spotted on electron microscope specimen screens covered by a collodion membrane (Wyckoff, 1949). All screens were air dried, shadowed with palladium and examined with an RCA EMU electron microscope. Four phages (82, 135, 51 L, 137) were examined by this method.

Routine procedure for phage typing. Agar overlay plates seeded with the bacterial host were prepared as described and spotted with phage suspensions by means of capillary pipettes. As many as twenty spots could be applied but ordinarily only a set of 4 phage preparations was used (a pool of phages 83, 135, 51 L, 24 L, 87). Since comparison of the lytic spectra of undiluted phage and phage diluted to the routine test dose revealed no differences, the former was used for typing.

RESULTS

Isolation of bacteriophage

Forty-three of the irradiated and spotted cultures lysed one or more of the 52 indicator strains of Listeria. Purified phage suspensions were obtained from 12 of these 43 by transfer from single plaques. No phage was isolated from strains of serotype 3 even after cross-testing a group of 10 such strains.

Demonstration of bacteriocines after irradiation

Thirty-one of the 43 previously mentioned lytic combinations failed to yield single plaques or phage when tested further. However, the lytic or inhibitory effect had a definite host range against the indicators. This effect was dependent on ultraviolet irradiation; unirradiated cultures did not cause lysis or inhibition of growth when spotted on indicators. This phenomenon was not further examined, but appeared to be referable to colicine-like agents. Such substances have been found in several bacterial species other than *Escherichia coli*, but have not been reported previously from *Listeria monocytogenes*. Jacob, Lwoff, Siminovitch & Wollman (1953) suggested the term 'bacteriocine' for substances possessing the general properties of colicines.

Electron microscopy

Plate 1, fig. 1, shows the morphology of phage 83. No morphological differences were found among the 4 phages examined. The phage heads appear to be about 85–90 m in diameter while the tails measure 265 × 15 mμ. A terminal knob on the tail (not shown here) was present in some preparations, but was never as pronounced as with some phages.

Effect of Listeria phages on heterologous species

To establish generic specificity undiluted stock phages 83, 135, 51 L, 24 L and 87 were spotted on the following heterologous bacterial species: *Corynebacterium pyogenes*, *C. pseudodiphtheriticum*, *C. pseudotuberculosis*, *Erysipelothrix rhusiopathiae*, *Lactobacillus arabinosus*, *L. brevis*, *L. buchneri*, *L. casei*, *L. fermenti*, *L. lycopersici*, *Staphylococcus aureus*, *Streptococcus equi*, *S. faecalis*. In no instance was lysis observed.

Lytic spectrum of phages against strains of Listeria

Eleven phage preparations (phages 23, 24L, 51L, 61, 71, 83, 87, 113, 132, 135, 137) were spotted on 149 strains of Listeria. The lytic spectrum of each preparation was usually restricted to strains of the serotype from which that phage had been obtained. Furthermore, there appeared to be two rather broad but distinct groups of strains with respect to phage susceptibility, namely, those lysed by one or more of the following phages: 23, 24L, 71, 83, 132, 135 and 137, and those lysed by one or more of phages 51L, 61 and 113. Since several of these phages possessed almost identical patterns of activity, only those with distinctly different spectra were used in developing a phage typing scheme. For this, a system of classification using only phages 83, 135, 51L, 24L and 87 was devised (Table 1). Phages 83 and 135 were quite similar, having only minor differences in their ranges of activity. Therefore these two were mixed into a single pool. The spectrum of phage 51L appeared to complement that of the pool (83+135). By spotting this battery of four phage preparations on an unknown strain, the latter could be classified in one of the eight groups listed in Table 1 or else was insensitive to all five phages. Only 22 out of 149 strains (14·7 %) were not sensitive to bacteriophage. Of these untypable strains, 2 were lysogenic and hence immune to phages related to their lysogenizing phage, and 19 were, colonially, non-smooth or frankly rough.

Table 1. *Bacteriophage sensitivity patterns of Listeria strains*

Phage type	Reactions with typing phages			
	83/135	51L	24L	87
1	+	+	+	.
2	+	+	−	.
3	+	−	+	.
4	+	−	−	.
5	−	+	+	.
6	−	+	−	.
7	−	−	+	.
8	−	−	−	+
Untypable	−	−	−	−

+ = lysis; − = no reaction.

Polyvalent pooled phage suspensions for generic identification of Listeria

A polyvalent pool of phages 83, 135 and 51L was spotted on the group of 149 strains mentioned above. The results obtained with the pool expressed the cumulative effect of the individual phage spectra on all strains tested and suggested that the phages could be usefully employed for identification of the genus *Listeria*.

Host and geographic distribution of phage types

An attempt was made to determine whether phage sensitivity showed any relation to the animal host or to the geographic origin of the strain (Tables 2, 3). None was found. Thus it appears that phage type, like the serological types described by Paterson (1939, 1940), is independent of source. However, certain patterns were noticeable within limited groups of strains. We found that two strains (44-17, 44-18)

isolated by Line & Cherry (1952) from infants in the same hospital ward had identical patterns of phage sensitivity. These workers suggested transmission from one mother to the other since the mothers shared a bathroom. We also found that 5 out of 6 strains isolated by Rome, Raitio & Vartiovaara (1953) in Finland from an outbreak of listeriosis in guinea pigs showed similar patterns of phage sensitivity. Similar tendencies were noted with other strains. It may then be possible to use this phage typing system for epidemiological purposes.

Table 2. *Bacteriophage type distribution of Listeria strains according to animal source*

Phage type	Strains		Animal source						
	No.	%	Human	Bovine	Ovine	Avian	Rodent	Porcine	Unknown
1	3	2·0	1	.	2	.	.	.	.
2	1	0·7	.	.	.	1	.	.	.
3	49	32·2	6	7	9	9	8	2	8
4	6	4·0	3	.	1	.	2	.	.
5	5	3·3	.	3	2	.	.	.	.
6	53	35·5	8	10	22	4	6	1	2
7	5	3·3	2	1	.	.	1	1	.
8	5	3·3	5	.	.	.	.	.	.
Untypable	22	14·7	10	3	6	1	.	1	1

Table 3. *Bacteriophage type distribution of Listeria strains according to geographic source*

Phage type	Geographic source			
	N. America	Europe	Asia	Australia
1	2	.	1	.
2	.	1	.	.
3	28	20	1	.
4	1	5	.	.
5	1	3	1	.
6	36	12	5	.
7	2	2	.	1
8	2	3	.	.
Untypable	7	14	1	.

Relationship of phage type to serological type

The phage type of 51 serologically typed strains was determined. The group of strains was selected to contain representative numbers of all the serotypes. There was almost perfect correlation between serotypes 1 and 2 and sensitivity to the pool of phages 83 + 135. Serologically, only the flagellar antigen 'D' separates these two serotypes, and strains of serotype 2 are very rare. Strains of both serotypes 1 and 2 belong to phage types 3 and 4. Most strains of serotype 3 are phage-resistant but those which are sensitive are lysed only by phage 87 and therefore belong to phage type 8. The phage-resistant serotype 3 strains are all intermediate or rough colonial types. Nearly all strains of serotype 4 are lysed by phage 51 L and therefore belong to phage types 5 and 6. Only one case of cross-activity between the pool of phages 83 + 135 and phage 51 L was noted in this group. Hence it appears that there is good correlation between phage sensitivity and serological type.

DISCUSSION

The ultraviolet irradiation procedure used in this study was effective in increasing the phage titre of susceptible lysogenic cultures. Carrier strains (Lwoff, 1953) and non-inducible lysogenic cultures should be unaffected by such a procedure. However, if these strains did produce phage it would be as readily detectable by this procedure as with conventional cross-testing procedures. All phages isolated in this study were from lysogenic strains since all responded to ultraviolet induction. Thus, all are 'temperate' rather than 'virulent' according to Lwoff's definition (Lwoff, 1953).

Examination of those strains which were not sensitive to the lytic action of phage disclosed that most of these were not smooth, frankly rough, or lysogenic. These differences in susceptibility suggest that if only freshly isolated smooth strains were used, the finding of apparently untypable strains should be considerably diminished.

The failure of these Listeria phages to lyse strains of bacteria of other genera confirms their generic specificity and indicates that they could, in a polyvalent pool, safely be used as a means of positive generic identification of fresh isolates. The high rate of host susceptibility and phage specificity, as well as the relative ease and economy of procedure as compared with serological methods suggest that this group of phages can profitably be used either as a substitute for serological typing or as an adjunct to it in epidemiological investigations of listeriosis.

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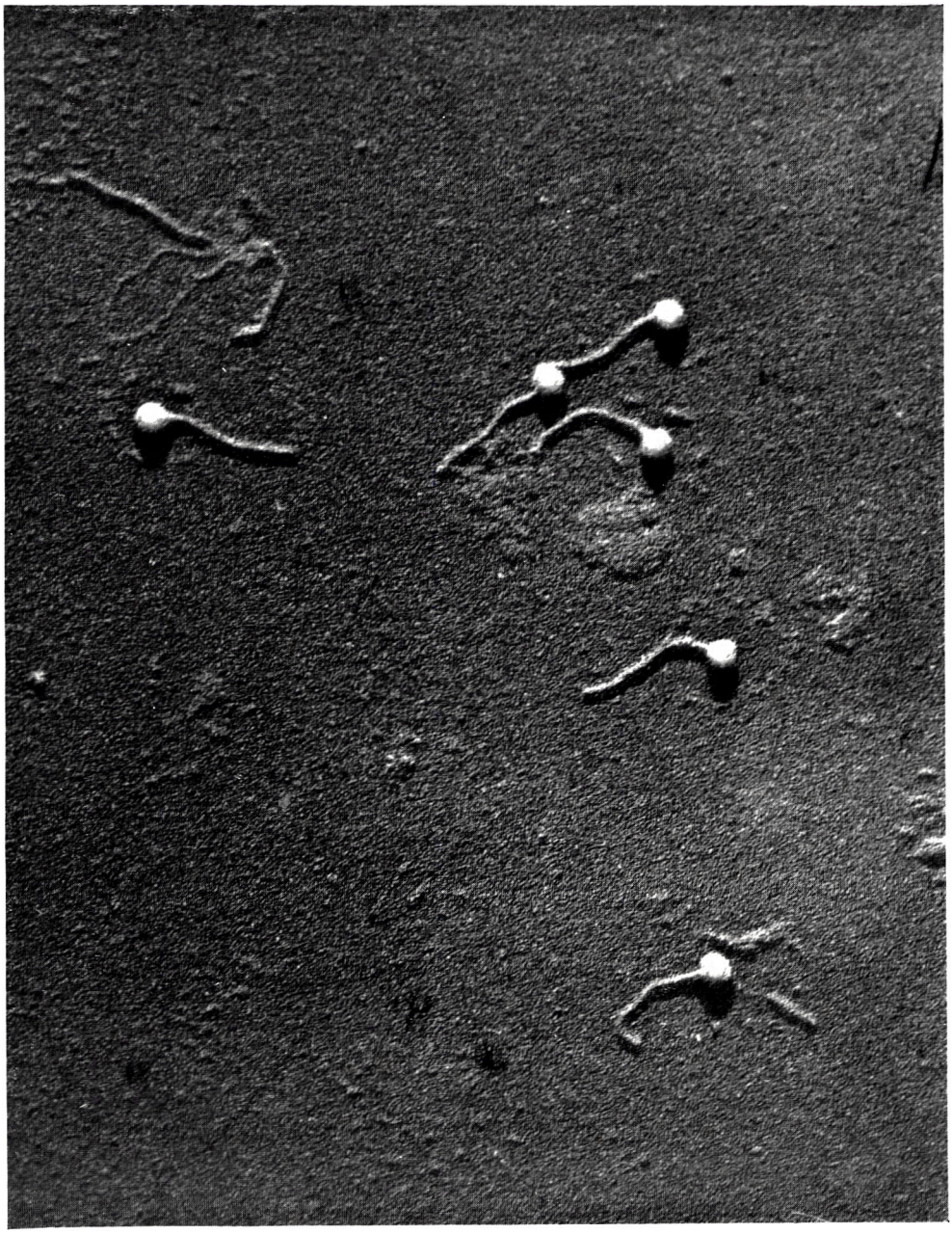
EXPLANATION OF PLATE

Fig. 1. Electron micrograph of 83: ×65,000.



C. P. SWORD AND M. J. PICKETT

(*Facing p.* 248)

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The Nutrition of a *Lactobacillus acidophilus* Variant Isolated from the Duodenum of a chick

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SUMMARY

Allen, Stephens, Jaffe & Wakelam (1959) isolated a lactobacillus which they found to require a growth factor present in malt distillers' dried solubles. We have identified the organism as belonging to the subgroup Thermobacterium Orla-Jensen. It was grown through many serial passages in a chemically defined medium containing thymidine, and had no residual indispensable requirement for unidentified nutrients.

INTRODUCTION

Malt distillers' dried solubles (MDDS), made from the liquor remaining in the whisky still after the spirit has been distilled, have been reported to contain unidentified factors which promote growth in chicks (Wakelam & Jaffe, 1959). The feeding of MDDS to chicks changed the composition of the bacterial flora of the duodenum and caused an increase in the numbers of an unidentified species of lactobacillus (Allen, Stephens, Jaffe & Wakelam, 1959). Allen *et al.* isolated this organism and found that it required a growth factor present in MDDS. Further tests with a variety of feed supplements showed that stimulation of chick growth was correlated with growth-promoting activity for the *Lactobacillus* sp. It seemed probable that the factor which stimulated growth in the chicks was the same as that required by the *Lactobacillus* sp. Through the kindness of Drs K. A. Allen and J. Stephens we have been able to make a further study of the nutrition of this organism.

METHODS

Organism. Cultures of the organism can be obtained from the National Collection of Dairy Organisms (NCDO) at the National Institute for Research in Dairying, Shinfield, Reading, as culture no. NCDO 1417. It will be referred to in this paper as strain 1417. We have found that it belongs to the subgroup Thermobacterium Orla-Jensen and has the cultural characteristics of *Lactobacillus acidophilus* (see Rogosa & Sharpe, 1959) except that it does not ferment trehalose.

Maintenance of organism. The organism was maintained by monthly transfer in the basal medium (Table 1) supplemented with 10 % (v/v) of an aqueous extract of MDDS. (To 50 g MDDS were added 400 ml. water and enough N-H_3PO_4 to bring to pH 6·5. The mixture was heated in flowing steam for 30 min., cooled and centrifuged. The supernatant fluid was diluted to 500 ml. with water.)

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Cultures were first grown for 18 hr. at 37° in the liquid medium and were then transferred as stabs to the same medium solidified with 1·5 % (w/v) agar. After incubation for 24 hr. these stab cultures were stored at 2°.

Preparation of inocula for tests. The cultures used to inoculate the tests were grown for 18 hr. at 37° in the basal medium supplemented with 10 % (v/v) of the MDDS extract. They were diluted 1/10 with sterile 0·9 % (w/v) NaCl solution and one drop added to each assay tube.

Basal medium. The composition of the basal medium is shown in Table 1. For some tests the medium was modified by using the following mixture of amino acids to replace the hydrolysed casein and Tryptone: L-leucine, L-isoleucine, L-valine, L-lysine, L-arginine, L-methionine, 500 mg. each; L-glutamic acid, L-asparagine, 1 g. each; L-alanine, glycine, L-serine, L-aspartic acid, L-tyrosine, L-proline, L-histidine, L-phenylalanine, L-threonine, L-tryptophan, 200 mg. each; in 200 ml. of 5 × single strength medium.

Table 1. *Basal medium. Composition to give 5 × the concentration of final medium*

Acid hydrolysed casein*	(g.)	5	Riboflavin	(mg.)	1
Charcoal-treated Tryptone†	(g.)	5	Thiamine	(mg.)	1
Glucose	(g.)	10	Nicotinic acid	(mg.)	1
KH_2PO_4	(g.)	3	Calcium pantothenate	(mg.)	1
K_2HPO_4	(g.)	3	Pyridoxal ethyl acetal hydrochloride	(mg.)	1
Diammonium citrate	(g.)	0·6	Biotin	(μg.)	10
Solution of mineral salts‡	(ml.)	10	Folic acid	(μg.)	1
Tween 80§	(ml.)	1	*p*-Aminobenzoic acid	(μg.)	1
Adenine	(mg.)	5	Cyanocobalamin	(μg.)	1
Guanine	(mg.)	5	Ascorbic acid	(g.)	0·5
Uracil	(mg.)	5	Cysteine	(mg.)	50
Xanthine	(mg.)	5	pH adjusted to 6·2, and water added to 200 ml.		

* Allen and Hanburys, Ltd. 'Vitamin free' grade.

† Prepared as follows: 50 g. Tryptone (Oxo Ltd.) were dissolved in 400 ml. water, and the solution adjusted to pH 4·0 with acetic acid. Charcoal (10 g. Sutcliffe and Speakman Ltd.; Grade 5) was added, and the whole stirred for 10 min. and then filtered. The filtrate was adjusted to pH 6·0 with 10 N-KOH solution, and a further 5 g. charcoal added. After stirring for 5 min. the mixture was filtered, and to the filtrate were added: DL-tryptophan (1 g.), L-tyrosine (200 mg.), L-phenylalanine (200 mg.), L-proline (100 mg.) and L-histidine (100 mg.). Finally, water was added to 500 ml.

‡ Contains $MgCl_2.6H_2O$, 20 g.; $CaCl_2$, 5 g.; $FeCl_3.6H_2O$, 0·5 g.; $ZnSO_4.7H_2O$, 0·5 g.; $MnSO_4.4H_2O$, 0·25 g.; $CoCl_2.6H_2O$, 0·25 g.; $CuSO_4.5H_2O$, 0·25 g.; VSO_4, 0·25 g.; Na_2MoO_4, 0·25 g.; dissolved in 1 l. distilled water with addition of N-H_2SO_4 to clear.

§ Polyoxyethylene sorbitan mono-oleate.

Test procedure. The growth promoting activities of the test preparations were judged by comparison with the activity of a 1/5 dilution of the MDDS extract. This 'standard' and the 'test' solutions were added to series of tubes in amounts of 1, 2, 4 and 8 ml., together with 2 ml. portions of 5 × single strength basal medium and water to make final volumes of 10 ml. The tubes were then capped and autoclaved at 115° for 10 min. After cooling to room temperature the tubes were inoculated and incubated at 37° for 24 hr. Growth responses were measured turbidimetrically with a Lumetron model 400 A photometer (Photovolt Corporation, 95 Madison Avenue, New York, 16 N.Y., U.S.A.).

RESULTS

Several proprietary extracts of yeast proved about as active as MDDS in promoting the growth of strain 1417. Yeast nucleic acid (British Drug Houses Ltd., Poole, Dorset, England) and herring sperm deoxyribonucleic acid (L. Light and Co., Ltd., Poyle Trading Estate, Colnbrook, Buckinghamshire, England) showed relatively little activity (about 2 % that of MDDS) but depolymerization of these compounds with cobra venom caused a considerable increase in their activity to equal, or slightly exceeding, that of MDDS. Ribose and deoxyribose were inactive. Adenosine, adenylic acid, guanosine, guanylic acid, cytidine and uridine were

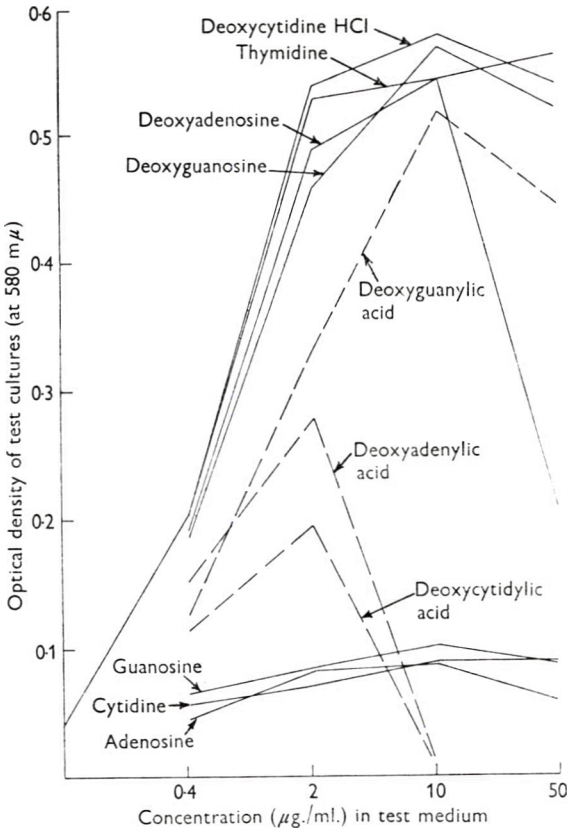

Fig. 1. Growth responses of strain 1417 to graded concentration of various nucleosides and nucleotides.

inactive or only very slightly active at 10 or 50 μg./ml. But deoxyadenosine, deoxyguanosine, deoxycytidine and thymidine were highly active, giving at 2 μg./ml. a growth response greater than that elicited by about 4 mg. MDDS/ml. Deoxyadenylic acid, deoxyguanylic acid and deoxycytidylic acid, at 0·4 and 2·0 μg./ml., were somewhat less active than the corresponding deoxynucleosides. At 10 and 50 μg./ml. deoxyadenylic acid and deoxycytidylic acid were inhibitory. The very slight activity of some of the ribosides tested might have been due to contamination

of these compounds with deoxyribosides. Figure 1 shows the growth responses of strain 1417 to graded concentrations of several of these compounds.

MDDS contains a high proportion of yeast and of the extracellular products of yeast metabolism. It undergoes a mild alkaline hydrolysis with lime at about pH 10·5 during the process of manufacture and would doubtless contain deoxynucleosides, whose presence might account for most of its growth-promoting activity for strain 1417.

The organism was grown through many serial passages in chemically defined medium supplemented with thymidine, and appeared to have no residual indispensable requirement for unidentified nutrients. The cultures grew faster, however, and to about 20 % greater population, in basal medium supplemented with 10 % (v/v) of the MDDS extract than in the same basal medium supplemented with any one or all four of the deoxynucleosides. It is evident that the extract of MDDS contributes stimulatory nutrients other than the necessary deoxyribosides. It provides additional buffering which alone might have permitted greater total bacterial growth.

Requirements for exogenous deoxyribosides have been noted for several of the lactic acid bacteria, among them 'Thermobacterium acidophilus R 26', which was recommended by Hoff-Jørgensen (1951) for the microbiological assay of deoxyribonucleic acid and deoxyribonucleosides, and is presumably of the same species as that studied by us. In both organisms the requirement for deoxyribosides persists in the presence of vitamin B_{12}. *Lactobacillus delbrueckii* also shows an absolute requirement for deoxynucleosides; but in certain other species the requirement is non-specific and is not apparent in the presence of vitamin B_{12} or enzymic digests of casein, or under reducing conditions induced by cysteine or ascorbic acid, or by growth in an atmosphere of hydrogen (Kitay, McNutt & Snell, 1949; Kitay, McNutt & Snell, 1950).

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A phage, $\phi\chi$, which attacks motile bacteria

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(*Received* 9 *December* 1960)

SUMMARY

A salmonella phage which attacks only flagellated bacteria (Sertic & Boulgakov, 1936*b*) has been studied. Tests with naturally occurring strains, and with artificial serotypes to which foreign H antigens had been transduced, have shown that sensitivity depends on the H antigen: bacteria with antigens of the *g*-complex are resistant, and with antigens *l*..., *e,h*, or Arizona *13*, are sensitive only to appropriate host-range mutants. Tests with non-motile and motile variants of the same strains showed that paralysed (non-motile H) as well as non-flagellated bacteria are resistant and thus that the flagella must be active as well as of correct antigenic type. Where resistance was due to absence of suitable flagella, it was associated with impaired adsorption of phage. Removal of the flagella from a sensitive strain led to diminished adsorption; a similar result was obtained when the bacteria were artificially paralysed in various different ways. No adsorption to detached flagella was detected, probably because they were inactive. Adsorption of the phage led to immobilization and agglutination of the bacteria, probably by a direct effect on the flagella. Electron micrographs showed phage particles attached to flagella, and infection could evidently follow adsorption to distal parts of a flagellum. The genome of the infecting particle may perhaps reach the bacterial body by being injected into an active flagellum at the point of initial attachment, and then travelling inside the flagellum.

INTRODUCTION

The susceptibility of a bacterium to phage infection primarily depends on whether or not the phage can adsorb to specific bacterial receptors (see Nicolle, Jude & Diverneau, 1953). Sensitivity is usually determined by structures on the surface of the bacterial body, which have sometimes been identified as somatic antigens (e.g. Burnet, 1930), or more superficial envelope antigens such as the Vi antigen (Craigie & Brandon, 1936; Sertic & Boulgakov, 1936*a*; Nicolle, Rita & Huet, 1951). However, a phage whose host-range appeared to depend on the presence of flagella was described by Sertic & Boulgakov (1936*b*). This phage, first named VIII-113, and later χ (Sertic & Boulgakov, 1936*c*), lysed a motile strain of *Salmonella typhi*, H 901, but not its non-flagellated variant, O 901 (Felix, 1930). This phage also selected resistant bacteria from H901 which were non-flagellated. Furthermore, it did not lyse H901 growing on agar containing 0·2 % (w/v) phenol, known to prevent the development of flagella (Braun, 1918), and also did not produce plaques on

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strains carrying abundant Vi antigen, known to interfere with motility. Flagella are structurally distinct from components of the cell proper, and the flagellar (H) antigen does not extend over the surface of the bacterial body (Craigie, 1931; Stocker & Campbell, 1959); thus, if the flagella are concerned in adsorption, it seems likely that the flagella themselves must be the structures to which the phage adsorbs.

The phage under discussion was further studied by Schiff & Bornstein (1940) who claimed that it specifically attacked salmonella species with H antigen *d*, characteristic of *Salmonella typhi*. Rakieten & Bornstein (1941) reported the isolation of five other phages whose host-ranges were associated with flagella. Apart from reports of the use of the χ phage for selecting non-flagellated mutants from motile strains (Stocker, Zinder & Lederberg, 1953), the only other description was given by London (1958), who tentatively concluded that its site of attachment was associated with the basal granules of the flagella. London's object was to identify the chemical groupings involved in adsorption of the phage by treating the bacteria in various ways and by altering the ionic composition and pH of the medium. In so far as our experiments have overlapped, our results have been broadly the same, with one notable exception: the present experiments have shown that paralysed (i.e. flagellated but non-motile strains; Edwards, Moran & Bruner, 1946; Hirsch, 1947; Friewer & Leifson, 1952), as well as non-flagellated strains, are resistant to the χ phage, and that it adsorbs very poorly to paralysed bacteria. London obtained good adsorption to bacteria washed and suspended in dilute salt solutions, a treatment which, in the present experiments, caused sufficient loss of motility for adsorption to be greatly decreased.

METHODS

Buffers. M/15 phosphate buffer (pH 7·0) containing 0·1 M-NaCl, 10^{-3} M-$MgSO_4$, and 10^{-4} M-$CaCl_2$ with gelatin 0·001 % (w/v). McIlvaine's citrate phosphate buffers pH 2·2, 2·6 and 3·0.

Media. Broth was that routinely made in this department from Tryptone (Oxoid), 10 g.; Marmite 5 g.; sodium glycerophosphate, 10 g.; potassium lactate 50 % (w/v) solution, 5 ml.; $MnSO_4.4H_2O$, 0·02 g.; $MgSO_4.7H_2O$, 0·2 g.; $FeSO_4.7H_2O_3$ 0·02 g.; with water to 1 litre. The pH was adjusted to 7·2 and sterile glucose solution was added to 0·2 % (w/v).

Nutrient agar consisted of broth solidified with 1·25 % (w/v) Davis N. Z. agar. In phage titrations by the overlay method, the bases and overlays contained 0·6 and 0·3 % (w/v) agar, respectively. Semi-solid motility medium (Edwards & Ewing, 1955) was dispensed in 8 ml. volumes in 2 in. diameter Petri dishes. All cultures were incubated at 37° without aeration.

Phage. The χ phage was originally obtained from Dr N. Boulgakov by Dr B. A. D. Stocker in 1952, and was usually grown and assayed on *Salmonella abortus-equi*, National Collection of Type Cultures (NCTC) 5727, a species not pathogenic for man. Before this strain had been found to be as good an indicator, Sertic & Boulgakov's usual propagating strain, *S. typhi* var Rough (now called SW 540) was used. Stocks were made by the overlay method (Swanstrom & Adams, 1951) with overlays and bases containing 0·3 and 1·25 % (w/v) agar, respectively. The homogenized overlays were clarified by centrifugation and the remaining bacteria were killed by heating at 56°–60° for 30 min. Titres of 5×10^{10}–2×10^{11} plaque-forming particles (p.f.p)/ml.

were readily obtained. In tests with a limited number of strains, the efficiencies of plating of stocks made on the two hosts, NCTC 5727 and SW 540, were similar.

Stocks of host-range variants of the χ phage. When the χ phage plated with low efficiency on a strain, one of the few plaques produced was diluted and replated with the strain to purify the phage, and a single plaque was then picked and grown with the strain concerned to make the variant stock. Stocks grown on NCTC 5727 were made from single plaques produced by the variant stocks on this strain. *Salmonella abortus-equi* strain NCTC 5727 was tested for possible lysogenicity with a phage whose presence in stocks might have been mistaken for a low efficiency of plating of the χ phage itself: supernatant fluids from cultures of NCTC 5727 treated in the same way as the χ phage stocks produced no plaques on any of the strains concerned. Nor did they produce plaques on strains of *Salmonella gallinarum*, a species sensitive to a wide range of salmonella phages (Dr E. S. Anderson, personal communication). To be sure that the phages with altered host-range were in fact variants of the χ phage, they were tested for neutralization by antiserum prepared against the wild-type phage: all were neutralized at the same rate as this was. Their activity against the strains which had selected them was neutralized to the same extent as their activity against NCTC 5727. They were also tested with motile and non-motile (O or paralysed) forms of a few different salmonella strains, and, like the wild-type phage, they attacked a strain only when it was motile.

Phage titrations. (1) Agar layer method. Satisfactory titrations giving consistent results were obtained by this method with *Salmonella abortus-equi*, NCTC 5727 or *S. typhi*, SW 540 as indicator strain, provided the volume of the overlay was not more than 2·5 ml. and the plates were kept strictly level. Salmonella strain NCTC 5727 tended to become less motile on subculture and the plaques then became hazy and small, but with passage of the indicator strain through semi-solid medium, the plaques again became larger and clearer.

(2) Surface method. Satisfactory plaques were usually obtained when drops of phage stocks were spotted on plates spread with indicator strain. Pipettes delivering drops of 0·02 ml. were used, and in general serial 10-fold dilutions were plated. With *Salmonella abortus-equi* strain NCTC 5727 as indicator, the same numbers of plaques were obtained by either the overlay or the surface method.

The agar layer method was suitable for use with strains of *Salmonella abortus-equi* or *S. typhi*; with other species, the number of plaques was greatly decreased when the concentration of agar in the overlay was high enough to give discrete plaques.

Where the titre of the phage is given, it refers to the number of plaque-forming particles (p.f.p.) on *Salmonella abortus-equi* strain NCTC 5727. The efficiency of plating (e.o.p.) on other strains was calculated using the titre on strain NCTC 5727 as denominator.

Total phage counts by fluorescence microscopy. Suspensions of phage were mixed with a bacterial suspension of known concentration. The mixture was examined by fluorescence microscopy (Anderson, Armstrong & Niven, 1959; Anderson, 1957) and the phage count estimated from the ratio of phage particles to bacteria.

Bacteria. The antigenic formulae for *Salmonella* are written according to the Kauffmann–White Scheme and no account has been taken of minor antigenic relationships or differences omitted from this scheme. For example, 1, 4, 5, 12 : *b* : *1,2*, the formula for *Salmonella paratyphi B*, indicates that the strain has somatic (O)

antigens 1, 4, 5, 12 and flagellar (H) antigens *b* and *1,2*, either of which may be present since they are subject to the rapid mutation and back-mutation known as phase variation (Andrewes, 1922, 1925). The formula for *S. typhi* is 9, 12 (Vi):*d*:–, and the formula for *S. abortus-equi* is 4, 12:–:*e,n,x*; these species are monophasic in phase 1 and phase 2, respectively.

The strains examined came from several sources which are indicated by the letters preceding their numbers: e.g. NCTC (National Collection of Type Cultures, Colindale Avenue, London, N.W. 9); SW, SL, SR, SY, or LT (Guinness–Lister Unit, Lister Institute of Preventive Medicine, Chelsea Bridge Road, London, S.W. 1); some of these strains were described by Stocker *et al.* (1953) or by Lederberg & Edwards (1953); A (Dr E. S. Anderson, Central Enteric Reference Laboratory, Colindale Avenue, London, N.W. 9). Other strains were provided by Dr Joan Taylor (Salmonella Reference Laboratory, Colindale), and by Dr P. R. Edwards (Communicable Disease Center, Chamblee, Georgia, U.S.A.). One of the latter is mentioned individually preceded by the letter E.

Tests were made on as many non-motile strains as possible, some of which were non-flagellated while others were 'paralysed', i.e. they possessed inactive flagella of normal serological and morphological structure. Examination of stained preparations and electron microscopy of the paralysed strains used here revealed no differences from normal motile strains either in number or appearance of flagella.

Motile variants of some non-motile strains were sometimes provided; otherwise these were isolated when possible by selection of mutants in semi-solid medium or by transduction using phage P22 (Stocker *et al.* 1953) grown on a motile strain, either *Salmonella typhimurium* strain LT2 (1, 4, 5, 12:*i*:*1,2*), or *S. enteritidis* strain SL 431(1, 9, 12:*g,m*:–).

One strain, SJ 30, isolated as a spontaneous mutant from *Salmonella abortus-equi* NCTC 5727 by Dr Tetsua Iino, had 'curly' flagella (Leifson, 1951; Leifson & Hugh, 1953) which have the usual sinuous form in fixed preparations, but whose wavelength is less than normal (1·5 μ as compared with 2·6 μ in the case of SJ 30 and NCTC 5727; Dr Iino, personal communication). Strain SJ 30 showed no translational motility, but very vigorous jerking and rotation; it had also a very marked tendency to clump, as has been observed with other bacteria having flagella of this type. Motile back-mutants with 'normal' flagella lose this tendency to clump.

Synthetic strains with foreign H antigens introduced by transduction (Lederberg & Edwards, 1953; Lederberg & Iino, 1956). Most of these strains came from the Guinness–Lister Unit. The phase 1 antigens *l*,z_{13}, z_{10} and *z*, were introduced into *Salmonella typhi*, H 901, from *S. napoli* (1, 9, 12:*l*,z_{13}:*e,n,x*) NCTC 6853, *S. ituri* (1, 4, 12:z_{10}:*1,5*) NCTC 8275 and *S. shubra* (4, 5, 12:*z*:*1,2*) SL 652, respectively. The phase 2 antigen *1,7* was introduced into *S. abortus-equi* NCTC 5727 from *S. kaapstad* (4, 12:*e,h*:*1,7*) SL136. Phage P22 was used as transducing phage, and bacteria of the recipient strain with the new antigen were isolated by means of Edwards motility medium containing either anti-*d* or anti-*e,n,x* serum. In the course of unsuccessful attempts to introduce other phase 2 antigens into NCTC 5727, bacteria of this strain were isolated in phase 1, with antigen *a*, which is usually suppressed (Edwards & Bruner, 1939). Attempts to introduce other antigens (z_{29}; z_{38}; *k*; z_6; *1,6*; *y*) into these hosts failed, usually because no strains naturally carrying these antigens were found which were sensitive to phage P22.

Selection of Vi-negative variants of Salmonella typhi. A pool of Vi phages I–VII (kindly provided by Dr E. S. Anderson) was spotted on lawns of the Vi-positive strains of *Salmonella typhi.* Resistant bacteria selected by this phage pool have lost the Vi antigen (Dr E. S. Anderson, personal communication). Single colonies obtained by subcultivation from the area of lysis were isolated, and identified as Vi-negative when they were resistant to the phage pool.

Selection of monophasic populations from diphasic strains. Whenever possible, suitable colonies were identified by slide agglutination; when this failed the strains were passed through semi-solid medium containing H antiserum to the unwanted phase.

Bacterial counts. Viable counts were made by spreading 0·2 ml. samples from dilutions of culture on the surface of nutrient agar plates. Total counts were done in a Helber counting chamber.

The proportion of motile bacteria. This was estimated by dark-field microscopy of broth cultures, usually with an ordinary slide and coverslip. Repeated counts of the same suspension gave comparable results. When special accuracy was needed, a Helber chamber was used, the estimate being based on separate counts of the total number of bacteria and of the number of motile or non-motile organisms (whichever was in a minority). Motility was recorded either as 'translational' when the organisms progressed across the field, or as 'rotational' when they rotated or tumbled about one spot.

Sensitivity tests. A loopful of an overnight broth culture of the test strain was spread over 1/3 of a nutrient agar plate. Drops of 0·02 ml. of three dilutions of the χ phage were spotted on this lawn, the drops containing *c.* 5×10^8, *c.* 5×10^3 and *c.* 5×10^1 p.f.p., respectively. After overnight incubation, the degree of clearing by the most concentrated suspension was recorded as $+++$, $++$ or $+$, or $(+)$ where the clearing was so slight as to be seen only when the plate was viewed obliquely. The effects produced by the other dilutions were recorded as $+$, semi-confluent plaques, or as an approximate estimate of the number of plaques with a brief description of their appearance. *Salmonella abortus-equi* strain NCTC 5727 was always included as a control. The conditions for plaque formation appeared to be more critical with some strains than with others, and the tests had then to be repeated several times to obtain an unambiguous result. In a few tests with such strains with 0·6 % (w/v) agar, the strains did not appear to be more sensitive than on the higher concentration.

Almost all the motile strains were passed through motility medium before testing, and with all strains, the overnight cultures which provided the inocula for sensitivity tests were examined for motility: all the strains classified as motile showed more than 50 % of motile bacteria; almost all showed more than 80 %. The non-motile strains were tested for phage-sensitivity in parallel with their motile relatives. The naturally occurring motile strains were not routinely checked for antigenic structure in these tests; but the synthetic strains with foreign antigens and the motile derivatives of non-motile strains were always tested by slide agglutination with H antisera. The non-motile strains were also tested in this way to determine whether they were non-flagellated or paralysed.

Isolation of stable χ phage-resistant variants of sensitive strains. Twenty-six salmonella strains were chosen, and an attempt was made to isolate 6 independent

resistant variants from each by exposing 6 separate single colony isolates to the phage on agar. Resistant colonies from the patch of lysis were purified by 4 successive single colony isolations. Many isolates reverted to sensitivity during purification and were discarded. When none of 20 colonies from a lysed patch remained resistant, the original strain was grown in broth with the phage, but this was little more successful than culture on agar. Six resistant variants came from the collection of the Guinness–Lister Unit.

Determination of phage adsorption. (1) Measurement of unadsorbed phage. Cultures in the late exponential phase of growth were used when the viable count was about $2–5 \times 10^8$ organisms/ml. Except where the effect of multiplicity was specifically measured, the ratio of phage (p.f.p.) to bacteria was always less than 0·1. After 5, 10 or 20 min. at 37°, the mixtures or their dilutions were centrifuged and the supernatant fluids titrated for free phage. (2) Measurement of infected bacteria. In these experiments, the ratio of phage to bacteria was 0·05 or less. The mixtures were diluted into antiphage serum at a concentration which inactivated more than 99% of free phage in 2 min. After 5 min., they were further diluted and plated with *Salmonella abortus-equi* strain NCTC 5727.

Electron microscopy. (1) Fixed, platinum-iridium-shadowed preparations. The material consisted of phage bacterium mixtures containing 10–40 p.f.p./bacterium; after 0·5–15 min., formalin was added to 10%(v/v). After standing overnight at room temperature, the mixtures were thoroughly washed in distilled water. It was calculated that, with the highest concentration of phage used, the washing was sufficient to leave less than 1 unadsorbed phage particle for every 15 bacteria. The specimens for electron microscopy were prepared and the electron microscopy performed by Mrs H. Ozeki at the Chester Beatty Research Institute. Phage stocks alone and a few bacterial strains without phage were also examined in this way. (2) Preparations negatively-stained with phosphotungstic acid (Brenner *et al.* 1959) were kindly made and examined by Dr E. H. Mercer and Dr M. Birbeck at the Chester Beatty Research Institute, using unfixed material consisting of phage alone or freshly made mixtures of phage and bacteria.

Blending. An M.S.E. Blendor (Measuring and Scientific Equipment Ltd., Spenser Street, London, S.W. 1) was used to stir 10–15 ml. volumes in 25 ml. 'Universal' screw-capped containers. With more than 1·5–2 min. blending of a bacterial suspension, no further decrease in the proportion of motile bacteria was observed. Blending for 2 min. did not alter the titre of a phage stock..

Preparation of detached flagella. Large crops of bacteria were grown on trays of nutrient agar incubated overnight at 37°. The growth was washed off in distilled water with minimal rubbing of the bacteria against the agar surface. The suspension was next treated in the M.S.E. Blendor in 10 ml. volumes followed by the removal of most of the bacterial bodies by centrifugation at 1100 *g*. The supernatant fluid was then centrifuged in a Spinco model L ultra-centrifuge at 20,000 *g* for 1 hr. to deposit the flagella. This deposit was resuspended in 30 ml. distilled water and centrifuged at 1100 *g* and the resulting supernatant fluid again centrifuged at 20,000 *g*. The deposit of flagella finally obtained was resuspended in about 2 ml. distilled water to give a grey opalescent suspension. When any bacterial bodies were seen microscopically in this suspension, they were removed by further low speed centrifugation.

Antiphage sera. Rabbits were injected intravenously with phage stocks grown on *Salmonella abortus-equi* strain NCTC 5727 and having titres of *c.* 2×10^{10} p.f.p./ml. The antisera were absorbed with the propagating strain before use.

Antibacterial sera. In most cases, slide agglutination alone was done to determine the flagellar antigens of a strain. Most of these tests were made with antisera prepared by the Standards Laboratory, Central Public Health Laboratory, Colindale. Antisera for inclusion in semi-solid motility medium were prepared here by inoculating rabbits intravenously with overnight broth cultures sterilized by heating for 1 hr. at 56°; where possible, monophasic strains were used.

RESULTS

General characteristics of the χ phage

The plaques of the χ phage on *Salmonella abortus-equi* NCTC 5727 and on *S. typhi* SW 540 with optimal plating conditions were *c.* 1 mm. in diameter, punched-out in appearance and containing only a little bacterial growth, usually in the form of a granular film. The conditions for plating were rather critical; small changes might cause the plaques to become either large, smeary and filled with growth, or so small as to be scarcely visible and greatly decreased in number. No plaque-type variants were obtained; differences in plaque appearance which were observed were accidental and were not inherited. Plaques similar to those produced in an agar layer, but usually rather smaller, were produced when the phage was spotted on full-strength (1·25% w/v) agar plates spread with strains NCTC 5727 or SW 540. There was no indication that the χ phage could lysogenize.

Almost clear plaques were also seen on other strains of *Salmonella abortus-equi* and on most sensitive strains of *S. typhi*, and the patch of lysis produced by drops of concentrated phage was similarly covered with only a thin film of bacterial growth. On the sensitive strains of other species the plaques and patches were usually much less thoroughly cleared and were often extremely shallow. Broth cultures were never cleared, in agreement with the findings of Dr Boulgakov (personal communication to Dr B. A. D. Stocker) and of Rakieten & Bornstein (1941) with their flagellar phages.

In one-step growth experiments, using *Salmonella abortus-equi* NCTC 5727 as host with a low multiplicity of infection, the minimum latent period was 55–60 min. and after the curve flattened *c.* 90 min. after infection, the average burst size was *c.* 200. With a multiplicity greater than one, the minimum latent period was about the same, but the burst size could not be estimated because of bacterial clumping, which will be described later.

Lysates with titres of $2–5 \times 10^{11}$ p.f.p./ml. were readily obtained. Filtrates through membrane filters were almost without activity; only $2{\cdot}5 \times 10^{-8} – 5 \times 10^{-7}$ of the original number of p.f.p. were obtained. Filtrates through Seitz filters were similarly inactive; less than 2×10^{-7} p.f.p. were present. With sintered glass filters, the filtrates contained *c.* 5% of the original number of p.f.p. The phage stocks which were used were freed from living bacteria by heating at 56°–60° for 30 min., although with 30 min. at 60°, the p.f.p. were decreased to 35–50%, and with 1 hr. to 12–20% of the original values.

The χ phage is about fifteen times as resistant to ultraviolet radiation as is phage T2 (Adams, 1959).

The χ phage was a good antigen; neutralizing sera with K values of 3500 were easily obtained.

Particles of the χ phage are tadpole-shaped. Electron micrographs of preparations negatively-stained with phosphotungstic acid showed a head about 675 Å in diameter and a long tail about 2300 Å in length and 125 Å in width (Pl. 1, figs. 1, 2). The tail showed fine transverse striations like those of phage T2 (Brenner *et al.* 1959), and in some particles the head appeared hexagonal in shape. A few particles with collapsed heads were seen in untreated phage stocks, but many more of these were seen in preparations which had been heated at 56°–60° for 30 min. When the phage had been deposited by high-speed centrifugation (16,000 ***g*** for 90 min.), 99·9 % of the original p.f.p. were inactivated and the heads of almost all the particles appeared to be collapsed.

The χ phage evidently multiplies intracellularly like other phages, for electron micrographs of sections of infected bacteria in plaques, kindly made by Dr E. H. Mercer using the technique he developed for phage T2 (Mercer, 1959), showed dense phage-like structures like those seen with phage T2 and thought to be the DNA of immature phage heads (Kellenberger, Séchaud & Ryter, 1959; Mercer, 1959). Also, the sequence of events after infection as observed by fluorescence microscopy was not qualitatively different from that seen with other phages (Anderson, Armstrong & Niven, 1959; Dr E. S. Anderson, personal communication). Thus, there is no reason to think that the χ phage is an agent active only against the flagella, as suggested by Rakieten & Bornstein (1941), a theory which would, moreover, presuppose that this phage constituted a completely new kind of bacterial parasite.

The susceptibility of highly motile strains of naturally occurring serotypes

The χ phage was tested against 524 naturally occurring salmonella strains of various serotypes to investigate the possible influence of antigenic structure on its host-range. The strains tested were highly motile on the assumption, taken from previous authors, that sensitivity was correlated with the presence of flagella. The identities of the strains are listed in the footnote to Table 1, which shows the results of the tests. The appearance of the plaques, i.e. size and degree of clearing, varied considerably from strain to strain, but with most strains on which any plaques could be seen, the efficiency of plating (e.o.p.) was over 0·05. Strains on which the phage had a much lower e.o.p. were:

(*a*) Nine strains of *Salmonella typhi*, (9, 12: *d*:–) comprising all of 8 strains of Vi-phage type 32 tested and the only strain of type B2; the former were completely resistant while Vi-positive. Plaques on Vi-negative derivatives of these strains were not regularly observed; when present, they were minute and the e.o.p. was *c.* 10^{-4}.

(*b*) all of 4 sensitive strains of *S. paratyphi A* (1, 2, 12: *a*:–), A. A203, A. BA528, NCTC 13, and NCTC 9452, the e.o.p. being 10^{-4}.

(*c*) one strain of *S. paratyphi B* (1, 4, 5, 12: *b*:–) SW 543 S (e.o.p. 10^{-4}), and 2 strains of *S. typhimurium* (1, 4, 5, 12: *i*: *1,2*), SW 964 S and SL 653 (e.o.p. 10^{-6}).

Strains on which the undiluted phage stock produced only discrete plaques were: *S. ness-ziona* (6, 7:*l*,z_{13}:*1,5*), NCTC 8717; *S. shanghai* (16:*l,v*:*1,6*), SL 651; *S. kasenyi* (38:*e,h*:*1,5*), NCTC 8278, (the plaques were on a background of slight clearing of degree (+), and dilutions of the phage sometimes also produced a much larger number of very indistinct plaques on this strain); *S. thomasville* (3, 15, 34:*y*:*1,5*), NCTC 9896; *S. harrisonburg* (3, 15, 34:z_{10}:*1,6*), NCTC 8258. A few of the strains which were classed as slightly sensitive may really have fallen into this category, as there was sometimes a suggestion of plaques in the partially cleared patch. These did not always appear on repeated testing and attempts to isolate phage from them which would give more definite plaques on the strain failed.

These host-range tests showed the following results:

(1) The H antigens could determine the sensitivity of a strain, for all strains which carried antigen *g* were resistant, regardless of whether this antigen was present as *g,m*, *g,p*, *g,s,t*, etc., (one slightly sensitive strain with antigen *m,t* constituted a weak exception to the resistance of strains with antigens of the '*g* complex'). Most strains with *g*-related antigens are monophasic, but there are a few diphasic serotypes (Kauffmann & Henning, 1952; Douglas, Taylor & McMath, 1951). Three such diphasic serotypes were tested and were found to be predominantly in the *g* phase and resistant when first tested; but by passing them through motility medium with antiserum it was possible to isolate bacteria in the alternative phase. When these strains were tested with the χ phage, one (with antigen *e,n,x*) was fully sensitive, and the other two (with antigens *e,n,x* and *1,5*, respectively) gave a (+) reaction.

The χ phage was also tested on 20 strains of the Arizona group (Edwards & Ewing, 1955; Edwards, Fife & Ramsey, 1959) carrying various O and H antigens. Eleven strains were sensitive. Some Arizona H antigens are shared with *Salmonella*; in particular, H antigen *13* cross-reacts with salmonella H antigen *g* (Edwards & Ewing, 1955; Edwards *et al.* 1959). Four of five Arizona strains carrying antigen *13* which were tested were χ phage-resistant, and the fifth, NCTC 7318, (O antigen 9:H antigen *13*, *15*) showed a few discrete plaques with the undiluted phage.

Strains carrying most of the other salmonella H antigens might be either sensitive or resistant, showing that susceptibility is not governed solely by H antigenic type.

(2) The somatic antigen probably did not determine sensitivity, for strains belonging to many different O Groups, as well as rough strains which had lost their O antigen (e.g. *Salmonella typhi* SW 540), could be sensitive. None of the strains belonging to O Groups E3, E4 or G which were tested were fully sensitive. Undiluted phage produced only discrete plaques on *S. harrisonburg* NCTC 8258 and *S. thomasville* NCTC 9896, belonging to Group E3. The results with some other somatic antigens do not carry much weight since so few strains were tested.

(3) The most sensitive species were *Salmonella typhi* and *S. abortus-equi*, both in the completeness of clearing in individual plaques and in patches produced by concentrated phage, and also in the proportion of strains found to be sensitive. One hundred and seventeen strains of *S. typhi* were tested, including 104 Vi-positive strains from 67 different Vi-phage types (Craigie & Yen, 1938), and 13 Vi-negative strains. Of 104 Vi-positive strains 82 fell into the fully sensitive category, but only 67 showed maximum clearing (+++), leaving 37 strains showing various degrees of resistance. Of these 37 strains, 21 owed their resistance either to the presence of the Vi antigen, as found by previous authors, or to poor motility. When the sensitivity

of the 117 strains was assessed, taking into account the reaction of these 37 strains either after loss of the Vi antigen or after passage through motility medium, only 17 of the 117 tested were not fully sensitive, with + + + clearing by undiluted phage.

Effect of Vi-phage type. The Vi-phage type of a strain was correlated with its reaction to the χ phage since: (*a*) 3/3 strains of Vi-phage type D4 were totally resistant; (*b*) the 5 strains giving a (+) reaction comprised 4/4 strains of type M (2 of type M1 and 1 each of types M2 and M3) and 1/1 strain of type 37; (*c*) the 9 strains showing plaques only irregularly and with low e.o.p. comprised 8/8 strains of type 32 and 1/1 strain of type B2.

Vi-phage typing depends on specific modifications in the host-range of Vi phage II (Anderson & Felix, 1953), but of the 67 types, only types D4 and M are resistant to all the *Salmonella typhi* Vi phages (I–VII) except phage III (Dr E. S. Anderson, personal communication). A correlation between χ phage resistance and Vi-phage type, especially if the type is rarely isolated and is restricted to a particular locality, might have no more significance than repeated tests on the same strain, but type D1 (4/4 strains), which is closely related to type D4, was fully sensitive to the χ phage. Clones of type D1 frequently arise in cultures of type D4, and such clones are sensitive to the χ phage while the parent culture remains resistant (Dr E. S. Anderson, personal communication). The change from type D4 to type D1 is not associated with loss of one of the recognized type-determining phages; types D4 and D1 carry the same type-determining phage, and type A (which is sensitive to all the adapted preparations of Vi phage II) remained fully sensitive to the χ phage after lysogenization with this phage isolated from either a type D1 strain or a type D4 strain. Thus the resistance of type D4 to the χ phage is not due to lysogenicity with a type-determining phage. One strain of type D4 absorbed the χ phage rapidly; therefore resistance in this strain at least was not due to failure of attachment.

The susceptibility of highly motile strains with artificial serotypes

The relation between χ phage sensitivity and H antigenic type was further examined by testing sets of artificial derivatives with differing H antigens prepared from single parental strains by transduction. The results in Table 2 allow the following conclusions.

(1) When a strain was originally sensitive, it kept its sensitivity with most of the foreign H antigens. The degree of clearing produced by the undiluted phage stock and the appearance of individual plaques were characteristic of the strain, and not of the H antigen it carried. Occasionally, a change of H antigen brought with it a small change in e.o.p. (*c.* five-fold) but without extensive tests it is impossible to be sure that this difference was greater than would have been found in tests on different clones of the same strain.

The low e.o.p. (*c.* 10^{-4}) of the phage on strains of *Salmonella paratyphi A* was characteristic of the bacterium and not of the H antigen *a*, for the phage still plated with an e.o.p. of 10^{-4} on derivatives of *S. paratyphi A*, strain A. 17689, in which antigens *i* or *r* had been substituted for *a*. SL 508 (*S. typhi* SY 79 with antigen *a* transduced from *S. abortus-equi* NCTC 5727) was fully sensitive, as was NCTC 5727 itself with antigen *a* in phase 1 which is normally suppressed (Edwards & Bruner, 1939).

Table 2. *Sensitivity of synthetic* Salmonella *strains with various flagellar antigens*

Strain	Antigen	Sensitivity	Donor of antigen	Sensitivity of donor*
S. typhi				
SY 79	*d* : –	+	.	.
Derivatives				
SW 520	*i* : –	+	*S. typhimurium* LT 7	+
SL 502	*b* : –	+	*S. abony* SW 803	–
SL 504	*r* : –	+	*S. heidelberg* SL 142	+
SL 505	*c* : –	+	*S. altendorf* SL 137	–
SL 508	*a* : –	+	*S. abortus-equi* NCTC 5727	+
SL 509	*1, 2* : –	+	*S. paratyphi B* SW 546†	+
SL 506	*e, h* : –	e.o.p. 10^{-6}‡	*S. chester* SL 139	–
SL 507	*e, h* : –	e.o.p. 10^{-6}‡	*S. kaapstad* SL 136	–
SL 503	*g, p* : –	–	*S. dublin* SW 553 (O)	–
SL 510	*g, m, s* : –	–	*S. hato* NCTC 9899	–
SL 511	*g, m* : –	–	*S. enteriditis* NCTC 4196	–
S. *typhi*				
SW 537 (H901)	*d* : –	+	.	.
Derivatives				
SW 569	*i* : –	+	*S. typhimurium* LT 2	+
SW 902	*c* : –	+	*S. altendorf* SW 825	–
SW 537.*z*	*z* : –	+	*S. shubra* SL 652	(+)
SW 537.z_{10}	z_{10} : –	+	*S. ituri* NCTC 8275	(+)
SW 668	*e, h* : –	e.o.p. 10^{-6}‡	*S. sandiego* SW 718	.
SW 537.*l*, z_{13}	*l*, z_{13} : –	e.o.p. 10^{-7}	*S. napoli* NCTC 6853	–
SW 667	*g, p* : –	–	*S. dublin* SW 553 (O)	–
S. abortus-equi				
NCTC 5727	– : *e, n, x*	+	.	.
Derivative				
NCTC 5727.*1, 7*	– : *1, 7*	+	*S. kaapstad* SL 136	–
S. paratyphi A				
A 17689	O strain	(–)	.	.
Derivatives				
SL 31	*i* : –	e.o.p. 10^{-4}	*S. typhimurium* LT 2	+
SL 37	*r* : –	e.o.p. 10^{-4}	*S. heidelberg* SL 28 (O)	+
S. paratyphi B (monophasic)				
SW 543	O strain	(–)	.	.
Derivatives				
SW 543 swarm	*b* : –	e.o.p. 10^{-4}	Self	.
SW 623	*i* : –	e.o.p. low	*S. typhimurium* LT 2	+
SW 940	*a* : –	e.o.p. low	*S. sendai* SW 771	.
SL 117	*r* : –	e.o.p. low	*S. heidelberg* SL 142	+
SW 633	*1, 2* : –	e.o.p. low	*S. paratyphi B* SW 546†	+
SL 163	*c* : –	e.o.p. low	*S. altendorf* SL 137	–
SL 159	*e, h* : –	–	*S. chester* SL 139	–
SL 165	*e, h* : –	–	*S. kaapstad* SL 136	–
SL 116	*g, p* : –	–	*S. dublin* SW 553 (O)	–
SW 679	*g, m* : –	–	*S. enteritidis* SW 764	–
S. typhimurium				
LT 2	*i* : *1, 2*	+	.	.
Derivatives				
SW 698	*i* : *e, n, x*	+	*S. abony* SW 803	–
SW 699	*b* : *1, 2*	+	*S. abony* SW 803	–
SL 141	*b* : *e, n, x*	+	*S. abony* SW 803 (two exposures)	–

Table 2 (*cont.*)

Strain	Antigen	Sensitivity	Donor of antigen	Sensitivity of donor*
S. typhimurium				
LL 22	*i* : *1, 2*	+	.	.
Derivatives				
SW 674	*g, p* : *1, 2*	.	*S. dublin* SW 553 (O)	−
Phase 1	*g, p*	−	.	.
Phase 2	*1, 2*	+	.	.
S. heidelberg				
SL 28	O strain	(−)	.	.
Derivatives				
SL 142	*r* : *1, 2*	+	Self	.
SL 118	*i* : *1, 2*	+	*S. typhimurium* SL 55	−
SL 121	*b* : *1, 2*	+	*S. paratyphi B* SW 609	(+)
SL 119	*g, p* : *1, 2*	.	*S. dublin* SW 553 (O)	−
Phase 1	*g, p*	−	.	.
Phase 2	*1, 2*	+	.	.
S. dublin				
SW 553	O strain	(−)	.	.
Derivatives				
SL 149	*g, p* : −	−	Self	.
SL 120	*b* : −	(+)	*S. paratyphi B* SW 543 (O)	e.o.p. 10^{-4}
SL 122	*r* : −	(+)	*S. heidelberg* SL 28 (O)	+
S. moscow				
NCTC 5768	*g, p* : −	−	.	.
NCTC 5768.*i*	*i* : −	+	*S. typhimurium* SL 375	+
S. essen				
NCTC 5723	*g, m* : −	−	.	.
NCTC 5723.*i*	*i* : −	+	*S. typhimurium* LT 2	+
S. hato				
NCTC 9899	*g, m, s* : −	−	.	.
SL 512.*i*	*i* : −	(+)	*S. typhimurium* SL 375	+
S. enteritidis				
SL 513	*g, m* : −	−	.	.
SL 513.*i*	*i* : −	(+)	*S. typhimurium* SL 375	+
S. enteritidis				
SL 433	*g, m* : −	−	.	.
SL 433.*i*	*i* : −	(+)	*S. typhimurium* LT 2	+
S. enteritidis				
SL 431	*g, m* : −	−	.	.
SL 431.*i*	*i* : −	−	*S. typhimurium* SL 375	+
S. enteritidis				
SL 432	*g, m* : −	−	.	.
SL 432.*i*	*i* : −	−	*S. typhimurium* SL 375	+

* Some donors were themselves nonmotile: this is indicated by (O) after the name of the strain and the sensitivity given is that of motile variants of the strain.

† SW 546 is a monophasic strain with *1, 2* as its phase 1 antigen (Lederberg & Edwards, 1953).

‡ Clear plaques (see text).

(2) The introduction of H antigen *g*... into a sensitive strain such as *Salmonella typhi*, SY 79 or SW 537, or the monophasic *S. paratyphi B*, SW 543, made the strain totally resistant. When antigen *g,p* replaced the natural phase 1 antigens *i* or *r* in the sensitive diphasic strains *S. typhimurium*, LT 22, or *S. heidelberg*, SL 28, respectively, the strains became resistant in this phase, while remaining sensitive in phase 2 (with antigen *1,2*). Six out of eight monophasic strains which naturally carried antigen *g*... and were resistant to the phage, became sensitive, or slightly sensitive, when another H antigen was substituted. Thus the resistance of some, although not all, natural strains carrying antigen *g*... is due to the presence of this antigen.

When antigen l,z_{13} was introduced into *Salmonella typhi* SW 537, concentrated phage produced only a small number of discrete plaques. When antigen *e,h* was introduced into *S. typhi* SY 79 or SW 537, the phage produced a small number of clear plaques with a much larger number of very indistinct plaques. The ratio of the former to the number of plaques produced on NCTC 5727 was about 10^{-6}, and that of the latter was about 10^{-3}–10^{-4}. When antigen *e,h* was introduced into the less sensitive *S. paratyphi B* SW 543, it made it totally resistant.

(3) A suitable H antigen was not the only requirement for sensitivity, for the reaction of the strain from which the antigen was transduced did not influence the reaction of the recipient, except where certain antigens, such as *g*..., l,z_{13}, or *e,h* were concerned.

Host-range variants

Several instances have been given where ordinary stocks of the χ phage had a very low e.o.p. on certain strains (e.g. 10^{-4}–10^{-7}) so that high concentrations of phage produced only relatively few plaques. The phage present in these plaques plated on the test strain concerned with almost maximum efficiency (e.o.p. 0·1–1). When the variants were again grown on *Salmonella abortus-equi* NCTC 5727, their behaviour divided them into two groups.

(1) The first group was that in which the e.o.p. on the test strain had again fallen to about the original value, so that the variant stocks had presumably shown only phenotypic variation and did not consist of mutants. The test strains concerned were:

Salmonella paratyphi A strains A 203; A. BA 528; NCTC 9542; SL 31 (with antigen *i* substituted for *a*); and SL 37 (with antigen *r* substituted for *a*); *S. paratyphi B* strain SW 543S; *S. harrisonburg* strain NCTC 8258.

(2) The second group consisted of those variants which were evidently host-range mutants since their stocks grown on *Salmonella abortus-equi* NCTC 5727 retained a high e.o.p. on the test strain. The bacterial strains concerned here were:

SW 537.*l*, z_{13} (*S. typhi* H 901 in which H antigen l,z_{13} had been substituted for *d*);

NCTC 8717 (*S. ness-ziona* 6, 7 : l,z_{13} : *1,5*). This strain was found to have been in phase 1 (with antigen l,z_{13}) when it was tested and the phage mutant was selected;

SL 651 (*S. shanghai* 16 : *l,v* : *1,6*). This strain was found to have been in phase 1 (with antigen *l,v*) when it was tested and the phage mutant was selected;

SL 507	(*S. typhi* SY 79 in which H antigen *e,h* had been substituted for *d*);
NCTC 8278	(*S. kasenyi* 38 : *e,h* : *1,5*);
NCTC 7318	(Arizona O antigen 9 : H antigen *13, 15*);
NCTC 9896	(*S. thomasville* 3, 15, 34 : *y* : *1,5*);
SW 964S	(*S. typhimurium*, the motile variant of a strain, SW 964, which was received in the O state);
SL 653	(*S. typhimurium* C 56 of Boyd, which carries phage A2*d*).

In tests with a limited number of strains, the mutants showed no loss of activity for strains sensitive to the wild-type phage; thus their host-range was enlarged rather than diminished. Their plaques on *S. abortus-equi* NCTC 5727 and other strains sensitive to the wild-type χ phage were similar in appearance to the plaques of the wild type phage.

Table 3. *Activities of the host-range mutants against strains on which mutants were isolated*

		e.o.p. mutant of χ selected on strains					
Strain	e.o.p. χ	537. l,z_{13}	8717	651	507*	8278*	7318
SW 537.l,z_{13}	10^{-6}–10^{-8}	0·5–1	0·5–1	0·5–1	$\leqslant\chi$	$\leqslant\chi$	$\leqslant\chi$
NCTC 8717, phase with l,z_{13}	10^{-6}–10^{-7}	0·5–1	0·5–1	0·5–1	$\leqslant\chi$	$\leqslant\chi$	$\leqslant\chi$
SL 651, phase with *l,v*	10^{-6}–10^{-7}	0·5–1	0·5–1	0·5–1	$\leqslant\chi$	$\leqslant\chi$	$\leqslant\chi$
SL 507 (*e,h*) (or SL 506, or SW 668)	10^{-6}*	$\leqslant\chi$	$\leqslant\chi$	$\leqslant\chi$	0·5	0·5–1	$\leqslant\chi$
NCTC 8278, phase with *e,h*	10^{-6}–10^{-7}	$\leqslant\chi$	$\leqslant\chi$	$\leqslant\chi$	0·01	0·2–0·7	$\leqslant\chi$
NCTC 7318 (*13, 15*)	10^{-7}–10^{-8}	$\leqslant\chi$	$\leqslant\chi$	$\leqslant\chi$	$\leqslant\chi$	$\leqslant\chi$	0·1–0·2

The phages were titrated by the modified Miles & Misra method, using preparations which had been grown on NCTC 5727 and whose titres on this strain were 10^{10}–10^{11}/ml. In some cases, a preparation grown on the strain which had selected the mutant was also tested, with similar results.

* Distinct, clear plaques (see text).

$\leqslant\chi$ the e.o.p. was no greater than that of wild-type χ phage.

Strains carrying H antigen l. The results of titrating the first six mutants on the bacterial strains by which each was isolated are summarized in Table 3. The results with the first three suggested that these strains had each selected a phage mutant which had gained the ability to attack bacteria carrying the H antigenic component *l*. Accordingly, strains with H antigen l,z_{13}, *l,v* or *l,w*, which had already been tested in the general host-range tests, were retested after separation into their two phases. Strains NCTC 8717 and SL 651, and all the strains which had appeared as fully sensitive were retested, together with most of the strains which had given a (+) reaction and a few of those which had appeared as resistant. Cultures in each phase were tested with the wild-type phage, and cultures, in the phase exhibiting antigen *l*... were tested with the mutant isolated from SW 537.l,z_{13} and grown on NCTC 5727; the results are shown in Table 4, Section A. A few strains were also tested with the mutants isolated from NCTC 8717 or SL 651, with similar results. The results supported the conclusion that the wild-type phage was unable to attack bacteria with H antigen *l*..., but could mutate to a form able to do so.

Strains carrying H antigen e,h. When the wild-type χ phage was plated with strains SL 506, SL 507 or SW 668 (*Salmonella typhi* in which antigen *e,h* had been substituted for *d*), very small indistinct plaques could occasionally be seen with the higher phage dilutions, the e.o.p. being about 10^{-3}. The lower dilutions produced partial clearing in which there were discrete clear plaques; the ratio of these to the total number of plaques which the preparation produced on NCTC 5727 (or on the same strains of *S. typhi* carrying antigen *d*) varied between 10^{-6} and 10^{-7}. The phage isolated from these plaques, or from plaques similarly produced on strain NCTC 8278 (*S. kasenyi* 38 : *e,h* : *1,5*) by the lower phage dilutions, gave clear plaques with high e.o.p. on the three *S. typhi* derivatives with antigen *e,h*, and also plated with high efficiency on strain NCTC 8278. Table 4, Section B shows the results when NCTC 8278 and 4 other strains with antigen *e,h* were tested in each phase with wild-type χ phage, and in the phase exhibiting *e,h* with the phage mutants isolated from SL 507 and NCTC 8278: these appear to be mutants which have gained activity for bacteria with H antigen *e,h*. The two mutants were not identical, for the one from NCTC 8278 plated with higher efficiency on these strains than the one from SL 507.

Table 4. *Sensitivity tests*

A. *Tests on naturally occurring strains carrying H antigens* l,v, l,w, *or* l,z_{13} *in one phase*

	No. of strains	Reaction to χ: Cultures in phase with antigen *l*...	Reaction to χ: Cultures in other phase	Reaction of cultures in phase with *l*... to mutant selected by SW 537. l,z_{13}
Strains with l,z_{13} as one antigen	1	Plaques	+	+
	1	–	(+)	+
	3	–	(+)	(+)
	2	–	–	–
Strains with *l,v* as one antigen	2	Plaques	+	+
	2	(+)	+	+
	4	–	+	+
	2	–	(+)	(+)
	3	–	–	–
Strains with *l,w* as one antigen	1	Plaques	+	+
	6	–	+	+
	2	–	–	–

B. *Tests on naturally occurring strains carrying H antigen* e,h *in one phase*

	No. of strains	Reaction to χ: Cultures in phase with antigen *e,h*	Reaction to χ: Cultures in other phase	Reaction of cultures in phase with *e,h* to mutants selected by NCTC 8278 or SL 507
Strains with *e,h* as one antigen	1	Plaques	+	+
	2	–	+	+
	2	–	(+)	(+)

Arizona strain NCTC 7318. Arizona NCTC 7318 has H antigen *13, 15*; since Arizona H antigen *13* closely resembles Salmonella H antigen *g* (Edwards & Ewing, 1955; Edwards *et al.* 1959), it seems likely that NCTC 7318 is resistant because it carries H antigen *13*, and that the mutant isolated on it is one which has gained activity for bacteria with this antigen. No other strains with antigen *13, 15* were available; but, on 2 of the 4 Arizona strains with antigen *13, 14* which were resistant to the wild-type phage, the mutant from NCTC 7318 had some activity in that concentrated phage gave some degree of clearing, although the dilutions produced no plaques. A number of salmonella strains with antigen *g*... were tested with the mutant from NCTC 7318 but all were resistant; these included the artificial strains SL 119 and SW 674 in the phase exhibiting antigen *g,p* (which were sensitive in the other phase), and strains NCTC 5723 and NCTC 5768 (which became sensitive when antigen *i* was substituted for *g*...). Thus, if the mutant isolated on NCTC 7318 is one which has gained activity for bacteria with Arizona antigen *13*, it can evidently distinguish between this antigen and the serologically related Salmonella antigen *g*. None of the other host-range mutants attacked bacteria with H antigen *g*.

Table 5. *Association between H antigenic type and reaction to the χ phage*

Antigen	Associated with	Evidence	
		Natural strains	Synthetic strains
a	Sensitivity	*S. abortus-equi* NCTC 5727 sensitive in phase 1 4 *S. paratyphi A* strains sensitive	*S. typhi* SL 508 sensitive
b	Sensitivity	3 strains *S. paratyphi B* sensitive in phase 1	*S. typhi* SL 502 sensitive *S. typhimurium* SW 699 sensitive in phase 1
c	Sensitivity	3 diphasic strains sensitive in phase 1	*S. typhi* SL 505 and SW 902 sensitive
d	Sensitivity	Most *S. typhi* strains sensitive	.
i	Sensitivity	*S. typhimurium* 1 monophasic strain and 2 diphasic strains sensitive in phase 1	*S. typhi* SW 520 and SW 569 sensitive
r	Sensitivity	2 diphasic strains sensitive in phase 1	*S. typhi* SL 504 sensitive
z	Sensitivity	2 diphasic strains sensitive in phase 1	*S. typhi* SW 537.*z* sensitive
z_{10}	Sensitivity	2 diphasic strains sensitive in phase 1	*S. typhi* SW 537.z_{10} sensitive
z_{36}	Sensitivity	1 monophasic strain sensitive	.
z_4, z_{23}	Sensitivity	5 monophasic strains sensitive	.
z_4, z_{24}	Sensitivity	2 monophasic strains sensitive	.
e,n,x	Sensitivity	4 *S. abortus-equi* strains sensitive	.

Table 5 (*cont.*)

Antigen	Associated with	Evidence: Natural strains	Evidence: Synthetic strains
1,2	Sensitivity	5 monophasic strains sensitive	*S. typhi* SL 509 sensitive
1,5	Sensitivity	2 monophasic strains sensitive	.
1,7	Sensitivity	1 diphasic strain sensitive in phase 2	*S. abortus-equi* NCTC 5727. *1,7* sensitive
z_6	Probably sensitivity	4 diphasic strains sensitive in phase 2	.
e,n,z_{15}	Probably sensitivity	3 diphasic strains sensitive in phase 2	.
1,6	Probably sensitivity	2 diphasic strains sensitive in phase 2	.
k	Probably sensitivity	2 diphasic strains sensitive in phase 1	.
l,z_{13}	Sensitivity to specific *l*... variant	See Tables 3 and 4	.
l,v	Sensitivity to specific *l*... variant		
l,w	Sensitivity to specific *l*... variant		
e,h	Sensitivity to specific *e,h* variant		
y	Sensitivity to variant	3/4 diphasic strains sensitive to variant in each phase, but sensitive to wild-type χ only when not exhibiting *y*	.
z_{29}	.	2/9 monophasic strains (+) reaction	.
z_{38}	.	2/4 monophasic strains (+) reaction	.
g...	Resistance	0/50 monophasic strains sensitive. (1 slightly sensitive strain with *m, t*) 3 diphasic strains in *g*...phase while sensitive in alternative phase	*S. typhi* SL 503, SL 510, SL 511, SW 667 resistant. SW 674, SL 119 resistant in phase with *g*... and sensitive in alternative phase. When *i* was substituted for *g*... naturally present, 6/8 strains became sensitive

The implications of the results with the last three strains are not clear. In the case of NCTC 9896 (*Salmonella thomasville* 3, 15, 34 : *y* : *1,5*), the H antigen appeared to be concerned, for this strain reacted differently in each phase, and the host-range of a phage mutant which it selected included (among other bacteria not sensitive to the wild-type phage) bacteria with H antigen *y*. When four strains with antigen *y* were tested, none was sensitive to the wild-type phage in the phase which exhibited it, but three were fully sensitive in the other phase; these three strains in the phase with *y* were sensitive to the mutant selected by NCTC 9896.

Strains SL 653 and SW 964S carry H antigens *i* : *1,2* which are associated with

sensitivity to the wild-type phage in other strains; cultures in each phase reacted in the same way, and the phage mutant which each strain selected had gained activity only against that strain itself.

Table 5 summarizes the results of testing the different H antigens. The antigens which occur naturally only in diphasic serotypes (those of Section 3 of Table 1) were tested in so far as possible after transduction to fully sensitive strains of *Salmonella typhi* (monophasic in phase 1), or *S. abortus-equi* (monophasic in phase 2). They were also tested by selecting predominantly monophasic clones from diphasic strains.

Association of sensitivity and motility

The association of sensitivity to the χ phage with the presence of flagella was investigated with (1) naturally occurring non-motile strains and with (2) phage-resistant variants selected from sensitive populations by the χ phage.

Naturally occurring non-motile strains. These comprised 62 strains of various serotypes. Motile derivatives of 45 of them were available and consisted either of spontaneous mutants or of derivatives obtained by transduction. The results are given in Table 6. All the non-motile strains were phage resistant and most of the motile derivatives were sensitive, with the consistent exception of strains which turned out to have H antigen *g*.... It will be noticed that the non-flagellated strains were not the only resistant ones; all of 10 paralysed strains were also resistant. The motile derivatives of these paralysed strains were sensitive. Table 6 therefore reveals a further condition determining sensitivity to the χ phage, namely, that the flagella must be active. This is well illustrated with the O strain of *Salmonella typhimurium*, SW 573, where mutation leading to flagella production (SL 43) was not enough for sensitivity; this only appeared when with further mutation the strain became motile (SL 43 swarm).

SJ 30, the 'curly' mutant of NCTC 5727 with short wavelength flagella, showed as marked clearing and as many plaques as NCTC 5727 itself. This variant was not truly paralysed for, although it did not exhibit translational motility, it did show very active jerking movement.

Experiments with Salmonella abortus-equi. The number of flagella formed by freshly isolated strains of this species, and hence their motility, depend on the pH of the medium; many flagella are formed at pH 6·5 and few at pH 8 (Kato, 1954). Three freshly isolated strains obtained by Dr B. A. D. Stocker from Dr Kato were tested and this observation was confirmed. Also, the strains were found to be sensitive to the χ phage on agar of pH 6·5 and resistant on agar of pH 8·0. Variants of these strains obtained by passage through semi-solid motility medium of pH 7·4 were as sensitive at pH 8·0 as at the lower pH.

Salmonella typhi: *effect of the Vi antigen*

Previous authors noted that Vi-positive strains showed weaker motility and lower sensitivity to the χ phage than Vi-negative strains. Broth cultures of all the Vi-positive strains used here contained over 50% motile bacteria and thus none was carrying sufficient Vi antigen to interfere seriously with its motility in broth. Nevertheless, 37 of 104 Vi-positive strains did not show maximum sensitivity to the χ phage, and in 21 of these, loss of the Vi antigen resulted in an increase of sensitivity.

Table 6. *Action of the χ phage on non-motile* Salmonella *strains and their motile derivatives*

	Nonmotile strain		Motile derivative*		
Strain	O or paralysed	Sensitivity	Strain	Antigen	Sensitivity
S. typhi					
SL 100	O	–	SL 100 S	*d* : –	+
SL 101	O	–	.	.	.
SL 102	O	–	.	.	.
SL 77 (0901)	O	–	SL 77 S	*d* : –	+
SL 436	O	–	SL 436 T	*d* : –	+
A C 6 6225	O	–	.	.	.
A T 820	O	–	A T 820 T	*d* : –	+
SL 232	Par. *d*	–	SL 232 S	*d* : –	+
S. enteritidis					
SW 971	O	–	SW 971 T	*g*, *m* : –	–
NCTC 203	O	–	.	.	.
NCTC 3045	O	–	.	.	.
NCTC 6676	O	–	NCTC 6676 S	*g*, *m* : –	–
S. dublin					
SW 553	O	–	SL 146 (S)	*g*, *p* : –	–
SL 76	O	–	NCTC 5766 (S)	*g*, *p* : –	–
SL 435	O	–	SL 435 T	*g*, *p* : –	–
Group D, unknown sp.					
SW 970	O	–	.	.	.
SW 972	O	–	.	.	.
S. paratyphi B					
SW 543	O	–	SW 543 S	*b* : –	low e.o.p.
SW 908	O	–	SW 908 S	*b* : *1*, *2*	+
SW 966	O	–	SW 966 T	*b* : *1*, *2*	(+)
SL 51	O	–	SL 51 T	*b* : *1*, *2*	+
A U 2	O	–	.	.	.
A U 15	O	–	A U 15 B (T)	*b* : *1*, *2*	+
A U 26	O	–	A U 26 M (T)	*b* : *1*, *2*	+
SR 107	O	–	SR 107 T	*b* : (*1*, *2*)	(+)
SL 368	Par. *b* : *1*, *2*	–	SL 368 T	*b* : (*1*, *2*)	(+)
SW 906	Par. *b* : *1*, *2*	–	SW 906 S	*b* : *1*, *2*	+
S. typhimurium					
SW 541	O	–	SW 541 T	*i* : *1*, *2*	–
SW 544	O	–	SW 544 S	*i* : *1*, *2*	+
SW 545	O	–	SL 89 (S)	*i* : *1*, *2*	+
SW 549	O	–	SW 594 (S)	*i* : *1*, *2*	+
SW 964	O	–	SW 964 S	*i* : (*1*, *2*)	+
SW 965	O	–	SW 965 S	*i* : (*1*, *2*)	+
SL 15	O	–	SL 15 T	*i* : (*1*, *2*)	+
A U 14	O	–	A U 14B (T)	*i* : *1*, *2*	+
A U 20	O	–	A U 20 M (T)	*i* : *1*, *2*	+
SL 56	O	–	SL 61 (S)	*i* : *1*, *2*	–
SW 573†	O	–	.	.	.
SL 43†	Paralysed from SW 573	–	SL 43 S	*i* : *1*, *2*	+
SL 499	Par. *i* : *1*, *2*	–	SL 499 S	*i* : *1*, *2*	+
SW 1153	Par. *i* : *1*, *2*	–	SW 1153 S	*i* : *1*, *2*	+
SW 578	Par. *i* : *1*, *2*	–	SW 582 (S)	*i* : *1*, *2*	+
SW 580	Par. *i* : *1*, *2*	–	SW 583 (S)	*i* : *1*, *2*	+
SJ 60	Par. *i* : *1*, *2*	–	SJ 60 S	*i* : *1*, *2*	+

Table 6 (*cont.*)

Strain	Nonmotile strain: O or paralysed	Nonmotile strain: Sensitivity	Motile derivative*: Strain	Motile derivative*: Antigen	Motile derivative*: Sensitivity
S. heidelberg					
AU 1	O	–	AU 1 T	*r* : *1, 2*	(+)
AU 21	O	–	AU 21 M (T)	*r* : *1, 2*	–
SL 28	O	–	SL 142 (S)	*r* : *1, 2*	+
S. paratyphi A					
A 17689	O	–	.	.	.
SL 14	O	–	.	.	.
NCTC 13	O	–	NCTC 13 S	*a* : –	low e.o.p.
NCTC 8052	O	–	.	.	.
NCTC 8285	O	–	.	.	.
NCTC 8388	O	–	.	.	.
NCTC 8389	O	–	.	.	.
S. paratyphi C					
SL 236	O	–	SL 236 S	*c* : *1, 5*	+
SL 437	O	–	SL 437 S	*c* : *1, 5*	+
S. cholerae-suis					
NCTC 5735	O	–	NCTC 5735 S	*c* : *1, 5*	+
Group C1, unknown sp.					
AU 23	O	–	.	.	.
Group C2, unknown sp.					
AU 25	O	–	.	.	.
S. riogrande					
NCTC 7399.3	Par. *b* : *1, 5*	–	NCTC 7399.3	*b* : (*1, 5*)	+
S. milwaukee					
NCTC 9890	O	–	NCTC 9890 S	*f, g* : –	–
S. abortus-equi					
SJ 30	'Curly' No translational motility	+	SJ 30 S	– : *e, n, x*	+
SL 220‡	.	–	SL 223 (S)	– : *e, n, x*	+
SL 221‡	.	–	SL 224 (S)	– : *e, n, x*	+
SL 222‡	.	–	SL 225 (S)	– : *e, n, x*	+

* Motile derivatives are designated S or T depending on whether they were obtained as spontaneous mutants or by transduction.

Antigens given in brackets were probably present in the strain although not detected in the culture tested.

† SL 43 was isolated from the non-flagellated *S. typhimurium* strain SW 573 as a flagellated but non-motile (paralysed) mutant (Stocker, Zinder & Lederberg, 1953).

‡ SL 220, SL 221, and SL 222 were strains of *S. abortus-equi* from Dr Kato described in the text. They produced few flagella and only a small proportion of the bacteria were motile in media of pH 7·2.

Resistant variants selected by the χ phage. Most of the strains found to be sensitive in the host-range tests were grown overnight in broth with the χ phage and examined microscopically the next day to estimate the proportion of motile bacteria. The strains fell broadly into four classes as shown in Table 7: class 1, 0–0·1% motile; class 2, 5–10% motile; class 3, 30–50% motile (over 80% of bacteria in control cultures were motile) and class 4 showing no decrease in the percentage motile.

Most strains fell into class 1. The three species *Salmonella typhi*, *S. paratyphi B* and *S. typhimurium*, of which a large number of strains were tested, behaved

differently in that most of the *S. typhi* and *S. paratyphi B* strains were in class 1 while the *S. typhimurium* strains were more evenly distributed amongst classes 1–4. Three of the strains of *S. typhimurium* tested more than once gave different results on different occasions, while the strains of the other two species gave consistent results in repeated tests.

Table 7. *Reduction of motility in overnight broth cultures infected with the χ phage*

Species	Number of strains in motility class *			
	1	2	3	4
S. typhi	79	5	3	0
S. paratyphi B	37	0	1	0
S. typhimurium	12 (+3)	12	14 (+3)	9
S. paratyphi A	5	0	0	0
Others	35 (+2)	11	4 (+1)	6 (+1)

* See text.

It was difficult to isolate stable phage-resistant mutants from most strains. The present experiments confirmed the finding of Rakieten & Bornstein (1941) that cultures which remained non-motile so long as phage was present could give rise to subcultures which regained motility when freed from the phage. This could be explained if all the genotypically motile bacteria present did not actually exhibit motility, and if phenotypic nonmotility was enough to make them resistant to the phage. Genotypically homogeneous salmonella strains have been studied in which not every bacterium is motile, but in which the proportion of motile organisms is a genetic characteristic of the strain (Quadling & Stocker, 1957). The phage might eliminate the motile bacteria, leaving those which were nonmotile either in genotype (none or a small minority) or merely in phenotype (the majority). Those that were merely phenotypically non-motile would give motile descendents. Some of the nonmotile bacteria in the culture may have been paralysed by the χ phage itself, as will be described later; but such bacteria might not give rise to colonies.

Table 8 shows the stable resistant variants that were obtained after attempts to isolate six variants from each of 26 strains. Some of these were flagellated but paralysed, again showing that flagellar inactivity leads to resistance. The resistant variants appeared to be of three types: non-flagellated; paralysed; slightly motile, i.e. broth cultures contained 25% or less of motile organisms often showing slow or irregular movement. As might be expected, variants belonging to the third class were not completely resistant; the most concentrated phage suspension often produced slight (+) clearing. Variants of this kind were chiefly found in strains of *Salmonella abortus-equi* but this was not due to reversion to the state described by Kato (1954) for freshly isolated strains, since these variants were not more motile, or more χ phage sensitive, at pH 6·5 than at pH 8·0. Examination of films from these cultures stained by Leifson's (1951) method showed that the mean number of flagella/bacterium was much lower than in their motile, phage-sensitive derivatives.

All the motile mutants of the resistant variants regained phage sensitivity.

Table 8. *Stable resistant variants selected by the χ phage from sensitive strains*

Strain	Stable resistants obtained: Total	Nature: O	Nature: Paralysed	Nature: Slightly motile	Swarms obtained*
S. typhi					
A. A Cr.	4	4	0	0	O
A. C8 6608	4	3	1	0	1 (from O)
A. E4 5839	2	1	1	0	O
A D1 5434	0	.	.	.	.
A. L2 131	0	.	.	.	.
SW 537 (H901)	7	4	3	0	4 (from O) 3 (from par)
SW 540	5	5	0	0	2 (from O)
S. paratyphi B					
A. 1927	3	3	0	0	2 (from O)
A. 3a 1	1	1	0	0	O
A. 1815	0	.	.	.	.
A. 1249	0	.	.	.	.
A. 1910	0	.	.	.	.
A. Workshop	0	.	.	.	.
SW 546 (*1*, *2* : –)†	1	1	0	0	1 (from O)
S. typhimurium					
SL 396	6	6	0	0	3 (from O)
LT 7	4	4	0	0	O
LT 2†	3	1	2	0	1 (from O) 1 (from par)
SW 593	6	6	0	0	5 (from O)
A. M4618	6	6	0	0	4 (from O)
A. U20M	6	6	0	0	3 (from O)
SL 394	0	.	.	.	.
SL 656	0	.	.	.	.
S. abortus-equi					
NCTC 5727	11	6	0	5	4 (from sl. mot.)
SL 224	6	4	0	2	2 (from sl. mot.)
S. stanley					
SW 536†	1	1	0	0	1 (from O)
S. cholerae-suis					
NCTC 5737	1	1	0	0	O
NCTC 5738	0	.	.	.	.
S. memphis					
NCTC 7402	4	3	0	1	2 (from O) 1 (from sl. mot.)
S. riogrande					
NCTC 7399	4	3	1	0	2 (from O) 1 (from par)

* All the swarms obtained from O, paralysed or slightly motile variants were highly motile and χ phage-sensitive.

† Resistant variants obtained from the collection of the Guinness–Lister Unit.

Adsorption experiments

The χ phage adsorbed slowly to many strains that were sensitive, there being no significant decrease in titre of free phage after 10–20 min. contact with the bacteria at concentrations of $2–5 \times 10^8$ organisms/ml. (Table 9). Thus, it was difficult to attribute the resistance of any given resistant strain to failure of adsorption. In

Table 9. *Adsorption of the χ phage to naturally occurring motile strains*

Species	Sensitivity	Number of strains with which phage in supernatant was reduced to <20 %	reduced to 20–60 %	not detectably reduced
S. typhi	+	6	2	0
	+(shallow plaques)	1	1	0
	(+)	1	1	1
	−	1	0	1
S. abortus-equi	+	2	0	0
S. paratyphi B	+	2	0	0
	+(shallow plaques)	1	0	0
	(+)	0	1	0
S. typhimurium	+	0	2	0
	+(shallow plaques)	0	0	6
	(+)	0	0	1
	−	0	0	1
S. stanley	+	1	0	0
	−	1	0	0
S. paratyphi A	−	1	0	0
Others, with H antigens other than *g*...	+	1	0	0
	+(shallow plaques)	1	2	3
	(+)	2	4	1
	−	1	1	5
With antigen *g*...	−	0	0	2

examining non-motile strains (either O or paralysed) only the results with strains whose sensitive motile variants absorbed the phage rapidly were considered to have any significance (Tables 10, 11) and the same considerations applied when the effects of different flagellar antigens were compared (Table 12).

A marked contrast was seen between:

(1) adsorption to naturally occurring non-motile strains and to their motile derivatives (Table 10);

(2) adsorption to non-motile or poorly motile derivatives isolated by the χ phage from naturally motile strains, and to motile forms of these strains (Table 11);

(3) adsorption to derivatives of a single strain carrying either an H antigen which prevents infection and of an H antigen which allows infection (Table 12). One of these strains, SW 674, gave very striking results since it absorbed rapidly in phase 2, with antigen *1,2*, but not in phase 1, with antigen *g,p*. Only the host-range mutant to which the bacteria were sensitive, not the wild-type phage or other host-range mutants, adsorbed perceptibly to bacteria with H antigens *l*..., *e,h* or Arizona *13* (Table 12).

As shown in Tables 10 and 11, no detectable adsorption occurred to any of the non-flagellated strains, but two paralysed strains (SL 232 and SW 537.1*a*), and perhaps a third (SL 478) did produce a significant drop in the titre of free phage. With the paralysed strains of *Salmonella typhimurium* SL 43 and *S. riogrande* NCTC 7399.3, no antibody-resistant phage could be detected, and in an experiment with SL 232 in which 65 % of the phage was sedimented with the bacteria, only 0·08 % of the attached phage gave rise to infective centres after passage through antiserum. Thus, even if the phage does attach to a paralysed bacterium, infection is very unlikely to follow.

Table 10. *Adsorption to non-motile strains and their motile variants*

Strain	State of the organisms	Sensitivity	Phage remaining in supernatant
S. typhi			%
SL 77	Non-flagellated	−	NL
SL 77 swarm	90 % motile	+	10·5
SL 100	Non-flagellated	−	NL
SL 100 swarm	95 % motile	+	16·6
SL 232	Paralysed	−	63, 35
SL 232 swarm	60 % motile (slow)	+	30, 10
S. paratyphi B			
SW 906	Paralysed	−	NL
SW 906 swarm	95 % motile	+	16·5
S. typhimurium			
SW 573	Non-flagellated	−	NL
SI 43	Paralysed	−	NL
SL 43 swarm	90 % motile	+	40
LT 2	80 % motile	+	33
SL 499	Paralysed	−	NL
SL 499 swarm	90 % motile	+	48
SW 1153	Paralysed	−	NL
SW 1153 swarm	70 % motile	+	48
S. riogrande			
NCTC 7399	60 % motile	+	17·5
NCTC 7399.3	Paralysed	−	NL
NCTC 7399.3 swarm	90 % motile	+	12
S. abortus-equi			
SL 220	20 % motile, poorly flagellated	−	NL
SL 224	98 % motile	+	6
NCTC 5727	95 % motile	+	7·5
SJ 30	'Curly' flagella; rotating movement	+	NL

The adsorption mixtures were held at 37° for 10–20 min. before centrifugation.
NL = no detectable loss of phage from the supernatant.

Resistance of a strain was not in all cases due to failure of adsorption. Four strains which appeared resistant in the usual sensitivity tests on agar, nevertheless, rapidly absorbed the phage. Two of these strains, *Salmonella stanley* NCTC 92 and *S. typhi* A D4 T 107, were also tested in broth; the results showed that although 85–95:% of the phage had adsorbed, only 0·4% in the case of NCTC 92 and 3%in the case of A D4 T 107 gave plaques after passage through antiserum.

Association of flagella and adsorption

The effect of inhibiting flagella formation and of removal of flagella. Salmonella abortus-equi NCTC 5727, in common with other salmonellas, does not form flagella when grown at 44°. At 44° this strain grew more slowly than at 37°, reached a lower final concentration after overnight incubation and produced many short filaments.

Adsorption was measured at 44° and at 37° to cultures grown at each of these temperatures. No translational movement was seen in the culture grown at 44° although 1 % of the organisms were rotating, and there was no detectable adsorption

Table 11. *Adsorption to sensitive strains and χ-selected variants*

Strain	State of the organisms	Sensitivity	Phage remaining in supernatant (%)
Salm. typhi			
SW 537 (H901)	95 % motile	+	8·5
SW 537/χ	Non-flagellated	−	NL
SW 537/χ swarm	90 % motile	+	23
SW 537.1a	Paralysed	−	44
SW 537.1a swarm	90 % motile	+	12
S. riogrande			
NCTC 7399	95 % motile	+	17·5
NCTC 7399.6a	Non-flagellated	−	NL
S. memphis			
NCTC 7402	90 % motile	+	50
NCTC 7402.3a	Non-flagellated	−	NL
S. typhimurium			
LT 2	95 % motile	+	33
SL 478	Paralysed	−	70
S. abortus-equi			
NCTC 5727	95 % motile	+	7·5
3 isolates	Non-flagellated	−	NL
3 isolates	< 2 % motile	−	NL
3 isolates	*c.* 40 % motile	(+)	30–70
SL 224	90 % motile	+	6
SL 224.5b	1 % motile	−	NL
SL 224.5a	50 % motile	(+)	27

The adsorption mixtures were held at 37° for 10–20 min. before centrifugation.
NL = no detectable loss of phage from the supernatant.

after 15 min. at either 37° or 44°. On the other hand, there was good adsorption to the culture grown at 37°, in which 90 % of the organisms were normally motile, 86 % of the phage being absorbed at 37° and 91 % at 44°.

Flagella can be detached from bacteria without killing the organisms, either by short exposure to acid, by rubbing cultures over stiff agar, or by treatment in a Blendor (Stocker & Campbell, 1959). Any of these methods greatly reduced the adsorption to *Salmonella abortus-equi* NCTC 5727 (Table 13), the blendor being least effective, probably because the method leaves short stumps of flagella on the bacteria (Stocker & Campbell, 1959). The presence of residual flagellar fragments was indicated here by observing rotational movement in the blended suspension; the acid-treated and the rubbed suspensions contained virtually no motile bacteria.

Regeneration of flagella. Actively growing bacteria largely regenerate their flagella within one doubling time after treatment in the blendor (Stocker & Campbell, 1959). The rate of adsorption of the χ phage to *Salmonella abortus-equi* NCTC 5727 increased progressively, while the bacteria were incubated in broth after blending (Fig. 1), and electron microscopy showed that during this time the flagella increased from small stumps to their normal length and also became more numerous.

Table 12. *Adsorption of wild-type χ phage, and host-range variants to bacteria with different H antigens*

	Wild-type phage		Host-range mutants		
Strain	Sensitivity reaction	Phage remaining in supernatant (%)	Mutant selected by:	Sensitivity reaction	Phage remaining in supernatant (%)
NCTC 5723 (*g,m*)	−	NL	.	.	.
NCTC 5723.*i* (*i*)	+	35	.	.	.
SW 674 *g,p* phase	−	NL†	.	.	.
SW 674, *1,2* phase	+	4	.	.	.
SW 537	+	15	.	.	.
SW 569 (*i*)	+	15	.	.	.
SW 667 (*g,p*)	−	NL‡	.	.	.
SW 537.l,z_{13} (l,z_{13})	Low e.o.p.	NL	SW 537.l,z_{13}	+	18
			NCTC 8717	+	14
			SL 651	+	7
			NCTC 8278	$<\chi$	NL
			NCTC 7318	$<\chi$	NL
SW 668 (*e,h*)	Low e.o.p.	NL	NCTC 8278	+	50
		NL*	NCTC 8278	.	28*
			SW 537.l,z_{13}	$\leqslant\chi$	NL
			NCTC 7318	$\leqslant\chi$	NL
SL 651 *l,v* phase	Low e.o.p.	NL	SW 537.l,z_{13}	+	18
			SL 651	+	40
S. victoria (1, 9, 12 : *l,w* : *1,5*)					
E 504, *l,w* phase	−	NL	SW 537.l,z_{13}	+	12
E 504, *1,5* phase	+	30	.	.	.
NCTC 7318	Low e.o.p.	NL	NCTC 7318	+	36
		NL*	NCTC 7318	.	10*
			SW 537.l,z_{13}	$\leqslant\chi$	NL

The absorption mixtures were held at 37° for 10–20 min. before centrifugation.

* Adsorption mixture incubated for 2 hr. in the presence of chloramphenicol before centrifugation.
NL = no detectable loss of phage from the supernatant.
† 0·07 % was found to survive exposure to antiphage serum, which probably represents the proportion of bacteria in the opposite phase.
‡ About 0·005 % survived exposure to antiphage serum, which was no more than in a control suspension without bacteria.

Tests for adsorption of phage to detached flagella

The results described so far suggest that the primary site of adsorption may be the flagella themselves. However, suspensions of flagella from sensitive strains detached from bacterial bodies neither inactivated the phage nor protected it from neutralization by antiphage serum (Table 14); nor did they promote infection of the

non-flagellated strain of *Salmonella typhi* O 901, whose flagellated and motile derivative, H 901, is fully sensitive. Moreover, the phage did not appear to adhere to free flagella so far as could be seen in experiments in which it was added to free flagella which were later removed from suspension by adding H antibody and formalinized bacteria (to which the phage no longer adsorbs) carrying flagella of the same antigenic type. On centrifugation the bacteria were deposited, presumably taking with them the free flagella; the titre of free phage remained unchanged.

Table 13. *Adsorption of the χ phage to bacteria whose flagella were removed by rubbing on stiff agar, by acid-treatment or by blending in an M.S.E. Blendor*

	Motility (%)	Viability (%)	Adsorbed phage (antibody-resistant) (%)	Unadsorbed phage (%)
Expt. 1				
Untreated	75	(*a*) '100'	90	14
		(*b*) '100'	91	18
		(*c*) '100'	87	15
Acid-treated (pH 2·6)	<0·1	(*a*) 25	0·4	85
		(*b*) 6·3	2·2	100
		(*c*) 12	.	95
Rubbed	<0·04	(*a*) 100	0·29	88
		(*b*) 100	0·1	96
		(*c*) 100	.	100
Blended	<0·05 translational (some rotators)	(*a*) 100	21	71
		(*b*) 100	38	60
		(*c*) 100	20	73
Expt. 2				
pH 2·2 ½ min.	<0·1	60	0·3	.
pH 2·6 ½ min.	<0·1	61	0·37	.
pH 3, 1 min.	<0·1	79	0·55	.
pH 7, 1 min.	80	'100'	75	.

S. abortus-equi NCTC 5727 was grown overnight on 1·25 % (w/v) agar plates. For the 'rubbed' preparation, bacteria were transferred by wire loop to a 4 % (w/v) agar plate and rubbed over it with a glass spreader for 3 min. They were then collected in distilled water and washed twice.

For the 'untreated', 'acid-treated' and 'blended' preparations, bacteria were gently soaked off in distilled water and washed once. The suspension was then divided into two parts; one part, which was to provide the untreated and acid-treated bacteria, was washed once again. The other part was blended for 3 min. and then washed once again. All the suspensions were adjusted to a total bacterial count of 5×10^{10}/ml., and dilutions in broth were examined for motility.

Acid treatment consisted in mixing 0·1 ml. of the previously untreated and well-washed suspension with 0·9 ml. of buffer pH 2·6 for 1 min. at 45°,* and then neutralizing with 9 ml. of either (*a*) buffer pH 7, (*b*) broth, or (*c*) broth containing 50 μg./ml. chloramphenicol. (Addition of either broth or buffer pH 7 raised the pH to about 6.) The neutralized suspensions were examined for motility. The 'untreated', 'rubbed' and 'blended' bacteria were similarly exposed to 45° for 1 min. using buffer pH 7; they were also tested after dilution into buffer pH 7, broth or broth with chloramphenicol.

Counts of viable bacteria were made, and adsorption of phage in each suspension was tested by mixing $7·5 \times 10^7$ phage particles with $6·5 \times 10^9$ bacteria, and counting: (1) the total number of plaques produced; and after 15 min. at 37° (2) the number of plaques after dilution through anti-phage serum (infected bacteria), and (3) the number of plaques produced by the supernatant after centrifugation.

In Expt. 2, the bacterial suspension, prepared as before, contained 3×10^{10} bacteria/ml. The acid was neutralized with buffer pH 7, and 3×10^9/ml. bacteria were present in the adsorption mixture.

* The rate of flagellar lysis is temperature-dependent (Weibull & Tiselius, 1945; Duncan, 1935).

Effect on adsorption of artificially paralysing Salmonella abortus-equi *NCTC* 5727

It is clear from the preceding experiments that genetically paralysed bacteria are resistant to the χ phage and that this is associated with impaired adsorption of the phage. This observation raises the question as to whether a phage-sensitive motile strain would become resistant to the phage and absorb poorly if it were immobilized without loss of flagella. This can be done in several ways, for example, by thoroughly washing the bacteria (Stocker & Campbell, 1959). Experiments with *Salmonella abortus-equi* NCTC 5727 showed that bacteria grown in broth were still motile when resuspended in 10^{-4}M-phosphate buffer containing 10^{-3}M-$CaCl_2$ which

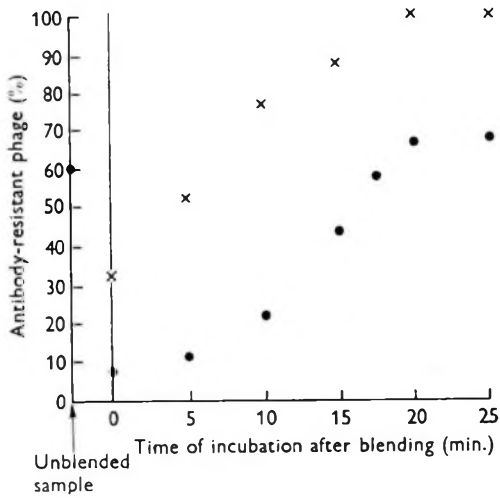

Fig. 1. Regeneration of flagella and adsorption. An actively growing culture of *Salmonella abortus-equi* NCTC 5727 was blended for 3 min. to detach the flagella and then incubated at 37° in a shallow layer of broth in a flask. Immediately after blending, and at 5, 10, 15, 17½, 20 and 25 min., samples were transferred to tubes containing 50 μg./ml. chloramphenicol (which arrests flagellar synthesis; Kerridge, 1959) and held at room temperature. The samples were examined for motility and total bacterial counts were made on the first and the last. *Sample taken immediately after blending*: total count, $2{\cdot}7 \times 10^8$/ml.; motility, 0·93 % transitional movement, 22% rotational movement.

Sample taken at 25 *min.*: total count, $4{\cdot}6 \times 10^8$/ml.; motility, 82 % translational movement. Samples taken at intermediate times showed progressively increasing proportions of motile bacteria; at first rotating bacteria predominated; at 15 min. 33 % of the bacteria showed translational movement which was rather slower than normal; and at 20 min. 60 % appeared to be fully motile.

When all the samples had been collected, phage was added to each to a concentration of $7{\cdot}5 \times 10^6$ p.f.p./ml. The numbers of infected bacteria (●) 2 min. and (×) 20 min. later were measured by plating the mixtures after exposure to antiphage serum.

must have contained only about 1 % (v/v) broth, and the phage adsorbed normally. However, after washing 3–4 times in buffer, no bacteria showed translational motility and less than 5 % were rotating. Phage adsorption to such suspensions was greatly decreased (Table 15); it increased again when the bacteria regained motility, which they did immediately after either broth or histidine were added to the buffer suspension (Stocker & Campbell, 1959).

Similar results were obtained with bacteria immobilized by several unrelated agents such as chloral hydrate or Merthiolate (Eli Lilley & Co) (Robertis & Peluffo, 1951), 2,4-dinitrophenol, aureomycin or erythromycin (Table 14). 2,4-Dinitrophenol at $2{\cdot}5 \times 10^{-3}$ M or more was needed; and its action was immediately

Table 14. *Tests for adsorption of the χ phage to detached flagella*

Mixtures		Total p.f.p.	Proportion of antibody-resistant phage (%)	Phage in supernatant
Expt. 1.				
Flagella – : *1,2*. Phage	15 min., 37°	$7{\cdot}1 \times 10^8$	$<0{\cdot}05$	.
Flagella *i* : *1,2*. Phage	15 min., 37°	$7{\cdot}1 \times 10^8$	$<0{\cdot}05$	.
Broth. Phage	15 min., 37°	$6{\cdot}7 \times 10^8$	$<0{\cdot}05$	.
Expt. 2.				
Flagella – : *1,2*. Phage	15 min., 37°	$1{\cdot}2 \times 10^9$	$<0{\cdot}09$	.
Buffer. Phage	15 min., 37°	$1{\cdot}1 \times 10^9$	$<0{\cdot}09$	.
Expt. 3.				
Flagella – : *1,2*; O 901 $2{\cdot}5 \times 10^8$/ml. Phage	20 min., 37°	.	$<0{\cdot}04$	.
Flagella *i* : *1,2*, O 901 $2{\cdot}5 \times 10^8$/ml. Phage	20 min., 37°	.	$<0{\cdot}04$	.
O 901 $2{\cdot}5 \times 10^8$/ml. Phage	20 min., 37°	.	$<0{\cdot}04$	.
Broth. Phage	20 min., 37°	$7{\cdot}9 \times 10^8$	$<0{\cdot}04$	.
Expt. 4.				
Flagella – : *1,2*. Phage	5 min., 37°	$7{\cdot}6 \times 10^7$	$<0{\cdot}013$	7×10^7
After 5 min. formalinized. SW 1061 added		.	.	.
Flagella – : *1,2*. Phage Formalinized SW 1061	10 min., 37°	$8{\cdot}7 \times 10^7$	$<0{\cdot}01$	$5{\cdot}1 \times 10^7$
Formalinized SW 1061. Phage	10 min., 37°	$6{\cdot}2 \times 10^7$	$<0{\cdot}01$	$6{\cdot}3 \times 10^7$
Broth. Phage	10 min., 37°	$6{\cdot}6 \times 10^7$	$<0{\cdot}01$	$5{\cdot}7 \times 10^{7}$*

In each individual experiment, the mixtures contained the same amount of phage, and after the time stated were assayed for total number of plaque-forming particles (p.f.p.) and by dilution through antiphage serum for antibody-resistant phage. Expt. 3 was made to see if the presence of flagella would promote infection of the non-flagellated strain of *S. typhi*, O 901. In expt. 4, a test was also directly made for physical adherence to flagella: formalinized bacteria of *S. typhimurium* SW 1061 (– : *1,2*) were added to the mixtures either at once or after 5 min., and H antibody *1,2* was subsequently added to agglutinate the free flagella (Craigie, 1931) and, it is presumed, to cause their attachment to bacteria whose flagella were of the same antigenic type. After centrifugation, the supernatant was assayed for unattached phage.

* Here there were no bacteria or flagella present, and the mixture was not centrifuged: the figure given is the plaque count after addition of anti-*1,2* serum, which did not affect the phage.

The flagellar suspension in Expt. 4 was used at a final dilution of 1/20, and a dilution of 1/2 in the first mixture before the addition of the formalinized bacteria. In Expts. 1–3 the suspensions were used at a dilution of 1/10, at which they showed a faint opalescence.

annulled on dilution to below 3×10^{-4} M. Chloral hydrate and Merthiolate have been reported to immobilize *Proteus vulgaris* reversibly, but in the present case chloral hydrate, and to a lesser extent, Merthiolate, at concentrations sufficient to immobilize, killed a considerable proportion of the bacteria. Aureomycin was more effective in immobilizing than erythromycin, but a high concentration of each was needed and both took about 30–40 min. at room temperature to produce their full

Table 15. *Adsorption to artificially paralysed* NCTC 5727

Bacteria treated with:	Motility	Phage in supernatant (%)	Motility restored by	Phage in supernatant (%)
Washing	0% translational 1–5% rotating	70–80	1/5–1/25 broth (motility 90–100%)	6–15
			1/75 broth (motility 5% slow)	50
			0·01 M histidine (motility 40% slow + 10% rotating)	34–47
			0·05 M histidine (50% slow)	40
Merthiolate				
9×10^{-3} M-3×10^{-3} M	1–10%	NL	.	.
$9{\cdot}8 \times 10^{-4}$ M	40%	31	.	.
$3{\cdot}4 \times 10^{-5}$ M	95%	12	.	.
Chloral hydrate				
0·12 M	0	75	.	.
2,4-dinitrophenol				
$2{\cdot}5 \times 10^{-3}$ M	0	80	Removal of drug (60	22
$3{\cdot}3 \times 10^{-3}$ M	2% rotating	76	80% motility)	14
Aureomycin				
0·5 mg./ml.	(*a*) 40 min., <1% translational, 5% rotating	60–100	.	.
	(*b*) 2 min., 90–98% motile	10–20	.	.
Erythromycin				
0·5 mg./ml.	40 min., 2% translational 5–50% rotating	60	.	.
Terramycin				
0·5 mg./ml.	98%	7	.	.
Streptomycin				
0·5 mg./ml.	98%	6	.	.
Chloramphenicol				
0·5 mg./ml.	95–98%	4–9	.	.

Adsorption was measured after 10 min. to late log or stationary phase bacteria at about 5×10^8/ml., with a phage multiplicity of less than 1. In a few experiments with bacteria treated by washing, dinitrophenol or aureomycin, the adsorption mixtures were also assayed after passing through antiphage serum to measure the proportion of infected bacteria. This agreed in all cases with the estimates obtained from assay of the supernatant. In each experiment, control bacteria which had not been treated left 10% or less of the phage in the supernatant.

Bactericidal effect of the drugs; survival after 15 min. exposure:

Merthiolate	9×10^{-3} M	10%	Aureomycin	1 mg./ml.	50%
	$1{\cdot}7 \times 10^{-3}$ M	50%	Erythromycin	1 mg./ml.	30%
Chloral hydrate	0·12 M	<0·1%	Terramycin	1 mg./ml.	90%
2,4-dinitrophenol	$2{\cdot}5 \times 10^{-3}$ M	85%	Streptomycin	1 mg./ml.	46%

Merthiolate, 3×10^{-3} M for 15 min., inactivated 40% of the phage. The phage was stable in the other drugs over the period of the experiments.

effect. Before it had affected the motility of the bacteria, aureomycin did not affect adsorption of the phage. The effect of aureomycin on motility may not have been reversible, but electron micrographs showed that the flagella were not destroyed. Terramycin, streptomycin and chloramphenicol did not affect motility or phage adsorption. It may be concluded from these experiments that bacteria which are merely phenotypically non-motile adsorb the χ phage more slowly, just as do genotypically paralysed strains.

Motility was irreversibly abolished by formaldehyde or by heating at 56° for 30 min.; no detectable adsorption occurred to cultures treated in either way. Neither treatment is supposed to alter the H antigen, but formaldehyde evidently denatures the flagellar protein (Astbury, Beighton & Weibull, 1955).

Electron microscopy

Electron micrographs of formalin-fixed, air-dried platinum + iridium-shadowed mixtures of *Salmonella abortus-equi* NCTC 5727 and the χ phage, which had been washed to remove the free phage, showed phage particles on the flagella, apparently attached by the tips of their tails (Pl. 2, figs. 3 and 4). The tails of some particles were curved as if they were genuinely attached in this way and had been bent while the specimens were prepared (Pl. 2, fig. 5). The bacteria could evidently absorb only a limited number of particles, as shown by estimates of the proportions of unadsorbed phage in an experiment where bacteria were mixed with varying concentrations of phage. The capacity appeared to be about 6 p.f.p.; if only about 1/5 of the total particles were plaque-formers, as the particle counts by fluorescence mieroscopy suggested, this would represent about 30 particles/bacterium, which was about the maximum number seen on electron microscopy.

It was impossible to say that there were no phage particles attached to the bodies of the bacteria, for phage particles were often seen near the body; but, as there was always a network of flagella in the region, they might as well have been attached to a flagellum as to the body itself. Control preparations of *Salmonella abortus-equi* NCTC 5727 with phage P22 (Pl. 2, fig. 6) showed many particles of this phage along the bodies of the bacteria, and none on the flagella, which strongly suggested that particles of the χ phage seen on the flagella were specifically attached. Attachment appeared to occur anywhere along the length of a flagellum, but groups of two, three, or more particles were commonly seen attached at or around a single point (Pl. 2, fig. 7). In contrast, phage stocks did not contain clumped particles except rarely, and then these were centred round a fragment of detached flagellum. The presence of these clusters suggests either that some portions of a flagellum favour phage attachment, or that the attachment of one particle encourages the attachment of others.

When the bacteria had been deflagellated in the blendor before mixing with the χ phage, an occasional phage particle was seen on the remaining flagellar stumps; none was seen on the bodies of the bacteria. When the bacteria were blended after mixing with the phage, fewer phage particles were seen than with bacteria which had been blended before exposure to the phage; this suggests that the distal parts of flagella which are removed by blending can compete for adsorption of the phage with the proximal parts which survive blending.

The synthetic strain SW 674 (*g,p*: *1,2*) was examined in both the phase

exhibiting *1,2*, which was phage sensitive, and the phase with *g,p*, which was resistant. The flagella of the two phases did not differ in appearance, but large numbers of particles were seen on the flagella exhibiting antigen *1,2* while none was seen with those exhibiting antigen *g,p*. Analogous observations were made on the following strains in either their paralysed or their normally motile forms: *Salmonella typhimurium* SW 1153, a paralysed variant of strain LT2; *S. riogrande* NCTC 7399.3 (*40 : b : 1,5*); *S. typhi* SW 537.1*a*. The latter is the only one of these three paralysed strains to which any adsorption of the phage was detected and here electron microscopy showed an occasional particle attached to the flagella. Individual flagella appeared normal in bacteria immobilized by aureomycin although the flagella tended to form skeins. The numbers of particles seen on the flagella decreased only when the motility had fallen, 30–40 min. after exposure to the drug.

Immobilization and clumping

There are few published observations on the effect of phage infection on motility, but infection by most phages seems to leave motility unaffected until the latent period is advanced, or even until lysis (Murphy, 1957). *Salmonella abortus-equi* NCTC 5727 is certainly not immobilized for some time after adsorption of a clear-plaque mutant of phage P22, and the same is probably true of a motile strain of *Escherichia coli* B (Furness & Rowley, 1955) and phage T2. (Bacteria of this latter organism, unlike salmonellas, tend to stop swimming in preparations held between slide and coverslip; however, no difference could be observed between bacteria which had absorbed phage T2 and control preparations of uninfected bacteria.) On the other hand, immediately after mixing salmonella NCTC 5727 with the χ phage at sufficiently high multiplicity, the bacteria became immobile and formed clumps of 10–20 bacteria. The effect was most striking, and could be readily observed when a drop of high titre phage stock was placed on a slide at one edge of a coverslip, a drop of highly motile culture placed at the opposite edge and the two allowed to mix gradually. Where they met, rapidly swimming bacteria came to an abrupt halt and then often joined other already immobilized bacteria to form clumps. Agglutination of infected bacteria was reported by Beardsley (1960), but only began when new phage started to be released; a loss of motility which this author also mentioned evidently did not immediately follow on infection.

Immobilization of *Salmonella abortus-equi* NCTC 5727 by the χ phage could be prevented either by treating the phage stock with antiphage serum or by removing the phage particles by centrifugation. However, phage inactivated by ultraviolet radiation or by over-centrifugation appeared to immobilize as efficiently as infective phage, suggesting that the effect was due to adsorption of the phage, which need not be followed by multiplication.

When the phage was diluted, progressively fewer bacteria were immobilized. The process went rapidly to completion with *Salmonella abortus-equi* NCTC 5727; with a concentration of phage which left some motile bacteria, the proportion of these did not alter perceptibly after the first few minutes. Serial dilutions of several different phage preparations were tested for their immoblizing activity by estimating the proportions of bacteria which still showed translational motility. In each experiment the immobilizing activity decreased more rapidly with dilution of the phage than would be expected if one phage particle per bacterium were sufficient. The

possibility is not excluded that an individual flagellum might be inactivated by adsorption of a single particle.

Other sensitive strains besides *Salmonella abortus-equi* NCTC 5727 were also immobilized by the χ phage, but generally the process was slower, and there was less clumping of the immobilized bacteria. Among the strains that were tested was SW 674 which was rapidly immobilized in the phase with H antigen *1,2*, but showed no loss of motility in the phase with *g,p*. Strains SW 537.l,z_{13}, NCTC 8717 exhibiting l,z_{13}, and SL 651 exhibiting *l,v*, were immobilized by the phage mutants which they had selected, but not by the wild-type phage or the other host-range mutants. Strain E 504 (*l,w* : *1,2*), in the phase with *1,2* was immobilized by either wild-type phage or the mutant selected by SW 537.l,z_{13}, but in the phase with *l,w* it was immobilized only by the latter. Analogous results were obtained with strains SL 507 (*S. typhi* carrying *e,h*) and NCTC 8278 (*e,h* : *1,2*) which was tested in each phase. Similarly, Arizona NCTC 7318 was slowly immobilized by the mutant which it had selected, but not by the wild-type phage or other mutants. Each of the host-range mutants rapidly immobilized *S. abortus-equi* NCTC 5727. Experiments were made with NCTC 5727 whose flagella had been removed by the blendor. Here, since the bacteria were necessarily immobile, only clumping could have been observed. None occurred. Thus it appears that the clumping is a sequel to attachment of the phage to the flagella. Immobilization and clumping following phage adsorption was not accompanied by morphological changes in the flagella so far as could be seen by electron microscopy.

DISCUSSION

Previous authors reported that the χ phage attacked only flagellated bacteria, but it is now clear that flagella alone do not lead to susceptibility; these must be both active and of a correct antigenic type. They must also be present in suitable bacteria, for not all motile strains with the correct H antigens were sensitive. Resistance in the latter case might be due to inability of the phage to multiply in the bacteria (particularly with the few strains to which the phage readily adsorbed); but when resistance was associated with absence of suitable flagella, it was evidently due to failure of adsorption. The importance of the H antigen in the control of susceptibility immediately suggests that the phage adsorbs to the flagella themselves, since the H antigen is present only on the flagella and does not extend over the surface of the bacterial body (Craigie, 1931; Stocker & Campbell, 1959). Removal of the flagella from sensitive bacteria impaired adsorption. Direct evidence for attachment of phage particles to active flagella of correct antigenic type was provided by electron micrographs.

The importance of the bacterial strain itself in the control of sensitivity was most clearly seen when two strains carrying the same H antigen differed in their reactions, although the H antigenic determinant of the one had been received from the other by transduction, when the antigenic complex is known to be transferred unaltered (Lederberg & Edwards, 1953). Presumably, either the phage could not multiply in the resistant strain or the flagella of the two strains differed significantly in characteristics, other than the H antigen, which affected adsorption or initiation of infection. Transduction of the H antigenic determinant is unlikely to change all the

genes concerned with flagella (Stocker *et al.* 1953); hence, in a hybrid resulting from transduction of an H antigen, the other flagellar characters would probably be those of the recipient and not those of the donor strain.

The phage presumably failed to adsorb to isolated flagella for the same reason as it failed to adsorb to paralysed strains, namely, because the flagella were not functioning. Flagella such as are carried by salmonellas are helical in shape (Leifson, Carhart & Fulton, 1955); in living preparations of moving bacteria, they appear as rotating spirals (Reichert, 1909; Pijper, 1938; Weibull, 1950, 1951), and in fixed preparations they are flattened into a sinuous form. It is currently thought (Astbury *et al.* 1955) that motility as well as the helical shape of flagella result from the passage of a spiral wave of contraction down the flagellum, the helical line of contraction which is continuously moving along being due to the transmission of contractile pulses in subfibrils of which the flagellum is probably composed. The reflexions seen in X-ray analysis of flagellar preparations have been interpreted to indicate the presence of polypeptide chains in two different states of folding, and it is thought that the undulations leading to active flagellar movement are brought about by a rhythmical change of length between one configuration and the other. The two polypeptide chain configurations are always present simultaneously in preparations of flagella detached from the bacterial body, and these, in addition, retain their wave shape; thus it is supposed that flagella detached in the course of transmitting the wave of contraction remain fixed exactly as they were at the moment they were broken off (Astbury *et al.* 1955). Paralysed and motile bacteria are morphologically and serologically similar. The flagella of paralysed bacteria, although not moving, show no difference in wave form from the flagella of motile ones, and no difference in X-ray diffraction pattern (Beighton, Porter & Stocker, 1958). In particular, flagella isolated from paralysed bacteria also possess the features which indicate that the protein is present in two different configurations. Thus motile and paralysed flagella may both have similar helical lines of contraction, but in a paralysed flagellum this is static and not transmitted as a wave. In other words, isolated flagella and flagella of paralysed bacteria are thought to have the same array of features as active flagella, which consequently do not possess any unique structures that could be tentatively identified with the phage receptor. Two possible explanations for the need for active flagella are either that the phage receptor becomes unmasked during active movement, or that functioning of the flagella is required *per se* for adsorption either to occur, or to be irreversible and to lead to infection. The slight adsorption which was detected with a few paralysed strains, which was not followed by infection, may mean either that attachment can occur with decreased efficiency, perhaps transiently, to inactive flagella, or that the flagella of these particular strains had perhaps some slight activity short of that required for motility. The degree of activity associated with motility is evidently necessary for initiation of infection, possibly for the injection of the phage DNA (Hershey & Chase, 1952).

It was suggested by London (1958) that the site of adsorption of the χ phage to the bacterium may be at the basal granule of the flagellum. However, the present electron micrographs show phage particles attached along the length of the flagella, distally as well as proximally. In addition, comparisons of untreated and blended bacteria have shown that phage particles adsorbed distally can certainly infect the bacterium:

(1) When the flagella have been reduced to short stumps by blending, not only is the rate at which the phage particles attach to the bacteria decreased, but so is the rate at which the bacteria become infected (as measured by antibody-resistant infective centres) and this returns progressively to normal as the flagella regenerate (Fig. 1). The alternative explanation for this finding, namely, that the motility of the flagellated bacteria increases the chances of collision between the phage and the proximal part of the flagella, seems intuitively unlikely to account for the magnitude of the difference.

(2) If phage particles which have attached to distal parts of a flagellum fail to infect, then a phage preparation should show a higher titre when titrated by adsorption to blended bacteria than to bacteria with long flagella, provided a sufficiently long time is allowed for adsorption; no difference in titre is observed.

If, therefore, the primary site of phage adsorption can be at any point along the flagellum, the phage genome might reach the bacterial body by any of the following routes:

(*a*) by passage of the entire phage particle up the outside of the flagellum until it reaches the junction with the body and there injects its DNA. Although no precise estimates have been made, electron micrographs of samples of phage-bacterium mixtures, which had been fixed at different times after mixing, gave no indication of a drift of phage particles up the flagella towards the bacterial bodies;

(*b*) by adsorption of the phage particles to the bacterial body in the usual way, following some kind of 'activation' by contact with a flagellum. If this were so, then a phage particle after attachment to a flagellum belonging to one bacterium might be able to infect a different bacterium, but there was no indication that this could occur;

(*c*) by being injected into the flagellum at the point of initial attachment, and then travelling in the flagellum into the bacterial body. The pattern common to larger flagella and cilia is evidently a cylindrical arrangement of 9 subfibrils enclosing 2 more, and theoretically this allows the latter to roll round in the cylinder (Astbury *et al.* 1955). If bacterial flagella are structurally similar, the χ phage DNA might pass along the channels or potential channels that this arrangement might provide. Electron microscopy has sometimes suggested that bacterial flagella are made up of subfibrils (Starr & Williams, 1952; Labaw & Mosley, 1955) although in most electron micrographs, including the present ones, no fine structure can be seen.

With fully motile bacteria, the phage genome evidently reaches the bacterial body not long after adsorption, for the stages could not be separated by removing the flagella: when bacteria were mixed with phage for 1 min. (when 50 % of the phage had adsorbed) and then blended for 1·5 min., there was no decrease in the total number of plaque-forming entities (i.e. infected bacteria + free phage) nor in the number of infected bacteria alone (measured by the number of antibody-resistant infective centres sedimented by centrifugation at 1100 *g*.) Nor was there any increase in number of non-sedimentable p.f.p. as might occur if phage in a state able to infect new bacteria were detached with the flagella.

The sudden loss of active flagellar movement upon adsorption of phage seems to be an effect peculiar to the χ phage. In general, phage infection interferes only with bacterial syntheses, not with energy-yielding processes (Cohen, 1949) which, together with the fact that more than one particle of the χ phage per bacterium is needed, may imply that the immobilization observed here is due to a direct effect on the flagella.

This might be either an effect on the energy supply or on the structure of the flagella. Attachment of coliphage T2 results in activation and liberation of phage enzymes, notably the phosphatase (Dukes & Kozloff, 1959; Kozloff & Lute, 1959), and the cell-wall lytic enzyme (Weidel & Primosigh, 1958) which can cause gross morphological changes in the bacterium. For example, adsorption of coliphage T2 causes isolated bacterial cell walls to shrivel (Williams & Fraser, 1956). Thus, by analogy, adsorption of the χ phage to the flagellum might well cause gross changes in flagellar structure and immediate loss of activity. Such changes could also account for the agglutination that follows phage adsorption, since flagella (Scholtens, 1938) and their H antigens (Ogonuki, 1940) are known to influence the stability of flagellated organisms in suspension. Agglutination is not due to immobilization *per se* since genetically and artificially paralysed bacteria do not clump. It is interesting to note that two strains with abnormal 'curly' flagella, *Salmonella abortus-equi* SJ 30 and a similar variant of a *S. typhimurium* strain (Dr Iino, personal communication), agglutinate spontaneously whereas their derivatives with normal flagella are stable.

I should like to thank Dr E. H. Mercer, Mrs H. Ozeki and Dr M. Birbeck for making electron micrographs; Dr B. A. D. Stocker for the χ phage and for bacterial strains; Dr E. S. Anderson for fluorescence microscopy, for Vi-phages and for bacterial strains; and Dr Joan Taylor and Dr P. R. Edwards for bacterial strains.

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. Particles of the χ phage negatively stained with phosphotungstic acid. $\times$ 163,000.

Fig. 2. Particles of the χ phage negatively stained with phosphotungstic acid. Some collapsed heads can be seen. $\times$ 130,000.

PLATE 2

Fig. 3. Particles of the χ phage on the flagella of NCTC 5727: fixed, shadowed preparation. $\times$ 24,300.

Fig. 4. Particles of the χ phage on the flagella of NCTC 5727: fixed, shadowed preparation. $\times$ 7800.

Fig. 5. Particles of the χ phage on a flagellum of NCTC 5727: the tail of one is bent (see text). Preparation negatively stained with phosphotungstic acid. $\times$ 270,000.

Fig. 6. Particles of phage P22 surrounding the bacterial bodies of NCTC 5727: fixed, shadowed preparation. $\times$ 8000.

Fig. 7. Cluster of particles of the χ phage (see text). $\times$ 8000.

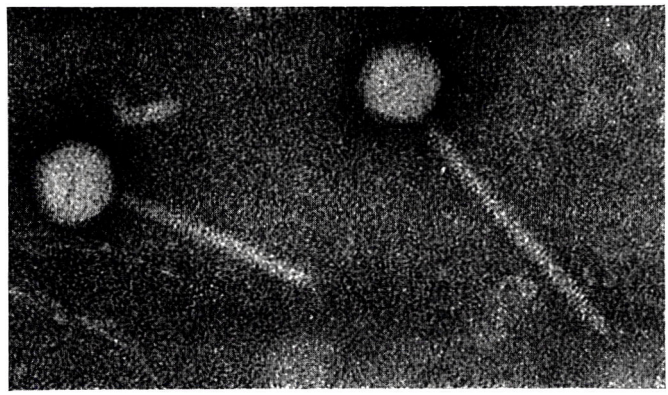


Fig. 1

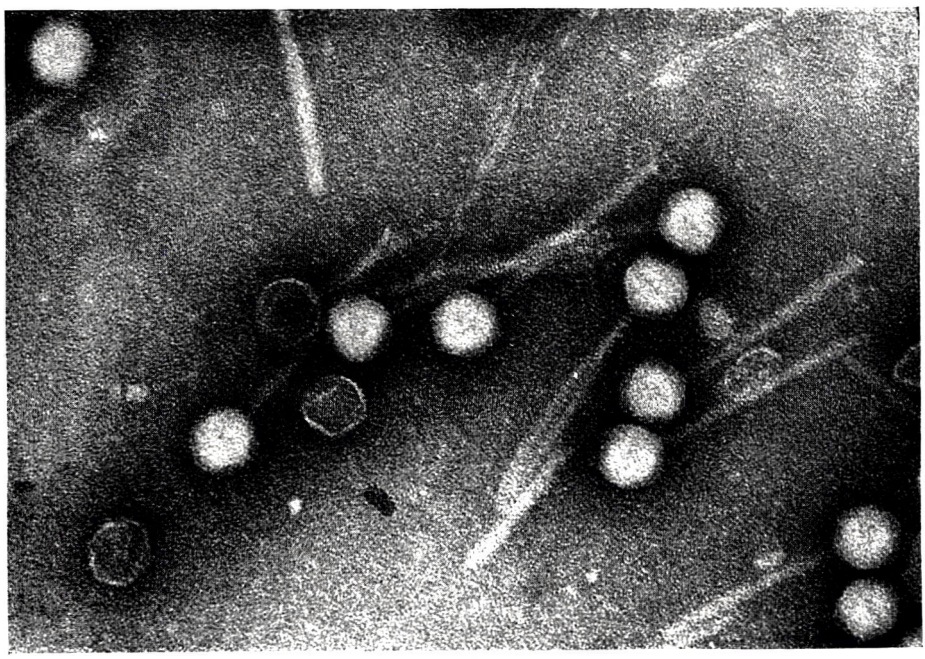


Fig. 2

(*Facing p.* 290)

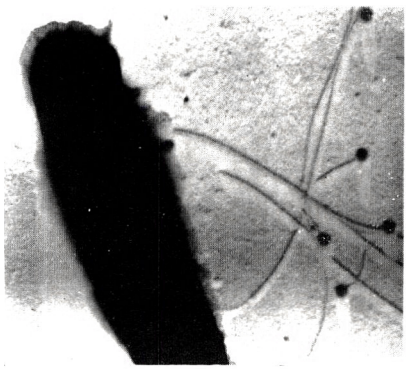
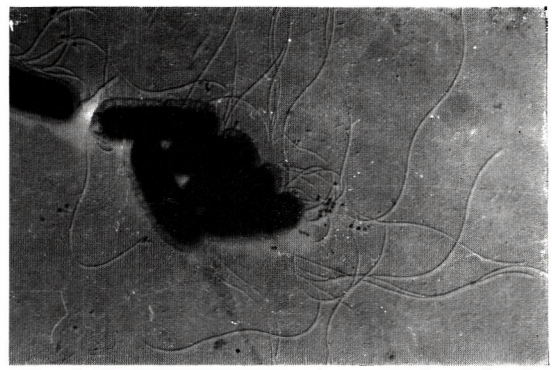


Fig. 3



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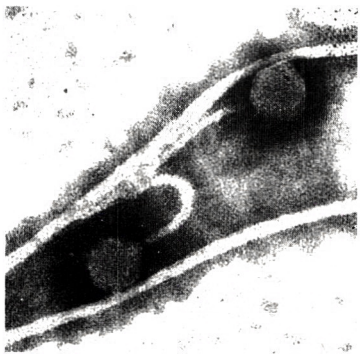

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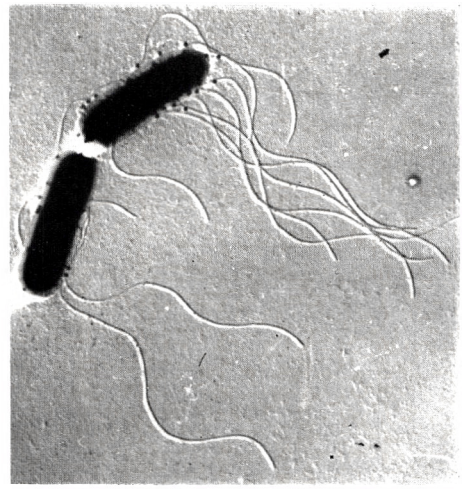

Fig. 6

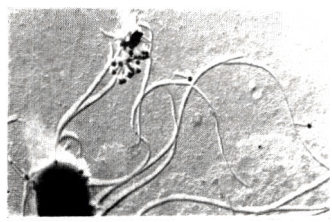

Fig. 7

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Substances which Protect Lyophilized *Escherichia coli* against the Lethal Effect of Oxygen

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SUMMARY

The lethal effect of oxygen on *Escherichia coli* organisms freeze-dried from water may be partly abolished by the addition of certain compounds before lyophilization. Three groups of such protective compounds were found: (i) thiourea, some of its derivatives and analogues; (ii) sugars, especially monosaccharides and some of their derivatives; (iii) some simple inorganic salts. The protective action was not unequivocally correlated with reducing power. Certain reducing agents (e.g. sodium dithionite, cysteine, reduced glutathione) even enhanced the lethal effects of oxygen.

INTRODUCTION

It was shown previously (Lion & Bergmann, 1961) that *Escherichia coli* organisms lyophilized from distilled water, were rapidly inactivated by air and oxygen, even at low pressures. The usual freeze-drying procedure in which primary drying is followed successively by admission of dry air into the system, followed by secondary drying, cannot therefore be applied to organisms suspended in distilled water. It is possible, however, to preserve successfully the viability of the organisms under these circumstances when the lyophilization is carried out in the presence of a protecting medium such as that proposed by Naylor & Smith (1946). The identity of the substance or substances in this medium, which protect the dried bacteria against oxygen, has now been studied. The results, as well as experiments with other compounds likely to possess protective power, are reported in the present paper.

METHODS

The methods for growing the bacteria, the determination of viability, and the freeze-drying technique were described previously (Lion & Bergmann, 1961).

The bacteria were harvested and washed well in distilled water. To make up the final suspension, 0·2 ml. of a solution of the compound to be tested, at 5 × the final concentration needed, was added to 0·8 ml. of the bacterial suspension in water, containing about $1{\cdot}5 \times 10^{11}$ bacteria. All the test solutions were sterilized by filtration.

Lyophilization was carried out at room temperature. At the end of the primary drying period, dried air was admitted to the system. The rubber tubes connecting the ampoules to the manifold were clamped off. The manifold was disconnected from the freeze-drying apparatus and transferred to a constant temperature incubator at 28°. The control ampoules to be kept under vacuum were clamped off before

air was admitted to the apparatus. Although flame sealing of ampoules under vacuum is much to be preferred, this is very difficult to carry out routinely on neutral glass ampoules, without constricting the neck. In practice, during 4 hr. (the duration of the experiments described here) the vacuum in the ampoules that were clamped-off was well maintained. As compared with the flame-sealed ampoules, sometimes a decrease in viability was found in the clamped-off ampoules, probably due to traces of air which diffused through the rubber connexions. However, this decrease in the controls was usually so small that it could be neglected in comparison with the mortality observed in the test ampoules when filled with air.

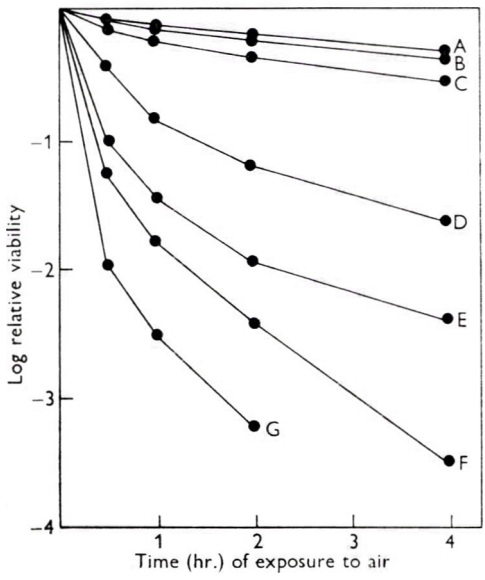

Fig. 1

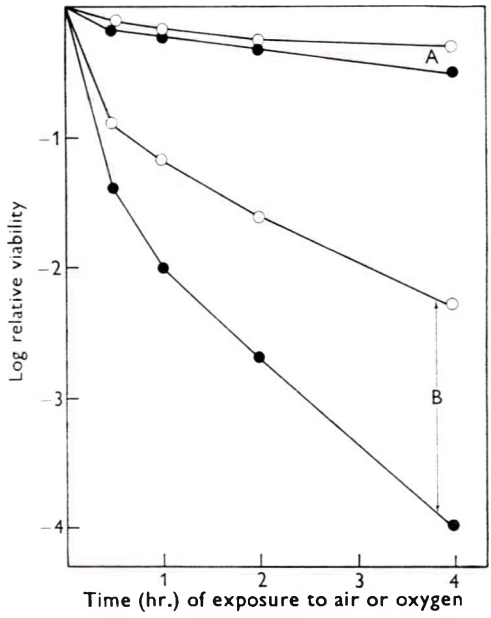

Fig. 2

Fig. 1. Viability of *Escherichia coli*, freeze-dried in single components of Naylor's medium, after exposure to dry air at 28°. A, complete medium; B, 1 % (w/v) thiourea; C, 0·5 % (w/v) thiourea; D, 0·5 % (w/v) ammonium chloride; E, 0·5 % (w/v) ascorbic acid neutralised to pH 6·5 with NaOH; F, distilled water; G, 2 % (w/v) dextrin.

Fig. 2. Viability of *Escherichia coli* strain B/r, freeze-dried from distilled water or 1 % (w/v) thiourea, after exposure to dry air or oxygen at 28°. A, bacteria dried in 1 % (w/v) thiourea; B, bacteria dried in water; ○, exposed to air; ●, exposed to oxygen.

RESULTS

The Naylor & Smith (1946) medium was first examined for the ability of its components to protect dried *Escherichia coli* against the oxygen of air. The Naylor & Smith medium is composed of (%, w/v): sodium ascorbate, 0·5; thiourea, 0·5; ammonium chloride, 0·5; dextrin, 2; in aqueous solution. Although ascorbic acid was originally contained in the Naylor & Smith medium as an antioxidant, its protective effect against air was negligible. Drying in dextrin gave worse results than drying from water, and only thiourea showed a protective power comparable to that of the complete medium. Increasing the concentration of thiourea from 0·5 % (w/v, as in the Naylor & Smith medium) to 1 % (w/v) permitted the replacement of the com-

plete medium by thiourea alone as far as the oxygen effect was concerned. Beside thiourea only ammonium chloride showed some protective action, although it was far less effective than thiourea. Fig. 2 shows the protection by 1 % (w/v) thiourea for dried *Escherichia coli*, strain B/r, against air and oxygen. Table 1 shows the protection afforded by various derivatives and analogues of thiourea. As it could be assumed that thiourea acted in its tautomeric form either as a reducing agent or as a sulphydryl compound or both, a few materials of these two classes were tested (Table 1, B). While ascorbic acid had hardly any protective action and cysteamine none at all, cysteine and especially sodium dithionite and reduced glutathione enhanced the lethal effects of oxygen considerably. The killing effect of these substances was pronounced in the presence of air only; dried bacteria *in vacuo* were not affected by them, either during lyophilization or after termination of the freeze-drying.

Ethylenediaminetetra-acetate (0·1 %, w/v, pH 7), tested as a representative chelating agent, did not show any protective action. Some materials containing high-molecular compounds, namely, nutrient broth (double strength), skimmed milk and gelatin were also examined. They had no protective effect.

Glucose has proved to be an effective protecting agent in lyophilization (Fry & Greaves, 1951). Considering that at least part of the killing of bacteria during freeze-drying is due to oxygen, one would expect glucose to protect against this gas. Indeed, glucose showed protective properties. Other sugars and sugar derivatives were also tested (Table 1). As it seemed possible that a correlation might exist between the protective effect of a sugar by the given bacterial strain and its fermentability, fermentation tests were carried out according to standard bacteriological procedures (Mackie & McCartney, 1945). Glucose, galactose, mannose, fructose and lactose were fermented; sucrose, maltose and α-methylglucoside were not. The protective power of α-methylglucoside (not fermented) was as high as that of glucose (fermented), but since no trace of fermentation of α-methylglucoside was observed even after incubation for 3 days, its protective action appears not to be related to the free 1-position of the monosaccharide.

As already mentioned, ammonium chloride had some protective effect. Naylor & Smith (1946) mentioned that ammonium chloride could to a certain extent be replaced by sodium chloride in their freeze-drying medium. We tested various inorganic salts for protective effects against oxygen. The results are given in Table 1, E. The iodides of sodium and potassium proved to be the most effective protective agents found so far; hardly any killing occurred in the dried organisms when exposed to air in the presence of iodide. Sodium and potassium nitrites and thiocyanates were also very effective. Not always, however, was the cation without influence. Sodium chloride, bromide and nitrate were much more effective protective agents than the corresponding potassium salts. Lithium, and especially magnesium chloride, showed strong protective action, while the chlorides of calcium, ammonium and rubidium were much less effective. The protective action of the magnesium and sodium ions disappeared with the sulphate and phosphates. Both potassium and sodium fluoride appeared to enhance the oxygen effect.

Table 1. *Viability of* Escherichia coli *exposed to dry air at 28° for various periods in the presence of different media*

Drying medium	Relative viability of bacteria (%)					
	At the end of drying*	Kept after drying at 28° in dry air†				4 hr. in vacuum
		0·5 hr.	1 hr.	2 hr.	4 hr.	
Group A agents (all 0·13 M)						
Thiourea	83	77	64	47	37	100
Monomethylthiourea	94	88	76	73	59	100
Symmetrical dimethylthiourea	81	67	52	49	39	91
Asymmetrical dimethylthiourea	62	19	11	9	4	62
Trimethylthiourea	64	36	22	8	6	78
Ethylisothiouronium bromide HBr	88	40	23	14	4	81
Urea	79	40	33	11	8	66
Urethane	45	16	12	4	1	69
Thioacetamide	75	49	38	16	5	91
Acetamide	74	20	12	3	1	26
Thiosemicarbazide	59	13	6	3	1	54
Group B agents						
Na ascorbate (pH 6·5, 0·5 %, w/v)	61	9	3	1	0·5	67
Na dithionite (0·05 M)	49	0·2	$< 10^{-3}$	$< 10^{-3}$	$< 10^{-3}$	42
Cysteamine (pH 6·5, 0·13 M)	77	13	3	0·4	0·01	78
Cysteine (pH 6·5, 0·06 M)	64	3	0·3	0·02	$< 10^{-3}$	87
Glutathione (0·16 M)	61	—	—	$< 10^{-4}$	$< 10^{-5}$	—
Glutathione (0·08 M)	60	—	—	$< 10^{-4}$	$< 10^{-5}$	—
Group C agents (all 0·11 M)						
D-Glucose	58	95	74	53	41	91
D-Galactose	70	57	47	38	31	97
D-Mannose	72	72	65	57	42	87
D-Fructose	60	40	35	36	25	93
L-Arabinose	91	58	51	38	31	75
Lactose	67	9	2	0·2	0·04	—
Sucrose	73	15	3	0·9	0·5	67
Maltose	66	35	1	0·3	0·2	50
Cellobiose	61	21	10	9	1	59
Melibiose	61	14	7	3	2	54
Group D agents (all 0·11 M)						
α-Methylglucoside	69	65	54	45	32	100
α-Methylmannoside	88	59	47	39	30	100
Mannitol	73	50	37	18	13	84
Inositol	76	33	23	11	3	62
Glucosamine HCl	87	71	65	51	42	98
Group E agents (0·16 M with exceptions)						
NaCl	63	81	62	40	30	75
KCl	76	7	2	0·8	0·2	80
NaBr	86	50	36	22	10	81
KBr	84	14	6	2	0·4	58
NaI	95	95	91	84	80	87
KI	92	93	86	79	82	96
NaF	37	$< 10^{-2}$	$< 10^{-3}$	$< 10^{-3}$	$< 10^{-4}$	10
KF	65	$< 10^{-2}$	$< 10^{-3}$	$< 10^{-3}$	$< 10^{-4}$	10
$NaNO_3$	89	82	69	63	54	90
KNO_3	82	17	9	4	0·7	48
$NaNO_2$	75	84	79	64	44	75
KNO_2	89	71	67	71	68	72
NaSCN	93	71	67	51	45	93
KSCN	80	76	65	62	52	96
Na_2SO_4	48	2	0·2	—	—	30
K_2SO_4	33	1	0·1	—	—	37
NaH_2PO_4 (M/15)+Na_2HPO_4	43	3	0·4	0·04	0·01	—
$MgCl_2$ (0·10 M)	66	73	63	43	36	67
$MgSO_4$ (0·10 M)	41	16	5	2	0·5	18
LiCl	77	46	31	21	12	74
RbCl	75	21	10	5	1	63
NH_4Cl	63	28	18	7	2	41
$CaCl_2$ (0·10 M)	53	21	13	5	3	—

* % viability referred to viability before drying.
† % viability referred to viability at the end of drying, before exposure to air.

DISCUSSION

The substances tested in this study which protected freeze-dried bacteria from atmospheric oxygen may be conveniently divided into three groups. The first group comprises thiourea and its derivatives and analogues. Comparing the effectiveness of the different methyl derivatives, one sees that the exchange of more than one hydrogen in one or both amino groups of thiourea caused a substantial decrease of protecting power. This was especially evident with the two isomeric dimethyl derivatives; the symmetrical compound was much more effective than the asymmetrical one. Derivatives of isothiourea seemed to possess much less protective ability than thiourea itself. The replacement of sulphur by oxygen, as in passing from thiourea to urea or from thioacetamide to acetamide, decreased the protective power. When one of the amino groups was replaced by methyl (as in thioacetamide) or the hydrazine radical (as in thiosemicarbazide) the protective effect was practically nil. It is difficult to draw definite conclusions from these data about the mode of action of this first group of agents. Perhaps it may be thought that the protective effect is based on the binding of the agent at the site which is sensitive to oxygen, e.g. through a chelation mechanism. It is obvious, in any event, that the reducing property of thiourea and the other compounds found to be effective is not involved in the mode of action, since other similar reducing sulphydryl compounds were either inactive (cysteamine) or even enhanced the deleterious effect of oxygen (dithionite, cysteine, glutathione).

The second group of protective agents comprised the sugars and sugar derivatives. The disaccharides were much less effective than the monosaccharides; at the concentration tested some of the disaccharides were quite ineffective. No correlation was found between the protective efficiency of a sugar and its chemical and steric structure, its fermentability by the bacteria and its reducing power. α-Methylglucoside was not fermented, is non-reducing and was an effective protective agent, while sucrose was not fermented, is non-reducing, and did not protect. Lactose is reducing, was fermented but inactive; glucose and galactose are reducing, were both fermented and possessed good protective power.

The inorganic salts constituted the third group of protective compounds. In some cases, the sodium salt was much more effective than the potassium salt; here the protective action might be ascribed to the cation. On comparing the different chlorides, the smaller cations (lithium, sodium, magnesium) were more potent than potassium, calcium, ammonium or rubidium. Nevertheless, the action of the cation was affected by the anion to which it was linked. This is evident when the protective sodium chloride, bromide and nitrate are compared with the inactive sodium sulphate and phosphate; the same trend was apparent as between magnesium sulphate and the chloride. With the iodides, which are the best protective agents found so far, the sodium and potassium iodides protected equally well. This was also true of the nitrites and thiocyanates tested. In these cases the anion seems to be responsible for the protective action. We are at present unable to suggest a theory that would interpret correctly the effects of all the protective agents found so far. One has to consider the possibility that the different compounds affect the outcome of the reaction between oxygen and dried bacteria at different stages of the process, so that their modes of protective action might be different.

This paper forms part of a thesis submitted by one of us (M. B. L.) to the Hebrew University in partial fulfilment of the requirements for the degree of Ph.D.

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The Oxidase Reaction as a Taxonomic Tool

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SUMMARY

The oxidase test of Kovacs (1956) was applied to 1660 strains of various genera, of which 210 gave a positive reaction and 64 gave a delayed positive reaction. Members of the Neisseriaceae and Pseudomonadaceae were generally oxidase-positive, whereas members of the Enterobacteriaceae and, with few exceptions, Gram-positive organisms, were oxidase-negative; equivocal results were obtained in the Brucellaceae and the genus *Bacillus*. Kovacs test is simple and quickly performed, but very sensitive. Some taxonomic implications of the results are discussed.

INTRODUCTION

The oxidase reaction, based upon the ability of certain bacteria to produce indophenol by the oxidation of dimethyl-*p*-phenylenediamine and α-naphthol, was introduced by Gordon & McLeod (1928) to aid identification of gonococci, although specificity for this organism was not claimed. A 1–1·5 % (w/v) solution of dimethyl-*p*-phenylenediamine hydrochloride was poured over suspected colonies grown on a suitable medium in a Petri plate; oxidase-positive colonies developed a pink colour which successively became maroon, dark red and black in 10–30 min. The use of the more sensitive and less toxic tetramethyl compound was advocated by Ellingworth, McLeod & Gordon (1929). Kovacs (1956) smeared bacterial growth on filter-paper impregnated with 1 % (w/v) aqueous tetramethyl-*p*-phenylenediamine dihydrochloride solution. Oxidase-positive organisms produced a purple coloration on the paper within 10 sec. Kovacs reported that, apart from *Pseudomonas aeruginosa*, the common Gram-negative rods did not produce this coloration. Gaby & Hadley (1957) introduced their 'cytochrome oxidase' test in which a broth culture was used. The appearance of a blue colour on addition of aqueous dimethyl-*p*-phenylenediamine oxalate and ethanolic α-naphthol solutions indicated the presence of this enzyme. This test was modified by Ewing & Johnson (1960) to permit the use of agar slope cultures. Kovacs (1956) oxidase test has now been applied to some of the organisms held in the National Collection of Type Cultures and the results are reported here.

METHODS

Organisms. A total of 1660 strains has been tested. The organisms were grown on the surface of solid media at their optimum temperature for 18–24 hr. or until distinct colonies were obtained.

Oxidase test. Growth was picked off the medium with a platinum loop and rubbed on filter-paper impregnated with 1 % (w/v) aqueous tetramethyl-*p*-phenylene-

diamine dihydrochloride solution. Production of a purple colour within 10 sec. was recorded as positive, its development in 10–60 sec. as delayed positive, and the absence of coloration or its still later development as negative.

Sensitivity of test. Traces of iron catalyse the oxidation of the phenylenediamine compound with the production of a purple colour, which may give rise to false reactions, so that it is essential to use a platinum loop or wire to remove the bacterial growth for testing. The reagent loses its sensitivity and becomes discoloured on storage. A fresh batch was prepared every two weeks and kept in a glass-stoppered bottle, protected from light, in a refrigerator.

RESULTS

Of the 1660 strains tested 210 gave positive and 64 gave delayed reactions (Table 1).

Table 2 gives details of genera in which prompt positive results were obtained. Delayed positive reactions were common in the genera *Bacillus*, *Brucella*, *Haemophilus*, and *Pasteurella*.

Table 1. *Distribution of oxidase activity among bacterial genera*

Total number of strains tested was 1660; of these 210 were positive, 64 were delayed positive and 1386 were negative.

Genus	No. of strains tested	+	(+)	−	Genus	No. of strains tested	+	(+)	−
Achromobacter	18	2	4	12	*Neisseria*	57	57	.	.
Actinobacillus	7	.	3	4	*Nocardia*	9	.	.	9
Actinomyces	13	.	.	13	*Pasteurella*	62	15	10	37
Aerococcus	6	.	.	6	*Proteus*	40	.	.	40
Aeromonas	5	5	.	.	*Pseudomonas*	33	32	1	.
Alcaligenes	7	7	.	.	*Salmonella*	97	.	.	97
Arizona	80	.	.	80	*Serratia*	12	.	.	12
Bacillus	84	2	21	61	*Shigella*	89	.	.	89
Bordetella	23	19	1	3	*Staphylococcus*	97	.	1	96
Brucella	31	14	6	11	*Streptococcus*	130	.	.	130
Chromobacterium	6	.	.	6*	*Streptomyces*	6	.	.	6
Citrobacter	17	.	.	17	*Vibrio*	35	32	.	3
Clostridium	54	.	.	54					
Corynebacterium	91	.	.	91	Miscellaneous bacteria				
Enterobacter†	17	.	.	17	*Bacteroides necrophorus*	1	.	.	1
Erysipelothrix	7	.	.	7	*Coccobacillus mycetoides*	1	.	.	1
Escherichia	107	.	.	107	*Flavobacterium meningosepticum*	1	1	.	.
Haemophilus	33	5	13	15	*Jensenia canicruria*	1	.	.	1
Hafnia	11	.	.	11	*Kurthia zopfii*	2	.	.	2
Klebsiella	274	.	.	274	*Leptotrichium dentium*	1	.	.	1
Lactobacillus	7	.	.	7	*Noguchia granulosis*	1	.	.	1
Leuconostoc	3	.	.	3	*Pediococcus cerevisiae*	1	.	.	1
Listeria	9	.	.	9	*Polysepta pedis*	1	.	.	1
Loefflerella	15	8	3	4	'Pseudomonas iodinum'	1	.	1	.
Lophomonas	4	4	.	.	*Sarcina* sp.	1	.	.	1
Moraxella	10	7	.	3	*Spirillum rubrum*	1	.	.	1
Mycobacterium	40	.	.	40	*Streptobacillus moniliformis*	1	.	.	1

* See discussion.

\+ = positive reaction within 10 sec.; (+) = delayed positive reaction in 10–60 sec.; − = negative reaction.

† The generic name *Enterobacter* was proposed by Hormaeche & Edwards (1960) to replace the generic name *Cloaca*. The genus as now proposed contains two species, *E. cloacae* and *E. aerogenes*.

Oxidase, catalase and aerobiosis

From the results in Tables 1 and 2 it is seen that the oxidase-positive strains were all aerobes or facultative anaerobes, with the exception of *Vibrio fetus* which is micro-aerophilic. Gordon & McLeod (1928) believed the oxidase reaction to be restricted to organisms which grew best in the presence of oxygen and which formed catalase. In a study of organisms resembling *Alcaligenes faecalis*, Moore & Pickett (1960) found 9 oxidase-positive strains which were catalase-negative, but of the organisms studied here all oxidase-positive strains produced catalase. None of the strict anaerobes showed any oxidase activity. Its absence from these organisms and from the Lactobacillaceae is in agreement with the lack of cytochrome in these organisms (Deibel & Evans, 1960).

Table 2. *Species within the oxidase-positive genera of bacteria*

Species	No. of strains	+	(+)	−	Species	No. of strains	+	(+)	−
Achromobacter anitratus	11	.	.	11	*Moraxella liquefaciens*	1	1	.	.
A. equuli	7	2	4	1	*M. lwoffii*	3	.	.	3
Aeromonas hydrophila	3	3	.	.	*Neisseria animalis*	1	1	.	.
A. liquefaciens	1	1	.	.	*N. catarrhalis*	7	7	.	.
A. salmonicida	1	1	.	.	*N. flavescens*	3	3	.	.
Alcaligenes denitrificans	1	1	.	.	*N. gonorrhoeae*	12	12	.	.
A. faecalis	5	5	.	.	*N. meningitidis*	32	32	.	.
A. viscosus	1	1	.	.	*N. pharyngis*	2	2	.	.
Bordetella bronchiseptica	12	12	.	.	*Pasteurella haemolytica*	6	6	.	.
B. parapertussis	3	.	.	3	*P. pestis*	10	.	.	10
B. pertussis	8	7	1	.	*P. pseudotuberculosis*	20	.	.	20
Brucella abortus	12	8	3	1	*P. septica*	26	9	10	7
B. melitensis	6	3	2	1	*Pseudomonas aeruginosa*	14	14	.	.
B. neotomae	4	.	.	4	*P. chlororaphis*	1	1	.	.
B. ovis	1	.	.	1	*P. diminuta*	1	1	.	.
B. paramelitensis	1	1	.	.	*P. fluorescens*	5	5	.	.
B. suis	7	2	1	4	*P. graveolens*	1	1	.	.
Haemophilus aegyptius	3	.	2	1	*P. mucidolens*	1	1	.	.
H. aphrophilus	4	.	.	4	*P. ovalis*	2	1	1	.
H. canis	2	.	1	1	*P. polycolor*	1	1	.	.
H. gallinarum	1	.	.	1	*P. syncyanea*	1	1	.	.
H. haemolyticus	1	.	1	.	*Pseudomonas* spp.	6	6	.	.
H. influenzae	16	4	7	5	*Vibrio choleraeasiaticae*	15	15	.	.
H. parainfluenzae	2	.	.	2	*V. el Tor*	10	10	.	.
H. suis	4	1	2	1	*V. fetus*	1	1	.	.
Loefflerella mallei	5	.	1	4	*V. metchnikovii*	1	.	.	1
L. pseudomallei	10	8	2	.	*V. paracholerae*	1	1	.	.
Lophomonas alcaligenes	4	4	.	.	*V. percolans*	1	1	.	.
Moraxella bovis	3	3	.	.	*V. proteus*	2	.	.	2
M. lacunata	3	3	.	.	*Vibrio* spp.	4	4	.	.

+ = positive reaction; (+) = delayed positive reaction; − = negative reaction.

Gram-positive organisms

With the exception of 'Pseudomonas iodinum', one strain of *Staphylococcus lactis*, and some *Bacillus* spp., oxidase activity was not found among Gram-positive organisms.

Oxidase activity in *Bacillus anthracis* and *B. subtilis* was recorded by Gordon & McLeod (1928) and Price (1929). With Kovacs technique, few prompt positives but

several delayed reactions were obtained among strains of the 22 species of the genus *Bacillus* tested here.

One strain of *Bacillus laterosporus* gave a positive reaction, one a delayed positive and one a negative reaction; with 7 strains of *B. licheniformis*, 1 positive, 2 delayed positive and 4 negative reactions were obtained. Delayed positive reactions were obtained with 5 strains of *B. sphaericus*, 2 of *B. alvei* and one each of *B. freundenreichii* and *B. lentus*. Some strains of the following species gave delayed positive reactions: *B. anthracis* (2 of 4 strains tested), *B. brevis* (1 of 3), *B. cereus* (1 of 7), *B. mycoides* (1 of 3), *B. subtilis* (4 of 14).

Oxidase activity was not observed in the following species: *Bacillus alcalophilus* (1 strain), *B. carotarum* (1), *B. circulans* (4), *B. coagulans* (3), *B. loehnisii* (1), *B. macerans* (4), *B. megaterium* (6), *B. pantothenticus* (4), *B. polymyxa* (2), *B. pumilis* (7) and *B. stearothermophilus* (1).

Gram-negative organisms

Oxidase activity was more common among the Gram-negative organisms. All strains tested of the genus *Pseudomonas* were oxidase-positive, as found by Buttiaux & Gagnon (1958); however, in one strain of *P. ovalis* the reaction was delayed. The observation of Gould & McLeod (1960) that young unpigmented growth of *P. aeruginosa* gave a more intense oxidase reaction than older colonies was confirmed, Köhler (1959) reported *P. fluorescens* to be oxidase-negative but, like Klinge (1960), I could not confirm this.

Only 5 strains of *Aeromonas* were tested, but all were oxidase-positive, a result confirming Ewing & Johnson (1960) who tested 36 strains. All strains of *Achromobacter anitratus* were oxidase-negative but 7 strains of *A. equuli* (*Actinobacillus equuli*) gave equivocal results. Buttiaux & Gagnon (1958) found fewer oxidase-positive strains of *Aeromonas* (5 of 24), but 12 of 23 strains of *Achromobacter* and none of 7 strains of '*B. anitratum*' were positive.

Strains of the genus *Alcaligenes* all gave positive reactions, although the colour produced on the reagent-impregnated paper intensified greatly after contact for 10 sec. Ewing & Johnson (1960) found the genus to be oxidase-positive but they too examined only a few strains. Gaby & Free (1958) considered Kovacs test to be over-sensitive because strains of *A. faecalis* gave reactions similar to those of *Pseudomonas aeruginosa*.

With the exception of *Vibrio metchnikovii* and *V. proteus*, all strains tested of the genus *Vibrio* were oxidase-positive. Oxidase activity was found by Ewing & Johnson (1960) in all of 115 strains of *V. choleraeasiaticae* and in 12 other strains of the genus. The four strains of *Lophomonas alcaligenes* were oxidase-positive, a character of the species (Galarneault & Leifson, 1956). In the genus *Neisseria*, all strains tested gave a positive reaction.

Because of their violet pigment, which was absorbed into the filter paper, difficulty was encountered in determining the oxidase activity of *Chromobacterium* species. Tests made with non-pigmented colonies and young cultures before the pigment was well developed failed to show definite oxidase activity. In Table 1 these strains have therefore been recorded as oxidase-negative, although it may be possible to observe oxidase activity by other test methods. The one strain of *Flavobacterium meningosepticum* gave a positive oxidase reaction, as originally

reported by King (1959). Reports of oxidase activity in this genus and in *Xanthomonas* are conflicting (Buttiaux & Gagnon, 1958; Ewing & Johnson, 1960), mainly because of the small number of strains tested.

Of the total of 1660 strains tested in the present work, 744 were members of the Enterobacteriaceae and none of these gave a positive or even a delayed positive reaction. Oxidase activity was not demonstrated in 800 strains of this family by Buttiaux & Gagnon (1958) nor in 1222 strains tested by Ewing & Johnson (1960). Gordon & McLeod (1928) reported *Serratia marcescens* to be oxidase-positive, but no confirmation of this has been obtained.

The Brucellaceae ('Parvobacteriaceae') was the family most heterogeneous in oxidase activity. Two strains of *Actinobacillus actinomycetemcomitans* and one strain each of *Bacteroides necrophorus*, *Noguchia granulosis* and *Streptobacillus moniliformis* were oxidase-negative. Of 5 strains of *A. lignieresi*, 3 gave a delayed positive reaction when colonies from blood agar plates were tested, but very weak or negative results when serum glucose agar was used.

All strains of *Bordetella bronchiseptica* and *B. pertussis* were oxidase-positive (although in one strain of the latter the reaction was delayed 15 sec.) whereas those of *B. parapertussis* were negative. Gordon & McLeod (1928) found that stock strains of *B. pertussis* on Dorset egg medium gave a positive reaction but that fresh isolates were negative. They noted that uninoculated Bordet-Gengou medium gave a positive reaction on addition of the test reagent. Using the tetramethyl compound (Ellingworth *et al.* 1929), *B. pertussis* was found to be oxidase-positive. Lacey (1960) reported that both *B. bronchiseptica* and *B. pertussis* gave positive oxidase reactions within 30 sec. when a loopful of the reagent was applied to the bacterial growth. Lautrop (1960) considered these two species to be oxidase-positive within 15 sec. by Kovacs method; he also noted the possibility of false positives due to the high proportion of blood in many media for *B. pertussis*. For the tests reported here, this organism was grown on Bordet–Gengou medium, except for two phase IV strains which were grown on Lemco agar.

The older species of *Brucella* gave equivocal results whereas strains of two proposed new species, *B. neotomae* and *B. ovis*, did not show oxidase activity. It was not possible to distinguish American and Danish strains of *B. suis* on their oxidase activity. *B. melitensis* was reported by Gordon & McLeod (1928) to be oxidase-positive. In a quantitative study Richardson (1957) showed that species of this genus differed markedly in their cytochrome oxidase activity; typical strains of *B. abortus* had twice the activity of *B. melitensis* and more than twice that of *B. suis* or thionin-resistant strains of *B. abortus*. Strong peroxidase activity was reported (Krčméry, 1959) in *B. suis*, and Smith (1954) noted that *p*-phenylenediamines might be oxidized by peroxidases. It is thus possible that an 'oxidase' activity in *B. suis* may be a reflexion of this side reaction rather than of oxidase proper.

Equivocal results were also obtained in the genus *Haemophilus*; over half the strains tested showed some oxidase activity. With dimethyl-*p*-phenylenediamine, *H. influenzae* was originally reported to be oxidase-negative, but use of the more sensitive tetramethyl compound showed it to be positive (Ellingworth *et al.* 1929). With the exception of *H. influenzae*, few strains of the other species of this genus were tested in the present work. The 4 strains of *H. aphrophilus*, which were grown in an atmosphere of CO_2, were all oxidase-negative.

Strains of *Loefflerella pseudomallei* were all oxidase-positive, although in two strains the reaction was delayed (15 sec.), whereas 4 strains of *L. mallei* gave a negative and 1 strain a delayed (30 sec.) positive reaction. Gordon & McLeod (1928) reported *L. mallei* as oxidase-positive and Fournier (1959) reported *L. pseudomallei* to be slowly positive by Gaby & Hadley's method. Miller *et al.* (1948) reported both species to be oxidase-positive. With the exception of *Moraxella lwoffii*, all members of the genus *Moraxella* were oxidase-positive, this is in agreement with the results of Henriksen (1952).

Strains of *Pasteurella pseudotuberculosis* and *P. pestis* were devoid of oxidase activity. *P. pestis* has been reported as weakly oxidase-positive (Gordon & McLeod, 1928). Strains of *P. haemolytica* gave positive reactions whereas variable results were obtained with *P. septica*.

DISCUSSION

The oxidase reaction was originally thought to depend on the presence of both peroxide and peroxidase (Gordon & McLeod, 1928). It is now believed to be due to the presence of a cytochrome oxidase, the enzyme which catalyses the oxidation of reduced cytochrome by molecular oxygen and which acts as the terminal stage in electron transfer. Most aerobic and facultatively anaerobic bacteria contain a cytochrome system which acts as an electron carrier in aerobic respiration. Many of these organisms also produce catalase. The cytochromes are a group of respiratory pigments comprising a number of related iron-porphyrin compounds. The distribution of these cytochromes varies with different bacteria; some have several while others have only one or none at all. Most workers have used the visual spectroscope to examine the absorption spectra of bacterial cytochromes, a technique which is not sufficiently sensitive to distinguish between cytochromes whose absorption maxima are close together. Smith (1954), in a review of bacterial cytochromes, pointed out the disagreement among various workers about the distribution of the cytochromes. Castor & Chance (1959) demonstrated the existence of four different cytochrome oxidases in various bacteria.

Many of the oxidase-positive organisms (Tables 1 and 2) have been reported in the past to contain cytochrome *c*, a component which is not autoxidizable but only oxidized in the presence of cytochrome *c* oxidase. The distribution, identity and nomenclature of the various cytochrome components remain confused, however. Consequently in this paper the term 'oxidase' is used without prejudice and no assumptions have been made about the possible identity of the material responsible for positive oxidase reactions with cytochrome *c* oxidase or any other component of the bacterial cell.

For a test to be of taxonomic value it is imperative that the results be reproducible with a standard technique. The oxidase test used at present differs in different laboratories both in the method and the reagents used. Gaby & Free (1958) claimed that the cytochrome oxidase test (Gaby & Hadley, 1957) was more accurate than Kovacs method, which they considered to be over-sensitive. Buttiaux & Gagnon (1958) found Gaby & Hadley's reaction to be suitable with *Pseudomonas* strains but not with other organisms which produced a slow coloration, making it difficult to decide whether it was a slow oxidase reaction or autoxidation of the reagent. Kovacs method, on the other hand, was rapid, simple and sensitive. Köhler (1959)

found a margin of error of up to 12 % in Gaby & Hadley's test and the observation time had to be increased to 20 min. Klinge is reported to have stated (Billing, 1960) that the number of strains of *P. aeruginosa* giving a positive reaction depended on the medium on which they were grown. Kovacs method, besides being simpler and quicker than the other methods, has the advantage that only a portion of a colony is needed for the test. It is undoubtedly a more sensitive method of determining oxidase activity than the other tests. The reagents used in the various methods include *p*-phenylenediamine hydrochloride, dimethyl-*p*-phenylenediamine (also known as *p*-aminodimethylaniline) hydrochloride or oxalate (Carpenter, Suhrland & Morrison, 1947) and tetramethyl-*p*-phenylenediamine dihydrochloride, all with or without the addition of aniline or α-naphthol. The instability of solutions of the *p*-phenylenediamines is a disadvantage common to all test methods.

Taxonomic implications

Although in many genera only a few strains have been examined, the taxonomic implications of the results need appraisal. The genus *Aeromonas* has many of the characters of the Enterobacteriaceae; it differs in being oxidase-positive and polarly flagellate. It has been suggested (Stevenson, 1959) that all members of the genus *Aeromonas* are unpigmented members of the genus *Serratia*. Oxidase activity has not been found in strains of *Serratia* and there is no evidence that the pigment prodigiosin plays any role in respiration in a manner analogous to that of 'oxidase'; Stevenson's hypothesis is therefore not supported.

The genus *Alcaligenes* is not yet satisfactorily delineated and the literature abounds with contradictory statements about its oxidase activity. Türck (1952) found that her strains formed two groups, oxidase-positive vibrio-like forms and oxidase-negative non-motile rods. Buttiaux & Gagnon (1958) considered that *Alcaligenes* should be included in *Achromobacter*, as they could not find *Alcaligenes faecalis* to be motile. Klinge (1958) thought that 'Bacterium anitratum', B 5 W organism, 'Diplococcus mucosus' and similar organisms should be classified as *Alcaligenes*. He rejected pleomorphism as a criterion for classification but stressed the importance of a negative oxidase reaction. Moore & Pickett (1960) questioned the validity of the genus *Alcaligenes* because they found few isolates of *A. faecalis* which resembled the original description in having peritrichous flagella. Of their 40 strains, 20 were oxidase-positive and 2 weakly positive and they consider they should be included in the genus *Achromobacter*. Haupt (1957) listed *Achromobacter equuli*, which gave equivocal results in my tests, as *Actinobacillus equuli*. Accepting this, it may be suggested that it would be expedient for oxidase-positive organisms now in the genera *Achromobacter* or *Alcaligenes* to be classified as *Alcaligenes* and oxidase-negative organisms as *Achromobacter*. A strain of *Alcaligenes faecalis* (NCTC 415) designated as 'type' by Winslow, Kligler & Rothberg (1919) is oxidase-positive as is the type strain of *A. denitrificans*.

The absence of oxidase activity in *Moraxella lwoffii* distinguishes it from the other recognized species of *Moraxella*. Henriksen (1952) discussed the taxonomy of this genus and considered that it might be closely related to *Neisseria*, in a relation analogous to that between lactobacilli and streptococci. In a later paper (1960) he stated that *M. lwoffii* did not belong to the genus *Moraxella*. Klinge (1958) would

include *M. lwoffii* in the genus *Alcaligenes*, but from my results it would seem better to place it in the genus *Achromobacter*.

Brindle & Cowan (1951) demonstrated that the type of flagellation of *Loefflerella pseudomallei* warranted its inclusion in the Pseudomonadaceae; they concluded, however, that it was closely related to *L. mallei* and the two species should form a genus which should not be combined with any then accepted genus of the Pseudomonadaceae. Wetmore & Gochenour (1956) agreed that *L. pseudomallei* could be included in this family. Fournier (1959), however, considered that there was no phylogenetic relation between this organism and *Pseudomonas aeruginosa*, and that *L. pseudomallei* and *L. mallei* were species of the same genus. *Bergey's Manual* (1957) listed these two organisms in different genera, as *Pseudomonas pseudomallei* and *Actinobacillus mallei*. The demonstration of oxidase activity in the former and its virtual absence from the latter is additional evidence for the inclusion of *L. pseudomallei* in the Pseudomonadaceae and the exclusion of *L. mallei* from this family.

'Pseudomonas iodinum' is a non-motile, non-sporing Gram-positive rod. Sneath (1960) did not consider that it should be included in the genus *Pseudomonas* as it has the morphology of a diphtheroid; he thought that it may belong to the genus *Corynebacterium* or *Brevibacterium*. Its oxidase activity excludes it from the first genus, however, if absence of oxidase activity is a character of the genus as a whole. (Only members of this genus which are of medical or veterinary interest were examined.) Reports have not been found in the literature about oxidase determinations in species of *Corynebacterium* from plant sources or in species of *Brevibacterium*. For the present it is felt that this organism should be retained in the genus *Pseudomonas* until an 'oxidase spectrum' has been determined.

In addition to the organism tested here, the oxidase activity of some other genera has been reported. Ewing & Johnson (1960) found only 3 slow and weak positives among 34 strains of *Erwinia* tested. Oxidase activity has been demonstrated in some strains of *Veillonella* (Berger, 1960), and in some leptospires (Goldberg & Armstrong, 1959; Faine, 1960) although Czekalowski, McLeod & Rodican (1953) obtained equivocal results with these organisms.

The results reported here confirm the claims made by various workers that the oxidase test is of value in distinguishing between different groups of organisms. Previously attention has been directed to the Enterobacteriaceae and the Pseudomonadaceae, but evidence is now accumulating that the test can be of value in other groups. Of the methods for carrying out the test that of Kovacs is reliable so long as freshly prepared reagent is used and a time limit for positives is set which is in accordance with the degree of sensitivity required.

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Inhibition of Enzyme Formation Following Infection of *Escherichia coli* with phage T2r$^+$

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SUMMARY

The synthesis of aspartate carbamyl transferase (ureidosuccinic synthetase), of dihydroorotic dehydrogenase and of alkaline phosphatase by a uracil-requiring strain of *Escherichia coli*, when the organisms were suspended in a minimal medium lacking uracil, was followed subsequent to infection of the cells with bacteriophage T2r$^+$ or with ghosts of the bacteriophage particles. The results were compared with the synthesis of these enzymes in uninfected cells. Following infection, the formation of all three enzymes was halted. Supplementation of the medium with casein hydrolysate and tryptophan or with 0·01 M-Mg^{++} + 10^{-3} M-spermine did not prevent the inhibition caused by bacteriophage infection.

INTRODUCTION

When *Escherichia coli* in logarithmic growth is infected with phage T2, the net synthesis of protein continues but at a linear rather than a logarithmic rate (Cohen, 1948). The majority of the protein synthesized appears to be phage protein or enzymes concerned with phage formation (Bessman, 1959; Sommerville, Ebisuzaki & Greenberg, 1959; Kornberg, Zimmerman, Kornberg & Josse, 1959; Keck, Mahler & Fraser, 1960). Whether a cell infected by a lytic phage is capable of synthesizing protein not concerned directly with phage synthesis was examined earlier by Monod & Wollman (1947) and by Benzer (1953). They found that β-galactosidase was not formed by these cells following the addition of the inducer, lactose. French & Siminovitch (1955) also found that this enzyme was not formed following infection with phage ghosts. Sher & Mallette (1954) also were unable to demonstrate the continued formation of the inducible lysine decarboxylase after addition of either phage T2r$^+$ or phage ghosts. However, even under conditions where limited amounts of protein can be synthesized, bacteria are able to synthesize relatively large amounts of enzymes in response to the removal of a repressor substance for these enzymes from the medium (Gorini & Maas, 1957; Vogel, 1957; Yates & Pardee, 1957; Levin & Magasanik, 1961). By using such a system, we have examined the effect of phage infection on the ability of *Escherichia coli* to synthesize the enzymes aspartate carbamyl transferase (ureidosuccinic synthetase), dihydroorotic dehydrogenase and alkaline phosphatase after removal of uracil and inorganic phosphate from the medium. These enzymes were chosen because under certain circumstances they appear to be essential for the formation of nucleic acid precursors.

METHODS

Organism. Strain B_{39}, a uracil-requiring mutant of *Escherichia coli* B, obtained from Dr F. C. Neidhardt (Harvard Medical School, Boston, Mass., U.S.A.), was used in these studies.

Bacteriophage. Coli phage strain T2r$^+$ maintained in this laboratory was used as the infecting agent and as the source of phage ghosts. Phage T2r$^+$ stocks were prepared from lysates of bacteria grown in the basal salts medium and were purified by the method of Hook *et al.* (1946).

The ghosts were prepared according to the procedure of Herriott & Barlow (1957). The protein content of ghost suspensions and of the starting phage preparation were measured; the % protein remaining in the ghost suspension multiplied by the phage titre was accepted as the ghost titre. The viable phage titre of the ghost suspension was less than 0·2 % of the original phage titre and was less than 0·5 % of the ghost titre.

Media. Bacterial cultures were maintained on agar slopes containing (%, w/v): Tryptone, 1·0; yeast extract, 0·5; $Na_2HPO_4.2H_2O$, 0·3; KH_2PO_4, 0·1; glucose, 0·5; agar, 2·0. The cultures were transferred to fresh medium at 2-week intervals.

The composition of the basal medium used for growing and infecting the bacteria was (%, w/v): NH_4Cl, 0·1; $Na_2HPO_4.2H_2O$, 1·05; KH_2PO_4, 0·45; $MgSO_4.H_2O$, 0·01; NaCl, 0·1. Glucose added to a final concentration of 0·2 % (w/v) was used as the carbon and energy source; uracil when added to the medium was at 50 μg./ml.

When a medium lacking inorganic phosphate was desired 2-amino-2-hydroxymethylpropane-1:3-diol (tris) adjusted to pH 7·4 with HCl was used to buffer the medium. The final concentration of tris was 0·1 M.

The phage particles were stored in a diluting fluid composed of (%, w/v): NaCl, 0·65; $MgCl_2.6H_2O$, 0·2; gelatin, 0·07.

Nutrient agar was used for performing phage assays according to the agar layer technique of Adams (1950).

Chemicals. Uracil and L-tryptophan were obtained from L. Light & Co., L-dihydroorotic acid and tris buffer were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A.; ureidosuccinic acid and paranitrophenylphosphate were obtained from British Drug Houses, Ltd, spermine tetrahydrochloride was obtained from Hofmann La Roche, Basle, Switzerland. Tryptone, yeast extract and casein hydrolysate were obtained from Oxo, Ltd. Dilithium carbamylphosphate was generously provided by Mr T. Gascoyne of this Department. All other chemicals were of reagent grade.

Preparation of suspensions of organisms. Escherichia coli B, strain B_{39}, was grown overnight at 37° with forced aeration in the basal medium containing uracil. Fifteen ml. of this culture were used to inoculate 600 ml. fresh medium of the same composition, and the culture was then grown for three to four generations under similar conditions. The organisms, which were growing logarithmically, were centrifuged down, washed twice with basal salts medium and then resuspended in one-half to three-quarters of the initial volume of basal medium. The viable counts of these suspensions were from 8×10^8 to 2×10^9 organisms/ml. Portions (25 ml.) of these suspensions were distributed into 100 ml. Erlenmeyer flasks and after the flasks were placed in a water bath at 37°, the cultures were incubated with forced aeration for the desired length of time and with the necessary additions. For the studies on alkaline phosphatase for-

mation, organisms were washed and resuspended in the tris buffer medium; subsequent steps were as described above.

Preparation of enzyme extracts and determination of activities. Portions (20–25 ml.) of culture were centrifuged, the deposit washed with cold distilled water and then resuspended in 5·0 ml. cold water. The suspension was then disrupted for 4–5 min. in a 19 kc. 60 W. sonic oscillator (Mullard-Measuring and Scientific Equipment Ltd., London). This preparation was then centrifuged for 5 min. at 12,000*g* and at 0–5° in an International refrigerated centrifuge. The supernatant solution was decanted and tested for its aspartate carbamyl transferase and dihydroorotic dehydrogenase activities by the procedures of Yates & Pardee (1957). Extracts for the determination of alkaline phosphatase activity were prepared and tested according to the procedure of Torriani (1960). Protein was measured by the method of Lowry, Rosebrough, Faar & Randall (1951).

RESULTS

Aspartate carbamyl transferase

Yates & Pardee (1957) demonstrated rapid synthesis of aspartate carbamyl transferase by uracil-requiring auxotrophs following exhaustion of uracil from the medium in which the organisms had been growing. When *Escherichia coli* B, strain B_{39}, was incubated in basal medium lacking uracil, there was an increase with time in the enzymic activity of the cell extracts. This increase in activity depended on the presence of a source of nitrogen in the basal medium (Fig. 1, curve A). More rapid synthesis of enzyme occurred following the addition of amino acids in the form of casein hydrolysate + tryptophan than occurred after the addition of the simple nitrogen source NH_4Cl (Fig. 1, curves E and B).

Table 1. *Aspartate carbamyl transferase activity of extracts from uninfected and infected cells of* Escherichia coli *B, strain* B_{39}

62·5 μg. of protein of an extract prepared from uninfected cells was used in A and C. 72·5 μg. of protein of an extract from infected cells was used in B and C. The reaction mixtures were incubated for 60 min. at 25°. The activity in C was 92·5 % of the sum of the activities of A and B.

Extract	Ureidosuccinic acid formed (μmole)
A. Uninfected cells	5·7
B. Infected cells	2·4
C. A + B	7·5

The effect of phage T2r$^+$ on the synthesis of the enzyme is shown also in Fig. 1. Addition of phage T2r$^+$ 5 min. after suspending the organisms in uracil-free medium prevented the continued synthesis of enzyme normally found in uninfected cultures. This complete inhibition could be demonstrated despite different rates of synthesis proceeding at the time of infection (Fig. 1, curves D and G). Attempts to increase the rate of enzyme synthesis at the time of infection also failed to prevent the complete inhibition (Fig. 1, curve C_1 compared with curve D). The addition of ghosts also caused a complete inhibition of enzyme synthesis (Fig. 1, curves C_2 and F).

The possibility that phage infection prevented the appearance of increased activity of extracts by causing the production of an inhibitor of enzyme action, rather than by

causing an inhibition of enzyme formation, proved unlikely since it was found that the addition of an extract from infected organisms to an extract from uninfected organisms changed only slightly the activity of the total preparation as compared with the sum of the two separate activities (Table 1).

That phage infection of *Escherichia coli* B, strain B_{39}, caused the formation of uracil, just as phage infection of *E. coli*, strain $15T^-$, caused the formation of thymine (Barner & Cohen, 1954), and that the uracil formed subsequently prevented the synthesis of the enzyme appeared unlikely since no change occurred in the total number of infective centres in a culture of *E. coli* B, strain B_{39}, infected in the absence of uracil and incubated for 95 min. (Fig. 2).

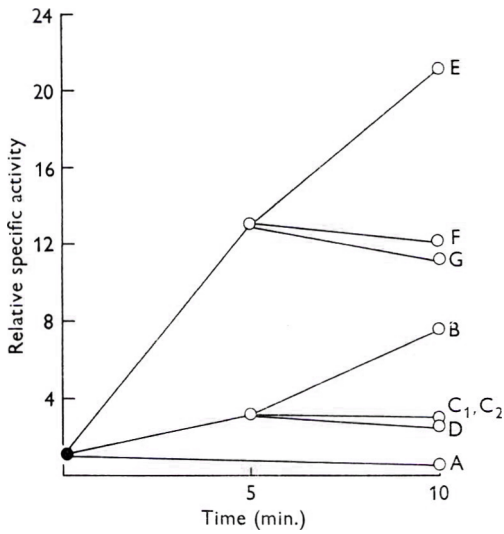

Fig. 1

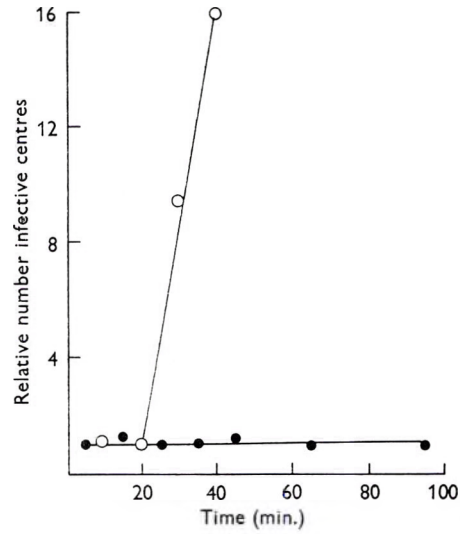

Fig. 2

Fig. 1. The effect of nitrogen sources, bacteriophage and ghosts on aspartate carbamyl transferase formation in *Escherichia coli* B, strain B_{39}. The values plotted are the specific activities of extracts relative to zero time. Specific activities are the μmole ureidosuccinic acid formed/mg. protein/hr. Curves: A, no nitrogen source added; B, NH_4Cl (0·2 %) added at zero time; C_1, $T2r^+$, casein hydrolysate (0·2 %, w/v) + tryptophan (0·1 %, w/v) added at 5 min.; C_2, ghosts added at 5 min.; D, phage $T2r^+$ added at 5 min.; E, casein hydrolysate (0·2 %, w/v) + tryptophan (0·1 %, w/v) added at zero time; F, ghosts added at 5 min.; G, phage $T2r^+$ added at 5 min. The multiplicities of infection were: C_1, 4·7; C_2, 6·0; D, 7·9; F, 2·4; G, 3·9.

Fig. 2. The total number of infective centres of phage $T2r^+$ relative to the number added to a culture of *Escherichia coli* B, strain B_{39}. (●), No uracil in the medium; (○), uracil added. Bacterial count = $1{\cdot}6 \times 10^6$ organisms/ml. Multiplicity of infection, 0·01. For other details of procedure see Burton (1955).

High multiplicities of infection can cause cell lysis (Delbrück, 1940) and thus release repressor molecules into the medium. Therefore, a medium was chosen in which one might expect the degree of cell lysis to be lower and consequently in which one might expect the apparent inhibition of enzyme formation caused by bacteriophage infection to be prevented. Infection was carried out in a medium containing 0·01 M-Mg^{++} and in a medium containing 0·01 M-Mg^{++} + 10^{-3} M-spermine. The addition of spermine to a basal medium has been shown to maintain spheroplasts of

Escherichia coli (Mager, 1959). In neither case was synthesis of enzyme carried out (Fig. 3). The inhibition of enzyme formation in the medium containing 0·01 M-Mg^{++} remained complete for as long as 90 min. (curve E) despite the fact that no viable phage particles were formed during this period.

Dihydroorotic dehydrogenase

Dihydroorotic dehydrogenase also has been found to increase in pyrimidine auxotrophs maintained in the absence of uracil (Yates & Pardee, 1957). The formation of this enzyme by infected and uninfected organisms of *Escherichia coli* B, strain B_{39}, was examined; results similar to those found with aspartate carbamyl transferase were obtained (Table 2).

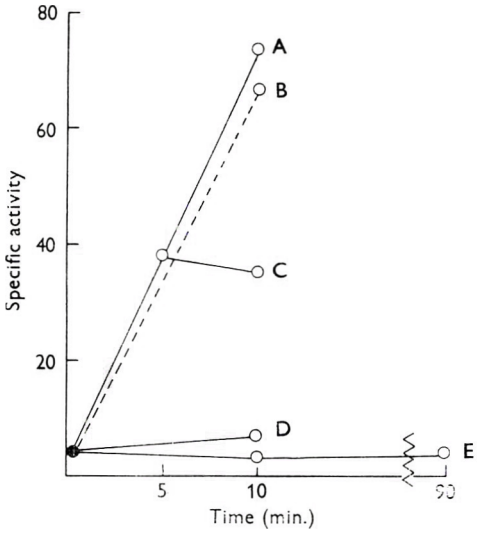

Fig. 3

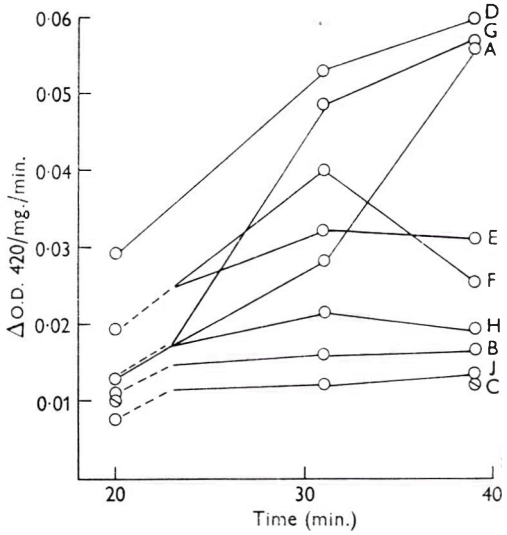

Fig. 4

Fig. 3. The effect of Mg^{++}, spermine and phage T2r$^+$ on aspartate carbamyl transferase formation in *Escherichia coli* B, strain B_{39}. Curves: A = Mg^{++}, 0·01 M; B = A + spermine 10^{-3} M, at zero time; C = A with phage T2r$^+$ added at 5 min.; D = B with phage T2r$^+$ added at zero time; E = A with phage T2r$^+$ added at zero time. The multiplicities of infection were 14·2 in curve C and 17·7 in curves D and E.

Fig. 4. Formation of alkaline phosphatase by *Escherichia coli* B, strain B_{39}.

Series 1. Multiplicity of infection = 35·8; no addition at zero time. Curve A, no addition; curve B, phage T2r$^+$ added; curve C, phosphate added, two points only, ⊘.

Series 2. Multiplicity of infection = 25·1; uracil added at zero time. Curve D, no addition; curve E, phage T2r$^+$ added; curve F, phosphate added.

Series 3. Multiplicity of infection = 32·6; no addition at zero time. Curve G, uracil added; curve H, uracil + phage T2r$^+$ added; curve J, phosphate + uracil added.

Additions were made at 23 min. The final concentration of phosphate was 80 μmole/ml.; of uracil was 50 μg./ml.

Alkaline phosphatase

The synthesis of alkaline phosphatase was examined as another example and also because it is controlled by a different repressor, namely inorganic phosphate (Torriani, 1960). When organisms of *Escherichia coli* B, strain B_{39}, were suspended in a medium which did not contain inorganic phosphate, it was found that the presence or

absence of uracil did not markedly affect ability to make alkaline phosphatase enzyme (Fig. 4, curves A, D, G). The addition of inorganic phosphate (curves C, F, J) or phage T2r$^+$ (curves B, E, H) caused inhibition of enzyme formation. It was found also that ghosts of phage T2r$^+$ caused an inhibition of enzyme formation.

Table 2. *Dihydroorotic dehydrogenase activity in extracts from uninfected and infected cells of* Escherichia coli *B, strain* B_{39}

Organisms were suspended in basal medium with the additions noted in the table. The multiplicities of infection were 14·2 in C and 17·7 in B and E.

	Additions	Time of addition of T2r$^+$ (min.)	Time of sampling (min.)	Orotic acid formed (μmole/mg./hr.)
A.	Mg^{++}, 10^{-2}M	—	0	5·3
			5	7·9
			10	11·8
B.	Mg^{++}, 10^{-2}M	0	10	4·5
C.	Mg^{++}, 10^{-2}M	5	10	6·7
D.	Mg^{++}, 10^{-2}M + spermine, 10^{-3}M	—	10	11·7
E.	Mg^{++}, 10^{-2}M + spermine, 10^{-3}M	0	10	4·1

DISCUSSION

Our results show that following infection with phage T2r$^+$ or with phage ghosts, organisms of *Escherichia coli* B, strain B_{39}, did not synthesize certain enzymes, where the formation of the enzymes had been repressed by previous growth conditions, even though the repressors had been removed from the medium shortly before the infection. That the action of the phage in stopping the formation of host protein was due to a more favourable competition of the infecting system for available amino acids seems unlikely in view of the findings obtained here that supplementation of the infecting medium with amino acids did not prevent the inhibition of enzyme formation. A medium of greater osmotic pressure which might have decreased the loss of cell constituents into the medium also did not affect the inhibition of enzyme formation caused by phage infection. Although in some of the experiments presented, very small increases were seen in enzyme activity over those at the time of addition of the phage, these increased activities represented maximum values of 0·5–3 min. of continued synthesis based on the rate of synthesis of uninfected cells. The techniques used in the present work would not be able to detect whether synthesis actually continued after infection for these lengths of time, or whether there might have been a delay in the adsorption or penetration of phage, leading to small increases in the amount of enzyme.

The effects of ^{32}P decay on the viability of bacteria and on their ability to form enzymes have been interpreted as suggesting that intact deoxyribonucleic acid (DNA) is necessary for the synthesis of bacterial enzymes (McFall, Pardee & Stent, 1958; Riley, Pardee, Jacob & Monod, 1960). This hypothesis would provide a plausible explanation of the results observed here, since there is extensive breakdown of the host DNA following phage infection (Weed & Cohen, 1951; Kozloff, Knowlton, Putnam & Evans, 1951; Hershey, Dixon & Chase, 1953; Burton, 1955). In another situation, McFall & Magasanik (1960) found that alkaline phosphatase was formed in a thymine-deficient culture of *Escherichia coli*, strain 15T$^-$, when the synthesis of

β-galactosidase and other proteins was inhibited. McFall & Magasanik suggested that the inhibition of β-galactosidase synthesis was a result, not of irreversible nuclear damage, but of repression. The possibility that the enzymes we have studied are not formed because of an accumulation of endogenous repressor substances seems to be unlikely, because the enzymes are involved in the synthesis of the precursors of DNA which are normally utilized by the phage-infected cell at an enhanced rate. However, other mechanisms are possible; one is suggested by the observations of the effect of phage infection on the permeability of the cell (Puck & Lee, 1954, 1955) and by the observation of Bessman & van Bibber (1959) that guanine deoxynucleotide kinase synthesized after phage infection requires different activating metal ions from the host enzyme which catalyses the same reaction. It is possible that the synthesis of host protein has different ionic requirements from those of the synthesis of protein in the phage infected cell.

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The Action of Tetanus Toxin in Frogs

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SUMMARY

The development of tetanus intoxication in the frog is dependent on the environmental temperature being above about 15°. The incubation period and time to death become shorter as the environmental temperature is raised. Cooling below about 15° prevents both the fixation of the toxin and its action after fixation but does not increase the speed at which the toxin is destroyed or excreted. The absorption of the toxin after injection into the dorsal lymph sac is not prevented by cooling. It is possible to produce local tetanus in frogs by the intramuscular injection of tetanus toxin, but the dose needed for this is very critical. In most cases either generalized tetanus develops or there are no signs of intoxication. However, local tetanus can regularly be produced when the toxin is given intramuscularly to frogs partially protected from generalized tetanus by an injection of tetanus antitoxin given via the dorsal lymph sac. The local tetanus so produced does not progress to involve the opposite limb. The muscular spasm is abolished by cutting the motor nerve or by general anaesthesia. Tetanus toxin appears to act on the central nervous system of the frog in the same manner as in mammals. However, frog brain tissue does not neutralize tetanus toxin in low concentrations as does mammalian brain tissue, but concentrated tetanus toxoid gives immediate protection to frogs in the same way as it does in mammals.

INTRODUCTION

The action of tetanus toxin on cold blooded vertebrates has not been extensively studied, but the majority of those species on which the toxin has been tested have proved susceptible. However, the dose of toxin required to produce tetanus is much greater than for even the most resistant of mammals and susceptibility is dependent on the environmental temperature being above a certain level. Courmont & Doyon (1893) were the first to observe that frogs became susceptible to tetanus toxin when kept at a temperature above 20°. Morgenroth (1900) endeavoured to analyse the mechanism of the temperature-dependent resistance of the frog to tetanus toxin. He inoculated frogs kept at 8° with tetanus toxin and found that they remained normal unless warmed to 32° when, after 2–3 days, they developed tetanus. However when, after being warmed for only 24 hr. and before signs of intoxication occurred, they were returned to the cold they remained in good health. On being warmed a second time, these frogs developed tetanus after a shorter interval than frogs similarly inoculated and warmed for the first time. Cold therefore arrests the development of tetanus even when part of the incubation process has taken place. It does not however annul the process of intoxication, because the sum of the two successive incubation periods remained the same as that of the single one which had been uninterrupted

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by any cooling. The only other amphibian about which the action of tetanus toxin has been reported is the axolotl, a larval salamander of the genus *Amblystoma*, which Metchnikoff (1897) found to be very sensitive. Of other poikilothermic vertebrates, there is one report on the effect of tetanus toxin on fish, by Lapenta (1932) who suggested that the gold fish *Carassius auratus* would be very suitable for the assay of tetanus toxin. The reptiles have been a little more fully investigated. Grasset & Zutendyk (1931), in South Africa, were able regularly to produce signs of tetanus in lizards, young crocodiles and snakes, but tortoises were only susceptible to intracerebrally injected toxin. Cowles & Nelson (1947) used the American crested lizard and were able to produce signs of tetanus. However, Metchnikoff (1897) failed to demonstrate tetanus in the European green lizard. With all the susceptible reptiles it was found that the development of tetanus intoxication depended on the environmental temperature. At low temperatures signs of tetanus either failed to appear or the incubation period was longer than at higher temperatures.

The present investigation was undertaken to see whether tetanus toxin appears to act in frogs in the same way as in mammals, or whether in these cold-blooded partially resistant animals it has some different mode of action.

METHODS

Frogs and aquaria. The frogs used in all the following experiments were of the common British species *Rana temporaria* and were obtained at intervals as required from Cornwall. They were of both sexes and varied in weight between 15 and 30 g. Galvanized iron tanks about 2 ft. square were used as aquaria. Different environmental temperatures were obtained by means of thermostatically-controlled low temperature electric immersion heaters or by placing the tanks in a refrigerated room. In most experiments control animals were kept in the same tank as the intoxicated ones. It was rare for a control frog to die from natural causes; they remained healthy over long periods at temperatures between 5° and 27°.

Blood samples from frogs. These were obtained by exposing the heart under ether anaesthesia and puncturing the ventricle with a fine glass pipette containing 10 $mm.^3$ heparin (Liquemin, Roche Products Ltd, 5000 i.u./ml.). The blood was centrifuged and about 0·25 ml. plasma obtained per frog.

Tetanus toxin. The toxin preparation was kept as a finely ground powder in a vacuum desiccator until dissolved in sterile saline a few minutes before use. Two preparations of tetanus toxin were used. The first was some of that described by Wright, Morgan & Wright (1950) and will be referred to as toxin A; the LD50 dose for mice, determined by the death time method of Ipsen (1941) was about 0·05μg. The second preparation of tetanus toxin, toxin B, was provided by Dr Mollie Barr (The Wellcome Research Laboratories); the LD50 dose for mice was 0·005μg.

Tetanus antitoxin. The antitoxin containing 1500 international units/ml. was supplied by Burroughs Wellcome and Co. Ltd.

Tetanus toxoid. A concentrated preparation of tetanus toxoid (TD 343 D) containing 1175 Lf doses/ml. was provided by Dr Mollie Barr.

RESULTS

Generalized tetanus in frogs

The concentration of toxin in the blood following inoculation. In most of the following experiments the inoculations were made into the dorsal lymph sac. Thorotrast (a thorium dioxide preparation) injected by this route reaches the blood stream very rapidly and then slowly passes from the blood into the tissue fluids (Foxon & Rowson, 1956). That tetanus toxin behaves in the same way seemed very probable but some experiments were made to confirm this and to determine the effect of different environmental temperatures on the concentration of toxin in the blood following injection into the dorsal lymph sac. As it is not possible to take samples of blood from the same frog at intervals, a group of similar frogs were each injected with 1·0 mg. tetanus toxin A in 0·5 ml. saline and bled at different times. The frogs were kept in three groups at 5°, 15° and 26°.

Table 1. *The plasma concentration of tetanus toxin following injection into frog dorsal lymph sac*

Time after injection of 1·0 mg. tetanus toxin (days)	Frogs kept at		
	5°	15°	26°
	Plasma level of tetanus toxin (in mouse LD_{50}/ml.)		
0·125	—	560	625
1	185 (2)	520	450
2	280 (2)	235	300
3	305 (2)	130	340 (2)
4	310	230	275
5	295	150 (2)	250
6	300	—	350 (2)
8	150	—	—
12	—	85	—
14	190	—	—
18	—	62	—
19	—	26	—
21	150	—	—
33	250	—	—

Values marked (2) are the average of two experiments.

Table 1 shows the amount of tetanus toxin found in the plasma of these three groups of frogs. At 15° and 26° the blood concentration was very high after only 3 hr. and then decreased fairly rapidly during the next 3–4 days as the toxin passed from the plasma into other tissues. At 5° the concentration in the blood increased slowly during the first 3 days, to reach about the same value as that found in the other two groups at this time. The frogs at 5° were lethargic, showed very little voluntary movement even when disturbed, and the lymph hearts beat at a decreased rate. Therefore it was not surprising that toxin injected into the dorsal lymph sac reached the blood stream more slowly in frogs at 5° than in those at higher temperatures. Because the toxin was entering the circulation at a diminished rate in frogs at 5°, there was time for it to become distributed throughout the body fluids while it was being absorbed from the dorsal lymph sac, with the result that the maximum concentration in the blood found in these frogs was not so high as in those kept at 15° or

26°. In the frogs at 26° death occurred before the seventh day, so the toxin concentration could only be followed for 6 days. However, in the other two groups clinical tetanus did not develop and the blood toxin concentration was followed and found to decrease very slowly, especially in the frogs kept at 5°, where even after 33 days there was a high proportion of toxin still circulating. At 15° the amount of toxin in the plasma decreased more rapidly and after 19 days had reached such a low value that the frogs could be warmed to 26° without developing tetanus.

From this experiment it is clear that tetanus toxin rapidly reaches the general circulation from the dorsal lymph sac, and that although cooling slows down the rate of entry it does not prevent the toxin reaching the blood stream. The lower maximum blood toxin concentration in the frogs at 5° cannot account for their insusceptibility to tetanus since the frogs at 18° were also unaffected, and their blood toxin concentration was initially as high as in the group at 26° which developed tetanus.

The physical signs of generalized tetanus. After the injection of tetanus toxin into the dorsal lymph sac Gumprecht (1895) found that the onset of tetanus was generalized, but Brunner (1894) reported that the onset was in the fore limbs even when the toxin was injected into a back leg. Zupnik (1905) produced ascending tetanus by an inoculation into the dorsum of the foot but Fröhlich & Meyer (1916) were only able to obtain this effect by inoculation into the cord. In the present experiments, the injection of tetanus toxin into the dorsal lymph sac produced a generalized condition in which all four limbs were affected at the same time. In the very early stages there was only a slight limitation of movement, most easily seen in the hind legs when the frog was raised from the water by a hand placed under the forepart of the body. A normal frog easily climbs onto the hand, whereas a frog with early tetanus cannot bring its hind legs far enough forward; there was a limitation of flexion. Soon the fore legs became fixed across the front of the chest (Pl. 1, fig. 1) and the frog could swim, able to make ever decreasing movements with his hind legs, which hung down in the water; eventually the hind legs became rigidly extended (Pl. 1, fig. 2) and the frog floated helplessly on the water. Reflex spasms occurred when the condition was well developed but were only obvious in the hind legs. The lymph hearts did not seem to be affected by tetanus toxin and continued to beat normally up to the end. It is in fact only by their continued beating in the final stages that the presence of life can be discerned.

The rate at which the intoxication progressed depended on the body temperature. At 26° death often occurred within 24 hr. of the first signs of tetanus, whereas at 18° the signs slowly progressed for 3 to 4 days and a frog might remain completely unable to move for a day or two before death. However, although cooling slowed its rate of progress it did not annul the process.

The relationship of dose of toxin and body temperature to time of death. Table 2 shows the median death time for groups of frogs, all kept at 26° but injected with different doses of tetanus toxin A into the dorsal lymph sac. The death time rapidly increased when the dose of toxin was below 0·5 mg. The figures in Table 2 give a curve very similar to that obtained by Ipsen (1941) with mice, except that the LD 50 dose for a frog is very much larger and must be in the region of 150 μg. as compared with 0·05 μg. for a mouse. Therefore at 26° the frog is about 3000 times as resistant as the mouse, but in the frog the lethal dose differs very much with the body temperature.

The effect of body temperature on the death time can be seen in Table 3, where the

median death time is shown for groups of frogs kept at different environmental temperatures but each injected with 0·5 mg. tetanus toxin A into the dorsal lymph sac. Below 20° the death time rapidly increased; at about 18° the frogs appeared to become completely resistant to the action of tetanus toxin in that dosage. Probably if the dose of toxin were increased, tetanus would develop in the frogs kept at a slightly lower temperature than 20°, but it is clear that cooling had a pronounced protective action. This was particularly remarkable in view of the fact that the frogs remained

Table 2. *The relationship between toxin dosage and death time at* 26°

Groups of four frogs were used at each dose value. Toxin injected into dorsal lymph sac.

Dose of toxin (mg.)	Median death time (days)
2	6·5
1	7
0·5	8·5
0·25	16

Table 3. *The relationship between body temperature and death time*

Groups of 10 frogs were used at each temperature. Tetanus toxin A (0·5 mg.) injected in dorsal lymph sac.

Temperature (° C.)	Median death times (days)
28	3·0
23	7·5
18	Over 21

Table 4. *The effect of antitoxin on the death time of frogs at* 27° *when given at different intervals after a standard dose of toxin*

Groups of 6 frogs were used. Dose of antitoxin: 0·2 ml. 300 international units. Dose of toxin: 0·5 mg. tetanus toxin A.

Interval between toxin and antitoxin (hr.)	Median death time (days)
24	Survived
48	8
72	4·5

quite active at temperatures down to 14° and only showed slight lethargy at 10°. The protective effect of a decrease in body temperature does not seem to be due to an alteration in the absorption or fate of the toxin in the body. Therefore the effect of environmental temperature on the fixation of toxin to, and action on, susceptible structures was studied.

Body temperature and the fixation of tetanus toxin. When tetanus toxin has passed to its site of action in the central nervous system and the development of tetanus cannot be prevented by the injection of antitoxin, it is said to have become fixed. In frogs living at 27° a lethal dose of toxin was fixed 48 hr. after injection as shown in Table 4.

This shows the results of an experiment in which three groups of frogs received a gross immunological excess of tetanus antitoxin (0·2 ml.) at different intervals after the injection of a lethal dose of tetanus toxin (0·5 mg. toxin A). By the end of 48 hr. although a lethal dose of tetanus toxin had been fixed the antitoxin was able to prolong the frog's life. Presumably this was because further toxin would have been fixed during the next 24 hr. After 3 days antitoxin has no effect on the death time. In contrast to the rapid fixation of tetanus toxin which took place at 27° was the complete failure of fixation at temperatures below about 18°. Frogs living at 15° were completely protected by antitoxin given a week or more after the inoculation of tetanus toxin, showing that fixation of a lethal dose had not occurred.

Table 5. *The effect of cooling from* 26° *to* 18° *for different periods beginning* 48 *hr. after the injection of tetanus toxin.*

Dose of tetanus toxin A: 1·0 mg.

Number of frogs	Duration of cooling (days)	Median death time (days)	Increase in median death time due to cooling (days)
7	Not cooled	5·5	—
7	3	8·5	3
6	6	10·5	5
6	9	13·0	7·5

Table 6. *The effect of cooling from* 26° *to* 5° *for different periods beginning* 48 *hr. after the injection of tetanus toxin*

Dose of tetanus toxin A: 1·0 mg.

Number of frogs	Duration of cooling (days)	Median death time (days)	Increase in median death time due to cooling (days)
6	Not cooled	7	—
6	6	14	7
6	9	16·5	9·5

Body temperature and the injurious action of tetanus toxin after fixation. From the observations recorded above it can be seen that cooling prevented the development of tetanus in the frogs and that the fixation of toxin was delayed and might not take place to the extent of a lethal dose. The action of toxin after fixation also seemed to be inhibited; this was shown by experiments in which groups of frogs were cooled after toxin had been allowed time to become fixed. All the frogs were inoculated with tetanus toxin (1·0 mg. toxin A) and kept at 26° for 48 hr. in order to ensure that a lethal dose of toxin had become fixed. In the first experiment, batches of these frogs were then transferred to a tank at 18° for different periods of time, at the end of which they were returned to the tank at 26°. Table 5 shows the length of time each group spent in the tank at 18° and their median death time. In column 4 of Table 5 the increase in the survival time over that of the control frogs is recorded, and it is clear that although the death time had been much prolonged by the cooling the increase was not quite equal to the duration of cooling. Therefore it appears that at 18° the

toxin was able to act, but only very slowly. In the second experiment frogs were cooled to 5° instead of to 18°. This more severe chilling prevented completely the injurious activity of the toxin (Table 6). The death time appeared to have been increased more than the duration of cooling (Table 6, column 4). If the action of the toxin was in fact annulled at this low temperature the increase in death time after cooling for 9 days ought to have been more than after 6 days, but it was not.

The effect of tetanus toxin given by different routes

The intramuscular route compared with inoculation into the dorsal lymph sac. It has been shown by several workers (Fildes, 1931, p. 308) that in mammals the minimal lethal dose of tetanus toxin by intravenous injection is about 5 times greater than the minimal lethal dose by intramuscular injection. An experiment was made to see whether this phenomenon could be shown in the frog. An inoculation of toxin via the dorsal lymph sac has been shown to be virtually equivalent to an intravenous injection, and so the effect of intramuscularly injected toxin was compared with the effect

Table 7. *The effect of tetanus toxin B given to groups of four frogs at 25° by different routes and in different dosages*

Dose of toxin B (mg.)	Median death time after inoculation into the	
	Dorsal lymph sac	Right gastrocnemius muscle
0·1	5 days	5 days
0·05	6 days	7 days
0·025	11 days	12 days
0·005	18 days	3 developed local tetanus
0·0025	Remained normal	Remained normal
0·0005	Remained normal	Remained normal
	Median death time after intracerebral injection	
0·01	2·5 days	
0·001	3·5 days	
0·0001	Remained normal	

of a similar dose given via the dorsal lymph sac. Two groups of 24 frogs were inoculated with tetanus toxin B, one group intramuscularly in the right gastrocnemius muscle, and the other via the dorsal lymph sac. Each group was divided into six subgroups, which received doses of toxin as shown in Table 7. The volume of toxin solution given to each frog was the same (0·1 ml.) and they were all kept at 25° after inoculation. With 0·025 mg. or greater doses of tetanus toxin B there was no significant difference in the median death time or in the signs of tetanus produced in the two groups; the frogs all died of generalized tetanus. However, with 0·005 mg. toxin B three of the four frogs inoculated intramuscularly developed a mild local tetanus in the inoculated limb. They were able to swim but there was a marked reduction of flexion at the knee, and to some extent at the hip, as compared with the normal leg. The opposite leg was not involved and the condition did not progress or regress during the 21 days for which these frogs were observed.

From Table 7 it appears that the minimal lethal dose of tetanus toxin was slightly larger by intramuscular injection than by inoculation via the dorsal lymph sac. This is the reverse of what has been described in mammals and may be due to the toxin

escaping from frog muscle more quickly than from mammalian muscle because of the greater blood capillary permeability in frogs. This view is supported by the failure to produce local tetanus by intramuscular injection of toxin, when the dose was more than 0·005 mg. With a large dose of toxin most of it escapes from the muscle but some passes up the motor nerve. The toxin reaches the central nervous system by two routes and this must be a less lethal process than when all the toxin is injected into the dorsal lymph sac. With a small intramuscular dose of toxin the amount which escapes is not enough to give generalized tetanus and the amount of toxin going up the nerve is only enough to produce a very mild local tetanus.

Intracerebral inoculation. With mammals tetanus toxin has always been found most lethal and dramatic in its action when injected into the central nervous system; this proved to be so with the frogs studied here. Frogs were anaesthetized with ether and 0·01 ml. toxin solution injected through the skull into the brain with a 31-gauge needle. From Table 7 it is clear that the dose of toxin required to kill was very much smaller when it was given intracerebrally than by either of the other two routes, and the death time for a given dose of toxin was much shorter. Cooling to 16° prevented the development of tetanus after an intracerebral injection of toxin. Two groups of three frogs were inoculated intracerebrally with 0·01 mg. tetanus toxin A and kept at 16°. After 6 days no signs of tetanus were observed. One group was then moved to a tank at 25° where they developed generalized tetanus after 24 hr. The second group was moved to the warm tank after 11 days in the cold and then developed tetanus after a further 24 hr. Tetanus following the intracerebral inoculation of toxin was very rapid in its progress and a frog which appeared normal in the morning might show gross signs of generalized tetanus in a few hours and die in the evening. In some cases after the intracerebral inoculation of tetanus toxin frogs showed signs of irritability and on being disturbed moved violently around the tank in a quite abnormal manner.

Intraneural inoculation of the sciatic nerve. In mammals the injection of a very small dose of toxin into a motor nerve will produce a severe local tetanus in the muscles supplied by the inoculated nerve. Some experiments were made to produce local tetanus in the frog by this technique. Under ether anaesthesia the sciatic nerve was exposed through a vertical incision in the back of the thigh. A piece of sterile filter paper was placed under the nerve and 0·01 ml. solution containing 0·2 mg. tetanus toxin A was injected with a 31-gauge needle. The frog's sciatic nerve is a very delicate structure and the amount of toxin which remained in the nerve was questionable as a large part appeared to leak onto the filter paper. The incision was closed and the frogs kept in a tank at 26°. In no case did local tetanus develop. A few frogs developed generalized tetanus, no doubt due to toxin which leaked from the nerve entering the general circulation. The intraneural inoculation could not be considered as satisfactory and no conclusions can be drawn from the results.

The production of local tetanus

It is possible to produce local tetanus consistently in the frog by the intramuscular injection of tetanus toxin. However the dosage is critical and the tetanus mild, for if too much toxin is given generalized tetanus develops as the first sign of intoxication. This is probably due to toxin leaking from the injection site into the general circulation. The local tetanus which follows the intramuscular injection of a suitable dose of

toxin is so lacking in severity that it may not be recognized and in a group of frogs similarly inoculated only a proportion will show local tetanus. Having observed that a slight local tetanus could be produced by intramuscularly injected toxin it seemed probable that with a larger dose of toxin a more severe local tetanus might be masked by the generalized tetanus which developed from toxin which had leaked into the general circulation. Therefore it was decided to try to neutralize toxin which entered the general circulation by giving tetanus antitoxin via the dorsal lymph sac a few minutes before the intramuscular injection of a large dose of toxin.

Table 8. *The effect of intramuscularly injected tetanus toxin in frogs protected by various doses of antitoxin in the general circulation. Similar doses of toxin and antitoxin were given to mice*

Dose of tetanus toxin B: 0·2 mg. in 0·05 ml. saline.

Dose of antitoxin (units)	Fate of frogs	Fate of mice
94	No intoxication	No intoxication
23	No intoxication	No intoxication
6	Local tetanus	No intoxication
1·5	Generalized tetanus	Generalized tetanus
0·4	Generalized tetanus	Generalized tetanus

Five pairs of frogs were given different doses of tetanus antitoxin, as shown in Table 8. Fifteen minutes later they were each inoculated with the same dose of tetanus toxin B (0·2 mg./0·05 ml. saline) into the right gastrocnemius muscle and put in a tank at 25°. The same quantities of toxin and antitoxin were mixed and injected intravenously into mice to determine whether or not antitoxin was in excess. The frogs which received 94 and 23 units of antitoxin were completely protected. However the pair given 6 units developed a well-marked local tetanus (see Pl. 1, fig. 3). The local tetanus was first apparent on the fifth day after injection of the toxin and did not significantly alter during the next 16 days. The mice which received 6 units of antitoxin survived. The smaller doses of antitoxin which were used did not protect and the mice and the frogs developed generalized tetanus.

In another experiment 10 frogs were given 6 units of tetanus antitoxin into the dorsal lymph sac and 15 min. later they were divided into two groups. One group received each 0·2 mg. tetanus toxin B in 0·05 ml. of solution intraperitoneally; the other group received the same dose of toxin into the right gastrocnemius muscle. The five frogs given toxin intraperitoneally showed no signs of intoxication during the 21 days for which they were observed, whereas 3 of the 5 frogs inoculated intramuscularly developed a marked local tetanus on the seventh day. Although the local tetanus was moderately severe there was no ascending involvement of the central nervous system and the opposite limb never showed any signs of tetanus.

General anaesthesia with ether abolished the muscular spasm in the affected limb and both legs became equally flaccid. On recovery from the anaesthetic the spasm returned. Two of the frogs with local tetanus were anaesthetized and the sciatic nerve, in the affected limb, cut. This produced a permanently flaccid limb, the spasm of local tetanus not returning on recovery from the anaesthetic. Local tetanus in the frog is therefore dependent, as in mammals, on an intact motor nerve supply and an unanaesthetized central nervous system.

It was suggested (Wright, 1953) that the force behind the neural transport of substances is the pressure produced inside contracting voluntary muscles. Frogs do not exhibit those frequent movements, typical of mammals. They will sit for long periods completely motionless and it seemed possible that the centripetal neural transport of tetanus toxin might only occur when the intramuscular pressure was raised during active movement. The toxin would therefore pass centrally more slowly in the frog than in mammals and would have a greater opportunity to escape from the muscle into which it had been injected. This would account for the rather poor development of local tetanus in the frog as compared with other laboratory animals.

Table 9. *The effect of exercise on the development of tetanus in frogs given 0·02 mg. toxin B and kept at 25°*

Group	Day of experiment on which death occurred
Exercise	6, 6, 7, 7, 7.
No exercise	6, 7, 7, 7, 8.

The above hypothesis was tested in two experiments. In the first, 8 frogs were given 0·005 mg. tetanus toxin B in 0·05 ml. solution, intramuscularly in the right gastrocnemius muscle. They were then divided into two groups; one group was put in a dark tank and not disturbed; the other group was placed in a tank of deep water where they had to keep swimming. Both groups were kept at 25° and after 24 hr. the 8 frogs were put together in one tank. On the eighth day of the experiment the frogs in both groups began to develop local tetanus which did not progress beyond the inoculated limb and was not more severe in one group than the other. In the second experiment a larger dose of tetanus toxin B was used (0·02 mg. in 0·05 ml. solution) and the group of frogs given exercise were kept swimming and hopping for 6 hr. before the two groups were put together in one tank at 25°. Again there was no difference between the two groups in survival time and none of the frogs showed local tetanus (Table 9).

The inactivation of tetanus toxin with brain tissue. The excised mammalian brain and spinal cord are capable of combining with large quantities of tetanus toxin; in doing so they inactivate the toxin (see Fildes, 1931, for an account of the Wassermann-Takaki reaction). The grey matter of the brain is more active than the white matter or the spinal cord in this reaction; no other body tissue shows any comparable neutralizing effect. The brains of birds and cold-blooded animals were found not to neutralize tetanus toxin by Metchnikoff (1898) and it was suggested by Knorr (1897) that their high degree of resistance to tetanus might be connected with the inability of the nervous tissue to combine with the toxin. The fact that many cold-blooded animals are susceptible to tetanus when warmed suggested that either there is a change in the combining power of the brain tissue on warming or that the phenomenon is unconnected with the development of tetanus.

The effect of mixing brain tissue with tetanus toxin was investigated by taking two equal samples of tetanus toxin, each dissolved in 1·0 ml. of saline and adding mashed brain tissue to one and an equal volume of saline to the other, which acted as a control. Penicillin (50,000 units) was added to each tube to prevent bacterial growth. After a period of incubation, the amount of free tetanus toxin in each specimen was then determined by inoculating mice intravenously with 0·2 ml. of the supernatant

fluid from each tube. In the first experiment 0·5 g. of brain tissue was placed in contact with 0·1 mg. tetanus toxin A at room temperature (18°) for 17 hr. Under these conditions mouse brain neutralized 90 % of the toxin, whereas with frog brain no neutralization could be demonstrated. Even when tetanus toxin + frog brain were incubated together at 26° or 37° no neutralization appeared to take place. The possibility that brain tissue from a frog kept at 26° for some days would react differently was examined. Three frogs were kept at 26° for 7 days before their brain tissue was removed and incubated as before with tetanus toxin at 26°. Again no neutralization was observed, although at this temperature the intact animals would have been susceptible to tetanus.

Another theory to explain the failure of frog brain tissue to neutralize tetanus toxin was that some accessory factor, not present in frog brain tissue, is required for the fixation of tetanus toxin. This hypothetical substance might be present in mammalian blood and would be mixed with mammalian brain brei in its preparation. This hypothesis was tested in two experiments. In the first, fresh rabbit serum was mixed with frog brain tissue and the mixture tested as above, no neutralization of tetanus toxin was demonstrated. In the second experiment the possibility that peritoneal fluid might contain the factor was tested by inoculating 3 mice intraperitoneally with a mixture containing 5 mouse LD 50 doses of tetanus toxin and 0·5 g. frog brain tissue. These mice developed tetanus and died, showing no increase in survival time as compared with control mice which received toxin only.

The specific precocious protective action of tetanus toxoid

The specific precocious protective action of tetanus toxoid first reported by Krech (1949) is probably due to toxoid molecules competing with toxin molecules for some receptor substance (Davies & Wright, 1955). As brain tissue from frogs cannot be induced to neutralize tetanus toxin it must be in some way different from that of mammals. It was therefore of interest to know whether tetanus toxoid would have any protective action in frogs. To demonstrate this phenomenon it is necessary to have a concentrated toxoid preparation; the one used contained 1175 Lf/ml. Seven

Table 10. *The protective action of tetanus toxoid in frogs at* 25°

Time of toxoid injection before the toxin (hr.)	No. of frogs	Period of survival after injection of toxin (days)
No toxoid	7	5, 6, 6, 6, 6, 6, 6.
$\frac{1}{4}$	4	10, 12, 15, 15.
24	3	11, 11, 16.

frogs were inoculated with 1·0 ml. of this concentrated toxoid via the dorsal lymph sac and placed with 7 uninoculated control frogs in a tank at 25°. After 15 min. the 7 controls and 4 of the previously inoculated frogs were each given 0·1 mg. tetanus toxin B via the dorsal lymph sac. On the next day (24 hr. after receiving the tetanus toxoid) the 3 remaining test frogs were given a similar dose of toxin. Table 10 shows the survival time of the three groups of frogs in this experiment. It can be seen that the

concentrated tetanus toxoid very considerably prolonged the survival time of the frogs but did not prevent the eventual development of tetanus. Tetanus toxoid seems to have the same protective action in the frog as in mammals.

DISCUSSION

Frogs can be kept at environmental temperatures between 4° and 28° but below about 10° they become noticeably lethargic and at 4° they remain motionless and only respond sluggishly to external stimuli. Above about 15° they are sensitive to the action of tetanus toxin but below this temperature they become resistant, although they remain active and respond readily to any disturbance. The change in sensitivity to tetanus toxin comes before there is any noticeable alteration in behaviour and before there is any demonstrable slowing in the rate of absorption of substances injected into the dorsal lymph sac. It seems clear from the experiments on the blood tetanus toxin concentration following its injection into the dorsal lymph sac, that the protective action of cooling is not due to a failure to absorb the toxin into the general circulation. These experiments also demonstrate that the toxin is circulating in an active form and has not been neutralized or toxoided by the frog tissues.

Tetanus toxin produces the same picture of unco-ordinated muscular contraction in the frog as in mammals, and the rate at which the intoxication progresses depends on the dose of toxin. However when using frogs in place of mammals there is an important additional dimension of experimental freedom: the environmental temperature. This might be of value for the analysis of the mode of action of the toxin. Cooling by a few degrees prevents both the fixation of the toxin and its action after fixation but it does not reverse either process. This protective effect of a low environmental temperature must be due either to an alteration in the frog's metabolism or to the toxin being able to act only above a certain temperature. It seems most unlikely that a change in body temperature which does not affect the normal activity of a frog could be accompanied by an alteration in neuromuscular physiology sufficiently profound to give complete resistance to tetanus toxin. It seems more probable that the effect of temperature is on the action of the toxin. Tetanus toxin is a protein and is active at a molecular concentration much below that required for most poisons. This suggests that it may be enzymic in nature and if so, below a certain temperature its combination with substrate and subsequent action might be so slow as to have virtually no effect. If this be true, mammals should also become resistant to tetanus toxin below a certain temperature. There are two reports which suggest that the environmental temperature has some slight effect on the resistance of mammals to tetanus toxin. A seasonal variation in the susceptibility of guinea pigs was reported by Herwick, Weir & Tatum (1936); they found that the lethal dose of tetanus toxin was 0·006 mg./kg. body weight in winter and 0·004 mg./kg. in summer. The observations were made over a period of two years and the authors pointed out that any change in the potency of the toxin would probably be a slow deterioration, whereas they found it to retain the same strength at the same season a year later. The increased resistance of these guinea pigs to tetanus toxin in the winter might be due to some cyclic physiological change not directly due to the environmental temperature. Ipsen (1951) observed a similar phenomenon in mice during hot and cold periods and made experiments in which mice were maintained continuously at environmental temperatures of 10°, 25°

and 35°. Different doses of tetanus toxin were tested and it was found that at 10° the survival time was generally longer than normal while at 35° it was shorter. Unfortunately in neither of these two series of experiments do we know the actual body temperature of the animals.

In mammals the site of action of tetanus toxin has been identified as a blocking of inhibitory hyperpolarization of the neurones which control the motor activities of the spinal cord (Brooks, Curtis & Eccles, 1957). In the frog the following evidence also points to a central site of action: (1) the dose of toxin required to produce generalized tetanus is smaller and the incubation period shorter when given intracerebrally than by other routes; (2) it is difficult to produce local tetanus in the frog by intramuscular injection of toxin, suggesting that it does not act on the nerve endings or muscles; (3) local tetanus is dependent on an intact motor nerve supply, which is at least consistent with the view that the toxin acts centrally.

Although tetanus toxin appears to have the same action in frogs as in mammals, the frog at its most sensitive is about 3000 times as resistant as a mouse. This might be due to a relatively impermeable blood brain barrier, but the frog is not very susceptible even to intracerebrally injected toxin and the ratio of lethal dose via the dorsal lymph sac to lethal dose intracerebrally is only about ten, a figure smaller than that reported for some mammals (Wright, 1955, p. 440). Therefore the frog's resistance must be due either to an insusceptible brain substance or to the fact that the toxin is operating at a temperature some 10° lower in the frog than in the mouse. If frogs could be kept at 37° they would almost certainly be more sensitive to the action of tetanus toxin than at 26°, but probably they would not be as susceptible as mice, for there must be other factors involved. It is relevant that birds, which have a high body temperature, are very resistant to tetanus toxin irrespective of the route by which it is given (Davies, Morgan & Wright, 1955).

It is not easy to produce local tetanus in frogs. This is probably partly due to toxin being washed from the site of injection by the very great lymph production in the frog's tissues (Isayma, 1924) and partly to the relatively high concentration of toxin required in the central nervous system to produce signs of intoxication. However, when the frogs are partially protected by antitoxin given via the dorsal lymph sac, a moderately severe local tetanus follows intramuscularly injected toxin. Circulating antitoxin will also protect them against a dose of toxin given intraperitoneally, but not against the same dose of toxin intramuscularly. A similar experiment was reported by Friedemann, Zuger & Hollander (1939) with guinea pigs. They found that when a dose of toxin was given intramuscularly it required between 5 and 8 times as much antitoxin to protect the guinea pigs as would have been necessary had the same dose of toxin been given intravenously. The development of local tetanus in frogs protected against circulating toxin is good evidence for the neural transport of tetanus toxin in the frog. Unfortunately the conclusive experiments involving nerve sclerosis (Baylis, Mackintosh, Morgan & Wright, 1952) and localized anaesthesia (Wright, Morgan & Wright, 1952) which have been applied to the rabbit to prove the central site of action of tetanus toxin in local tetanus, cannot for technical reasons be used to elucidate the pathology of local tetanus in the frog.

It has not proved possible to demonstrate any neutralization of tetanus toxin by frog brain tissue, using low concentrations of toxin, and in this frogs may resemble birds. Metchnikoff (1898) was unable to demonstrate the neutralization of tetanus toxin by

bird brain tissue, but recently van Heyningen (1959*a*) found chicken brain to be as active as mammalian brain tissue in the absorption of tetanus toxin. The reaction between brain tissue and tetanus toxin is a reversible absorption process (Fulthorpe, 1956), the amount of toxin absorbed depending on the concentration of toxin. Metchnikoff may well have failed to demonstrate absorption of the toxin by bird brain because he used too low a concentration of toxin. The active substance in brain tissue has recently been identified (van Heyningen, 1959*b*) as a ganglioside and it seems probable that the combination of the toxin with this substance may be the first step in the action of the toxin. If this be so the high resistance of birds and cold-blooded vertebrates to tetanus toxin might be due to their brain tissue not combining with low concentrations of the toxin.

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Fig. 1

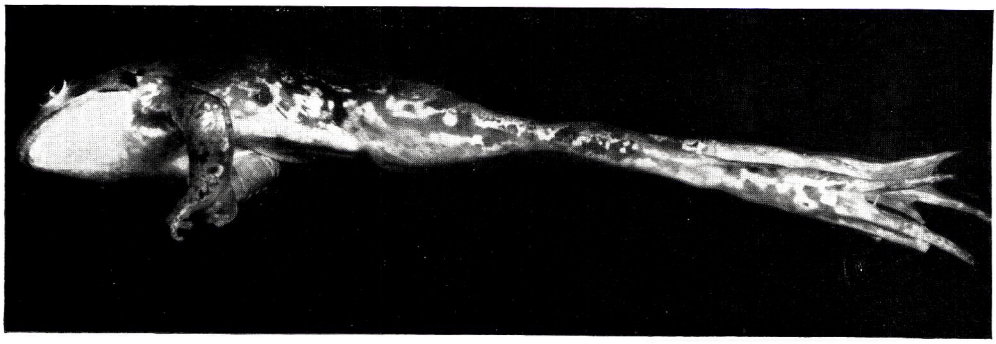

Fig. 2

Fig. 3

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EXPLANATION OF PLATE 1

Fig. 1. A frog with generalized tetanus showing the fore legs fixed across the front of the chest.

Fig. 2. The same frog as in Fig. 1 showing the extreme extension of the back legs.

Fig. 3. Two frogs showing local tetanus of the right leg.