A SYMPOSIUM ON SOME COMPARATIVE ASPECTS OF INTERMEDIARY METABOLISM IN MICRO ORGANISMS

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Comparative Aspects of Alcohol Formation

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The ability to produce ethanol from glucose is widely distributed in the microbial kingdom, and additionally, higher alcohols are formed by some micro-organisms. The yields vary considerably, from almost 2 moles of ethanol per mole of glucose fermented, characteristic of yeast, to the very much smaller amounts formed by many bacteria. These large variations are attributable to the operation of different metabolic pathways and, at present, four different routes of ethanol formation have been recognized, three of which involve pyruvic acid as an obligatory intermediate.

Pyruvate may be formed from glucose by different metabolic sequences (e.g. Embden–Meyerhof glycolysis or Entner–Doudoroff cleavage) and subsequently may be converted to a C_2 unit by one of two pathways, namely decarboxylation to acetaldehyde or by thioclastic reaction to acetylcoenzyme A. Reduction of either C_2 moiety yields ethanol. The heterolactic fermenters employ an entirely different mechanism: glucose is converted to xylulose-5-phosphate which is split by the enzyme phosphoketolase to acetylphosphate and glyceraldehyde-3-phosphate, the former undergoing reduction to ethanol.

The four different combinations of pathways are:

Type 1. Glycolysis and pyruvate decarboxylase ('carboxylase': yeast, fungi, protozoa and a few bacteria).

Type 2. Glycolysis and thioclastic reaction (Enterobacteriaceae, Clostridia, Zymosarcina).

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Type 3. Entner–Doudoroff and pyruvate decarboxylase (*Zymomonas mobilis*, *Z. anaerobia*)

Type 4. Phosphoketolase (heterolactic bacteria).

To date, the combination of Entner–Doudoroff and thioclastic reactions has not been discovered.

The reductive formation of ethanol, common to all four types, must be coupled with oxidative reactions that occur in the fermentations. Nicotinamide adenine dinucleotides (NAD or NADP) mediate this coupling.

In microbial fermentations the further metabolism of pyruvate can give rise to a variety of products and consequently the occurrence of such reactions (in Types 1, 2 and 3) will divert pyruvate from ethanol formation with corresponding decreases in yield. Controlling factors must be the enzymic constitution and/or the enzymic activity of the organism, regulated by repression, pH or other environmental conditions.

Type 1. Glycolysis may be formulated as:

$$\text{Glucose} + 2\,\text{NAD} + 2\,\text{ADP} + 2\,\text{P}_i \rightarrow 2\,\text{Pyruvate} + 2\,\text{NADH}_2 + 2\,\text{ATP}$$

which reveals an effective reducing power equivalent to 4H, and a net energy yield of 2ATP. The carboxyl carbons of pyruvate are derived from C-3 and C-4 of glucose. In yeast two enzymes—pyruvate decarboxylase and ethanol dehydrogenase—convert pyruvate to ethanol:

$$\underset{\text{(Carboxylase)}}{CH_3COCOOH \xrightarrow[Mg^{2+}]{\text{Thiamine pyrophosphate}} CH_3CHO + CO_2} \xrightarrow{NADH_2} \underset{\text{(Ethanol dehydrogenase)}}{CH_3CH_2OH}$$

Two moles of ethanol can thus be formed per mole of glucose. Ethanol is also formed by these reactions in some fungi (Foster, 1949) and protozoa (Bauchop, 1962), but in very few bacteria, e.g. *Zymosarcina ventriculi* (Bauchop & Dawes, 1959). The only other examples of ethanol-forming bacteria which possess a carboxylase occur in Type 3.

Type 2. The Enterobacteriaceae and Clostridia cleave pyruvate to acetyl CoA by the following 'thioclastic' reactions:

$$CH_3COCOOH \xrightarrow[Mg^{2+},\ \text{coenzyme A}]{\text{Thiamine pyrophosphate}} CH_3COSCoA + \begin{cases} HCOOH & \text{Enterobacteriaceae} \\ H_2 + CO_2 & \text{Clostridia} \end{cases}$$

The mechanisms are still obscure but the evidence of Mortlock, Valentine & Wolfe (1959) suggests that the C_2 fission product in *Clostridium butyricum* is at the aldehyde level of oxidation.

Despite earlier suggestions to the contrary (Tikka, 1935), pyruvate is an obligatory intermediate for ethanol formation in *Escherichia coli*, and acetylcoenzyme A is reduced to acetaldehyde by a coenzyme A-dependent acetaldehyde dehydrogenase, followed by reduction to ethanol by ethanol dehydrogenase (Dawes & Foster, 1956).

$$CH_3COSCoA \xrightarrow{NADH_2} \underset{+\ \text{CoASH}}{CH_3CHO} \xrightarrow{NADH_2} CH_3CH_2OH$$

As the reduction of 1 mole of pyruvate to ethanol requires 4H the maximum possible

ethanol yield by Type 2 reactions would be 1 mole/mole glucose; other reactions which compete for $NADH_2$ decrease the yield and the highest recorded is 0·8 mole (Stokes, 1949). When glycerol is the substrate, however, the fermentation approximates to

$$\text{Glycerol} \rightarrow \text{ethanol} + \text{formate},$$

since the conversion of glycerol to pyruvate furnishes the 4H necessary for quantitative ethanol formation. This observation emphasizes a general feature of alcohol production, namely that the yield is higher the more reduced the substrate fermented.

At present, *Zymosarcina ventriculi* is the only organism known to possess both decarboxylase and thioclastic enzymes for pyruvate (Arbuthnott, Bauchop & Dawes, 1960).

Some Clostridia produce butanol when the pH of glucose fermentation has fallen to about 4. The reactions occurring are probably analogous to those in *Escherichia coli*: butyrylcoenzyme A (formed from acetylcoenzyme A via acetoacetylcoenzyme A and reduction) may be reduced to butanol via butyraldehyde (for review, see Barker, 1956). In *Clostridium butylicum* acetone is reduced to isopropanol by the appropriate dehydrogenase.

$$CH_3COCH_3 + NADH_2 \rightleftharpoons CH_3CHOHCH_3 + NAD.$$

Type 3. *Zymomonas mobilis* gives a similar fermentation balance to yeast but ethanol derives from C-2, C-3 and C-5, C-6 of glucose, characteristic of the Entner–Doudoroff (1952) pathway (Gibbs & DeMoss, 1954).

$$\text{Glucose} \xrightarrow{\text{ATP}} \text{glucose-6-phosphate} \xrightarrow{\text{NAD}} \text{gluconate 6-phosphate} \xrightarrow{-H_2O}$$

$$\text{2-oxo-3-deoxygluconate-6-phosphate} \rightarrow \begin{cases} \text{pyruvate} \\ + \\ \text{glyceraldehyde-3-phosphate} \\ \downarrow \\ \text{pyruvate} \end{cases}$$

The net reaction is described by the equation

$$\text{Glucose} + 2\,\text{NAD} + \text{ADP} + P_i \rightarrow 2\ \text{pyruvate} + 2\,NADH_2 + \text{ATP},$$

which reveals that, effectively, it carries out the same overall reaction as glycolysis but with half the energy yield, a conclusion verified experimentally by Bauchop & Elsden (1960). The organism possesses a pyruvate decarboxylase.

Zymomonas anaerobia displays a similar fermentation balance (Shimwell, 1950; Millis, 1956), it possesses Entner–Doudoroff and pyruvate decarboxylase enzymes, and has a molar growth yield similar to that of *Z. mobilis* (McGill, Ribbons & Dawes, unpublished).

Type 4. The heterolactic organisms, e.g. *Leuconostoc mesenteroides*, ferment glucose as follows:

$$\text{Glucose} \rightarrow \text{lactate} + \text{ethanol} + CO_2,$$

ethanol being derived from C-2 and C-3. The mechanism was illuminated by the discovery of an enzyme phosphoketolase (Heath, Hurwitz & Horecker, 1956) which cleaves xylulose 5-phosphate in accordance with the equation

$$\text{Xylulose 5-phosphate} + P_i \rightarrow \text{acetyl phosphate} + \text{glyceraldehyde 3-phosphate}$$

The triosephosphate is converted to pyruvate, by reactions common to glycolysis, and then reduced to lactate. The 4H made available in the conversion of glucose to

xylulose-5-phosphate are utilized for reduction of acetylphosphate to ethanol, a reaction associated with the loss of biologically useful energy to the organism. A similar loss occurs in the reduction of acetylcoenzyme A (Type 2). When pentose is fermented acetylphosphate cannot be reduced to ethanol and an extra mole of ATP is conserved.

Other reactions. Some micro-organisms convert pyruvate to the alcohols acetylmethylcarbinol (acetoin) and 2,3-butanediol by one of two pathways:

(*a*) organisms lacking carboxylase, e.g. *Aerobacter aerogenes*, *Streptococcus faecalis*

$$2 \text{ pyruvate} \rightarrow CO_2 + \alpha\text{-acetolactate} \rightarrow CO_2 + \text{acetoin};$$

(*b*) organisms having carboxylase, e.g. yeast

$$\text{Pyruvate} + \text{acetaldehyde} \rightarrow CO_2 + \text{acetoin}.$$

The Acetobacter are unique in possessing enzymes for both pathways (De Ley, 1959). Those micro-organisms which possess a 2,3-butanediol dehydrogenase can reduce acetoin to 2,3-butanediol. Clearly these reactions will divert pyruvate from ethanol formation.

The anaerobe *Vibrio cholinicus* (subsequently shown to be indistinguishable from *Desulfovibrio desulfuricans*, Baker, Papiska & Campbell, 1962) ferments choline to trimethylamine, acetate and ethanol:

$$2(CH_3)_3\overset{+}{N}CH_2CH_2OH - H_2O \rightarrow 2(CH_3)_3\overset{+}{N}H + CH_3COOH + CH_3CH_2OH.$$

Hayward (1960) demonstrated that the C_2 moiety of choline is transformed to acetaldehyde, and that the acetaldehyde then undergoes a dismutation to ethanol and acetylcoenzyme A catalysed by NADP-dependent ethanol and acetaldehyde dehydrogenases respectively. Acetylcoenzyme A yields acetate and ATP via acetylphosphate. Since the organism grows on choline as the sole carbon and energy source some molar growth yield experiments would be instructive.

Fusel oils. These are higher alcohols produced by yeast from amino acids, in the presence of glucose, and without the release of ammonia. SentheShanmuganathan & Elsden (1958) showed that tyrosol was produced from tyrosine in a sequence of three reactions: (*a*) transamination between L-tyrosine and 2-oxoglutarate (formed from glucose) to yield *p*-hydroxyphenylpyruvate and L-glutamate; (*b*) decarboxylation of *p*-hydroxyphenylpyruvate to *p*-hydroxyphenylacetaldehyde; and (*c*) its reduction to tyrosol in the presence of alcohol dehydrogenase and $NADH_2$. *n*-Propanol is formed from α-oxobutyrate, an intermediate in isoleucine synthesis, by decarboxylation and subsequent reduction (Guymon, Ingraham & Crowell, 1961).

Regulation. In all reactions leading to alcohol formation the reduction of an aldehyde is the final step, catalysed by an alcohol dehydrogenase. The presence of such an enzyme is therefore essential for alcohol formation and its regulation might be expected under conditions where the alcohol yield is altered. McPhedran, Sommer & Lin (1961) have shown that the ethanol dehydrogenase of *Aerobacter aerogenes* is repressed under anaerobic conditions when a hydrogen acceptor such as fumarate is added to the growth medium; ethanol, acetaldehyde and acetate were without effect. It would be interesting to know if this repression extends to acetaldehyde dehydrogenase.

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The Breakdown and Biosynthesis of Glutamic Acid

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Glutamic acid is important in the carbon and nitrogen metabolism of micro-organisms; it participates in the structure of the folic acid coenzymes, and is a component of the bacterial cell wall. Although a number of micro-organisms, including some of the lactic acid bacteria, require glutamate as an essential amino acid, most micro-organisms synthesize glutamate from simple carbon sources.

The biosynthesis of glutamic acid has been investigated by isotopic tracer techniques, by the use of auxotrophic mutants, and by the study of the enzymes involved in the biosynthetic pathway. The isotope technique was applied to a number of micro-organisms using differentially labelled acetate $^{13}CH_3.^{14}COOH$ by Ehrensvärd and co-workers (Cutinelli *et al.* 1951*a*; Ehrensvärd *et al.* 1951; Anderson-Kottö *et al.* 1954). The glutamate was not degraded completely and unequivocally. Nevertheless, the results with the aerobic micro-organisms they examined were consistent with the incorporation of acetate into glutamate via the tricarboxylic acid cycle: that is through the successive formation of citrate, aconitate, isocitrate, oxalosuccinate and α-oxoglutarate. Wang, Christensen & Cheldelin (1953) examined the incorporation of [1-^{14}C]-acetate and [2-^{14}C]-pyruvate into glutamate by *Saccharomyces cerevisiae* with results consistent with the tricarboxylic acid cycle. This technique has also been applied to glutamate biosynthesis in higher plants and animals (Bilinski & McConnell, 1957; Koeppe & Hill, 1955; Black & Kleiber, 1957; Black *et al.* 1957).

Ehrensvärd and co-workers extended their studies to the anaerobic photosynthetic bacterium *Rhodospirillum rubrum* using $^{13}CH_3.^{14}COOH$ and $NaH^{14}CO_3$ (Cutinelli *et al.* 1951*b*). Again the isolated glutamate was not completely and unequivocally degraded although the results were different from those found with the aerobic bacteria. Alanine was also isolated and degraded and was found to be specifically labelled in a manner different from that found in aerobes (Table 1). Assuming the alanine to be derived directly from pyruvate, the glutamate degradation data were consistent with its formation via the following series of reactions including part of the tricarboxylic acid cycle:

$$\text{pyruvate} + CO_2 \rightarrow \text{oxalacetate}$$
$$\text{oxalacetate} + \text{acetate} \rightarrow \text{citrate} \rightarrow \text{isocitrate} \rightarrow$$
$$\text{oxalosuccinate} \rightarrow \alpha\text{-oxoglutarate} \rightarrow \text{glutamate}.$$

The final step in the above reaction sequence could be carried out by glutamic dehydrogenase or transaminases. Hug & Werkman (1957) showed that *R. rubrum* possessed transaminases capable of catalysing the final step in the reaction sequence.

The isotope technique was extensively used in studies of amino acid biosynthesis in *Escherichia coli* by Roberts *et al.* (1955), and their successful application of the 'isotopic competition' method showed that glutamic acid, formed via the tri-

carboxylic acid cycle, was a precursor of other amino acids including proline and arginine; results which were consistent with earlier studies on the biosynthesis of these amino acids in micro-organisms (notably work with auxotrophic mutants of *Neurospora crassa*). Similarly, aspartic acid was shown to be a precursor of threonine, isoleucine, methionine and lysine. It was thus clear that the tricarboxylic acid cycle in *E. coli* was an important source of cell constituents, especially amino acids. These findings supported the earlier conclusions of Krebs, Gurin & Eggleston (1952) as a result of their studies on acetate metabolism in yeast. However, it yet remained to be established that the tricarboxylic acid cycle was the only significant source of glutamate in micro-organisms. This was demonstrated very clearly by Gilvarg & Davis (1956) with auxotrophic mutants of *E. coli* and *Aerobacter aerogenes*. These mutants were blocked at the citrate condensing enzyme and had an absolute requirement for glutamate, and were unable to oxidize acetate.

A number of bacteria and fungi accumulate α-oxoglutarate, particularly when grown with nitrogen as the growth-limiting constituent (Walker, Hall & Hopton, 1951; Hockenhull, Wilkin & Winder, 1951; Dagley, Fewster & Happold, 1952). These results suggest that α-oxoglutarate is the precursor of glutamate in these micro-organisms. α-Oxoglutarate accumulation has been found with species of Pseudomonas (Lockwood & Stodola, 1946; Kogut & Podoski, 1953) for which there is independent evidence for the operation of the tricarboxylic acid cycle which could give rise to α-oxoglutarate (Clarke & Meadow, 1959).

The tricarboxylic acid cycle may not be involved in α-oxoglutarate synthesis, and hence glutamate formation, in all aerobes: α-oxoglutarate can be formed from L-arabinose by a route independent of the tricarboxylic acid cycle in *Pseudomonas saccharophila* (Weimberg & Doudoroff, 1955). Some species of acetic acid bacteria, including *Acetobacter suboxydans* and *A. melanogenus*, can synthesize glutamic acid although they appear to be deficient in key enzymes of the tricarboxylic acid cycle (Rao, 1957). Glutamate biosynthesis in *A. suboxydans* has recently been investigated by Sekizawa *et al.* (1962) and a novel mechanism for its biosynthesis has been proposed. Kinetic studies with the green alga *Chlorella pyrenoidosa* (Bassham & Kirk, 1960; Smith, Bassham & Kirk, 1961) suggest that glutamate may not be synthesized via citrate. It was found that the rate of formation of citrate in an actively photosynthesizing cell suspension was considerably less than the rate of formation of glutamate.

There is now little doubt that novel mechanisms of amino acid synthesis operate in some strict anaerobes. This was first clearly demonstrated for the biosynthesis of alanine and glutamic acid in *Clostridium kluyveri* (Tomlinson, 1954*a*, *b*). Recent studies on rumen anaerobes, e.g. *Ruminococcus flavefaciens*, confirm novel pathways for the biosynthesis of valine and leucine (Allison, Bryant & Doetsch, 1962). The glutamate formed in *C. kluyveri* from [1-^{14}C]-acetate and from $NaH^{14}CO_3$ was degraded unequivocally and the isotope distribution was not consistent with any known metabolic reaction sequence (Table 1). As yet no enzyme studies have been carried out to elucidate the mechanism of glutamate biosynthesis in *C. kluyveri*. A common feature of the amino acids synthesized by the above anaerobes and by *R. rubrum* is the specific incorporation of bicarbonate into a carboxyl group.

Recent studies on the photoassimilation of acetate by *Rhodospirillum rubrum* stimulated a re-investigation of the isotope incorporation of acetate into glutamate

in this organism ((Hoare, 1962*a*, *b*). In short exposure experiments in which washed cell suspensions of *R. rubrum* were exposed in the light to [1-^{14}C]- or [2-^{14}C]-acetate in the presence of bicarbonate, one of the earliest detectable products of photoassimilation was found to be glutamic acid. This appeared to be formed before one could detect intermediates of the tricarboxylic acid cycle. The failure to detect the latter at the shortest exposures might have been due to their small pool size, although this seemed unlikely since they were readily detected when $Na_2{}^{14}CO_3$ was assimilated in the light in the presence of acetate. The glutamate formed under the above conditions was degraded and the isotope distribution differed from that found in *Clostridium kluyveri* and from that found in aerobic organisms in which glutamate is formed via the tricarboxylic acid cycle (Table 1).

Table 1. *Isotope incorporation into alanine and glutamic acid*

b = carbon atoms derived from bicarbonate. c = carbon atoms derived from carboxyl (C-1) of acetate. m = carbon atoms derived from methyl (C-2) of acetate.

	Alanine $CH_3.CH(NH_2).COOH$			Glutamic acid $HOOC.CH(NH_2).CH_2CH_2.COOH$					Reference
Aerobes*	m	m	b	b	m	m	m	c	Ehrensvärd (1955)
Rhodospirillum rubrum	m	c	b	b	(m	c	m)	c	Cutinelli *et al.* (1951*b*)
				b	c	m	m	c	Hoare (1962*b*)
Clostridium kluyveri	m	c	b	c	m	c	m	b	Tomlinson (1954*a*, *b*)

* Assuming the biosynthesis proceeds via the tricarboxylic acid cycle.

Studies of Cutinelli *et al.* (1951*b*) did not establish an unequivocal distribution of carbon atoms 2, 3 and 4 of glutamic acid.

In aerobic bacteria it is generally assumed that the breakdown of glutamic acid proceeds via α-oxoglutarate and the tricarboxylic acid cycle. The wide distribution of transaminases and of glutamic dehydrogenase is in accord with this view. A number of anaerobic bacteria degrade glutamic acid to carbon dioxide and volatile fatty acids including mainly acetate and butyrate. Conversion of glutamate to acetate via the tricarboxylic acid cycle should result in the formation of three moles of CO_2 per mole of glutamate. Several strict anaerobes, including *Clostridium tetani* (Clifton, 1942), *C. saccharobutyricum* (Cohen, Nisman & Cohen-Bazire, 1948), *C. tetanomorphum* (Woods & Clifton, 1937; Barker, 1937), *Micrococcus aerogenes* (Whiteley, 1957) and *Fusobacterium nucleatum* (Jackins & Barker, 1951), ferment glutamate with the formation of only 1 mole of CO_2. The fermentation of glutamate has been studied very thoroughly with *C. tetanomorphum*. Isotopic tracer studies showed that the CO_2 was derived exclusively from C-5 of glutamic acid, acetate mainly from C-1 and C-2 and butyrate from C-3 and C-4 (Wachsman & Barker, 1955; Wachsman, 1956). The enzyme steps involved in the degradation of glutamate by *C. tetanomorphum* have now been identified and studied in some detail. The initial enzyme step catalysing the isomerization of glutamic acid to β-methylaspartic acid was found to involve a coenzyme derivative of vitamin B_{12} (Barker, Weissbach & Smyth, 1958). It is not yet known whether glutamate fermentation by *C. tetani*, *C. saccharobutyricum* and *F. nucleatum* proceeds by a similar mechanism to that in *C. tetanomorphum*.

Clearly the anaerobic bacteria merit a more extensive investigation with the possibilities of finding new pathways in the biosynthesis and degradation of amino acids. It is by no means established that the tricarboxylic acid cycle functions in strict anaerobes and, since such organisms may not depend upon it for a source of reducing power, they may prove to have evolved some alternative mechanism for providing intermediates for amino acid biosynthesis. A number of strict anaerobes, in addition to *Clostridium kluyveri* (which is the only one to be examined so far), use two carbon compounds as sole carbon sources and an examination of glutamate biosynthesis in these organisms would be of particular interest. Some of the methane bacteria and several organisms fermenting nitrogenous compounds (*Streptococcus allantoicus*, *Peptococcus glycinophilus*, *Clostridium cylindrosporum* and *Clostridium acidi-urici*) fall in this category.

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The Assimilation of 1-C Compounds

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A wide variety of micro-organisms can grow on 1-C compounds as sole carbon source; the growth substrates range in oxidation level from carbon dioxide to methane. The organisms may be subdivided into three main classes (Table 1).

Group A is comprised of the photosynthetic and chemosynthetic autotrophs. The elucidation of the way in which cell constituents could be made *de novo* from carbon dioxide came principally from Calvin's laboratory in 1954 (Bassham *et al.* 1954). These authors suggested a cycle of reactions which may be termed the ribulose diphosphate cycle of carbon dioxide fixation. Variants on the basic theme of the cycle may permit synthesis of triose-, tetrose- pentose-, or hexose phosphates from carbon dioxide (Elsden, 1962). The operation of the cycle has been well demonstrated in various photosynthetic tissues but there are still one or two areas of uncertainty, e.g. the considerable discrepancy between the activity of some of the cycle enzymes as measured *in vitro* and the rates of carbon dioxide fixation observed in intact cells (Peterkofsky & Racker, 1961). This may, however, only reflect a greater activity of the enzymes when organized within the chloroplast than when extracted.

Table 1. *Subdivision of micro-organisms capable of growth on 1-C compounds*

Group	Source of energy	Growth conditions	Carbon source
A	Inorganic oxidation, light	Aerobic or anaerobic	CO_2
B	Organic oxidation	Aerobic	Reduced 1-C compounds
C	Organic dismutation	Anaerobic	CH_3OH, HCO_2H, CO

Following the discovery of the ribulose diphosphate cycle in 1954, its occurrence in a variety of non-photosynthetic autotrophs has been established (for review, see Quayle, 1961). Such work indicates that, so far, autotrophic growth may be equated with the ribulose diphosphate cycle, and that a cycle of this type is probably responsible for *de novo* synthesis of polycarbon compounds from carbon dioxide, whatever the energy source.

Less is known about the metabolism of micro-organisms capable of aerobic growth on reduced C_1 compounds. Some of the better authenticated species of such organisms are given in Table 2. There are so many similarities between the coloured organisms that they have been tentatively bracketed together as being related to *Pseudomonas methanica*, an organism re-isolated by Dworkin & Foster (1956), 50 years after its first isolation as *Bacillus methanicus* by Söhngen (1906). Of the remaining four organisms in Table 2, one of these, *P. aminovorans* described by den Dooren de Jong (1926), does not appear to be available now. Hence it is not possible to decide whether it might be related to *P. methanica*. The other three organisms in Table 2, however, are quite distinct.

Up to 1958, very little was known of the mode of biosynthesis of cell constituents from reduced 1-C growth substrates. Bhat & Barker (1948) and van Niel (1954) had pointed out the possibility that an organism growing on a highly oxidized 1-C substrate, such as formate, might utilize an autotrophic type of metabolism, in which the energy of oxidation of the substrate is coupled to the assimilation of carbon dioxide. In fact, several authors, e.g. Thimann (1955), have assumed that this takes place without there being any proof that it does. However, of all the organisms tested so far, only one has proved to be autotrophic, viz. formate-grown *Pseudomonas oxalaticus*. Isotopic work with whole cells and enzymic studies with cell-free extracts point to growth of this organism on formate as being a strictly autotrophic process in which the bulk of the carbon is assimilated by the ribulose diphosphate cycle of carbon dioxide fixation, the necessary energy being derived from oxidation of formate (Quayle & Keech, 1959*a*, *b*).

Table 2. *Some micro-organisms capable of growth on* 1-*C compounds*

Organism*	Characteristic features	Oxidation level of 1-C growth substrates
Pseudomonas methanica (over 30 strains) *Pseudomonas* } *extorquens* *Flavobacterium* } *Pseudomonas* FRL-W4 *Pseudomonas* AM1 *Protaminobacter ruber* *P. alboflavus* } *P. methanica*?	Mostly pink. Yellow, brown and colourless strains of some of them are known	Ranges from $CH_4 \rightarrow HCO_2H$. Some organisms are 1-C specific
Hyphomicrobium vulgare	Colourless. Slow growing, 'stalked' cells	CH_3OH, HCO_2H. 1-C specific
Bacillus sphaericus	Colourless	*N*-methyl urea. Not 1-C specific
Pseudomonas aminovorans	Colourless to yellow, probably needs re-isolating	CH_3OH, HCO_2H. Not 1-C specific
P. oxalaticus	Colourless	HCO_2H. Not 1-C specific

* For bibliography, see Quayle (1961) and Peel & Quayle (1961).

It would appear to be energetically wasteful to assimilate carbon at the level of carbon dioxide and reduce it to the level of cellular material when the substrate itself presents the cell with reduced carbon. When the substrate is as highly reduced as methane, the wastage is very obvious. It is thus not surprising to find evidence now accumulating of the occurrence of a heterotrophic type of growth on reduced 1-C compounds in which a substantial portion of the carbon is taken in at a reduction level higher than that of carbon dioxide. The first direct evidence for this type of metabolism was the finding by Leadbetter & Foster (1958) that the specific radioactivity of cells of various strains of *Pseudomonas methanica* grown under an atmosphere of methane + air + $^{14}CO_2$ was always much less than that of the exogenous $^{14}CO_2$. This showed that the cells could not have been synthesized exclusively from exogenous carbon dioxide or from respiratory carbon dioxide in equilibrium with the exogenous carbon dioxide. Kaneda & Roxburgh (1959) incubated methanol-grown Pseudomonas strain FRL-W4 with [^{14}C]-methanol and $^{14}CO_2$ and found that

the first stable intermediate in methanol metabolism was serine. They concluded that the metabolism was non-autotrophic. Large, Peel & Quayle (1961) carried out a kinetic analysis of the course of entry of [^{14}C]-substrate and $^{14}CO_2$ into methanol- and formate-grown Pseudomonas AM 1, and methanol-grown *Hyphomicrobium vulgare*. These studies showed that serine is a primary product of [^{14}C]-methanol or [^{14}C]-formate incorporation (and glycollate in *H. vulgare*) and that malate or aspartate is a primary product of $^{14}CO_2$ incorporation. With all tracers, glycine too, was an early labelled product; 50 % of the cell carbon was found to be exchangeable with exogenous carbon dioxide during growth of Pseudomonas AM 1 on [^{14}C]-methanol. Leadbetter recently analysed the kinetics of entry of [^{14}C]-methylamine and $^{14}CO_2$ into a pink, methylamine-grown organism and found very similar results. Labelling patterns of glycine, serine, phosphoglycerate and malate have been determined in methanol-grown Pseudomonas AM 1 incubated with [^{14}C]-methanol or $^{14}CO_2$ (Large, Peel & Quayle, 1962). On the basis of these data a scheme was proposed for a heterotrophic type of metabolism in which hydroxymethylation of glycine to give serine serves as a major pathway for synthesis of 3-C compounds from 2-C compounds. The crucial problem of the necessary synthesis of glycine, or its precursors, from 1-C units is unsolved.

Virtually nothing is known of the metabolism of the anaerobic organisms in Group C. Lack of knowledge is due largely to the difficulty of isolating and handling these bacteria.

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The Assimilation of 2-C Compounds other than Acetate

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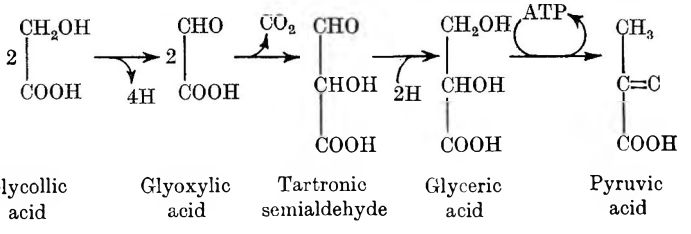
The tricarboxylic acid cycle is both the main terminal respiratory pathway in micro-organisms and a source of precursors for the net biosynthesis of cell constituents. Intermediates drained away from the cycle for these biosynthetic purposes must be replenished; a process accomplished by carboxylation reactions when suitable CO_2-acceptor molecules (e.g. pyruvate and phosphopyruvate) are available. Any organism that utilizes a 2-C compound as sole source of carbon for growth must therefore be endowed with a means of forming from this substrate either the TCA cycle intermediates themselves or such 3-C CO_2-acceptors.

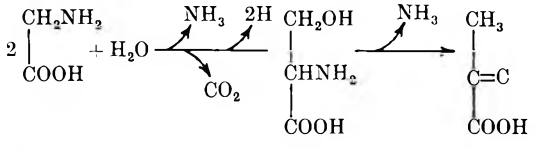
The glyoxylate cycle (Kornberg & Krebs, 1957; Kornberg & Madsen, 1958) in which isocitrate lyase (Smith & Gunsalus, 1954; Olson, 1954) and malate synthase (Wong & Ajl, 1956) operate in conjunction with enzymes of the TCA cycle to form 1 mol. unit of succinate from 2 mol. units of acetate, is the best documented of all such pathways. However, this biosynthetic route is not available to organisms utilizing 2-C compounds more highly oxidized than acetate. A number of pathways all involving glyoxylate or glycine or both have now been described whereby net synthesis of pyruvate from such compounds may be accomplished.

In micro-organisms such as *Escherichia coli* or Pseudomonas growing on glycollate, the necessary net formation of TCA cycle intermediates is effected via the 'glycerate pathway' (Kornberg, 1961). In this pathway glyoxylate (derived from the oxidation of the glycollate) undergoes a condensation reaction in which 2 mol. units of glyoxylate yield 1 mol. unit each of tartronic semialdehyde and carbon dioxide (Krakow & Barkulis, 1956; Krakow, Barkulis & Hayashi, 1961; Kornberg & Gotto, 1959, 1961): the enzyme catalysing this process has been named glyoxylic carboligase (Krakow, Barkulis & Hayashi, 1961). The tartronic semialdehyde thus formed is subsequently reduced to glyceric acid by tartronic semialdehyde reductase (Kornberg & Gotto, 1959, 1961; Krakow, Udaka & Vennesland, 1962). In the presence of ATP and Pseudomonas extracts, glyceric acid has been shown to yield pyruvate, presumably via the well-established Embden–Meyerhof sequence. The net effect of these reactions is to transform 2 mol. units of glycollate to 1 mol. unit of carbon dioxide and one of pyruvate which can either be carboxylated to oxaloacetate, or, after oxidation to acetylcoenzyme A, condensed with a third mol. unit of glyoxylate to yield malate (Kornberg & Sadler, 1961); in either case, the *de novo* formation of intermediates of the TCA cycle from glycollate has been effected.



The glycerate pathway operates not only during growth on glycollate but also on a number of 2-C compounds that first give rise to glyoxylate. Thus Callely & Dagley (1959) reported that glyoxylic acid carboligase and tartronic semialdehyde reductase were present in large amounts in extracts of a pseudomonad grown on glycine. *Pseudomonas oxalaticus* also formed these enzymes during growth on oxalate; this provided evidence for the initial reduction of oxalate to glyoxylate by this organism (Quayle & Keech, 1959). In this process oxalate is activated to oxalylcoenzyme A (Quayle, Keech & Taylor, 1961) which is reduced to glyoxylate by glyoxylic dehydrogenase and $NADPH_2$ (Quayle & Taylor, 1961). Thus pseudomonads growing on glycollate, glycine or oxalate are all in effect growing on glyoxylate and a source of 'reducing power'.

It is probable that in some organisms an alternative route for glycine utilization is available which does not require its preliminary deamination. Kornberg & Morris (unpublished) observed that serine, alanine and glutamate were early-labelled products during growth of *Arthrobacter globiformis* on [^{14}C]-glycine. Extracts of the organism formed pyruvate, alanine and CO_2 from glycine in the presence of reduced pyridine nucleotides, pyridoxal phosphate and tetrahydrofolic acid. It would seem that in such coryneform organisms 2 mol. units of glycine might undergo a condensative decarboxylation in the presence of pyridoxal phosphate and a folic acid derivative to give serine, which would readily yield pyruvate through the action of serine dehydratase. This reaction sequence has been convincingly demonstrated in anaerobic organisms capable of fermenting glycine with acetate as end product, e.g. *Clostridium acidi-urici* (Hadin & Barker, 1953; Sagers & Beck, 1956), *Diplococcus glycinophilus* (Barker, Volcani & Cardon, 1948; Sagers & Gunsalus, 1961). A similar route may be used for the metabolism of glycollate in plants (Rabson, Tolbert & Kearney, 1962).

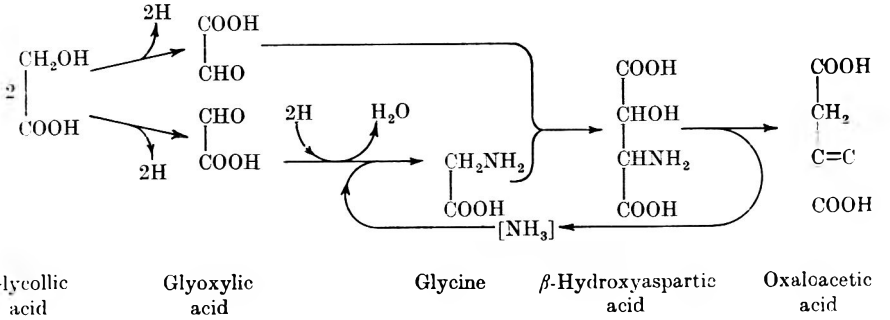


Glycine Serine Pyruvic acid

Here, as in the glycerate pathway, 2 mol. units of the 2-C substrate undergo a condensation reaction wherein one 3-C product is formed with the loss of the carbon atom derived from one carboxyl group as carbon dioxide.

A novel metabolic route which avoids this loss of CO_2 in the initial formation of a compound containing more than 2 carbon atoms operates during growth of *Micrococcus denitrificans* on substrates catabolized to glyoxylate. Here both glycine and glyoxylate are required for the nett formation of TCA cycle intermediates. When *M. denitrificans* was grown on glycollate as sole carbon source, no glyoxylic carboligase activity was present despite initial oxidation of the substrate to form glyoxylate. Short-term incubation of growing cultures with [1-^{14}C]-glycollate resulted in the appearance of isotope first in glycine, malate and aspartate. In contrast to the results obtained with Pseudomonas and *Escherichia coli*, no isotope was detected in glycerate or phosphoglycerate. Evidence was obtained that during growth on glycollate two hitherto unreported enzymes were inducibly formed by *M. denitri-*

ficans (*a*) an aldolase-type enzyme which catalysed the condensation of glycine and glyoxylate to form *erythro*-β-hydroxyaspartate, and (*b*) a β-hydroxyaspartate dehydratase capable of forming oxaloacetate from the *erythro* form of β-hydroxyaspartate (Kornberg & Morris, 1962*a*, *b*). Acting in conjunction with a mechanism for the production of glycine from glyoxylate these enzymes could account for the formation of 1 mol. unit of a 4-C compound (oxaloacetate) from 2 mol. units of glyoxylate without concomitant loss of CO_2 (though the subsequent utilization of oxaloacetate for biosyntheses would, of course, be accompanied by loss of CO_2 (Kornberg & Morris, 1963).



Other sources of glyoxylate, e.g. allantoin and ethyleneglycol, are also utilized by *Micrococcus denitrificans* via this β-hydroxyaspartate pathway.

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Carbon and Energy Storage in Bacteria

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Many compounds have been assumed to act as storage materials in bacteria. Those most commonly implicated as specialized carbon and energy reserves are intracellular polysaccharides particularly polyglucoses such as glycogen and starch, and lipids, particularly poly-β-hydroxybutyrate (reviewed by Wilkinson, 1959). The evidence available suggests that polyglucoses and lipids act as alternative reserves depending upon the bacterial species and upon the nature of the carbon and energy source in the environment. Thus all the enterobacteria can store glycogen while most Bacillus species can store poly-β-hydroxybutyrate. On the other hand, Stanier, Doudoroff, Kunisawa & Contopoulou (1959) have shown that the product of photosynthetic assimilation in *Rhodospirillum rubrum* depends on the substrate used. Acetate and butyrate were converted to poly-β-hydroxybutyrate while malate or succinate formed a polyglucose. It is probable that the ability to form such alternative reserves is common among bacteria. Dagley & Johnson (1953) studied lipid and polysaccharide production in *Escherichia coli* and showed that while acetate stimulated lipid production and depressed polysaccharide production, glucose had the opposite effect. Similarly, *Bacillus megaterium* synthesizes an appreciable amount of glycogen as well as poly-β-hydroxybutyrate (Barry, Gavard, Milhaud & Aubert, 1953). It can be expected that a carbon and energy reserve will be synthesized or broken down depending upon whether there is an excess or a deficiency of the carbon and energy sources available. As examples, glycogen synthesis in *E. coli* and poly-β-hydroxybutyrate synthesis in *B. megaterium* will be considered.

Synthesis of glycogen and poly-β-hydroxybutyrate

Both glycogen and poly-β-hydroxybutyrate can accumulate in large amounts within the cell, particularly in the stationary phase when growth is limited by a deficiency of some factor other than the carbon and energy source. Thus, in a nitrogen-deficient medium, *Escherichia coli* can synthesize as much as 20% of its dry weight as glycogen (Holme & Palmstierna, 1956*b*) and *Bacillus megaterium* as much as 40% of its dry weight as poly-β-hydroxybutyrate (Macrae & Wilkinson, 1958*a*). This accumulation is the result of an unbalanced growth in the stationary phase and is equivalent to conditions in a washed suspension provided with a suitable carbon and energy source. However, these large amounts are produced only under abnormal conditions analogous to what Foster (1947) has called a 'hothouse' environment. Do glycogen and poly-β-hydroxybutyrate occur in appreciable quantities in cells grown under conditions analogous to those occurring in nature? It is difficult to reproduce in the laboratory natural conditions for organisms like *E. coli* and *B. megaterium*. For example, the supply of the carbon and energy source will probably be intermittent. However, glycogen and poly-β-hydroxybutyrate

can accumulate in the exponential phase of growth as evidenced by continuous culture experiments.

In most of the published work on polysaccharide production in continuous culture, it is impossible to distinguish between intracellular polyglucoses and extracellular structural polysaccharides. However, Holme (1957) has studied glycogen production in *E. coli* growing at various dilution rates (i.e. growth rates) in a

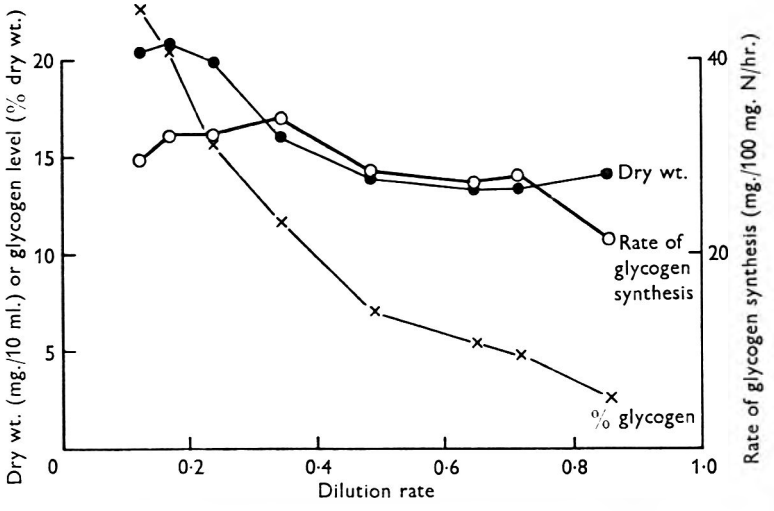

Fig. 1. The effect of the dilution rate (growth rate) in nitrogen-deficient continuous cultures of *Escherichia coli* upon the dry weight of the culture, the percentage of glycogen in the cells and the rate of glycogen synthesis. ●, Dry weight; ×, % glycogen; ○, rate of glycoger synthesis. From Holme, 1957.

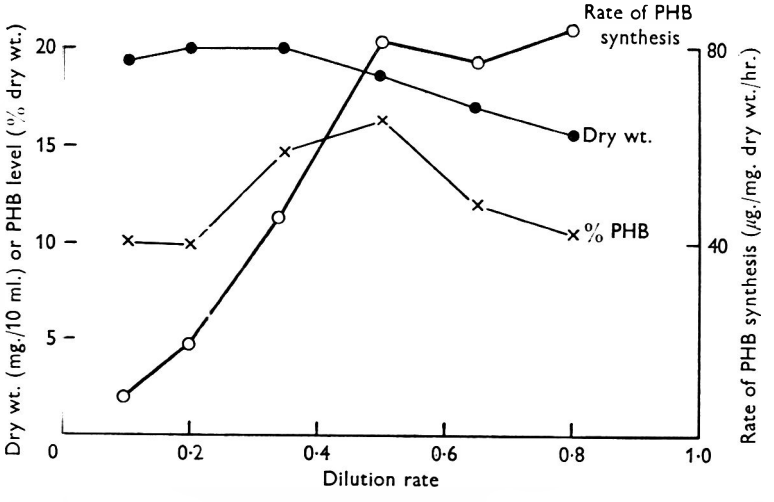

Fig. 2. The effect of the dilution rate (growth rate) in nitrogen-deficient continuous cultures of *Bacillus megaterium* upon the dry weight of the culture, the percentage of poly-β-hydroxybutyrate in the cells and the rate of poly-β-hydroxybutyrate synthesis. ●, Dry weight; ×, %, poly-β-hydroxybutyrate; ○, rate of poly-β-hydroxybutyrate synthesis. From Munro & Wilkinson (unpublished results).

nitrogen-deficient simple defined medium with glucose as the carbon and energy source. Some of his results are shown in Fig. 1. It is evident that the rate of glycogen production per unit amount of cell nitrogen was roughly constant at all growth rates. Glycogen production under balanced growth must occur at a similar rate to that under unbalanced growth, resulting in a higher content of glycogen at low dilution rates. We have studied poly-β-hydroxybutyrate production in nitrogen-deficient cultures of *B. megaterium* (Munro & Wilkinson, unpublished data) Typical results are given in Fig. 2 and show that appreciable amounts of poly-β-hydroxybutyrate were produced at all growth rates although the rate of synthesis was higher at high dilution rates. In both *E. coli* and *B. megaterium*, the level of the storage product at high dilution rates corresponded to that in the exponential phase of growth in batch culture in a simple synthetic medium with glucose as the carbon and energy source. Presumably in such a medium, some factor other than the rate of utilization of the carbon and energy source is limiting the rate of growth. Under these circumstances, intermediates of the main pathway for the breakdown of the carbon and energy source are either excreted by the cell (e.g. pyruvic and oxoglutaric acids) or are converted to storage polymers. Unfortunately there are no figures available for glycogen synthesis in carbon and energy-deficient continuous culture of *E. coli* although preliminary experiments in this laboratory with *B. megaterium* suggest a very low level for both poly-β-hydroxybutyrate and glycogen at low dilution rates.

Breakdown of glycogen and poly-β-hydroxybutyrate

The subject of endogenous metabolism has been reviewed recently by Dawes & Ribbons (1962). It is evident that many substances can act as substrates for endogenous metabolism depending upon the micro-organism and upon the conditions of growth, but it is certain that glycogen and poly-β-hydroxybutyrate can be broken down at a rapid rate by 'resting' cells and can act as primary endogenous substrates. Thus Ribbons & Dawes (1963) have concluded that glycogen is the primary endogenous substrate for *Escherichia coli* and that only when it is exhausted will net degradation of other substrates such as ribonucleic acid and protein occur. The term 'net degradation' must be emphasized since Mandelstam (1960) has pointed out that turnover of ribonucleic acid and protein occurs as soon as balanced growth ceases. Poly-β-hydroxybutyrate can also be a major substrate for endogenous respiration in *Bacillus megaterium* (Macrae & Wilkinson, 1958*b*), although its breakdown is rarely complete or unique.

Since glycogen and poly-β-hydroxybutyrate can be accumulated and degraded, what determines their amount in a cell? Is it simply a measure of the steady-state level of the monomer donor or is there some degree of control over the rate of synthesis or breakdown? We do not know the pathway of glycogen metabolism in *Escherichia coli* but in *Agrobacterium tumefaciens* there is a glycogen cycle similar to that in animal tissues (Madsen, 1961*a*); it is shown in Fig. 3. Control seems to be exerted by the intracellular concentration of uridine diphosphate glucose which, if high, acts as a substrate for glycogen synthetase and inhibits glycogen phosphorylase (Madsen, 1961*b*). Poly-β-hydroxybutyrate metabolism in bacteria has been studied by Merrick & Doudoroff (1961), the pathways for synthesis and breakdown being summarized in Fig. 4.

In order to demonstrate a storage function for a compound, it is necessary to show that the products of breakdown can be used for some purpose that gives the cell an advantage in the struggle for existence over other organisms not so endowed. There is evidence that both glycogen and poly-β-hydroxybutyrate can provide intermediates for the synthesis of proteins (Holme & Palmstierna, 1956*a*; Doudoroff & Stanier, 1959). This may allow an increment of growth and division which may well be important, particularly if the supply of the carbon and energy source is sporadic. It may also allow a more rapid adaptation to a different environment by the production of inducible enzymes or permeases. Further, poly-β-hydroxybutyrate breakdown has been shown to provide energy and intermediates for the process of sporulation (Slepecky & Law, 1961). The breakdown of a reserve may help to preserve viability by providing the so-called energy of maintenance, the necessity of which has been shown by Mallette (1963) and by Marr, Wilson & Clark (1963). It is

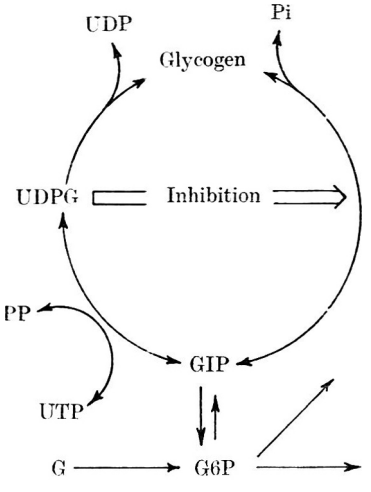

Fig. 3

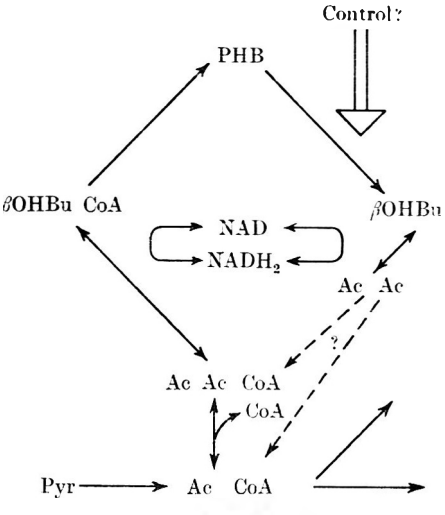

Fig. 4

Fig. 3. The glycogen cycle in *Agrobacterium tumefaciens*. G = glucose; G6P = glucose-6-phosphate; G1P = glucose-1-phosphate; UTP = uridine triphosphate; UDP = uridine diphosphate; UDPG = uridine diphosphate glucose; PP = inorganic pyrophosphate; Pi = inorganic orthophosphate. From Madsen (1961*a*, *b*).

Fig. 4. The metabolism of poly-β-hydroxybutyrate in *Bacillus megaterium*. Pyr = pyruvate; Ac = acetate; Ac Ac = acetoacetate; βOHBu = β-hydroxybutyrate; CoA = coenzyme A; PHB = poly-β-hydroxybutyrate. From Merrick & Doudoroff (1962) and Merrick, Doudoroff & Wilkinson (unpublished results).

obvious, however, that the question of the preservation of viability is a complex one. Strange, Dark & Ness (1961) have shown that death of *Aerobacter aerogenes* populations was preceded by degradation of protein, ribonucleic acid and polysaccharide and Postgate & Hunter (1962) have analysed in great detail one of the factors involved in the survival of starved bacteria. It is evident that polymers such as protein and nucleic acid can be metabolized to provide carbon and energy without loss of viability. Indeed, Campbell, Gronlund & Duncan (1963) have suggested that *Pseudomonas aeruginosa* synthesizes no specialized reserves. On the other hand, loss

of viability of *Micrococcus halodenitrificans* can follow closely the loss of stored poly-β-hydroxybutyrate (Sierra & Gibbons, 1962).

In conclusion, we can assume that a cell will tend to store any polymers that can be accumulated without decreasing the rate of growth. The nature of these polymers will depend primarily upon the rate-limiting step in growth and, therefore, upon the nature and level of nutrients in the medium. Probably the rate-limiting factor will either be in the synthesis of proteins and nucleic acids when reserves containing carbon, hydrogen and oxygen only will accumulate, or it will be in the primary degradative pathway of the carbon and energy source when no specialized carbon and energy reserves will accumulate.

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The Microbiological Degradation of Aromatic Compounds

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The breakdown of aromatic compounds by ring cleavage is an essential biochemical step in Nature's 'carbon' cycle and is performed by several kinds of microorganism. Bacteria are the most versatile in this respect, but several yeasts and fungi are able to degrade a more limited range of benzenoid structures (see list of Reviews on p. 183).

Among the eubacteria, representatives of the families Coccaceae, Mycobacteriaceae, Pseudomonadaceae, Spirillaceae, Bacteriaceae and Bacillaceae are active in this respect. The yeasts Oospora, Candida, Debaromyces, Pichia and Saccharomyces grow in media with catechol as sole carbon source. Certain higher fungi, e.g. Aspergillus, Penicillium and Neurospora attack aromatics and a variety of soil and wood-rotting fungi dissimilate the aromatic polymer lignin, as well as other plant phenolics. In all the above cases of aromatic ring metabolism, molecular oxygen is an obligatory oxidant.

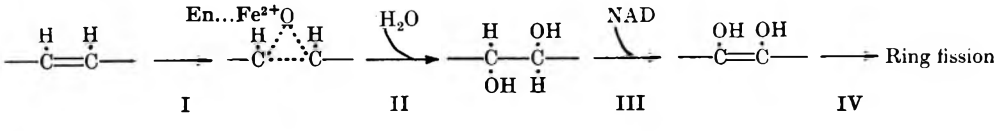
The photosynthetic bacteria Rhodopseudomonas and some Rhodospirillum strains utilize benzoate under strictly anaerobic conditions in the light. Finally, there exists an anaerobic type of aromatic ring metabolism—e.g. the so-called methane fermentation of benzoate, although it is doubtful whether these methane bacteria are in pure culture.

These microbes produce, mostly as a result of induction, a whole sequence of enzymes which convert aromatic substrates into an *ortho* or *para* dihydroxyphenol derivative, followed by cleavage of the ring to aliphatic acids; these ring fission products are then funnelled into the Krebs cycle through a variety of pathways, depending on the organism and cultural conditions.

The distinctive biochemical step is ring cleavage. There are many instances of microbes being able to modify aromatic compounds, either by hydroxylation, or elimination of substituent groups, without necessarily causing fission. In the case of the aromatic amino acids, phenylalanine, tyrosine and tryptophan, pathways of metabolism exist that have much in common in all forms of life. It is only possible to sketch the salient features of microbial aromatic ring metabolism here, since the topic has, of recent years, greatly increased in scope.

(1) *Aromatic hydroxylating enzymes*

Aromatic hydrocarbons undergo a perhydroxylation to the *trans*-dihydroarenediols, which are then dehydrogenated to the *o*-dihydroxyphenol prior to ring fission, thus:

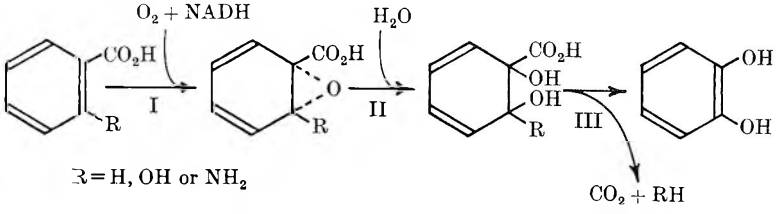


A similar system exists in liver microsomes. The bacterial enzymes are extremely labile and difficult to separate; parts of this scheme are therefore still hypothetical. The corresponding 'diol' intermediate has been isolated from cultures of naphthalene (Walker & Wiltshire, 1953) and phenanthrene (Colla, Fiecchi & Treccani, 1959); evidence for its formation also exists in the case of benzene (Marr & Stone, 1959, 1961) and anthracene (Colla *et al.* 1959). Ayengar, Hayaishi, Nakajima & Tomida (1959) studied reaction III, using sonic extracts of *Aerobacter aerogenes* known to contain a specific myoinositol dehydrogenase, but conveniently lacking the enzymes catalysing reactions I, II, and IV. With *trans*-3,5-cyclohexadiene-1,2-diol as substrate, catechol was formed; NAD and a regenerating system were necessary for maximal activity.

The perhydroxylation step is Fe^{2+} dependent, and to account for the invariable formation of *trans*-dihydrodiols, the enzyme + oxygen + substrate complex is visualized as being of the epoxide type; second-order nucleophilic attack by a hydroxyl anion would yield the *trans*-isomer.

Polynuclear aromatic hydrocarbons attach to the relevant bacterial enzymes at C–C bonds of high electron density (the K regions of Pullman & Pullman, 1955). If they have a linear configuration, e.g. naphthalene, anthracene, attachment and ring splitting takes place on the same ring. Angular aromatic compounds such as phenanthrene afford attachments to an enzyme at a bond in a ring other than the one containing the ring-splitting site (Rogoff, 1962).

Benzoate, salicylate and anthranilate are common intermediates in degradative pathways of more complex aromatic compounds. These acids are converted to catechol by bacterial oxidases before ring fission; recently much light has been shed on these particular enzymes. Ichihara, Adachi, Hosokawa & Takeda (1962) prepared active cell-free systems which oxidatively decarboxylated these acids and caused ring cleavage of the resulting catechol. If *meta*-substituted benzoates were used as substrates, the oxidase preparations converted them to the relevant catechols only, since the specificity of the fission enzymes prevented them from being metabolized further; in this way they were able to study the initial enzyme reactions. Another successful approach has been the purification of the relevant enzyme systems. Thus Hosokawa, Nakagawa & Takeda (1961) fractionated crude anthranilate oxidase preparations into two indispensable protein components. Katagiri, Yamamoto & Hayaishi (1962) separated salicylate hydroxylase activity from the catechol cleavage enzyme. Results show these enzymes to be Fe^{2+}-dependent requiring NADH or NADPH (and regenerating systems, e.g. ethanol and alcohol dehydrogenase). In the case of salicylate hydroxylase, an absolute requirement for flavin adenine dinucleotide (FAD) was demonstrated in addition.



The mode of action of reduced pyridinenucleotides is not yet clear, but as suggested by Mason (1957) for a mixed function oxidase, one atom of oxygen might be

used to hydroxylate the substrate, and the second be reduced by the NADH (or NADPH). A variety of electron donors function in different hydroxylating systems, e.g. NADH, NADPH, $FADH_2$ in the case of the bacterial enzymes; ascorbic acid with phenolases; tetrahydropteridine in the phenylalanine hydroxylase of liver, although NADPH serves as a reductant indirectly to reduce the oxidized pteridine derivative.

Brief mention must be made at this point of the fate of the other monohydroxybenzoates. Yano & Arima (1958) succeeded in extracting from some bacteria hydroxylase A, which converted *m*-hydroxybenzoate to protocatechuate, and from other organisms a hydroxylase *B*, which gave gentisate. Both hydroxylases required NADPH as co-factor. The subsequent pathways of these two dihydroxybenzoates differ (Figs. 1, 2). *p*-Hydroxybenzoate gives protocatechuate in all organisms studied.

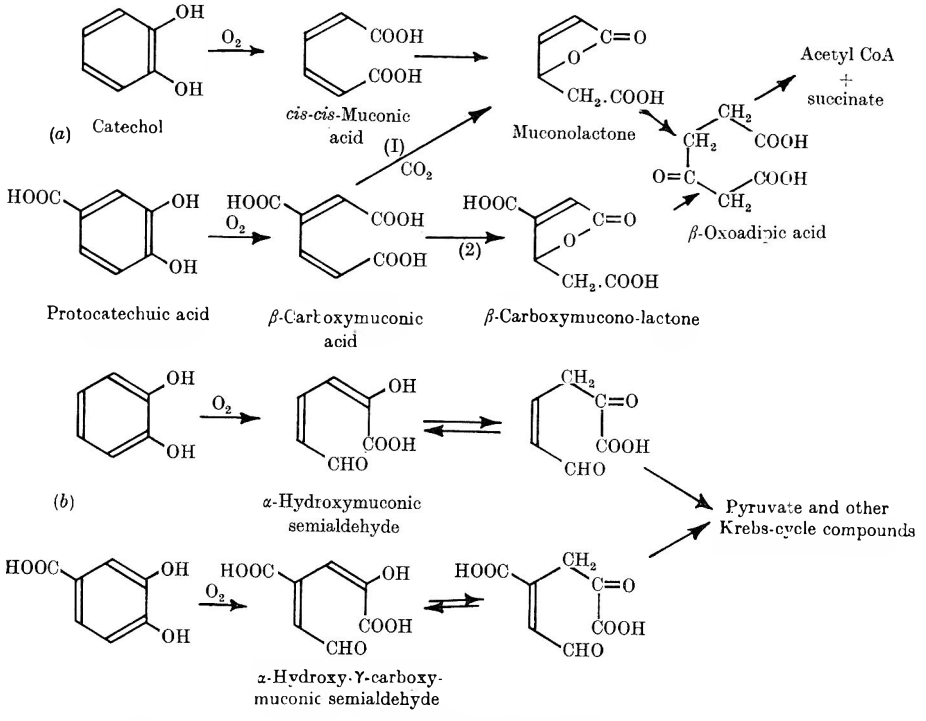


Fig. 1. Oxidative fission of *o*-dihydroxyphenols by micro-organisms.

(2) *Aromatic ring-cleavage enzymes**

The enzyme molecule contains Fe^{2+} and sulphydryl groups, the integrity of both being essential for activity; molecular oxygen is obligatory.

In the case of *o*-dihydroxyphenol derivatives, two distinct cleavage enzymes are known. Their action may be illustrated with respect to catechol and protocatechuate, two central aromatic intermediates (Fig. 1).

(*a*) Pyrocatechase (catechol 1,2-oxygenase) and protocatechuic acid oxidase (protocatechuate 3,4-oxygenase) open the C–C bond between the two hydroxyls.

* Dagley, Evans & Ribbons (1960).

The ring fission product, a *cis-cis* muconic acid, is then lactonized by a Mn^{2+} dependent enzyme; hydrolytic cleavage of this to β-oxoadipate is achieved by a delactonizing enzyme. An enzymic C4–C2 split of this oxo-acid in the presence of coenzyme A and catalytic amounts of succinyl-coenzyme A provides the substrates for the tricarboxylic acid cycle, thus:

$$\begin{array}{rcl} \beta\text{-oxoadipate} & \overset{\text{succinyl-CoA. thiophorase}}{\rightleftharpoons} & \text{acetyl-CoA.} \\ \text{succinate} & & \overset{\text{thiolase}}{\rightleftharpoons} \ \beta\text{-oxoadipyl-CoA} \end{array} \quad \text{CoA.}$$

(*b*) Catechol 2,3-oxygenase (metapyrocatechase) and protocatechuate 4,5-oxygenase open the C–C bond adjacent to a hydroxyl. The muconic semialdehyde so formed can undergo a non-enzymic reaction with NH_4^+ ions to give a pyridine nucleus, or be metabolized to Krebs cycle acids, one of which is certainly pyruvate. In their announcement of the new catechol pathway, Dagley & Stopher (1959) suggested a route of metabolism of α-hydroxymuconic semialdehyde to pyruvate without gaseous exchange; subsequently, Nishizuka, Ichiyama, Nakamura & Hayaishi (1962) have suggested the following route:

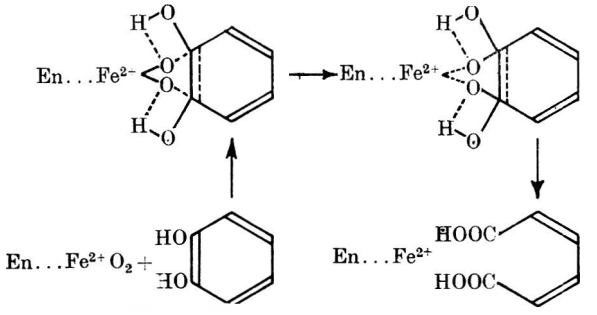
$$\begin{aligned} \text{catechol} &\xrightarrow{O_2} OHC{-}CH{=}CH{-}CH{=}C(OH){-}CO_2H \\ &\xrightarrow{NAD} HO_2C{-}CH{=}CH{-}CH_2{-}CO{-}CO_2H \\ &\xrightarrow{H_2O} CO_2 + CH_3{-}CH(OH){-}CH_2{-}CO{-}CO_2H \\ &\xrightarrow{NAD} (CH_3{-}CO{-}CH_2{-}CO{-}CO_2H) \\ &\xrightarrow{H_2O} CH_3{-}CO_2H + CH_3{-}CO{-}CO_2H \end{aligned}$$

It is interesting to note that Gholson *et al.* (1962) have recently implicated α-hydroxymuconic semialdehyde in the catabolism of tryptophan by mammalian liver. Ribbons & Evans (1962) adduced evidence in favour of pyruvate and malate as the products of protocatechuate metabolism via γ-carboxy-α-hydroxymuconic semialdehyde.

In the case of *p*-dihydroxyphenols, rupture of the bond between the carbon atom bearing a hydroxyl and an adjacent carbon atom carrying a side chain or carboxyl, occurs. For homogentisate, this method is common to micro-organisms (Chapman & Dagley, 1962) and mammals, being the pathway of tyrosine metabolism. Both homogentisicase and gentisicase give rise initially to the aliphatic oxo-acids with the *cis* (maleyl) configuration, but a glutathione dependent isomerase converts them to the *trans* (fumaryl) isomers; a hydrolase then catalyses fission of these to fumarate and acetoacetate, and fumarate and pyruvate respectively (Fig. 2).

There are two routes of tryptophan metabolism found among Pseudomonads. The 'anthranilate' pathway involves oxidative fission of the pyrrol ring to formylkynurenine, elimination of the formyl group and a non-oxidative cleavage of the kynurenine to alanine and anthranilate (subsequently to be metabolized via catechol). In the 'quinoline' pathway, kynurenic acid is formed, which is then hydroxylated in the C7, 8, positions; fission of the aromatic nucleus apparently occurs in two ways. Hayaishi & collaborators (Kuno *et al.* 1961) initially suggested a split between the two hydroxyls, then revised this view (Kojima, Itada & Hayaishi, 1961) in favour of cleavage between C8 and the angular C atom. The substituted pyridine rings so formed give rise to glutamate in both cases.

Mechanism of action of bacterial oxygenases. These all belong to the class of enzymes called 'oxygen transferases' by Mason (1957). A tracer study with ^{18}O proves that the two oxygen atoms in the ring fission product are derived from molecular oxygen (Hayaishi, Katagiri & Rothberg, 1957). It is thought that the first step is the formation of an oxyferro-oxygenase. Electrons have been donated to the oxygen from the iron, allowing the O—O bond to be weakened (Ingraham, 1962);



association of the perferryl complex with the *o*-dihydroxyphenol group leads to a redistribution of electrons and fission of the C–C bond. Oxygenases are labile in oxygen; Taniuchi *et al.* (1962) were able to restore catechol 2,3-oxygenase (metapyrocatechase) activity with sodium borohydride (and other reducing agents to a certain extent) best under anaerobic conditions. $FADH_2$, FMN and riboflavin in the presence of NADH were also effective, and they suggest a physiological role for flavin coenzymes in reductive enzyme reactivations.

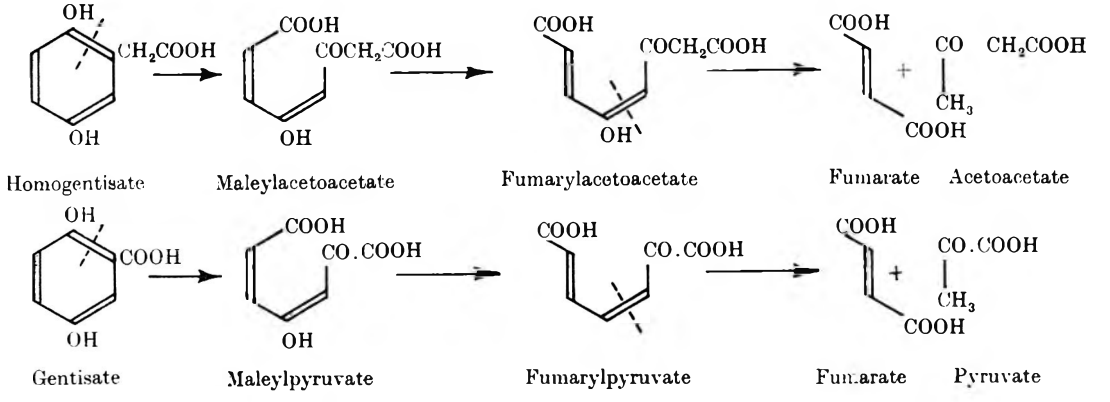


Fig. 2. Oxidative fission of *p*-dihydroxyphenols by micro-organisms and mammals.

(3) *Fate of substituent groups on monocyclic aromatic rings before cleavage*

There are obviously a great many biochemical reactions which ring-splitting micro-organisms perform on substituted benzenoid structures before nuclear fission. A few of these may be illustrated.

(i) Aromatic methyl ethers are demethylated to the corresponding hydroxy-derivative. Semicarbazide traps the methoxyl C as formaldehyde; in its absence it is oxidized terminally to CO_2 (Woodings, 1961).

(ii) Methyl substituents may be oxidized step-wise to the carboxylic acid (e.g. the cresols), but in some cases it remains intact until after ring fission (e.g. MCPA metabolism, Evans, Gaunt & Davies, 1961).

(iii) Chloro-substituents usually remain on the ring unless removal is obligatory for the introduction of hydroxyls, which are necessary for fission.

(iv) Nitro and sulphonic acid groups can be eliminated and replaced by hydroxyl.

(v) A carboxyl may be oxidatively decarboxylated; in some cases anaerobic decarboxylation occurs, e.g. of 4,5-dihydroxyphthalate to protocatechuate (Ribbons & Evans, 1960).

(vi) Aliphatic side-chains are dealt with in a variety of different ways. They may be eliminated, by β-oxidation (Webley, Duff & Farmer, 1955) or other mechanisms familiar to biochemistry, or even remain intact—e.g. phenylpropionic acid (Coulson & Evans, 1959; Dagley & Chapman, 1961, private communication).

(4) *Metabolism of polynuclear aromatic hydrocarbons*

Perhydroxylation followed by dehydrogenation produces 1,2-dihydroxynaphthalene, 1,2-dihydroxyanthracene, and 3,4-dihydroxyphenanthrene from their parent hydrocarbons. The actual site of cleavage in the naphthalene nucleus is the bond between the angular C and C1 which also carries a hydroxyl (Davies & Evans, 1962). There is strong evidence that it takes place at the analogous bond in 1,2-dihydroxyanthracene and 3,4-dihydroxyphenanthrene (Fernley & Evans, 1958; Griffiths & Evans, 1963, personal communication).

The case of naphthalene will serve to illustrate this pathway: A Fe^{2+} oxygenase converts 1,2-dihydroxynaphthalene to *o*-hydroxybenzalpyruvate; a hydrolytic split converts this to salicylaldehyde and pyruvate; a NAD dependent dehydrogenase produces salicylate from the aldehyde; a salicylate hydroxylase gives catechol, which is ruptured to α-hydroxymuconic semialdehyde by the catechol 2,3-oxygenase also present in the cell-free extract.

(5) *Anaerobic metabolism of certain aromatic compounds*

Hitherto, we have considered only the aerobic metabolism of aromatic compounds by micro-organisms. Although much less is known about them, certain derivatives of benzene are dissimilated under anaerobic conditions by some bacteria.

Photosynthetic bacteria require accessory hydrogen donors for anaerobic photosynthesis in light; although evolution of oxygen has never been observed, an oxidizing entity of sufficient positive potential to perform the peroxidation of benzoate is produced by several strains of non-sulphur photosynthetic bacteria, e.g. Rhodopseudomonas and Rhodospirillum. According to Proctor & Scher (1960) sequential induction evidence supports the following tentative scheme:

Benzoate → protocatechuate → catechol → unidentified oxo-acids.

Clarke & Fina (1952) confirmed the utilization of benzoate by methane-producing cultures; catechol and protocatechuate could not be metabolized by these cultures, and exogenous CO_2 was not reduced mainly to methane. In subsequent tracer studies (Fina & Fiskin, 1960; Roberts, 1962) it was determined that the carboxyl-C and C4 of benzoate behaved the same as exogenous CO_2. They were not reduced to methane primarily; on the other hand, C1 of the ring was converted almost entirely

to methane. Benzoate utilizing cultures did not produce methane and CO_2 from fumarate, acrylate or isobutyrate without very long adaptive lag periods. Cyclohexane carboxylic acid, butyric and propionic acids, however, produced gas readily with no lag period. Propionate, containing C4 of the ring, was isolated from the benzoate culture liquor; it did not contain C1 or C7. One can thus speculate that there is a preliminary saturation of the ring followed by a rupture between C1 and C2 before propionate is released. No other organic acid has yet been isolated.

REVIEWS

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The Classification of some Violet-Pigmented Micrococci

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SUMMARY

Seven strains of micrococci which produce violet pigment were studied. We recommend that the names *Staphylococcus flavocyaneus* Knaysi and *Micrococcus polychromus* Makarova should be regarded as synonyms of *M. luteus* (Shroeter) Cohn, and consider that *M. violagabriellae* Castellani belongs to the species *Staphylococcus epidermidis*. For the practical diagnosis of micrococci we recommend that those strains which form a violet pigment, which do not give acid in glucose peptone water and do not form acetylmethylcarbinol, should be identified as atypical strains of *Micrococcus luteus*.

INTRODUCTION

Several species of micrococci which produce violet pigment have been described but their classification is not satisfactory; *Micrococcus cyaneus* (Schroeter) Cohn (1872), and *Staphylococcus flavocyaneus* Knaysi (1942) are examples. Hucker (1928) thought that they probably belong to the species *M. flavus* and therefore they are not described in the 7th edition of *Bergey's Manual* (1957). Krasilnikov (1949) did not mention any species of violet-pigmented cocci in his guide. Shaw, Stitt & Cowan (1951) considered that *S. flavocyaneus* is identical with *S. afermentans*. The more recently described cocci, *M. polychromus* Makarova (1949) and *M. violagabriellae* Castellani (1955) are not classified in the 7th edition of *Bergey's Manual*. We have studied seven strains of micrococci which produce violet pigment obtained from various collections.

METHODS

The methods used were those described previously (Kocur & Martinec, 1959). The strains studied were strains BS 622, 851, 853, *Micrococcus flavocyaneus*; strain BS 856, *M. cyaneus*; strain BS 852, *Micrococcus* sp.: all from C. B. van Niel (Hopkins Marine Station of Stanford University, Pacific Grove, California, U.S.A.), strain BS 247, *Staphylococcus flavocyaneus* Knaysi NCTC 7011 from National Collection of Type Cultures, London; strain BS 575, *M. violagabriellae* from A. Castellani, Institute for Tropical Medicine, Lisbon, Portugal.

RESULTS

Morphology. All the strains showed Gram-positive cocci of diameter 1·2–2 μ, occurring in clumps; cubical packets were formed only by strains 247, 622 and 851. All the strains were non-motile.

Cultural characteristics. On nutrient agar all the strains formed round slightly

Table 1. *Biochemical characteristics of violet-pigmented strains compared with those of known species of micrococci*

The tests were made as described by Kocur & Martinec (1959).

Test	Strain no. 247	622	851	852	853	856	*Micrococcus luteus**	*Micrococcus polychromus* Makarova, 1949	*Micrococcus violagabriellae*, strain 575
Acid from									
Glucose	−	−	−	−	−	−	−	−	+
Lactose	−	−	−	−	−	−	−	−	+
Sucrose	−	−	−	−	−	−	−	−	+
Maltose	−	−	−	−	−	−	−	−	+
Galactose	−	−	−	−	−	−	−	0	+
Fructose	−	−	−	−	−	−	−	0	+
Rhamnose	−	−	−	−	−	−	−	0	−
Mannose	−	−	−	−	−	−	−	0	+
Inulin	−	−	−	−	−	−	−	0	−
Xylose	−	−	−	−	−	−	−	−	−
Arabinose	−	−	−	−	−	−	−	0	−
Glycerol	−	−	−	−	−	−	−	0	+
Adonitol	−	−	−	−	−	−	−	0	−
Sorbitol	−	−	−	−	−	−	−	0	−
Mannitol	−	−	−	−	−	−	−	0	+
Dulcitol	−	−	−	−	−	−	−	0	−
Salicin	−	−	−	−	−	−	−	0	−
Aesculin hydrolysis	−	−	−	−	−	−	(−)	0	−
Starch hydrolysis	−	−	+	−	+	−	(−)	0	−
Sodium citrate utilized	−	−	−	−	−	−	(−)	0	−
Acetoin formed	−	−	−	−	−	−	−	0	+
Methyl red	−	−	−	−	−	−	−	0	−
Gelatin hydrolysis	+	+	+	+	+	+	(+)	+	−
Casein hydrolysis	+	+	+	+	+	−	(+)	0	−
Nitrate reduced to nitrite	+	+	+	+	+	+	(−)	−	+
Hydrogen sulphide	−	−	−	−	−	−	(−)	0	−
Indole	−	−	−	−	−	−	−	0	−
Milk coagulation	−	−	−	−	−	−	(−)	+	−
Phenylalanine deaminated	−	−	−	−	−	−	−	0	−
Catalase	+	+	+	+	+	+	+	+	+
Haemolysis	−	−	−	−	−	−	−	−	−
Coagulase	−	−	−	−	−	−	−	0	−
Phosphatase	−	−	−	−	−	−	−	0	−
Urease	−	+	+	+	+	−	(−)	0	+
Lipase	−	−	−	−	−	+	(−)	0	+
Yellow pigment on nutrient agar	+	+	+	+	+	−	(+)	+	−
Sensitivity to									
Penicillin 10 units/ml.	+	+	+	+	+	+	(+)	0	−
Streptomycin 20 μg./ml.	+	+	+	+	+	+	(+)	0	+
Chloramphenicol 20 μg./ml.	+	+	+	+	+	+	(+)	0	+
Chlortetracycline 100 μg./ml.	+	+	+	+	+	+	(+)	0	+
Terramycin 50 μg./ml.	+	+	+	+	+	+	−	0	+
Tetracycline 50 μg./ml.	+	+	+	+	+	+	(+)	0	+
Erythromycin 20 μg./ml.	+	+	+	+	+	+	(+)	0	+
Neomycin 40 μg./ml.	+	+	+	+	+	+	(+)	0	+
Tyrothricin 40 μg./ml.	−	−	−	−	−	−	−	0	−
Nystatin 20 units/ml.	+	+	+	+	+	+	(+)	0	−
Bacitracin 5 units/ml.	+	+	+	+	+	+	(+)	0	−
Lysozyme	+	+	+	+	+	+	(+)	0	−

Explanations: + = positive; (+) = most strains positive; − = negative; (−) = most strains negative; 0 = data not given; * = data from Kocur & Martinec (1962).

convex, smooth colonies with an entire, or slightly modulate margin. In five strains the colour of the colonies was yellow, but they also formed another pigment which diffused into the medium and gave it a light brown colour. Strain 856 formed violet-black colonies. Strain 575 (*Micrococcus violagabriellae*) did not produce pigment on any of the media used. The strains formed colonies which were similar in form and colour to the colonies grown on nutrient agar, except that they produced a violet pigment which diffused into the medium on glucose yeast agar (Rosypal, Kocur & Hodák, 1963). Strain 856 formed rough dark-violet colonies on this medium but the pigment diffused only slightly into the medium. All the strains except *M. violagabriellae* produced pigment on potato and on most of the usual diagnostic media (starch agar, fat agar, etc). All the strains formed a slight turbidity and sediment in nutrient broth.

Biochemical characteristics. These are shown in Table 1.

DISCUSSION

With the exception of *Micrococcus violagabriellae* BS 575 all the strains had similar biochemical properties (Table 1). A detailed comparison showed that all the strains other than BS 575 could be classified as the species *M. luteus*. We came to this conclusion by comparing the present results with data from 149 strains of yellow-pigmented cocci and with the characteristics of *M. luteus* (Kocur & Martinec, 1962). The only constant difference between these strains and *M. luteus* was in pigmentation. However, on nutrient agar these strains formed yellow or greenish yellow colonies similar in colour to those of *M. luteus*. Only when cultivated on glucose yeast agar did these strains produce a violet diffusible pigment not observed on other diagnostic media (e.g. gelatin, starch agar, Simmons's citrate medium, blood agar, etc.).

The production of this violet diffusible pigment is not only dependent on the medium, since Rosypal *et al.* (1963) have shown that *Staphylococcus flavocyaneus* and other strains of violet cocci can lose the ability to produce it, but no change in the production of the yellow pigment was noticed on nutrient agar. For these reasons we do not consider the production of a violet pigment is a suitable criterion for the differentiation of micrococci into species. Strain BS 856 differed somewhat from the others; we think that it is an atypical strain of *M. luteus*.

Another species, which produced a violet pigment, the validity of which is questionable, is *Micrococcus polychromus* described by Makarova (1949). We have not studied a strain of this species, but the description of the biochemical properties of the pigment is in agreement with that of strains which we studied. We think it unlikely that it forms a new species and that it is probably a strain of *M. luteus*.

On the basis of these results we recommend that those micrococci which produce a violet pigment and which do not acidify glucose peptone water nor form acetylmethylcarbinol should be classified as *Micrococcus luteus*.

Micrococcus violagabriellae, strain BS 575, differed substantially from the other strains we studied. According to the original description this species is supposed to produce a violet pigment on glucose agar and on potato. The strain which we received directly from Dr A. Castellani, however, and also a strain from the American Type Culture Collection (ATCC 12328) did not produce pigment on either of these

media nor on any other diagnostic media. They may have lost their pigment-forming ability during maintenance. In contrast to Castellani's data the strain we studied formed acetylmethylcarbinol, gave acid in lactose peptone water and in mannitol peptone water, and did not liquefy gelatin. These apparent differences may have been due to the use of different methods. *M. violagabriellae* is apparently not a species of the genus *Micrococcus*, but appears to belong to the species *Staphylococcus epidermidis* (Winslow & Winslow, 1908) Evans. Similar conclusions were drawn by Sneath (1960).

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A Study of Violet-pigmented Micrococci. Yellow-pigmented Mutants of *Staphylococcus flavocyaneus*

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SUMMARY

Staphylococcus flavocyaneus produces at least two pigments on glucose yeast-extract agar; a violet diffusible pigment and a yellow non-diffusing pigment. On some media the production of violet diffusible pigment is inhibited. In broth cultures of this organism a mutation takes place giving rise to yellow-pigmented mutants which have lost the ability of producing the violet pigment. A considerable accumulation of these mutants occurs in cultures on both solid and liquid media. No reversion to the violet-pigmented state or other change in pigmentation in the mutants has been observed.

The yellow-pigmented mutants do not differ from strains of *Micrococcus luteus* with the exception of some characteristics which are variable within that species. This supports the conclusions of Kocur & Martinec (1963) that *Staphylococcus flavocyaneus* Knaysi should be regarded as a strain of *Micrococcus luteus* (Schroeter) Cohn.

INTRODUCTION

Kocur & Martinec (1963) concluded that the violet-pigmented cocci named *Staphylococcus flavocyaneus* Knaysi, *Micrococcus cyaneus* (Schroeter) Cohn, and *M. polychromus* Makarova are identical with *M. luteus* (Schroeter) Cohn. Knaysi (1942) observed that one of these cocci described under the name *S. flavocyaneus* dissociated spontaneously into a lemon-yellow strain. Under our cultural conditions on the glucose yeast-extract agar *S. flavocyaneus* produced, according to Knaysi's observation (1942), two pigments, one violet and diffusible, the other yellow and non-diffusing. In preliminary experiments we found that the accumulation of yellow-pigmented variants occurred in the broth cultures of *S. flavocyaneus* even if we initiated the cultures with very small inocula that did not contain the yellow variants. In addition the proportion of yellow-pigmented variants varied significantly between similar broth cultures of this organism. According to these findings, which will be described in detail in a subsequent paper, we consider the yellow variants of *S. flavocyaneus* to be spontaneous mutants. Here we describe the population changes in broth cultures of *S. flavocyaneus* due to the accumulation of the yellow-pigmented mutants, and the taxonomic significance of this in the classification of *S. flavocyaneus* will be considered.

METHODS

Most of the observations described in this article were made with the strain BS 852 designated by us as *Staphylococcus flavocyaneus* and received from Professor C. B. van Niel. Other experiments were made with the original strain of Knaysi *S. flavocyaneus* (NCTC 7011), and with strains BS 851 and BS 856 (see Kocur & Martinec, 1963). We found the same phenomenon in Knaysi's type strain. The strains given in Table 3 come from our collection.

Gurr's nutrient broth was used for the studies of the accumulation of mutants. Viable counts were made by plating 0·1 ml. of appropriate dilutions on glucose yeast-extract agar (g./l. distilled water: yeast extract, 5; peptone, 5; glucose, 10; agar, 10; adjusted to pH 7·2). The following media were also used: Difco nutrient agar; nutrient agar with gelatin (Difco nutrient agar, 900 ml.; 10 % (w/v) gelatin, 10 ml.; adjusted to pH 7·2); Difco nutrient agar with 2 % (w/v) soluble starch (adjusted to pH 7·2); yeast-extract agar with phenylalanine (g./l. distilled water: yeast extract, 3; DL-phenylalanine, 2; Na_2HPO_4, 1; NaCl, 5; agar, 10); glucose yeast-extract agar with beef extract (g./l. distilled water; yeast extract, 3; beef extract, 3; glucose, 20; $CaCO_3$, 20; agar, 20).

Serial transfer of BS 852 was carried out as follows: Erlenmeyer's flasks, each containing 10 ml. broth, were inoculated with $878 \pm (ts_{\bar{x}} = 65, P = 0{\cdot}05)$ cells of strain BS 852. Control platings showed that there were no yellow-pigmented mutants in the inocula. The flasks were incubated for 96 hr. at 30° and 0·1 ml. was transferred into flasks containing fresh broth. Similar transfers were repeated ten times, but at 48 hr. intervals. After the second, fourth, sixth, eighth and tenth transfers platings of suitable dilutions were made on glucose yeast-extract agar and incubated at 30° for 72 hr. to determine the percentage of yellow-pigmented mutants. The same procedure was used with strains NCTC 7011, BS 851 and BS 856.

To estimate the mutants in cultures on solid media a loopful of an agar slant culture grown for 48 hr. at 30° was suspended in a sterile physiological saline and plated as above.

Growth rates were determined from viable counts on populations allowed to grow for 22 hr. at 30°. As the growth constant we used the mean division rate (r), calculated according to Monod (1949).

The biochemical tests were the same as in previous works (Kocur & Martinec, 1959, 1963).

Each experiment described in this paper consists of several parallel observations from which the mean was calculated. With these parallels it was several times repeated. The results given involve the values of successful experiments which were analysed by conventional statistical methods, e.g. by the analysis of variance or by estimating the standard error ($s_{\bar{x}}$). The value of δ was calculated according to the equation

$$\delta = t\sqrt{\frac{2 \times \text{residual variance}}{m}},$$

where m is the number of members in the analysed group.

RESULTS

Table 1 shows that after the second transfer the number of yellow-pigmented mutants reaches 8·73 % of the *Staphylococcus flavocyaneus* population. After the eighth transfer the number of mutants reaches over 90 %.

Strains NCTC 7011, BS 851 and BS 856 were similarly examined. After the tenth transfer there were on the average 8·35 % of yellow-pigmented mutants in strain NCTC 7011 and 19·1 % in strain BS 851. In BS 856 no yellow-pigmented mutants were found.

Table 1. *Accumulation of yellow-pigmented mutants of* Staphylococcus flavocyaneus *during serial transfers in nutrient broth*

Each population was initiated with $878 \pm (ts_{\bar{x}} = 65, P = 0{\cdot}05)$ cells of strain BS 852 and then after 48 hr. incubation at 30° 0·1 ml. of each population was transferred into fresh nutrient broth. The figures are the averages of six experiments.

No. of transfers	Average percentage of mutants
2	8·73
4	30·68
6	70·16
8	91·50
10	97·33

Table 2. *Mean division rates of strain* BS 852 *and its yellow-pigmented mutant in mixed and non-mixed populations*

Mean division rates were estimated for the time interval of growth 0–22 hr. at 30°.

	Mixed populations		Non-mixed populations	
	Parent	Mutant	Parent	Mutant
Inocula	212×10^4	356×10^2	266×10^4	503×10^4
No. of cells per 1 ml. $\pm ts_{\bar{x}}$; $P = 0{\cdot}05$	$\pm 32 \times 10^4$	$\pm 84 \times 10^2$	$\pm 41 \times 10^4$	$\pm 34 \times 10^4$
Mean division rates per hour (averages of six experiments)	0·242	0·545	0·227	0·274

The proportion of mutants in cultures grown on glucose yeast-extract agar slants and plates was on the average 0·21 %.

What causes the accumulation of the yellow-pigmented mutants? To answer this we determined the mean division rates of one of the mutant strains and of the parental strain. We used BS 852/17, isolated from BS 852, as the yellow mutant strain. From Table 2 it is seen that the mean division rate of the mutant strain in non-mixed populations is only slightly higher than that of the parental strain. Nevertheless, the difference is significant ($\delta = 0{\cdot}030$ calculated from the analysis of variance). However, the mean division rate of the mutant strain increased to approximately double in mixed populations. At the same time the mean division rate of the parental strain in the mixed populations was the same as that in the non-mixed populations (the difference was not significant statistically).

As to the phenotypic expression of the pigmentation by the parent and mutant

strain, on some media the parent culture produces no violet diffusible pigment, or produces only very small amounts. The production of this pigment is slight or absent on nutrient agar, nutrient agar with starch, nutrient agar with gelatin and on yeast-extract agar with phenylalanine. On plates of these media the colonies of the parent culture appeared to be greenish yellow. On glucose yeast-extract agar with or without beef extract it produces both pigments. The mutant strain BS 852/17 produces yellow pigment on all these media.

Thirty yellow-pigmented mutants from BS 852 were serially transferred in order to ascertain whether they lose the ability to produce the yellow pigment. No such loss was found even after fourteen transfers. We have been working with these mutants for 2 years (transfers into fresh media have been made at least once every two months) and we have not found any reversion to the violet-pigmented state or any other change in pigmentation.

Table 3. *Taxonomic comparison of a yellow-pigmented mutant of strain* BS 852 *with other strains of* Micrococcus luteus

Name as received and no. of strain	Clumps	Packets	Violet diffusible pigment	Yellow pigment	Acid from: Glucose	Starch	Salicin	Esculin	Casein hydrolysis	Gelatin hydrolysis	Simmons' citrate	Acetylmethylcarbinol	Methyl-red test	Nitrate reduction	Indole	Hydrogen sulphide	Phenylalanine	Catalase	Lipase	Urease	Coagulase	Haemolysis	Lysozyme sensitivity
	Tests																						
Staphylococcus flavocyaneus BS 852	+	−	+	+	−	−	−	−	+	+	−	−	−	+	−	−	−	+	−	+	−	−	+
Yellow-pigmented mutant BS 852/17	+	−	−	+	−	−	−	−	+	+	−	−	−	+	−	−	−	+	−	+	−	−	+
Micrococcus luteus BS 810	+	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	+
M. lysodeikticus BS 1335	+	−	−	+	−	−	−	−	+	+	−	−	−	−	−	−	−	+	−	−	−	−	+
M. sodonensis BS 144	+	−	−	+	−	−	−	−	+	+	−	−	−	−	−	−	−	+	+	−	−	−	+
Staphylococcus afermentans BS 855	−	+	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−	+
Sarcina lutea BS 310	−	+	−	+	−	+	−	−	−	−	−	−	−	+	−	−	−	+	−	−	−	−	+
S. flava BS 309	−	+	−	+	−	−	−	−	+	+	−	−	−	−	−	−	−	+	−	−	−	−	+
S. citrea BS 248	+	−	−	+	−	−	−	−	+	+	−	−	−	−	−	−	−	+	−	−	−	−	+
S. aurantiaca BS 686	−	+	−	+	−	+	−	−	+	+	−	−	−	−	−	−	−	+	+	−	−	−	+
S. subflava BS 559	−	+	−	+	−	+	−	−	−	+	−	−	−	−	−	−	−	+	−	−	−	−	+
S. pelagia BS 331	+	−	−	+	−	−	−	−	+	+	−	−	−	+	−	−	−	+	−	−	−	−	+

None of the thirty yellow-pigmented mutants differs except in pigmentation from the parental strain BS 852 in morphological, cultural and biochemical characters commonly used in taxonomy. One mutant strain, BS 852/17, is compared with other yellow-pigmented cocci in Table 3. The names in Table 3 are commonly regarded as names of species, but we regard them all as strains of *Micrococcus luteus* (Kocur & Martinec, to be published). The mutant strain only differs from the other strains in characteristics which (Kocur & Martinec to be published) consider to be variable in *M. luteus*.

DISCUSSION

Metabolism of the organism can cause selection against the parent cells and favour other types (Braun *et al.* 1951; Braun, Firshein & Whallon, 1957; Braun, 1958; Firshein & Braun, 1958, 1960). Similarly in the broth cultures of *Staphylococcus flavocyaneus* the metabolism of parent cells may cause the selection of yellow-pigmented mutants so that the parent cells are outgrown by the mutant ones. Since the mean division rate of the mutant strain in unmixed populations was only slightly higher than that of the parent strain it follows that in broth cultures of *S. flavocyaneus* conditions exist that favour the growth of yellow-pigmented mutants. These conditions created by the parent strain favour the survival of the mutants which outgrow the parent population.

The mutants did not revert to the violet pigmentation state. The production of yellow pigment by the mutants did not undergo phenotypic modification on different media, unlike the production of violet pigment by the parent culture, which was inhibited on some media. A convenient medium for the production of violet pigment seems to be glucose yeast-extract agar; on this and on other media, however, the yellow-pigmented mutants did not produce any violet pigment. This indicates that, as to pigmentations, the mutants differ genetically from the parent strain; they have lost by mutation the ability to produce the violet diffusible pigment. It was not possible, however, to measure the mutation rate by the methods of Stocker (1949) or Luria & Delbrück (1943), because of the different growth rates and the absence of a selective medium for the yellow-pigmented mutants.

Yellow-pigmented mutants were not formed by BS 856 under our conditions of cultivation. This strain is a rough (R) form, whereas the strains BS 852, BS 851, and NCTC 7011 are smooth (S), and the environmental conditions for accumulating yellow-pigmented mutants may possibly be different.

Since the violet-pigmented cocci spontaneously throw off yellow variants that do not differ from other strains of *Micrococcus luteus*, except in a few characters that are variable in this species, we conclude that *Staphylococcus flavocyaneus* Knaysi is so closely related genetically to *M. luteus* (Schroeter) Cohn that it should be regarded as a strain of this species.

The authors wish to thank Miss Zdenka Vysloužilová for her excellent technical assistance in this work.

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Environmental Control of the Course of Development in *Dictyostelium discoideum*

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SUMMARY

Dictyostelium discoideum uses a wide variety of extracellular materials to accelerate the rate of morphogenesis. The stimulants of morphogenesis do not appear to exert their effect through the action of such factors as buffering, ionic strength, tonicity of the medium, or chelation.

Both glucose and histidine stimulate the rate of incorporation of amino acids into protein but at differing stages of development. Glucose stimulates throughout differentiation while histidine shows maximal stimulatory ability at preculmination (i.e. the stage just prior to complete fructification). The two compounds exhibit a mutual antagonism when added together.

It is concluded that glucose is probably acting as a primary energy source, whereas histidine is not acting in this manner or as a limiting amino acid for protein synthesis.

INTRODUCTION

During the life cycle of the cellular slime mould *Dictyostelium discoideum*, individual myxamoebae multiply on a rich medium at the expense of a bacterium such as *Escherichia coli*. This is the only stage of the life cycle in which growth, or an increase in protoplasm, occurs. When the bacterial portion of the food supply is gone, thousands of individual amoebae aggregate to form a multicellular pseudoplasmodium. After a period of migration, the pseudoplasmodium culminates to form the final fruiting body, or sorocarp, which is composed of a mass of spores resting on a stalk of cellulose-encased cells. For a detailed discussion of the morphogenesis of *D. discoideum* see Bonner (1959).

The investigations described here are an outgrowth of those of Bradley, Sussman & Ennis (1956), who found that histidine enhanced both the efficiency (i.e. the number of aggregation centres formed, per unit of cell population, as well as the minimum population density required for any centre formation) and rate of the aggregative process.

METHODS

Microbiological. The myxamoebae of *Dictyostelium discoideum* were grown on a complex solid medium (Bonner, 1947) on living *Escherichia coli*. After the growing amoebae had essentially cleared the agar surface of bacteria they were washed off

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with cold distilled water, separated from residual bacteria (as determined microscopically) by repeated light centrifugation and resuspended in cold distilled water before transfer to 2·5% agar plates. When it was desired to incubate the cells further in an aqueous environment (as opposed to the agar surface), the myxamoebae were finally suspended in a cold salt solution (Bonner, 1947) instead of water. Small amounts of amoebae were harvested from standard Petri dishes while larger numbers of cells were obtained from covered aluminium cookie sheets measuring $15 \times 10 \times 1$ in.

Morphogenetic experiments. In testing the effect of a substance on the developmental process, the material was incorporated into 10 ml. of either 2·5% washed agar (10 volumes of distilled water) (Bradley *et al.* 1956) or 2·5% Difco Noble agar. On the day preceding the actual experiment, the agar was poured into a standard 9 cm. diam. Petri dish. The vegetative amoebae obtained from one Petri dish culture, prepared as outlined above, were suspended in a final volume of 10 ml. of cold distilled water. Six droplets of 0·01 ml. each were placed on the agar surface of each test plate without being spread. Thus the cell population and density could be maintained constant (Bradley *et al.* 1956). The pH was initially 6·2 except where noted.

The test plates were incubated at 22° for a time period (usually 20–24 hr.) such that only a small fraction of the ultimately possible mature sorocarps had formed on the surface of the control plates (i.e. those with nothing added to the 2·5% agar). After incubation, the number of sorocarps as well as the total number of bodies at all stages of development (from definite aggregation through mature sorocarp formation) were counted in each spot and totalled for each plate.

The greatest contrast in the degree of sorocarp formation between control and experimental plates is observed when the control plates have just commenced fruiting. Since the fruiting process is not complete when the counts are made, any change in the rate of development would be reflected in the number of sorocarps observed. In most cases studied, the stimulatory effect of a given material was qualitatively the same regardless of whether the total number of mature sorocarps, the total number of developing centres, or the ratio of sorocarps to centres was considered. (The few exceptions noted are pointed out in the Results section). For all three criteria to yield the same qualitative results, changes in the rate of development as a whole must be a major factor in the observed results. Even though the population densities were not adjusted to a fixed value from experiment to experiment, the standard procedure used for preparing the amoebae suspensions gave populations which operationally fell well within the limits given by Bonner & Dodd (1962).

If one waits long enough, the number of sorocarps should be the same in all plates when fruit formation is complete. This was found to be so with most of the conditions used in this study. An example may be found in the data in Fig. 4. On plates containing 0·04 M-histidine plus 4×10^{-3} M or more phosphate as well as the plate containing 1×10^{-2} M-phosphate alone, fruit formation was complete. The number of sorocarps is essentially the same in all cases. Therefore, differences in numbers of sorocarps prior to complete fructification would reflect changes in rate of morphogenesis

The average % standard error for all the data presented in this paper was found

by: (*a*) computing the standard error of the mean of the sorocarps found in the six drops on each plate; (*b*) converting this error to a % of each mean; (*c*) averaging these % errors by multiplying together and taking the *N*th root. By this method, the average % error was found to be a maximum of 23·6%. This figure was derived by assuming that all the plates exhibiting no mature sorocarps had a % standard error of 100%. If these samples are omitted from the calculation, then the average % error is 19%. As might be expected, those plates exhibiting the greatest number of mature sorocarps (i.e. those stimulated to the greatest extent) were most reliable, having an average % standard error (with more than 50 sorocarps) of 8–15%.

Isotope incorporation experiments. Two types of incubations were performed to test the effect of histidine and/or glucose on the ability of *Dictyostelium discoideum* to incorporate isotopically-labelled amino acids into protein. In all experiments, approximately 4% of the washed amoebae obtained per cookie sheet were incubated in each Petri dish. The cells were either: (*a*) suspended in Bonner's salt solution (0·6 g. NaCl; 0·75 g. KCl; 0·3 g. $CaCl_2$ per litre of distilled water) and all materials subsequently added were also dissolved in the salt solution, or (*b*) 0·4 ml. of cells suspended in cold distilled water were spread on the surface of washed agar and distilled water was used as the diluent for all substances. In the former system differentiation could proceed only through the aggregation stage, so that long term experiments were carried out in the agar system. Where comparable experiments were performed, no qualitative differences were observed between the two incubation systems.

After pre-incubating the organisms for varying lengths of time, the substance to be tested was added along with the tracer materials (0·5 μC./sample; 0·65 μg. amino acids). Incubation was at either 19° or 22° for the times indicated, and when complete an equal volume of 6% trichloroacetic acid was added. The protein was purified by the method outlined by Wright & Anderson (1960*b*). When incorporation of tracer into the organism's soluble pool was to be determined, the cells were washed three times with the salt solution before the trichloroacetic acid was added. In this case, the acid supernatant was retained for counting.

It should be pointed out that such incorporation does not represent a net protein synthesis. While both protein synthesis and protein degradation take place during morphogenesis, the net effect is one of degradation (Wright & Anderson, 1960*a*). Although the term 'protein synthesis' will be used hereafter, it is obvious that any effects on the amounts of isotopically labelled amino acids in protein are a resultant of effects on both protein synthesis as well as degradation.

The incorporation of isotopically labelled amino acids into protein in the continued presence of histidine was found to be highly variable and confusing when the simple specific activities were considered. This appears to be due to an inhibition by exogenous histidine, at high levels, of the incorporation of extracellular amino acids into the free pool of the cells. As shown in Table 1, the presence of histidine results in a lower ^{14}C level in the pool. Glucose has little or no effect in this regard. The combination of glucose and histidine is the same as histidine alone. The data of Table 2 indicate that the levels of incorporation of exogenously supplied amino acids were found to correspond to the levels in the pool after 1 hr. incubation. The contribution of permeability on the observed protein synthesis can be eliminated

by considering only the rate of labelled amino acids incorporated into protein. Therefore, all further data will be presented as the percentage change in protein specific radioactivity over a given period of time.

Table 1. *Effect of histidine and/or glucose on free amino acid pool size*

Conditions were those of Fig. 5 except that (*a*) the final concentrations of glucose and histidine were 0·06 M and 0·04 M, respectively, (*b*) instead of isolating the protein, the cells were washed three times with cold Bonner's salt solution before addition of trichloroacetic acid. The acid supernatant was retained for counting.

Additions	Stage*		
	Fruit	Preculmination	Preculmination
	C.p.m. in TCA supernatant		
None	380	332	501
Histidine	313	212	173
Glucose	375	—	414
Histidine + glucose	278	—	155

* The amoebae were allowed to differentiate to the stage noted before any additions were made.

Table 2. *Effect of pool size on amino acid incorporation into protein*

The data herein presented were derived from the experiments described in the last column of Table 1 as well as Fig. 5. The stage of differentiation was preculmination.

Additions	Pool*	Protein†
	Relative specific activity	
None	100	100
Histidine	35	21
Glucose	83	85
Histidine + glucose	31	22

* The specific activity (c.p.m./ml.) of the acid supernatant with no additions was taken as 100.
† The specific activity (d.p.m./mg. protein) of the sample without added glucose or histidine was taken as 100.

Protein was determined by the method of Sutherland, Cori, Haynes & Olsen (1949). Radioactivity measurements were done in the Packard Tri-Carb Scintillation Spectrometer by an anthracene two-phase method (Steinberg, 1960) or a gel method (Gordon & Wolfe, 1960; Kinard, 1957). Whenever necessary, the results of counting were corrected for quenching.

Mixed amino acids (hydrolysed Chlorella protein) were obtained from Isotopes Specialties Company, Burbank, California.

RESULTS

Non-specificity of stimulation of morphogenesis. When tested by the method outlined in the previous section, histidine stimulated the rate of development (Fig. 1) with the optimum concentration in the range of 0·02–0·05 M. It was found that a number of materials besides histidine, such as glucose, inorganic salts, and other amino acids, could stimulate fruit formation. Specific details are given in later sections. Possible mechanisms of action for these effects include ionic strength, buffer capacity, chelation, energy generation, and protein synthesis.

Ionic strength. Since sulphuric acid was used to adjust the pH of the histidine solutions, sulphate ion was tested for its ability to stimulate sorocarp formation. Figure 2 shows that sodium sulphate stimulated only slightly at a concentration of 0·01 M and not at all at the other concentrations tested. Potassium sulphate was quite stimulatory and sodium chloride enhanced fruit formation to a small extent. Magnesium ion and a mixture of sodium, potassium and calcium ions had a striking stimulatory effect at quite low molar concentrations. It should be pointed out that

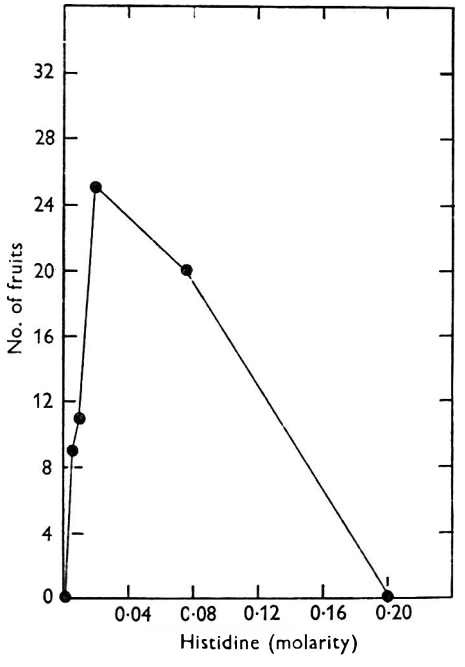

Fig. 1

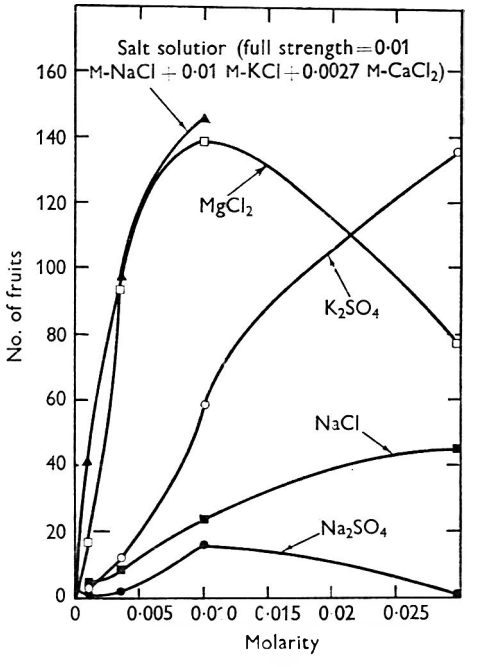

Fig. 2

Fig. 1. Stimulation of sorocarp formation by histidine. The procedure was that described under Methods except that three 0·01 ml. drops were added per Petri dish instead of six drops.

Fig. 2. Effect of various inorganic salts on sorocarp formation. The NaCl, KCl, $CaCl_2$ solution used was that described by Bonner (1947).

0·03 M-Mg^{2+} was extremely stimulatory towards the rate of fruit formation but the number of aggregative centres was markedly decreased so that apparent maximum stimulation occurred at a concentration of 0·01 M-Mg^{2+}. In separate experiments (data not shown) KCl and $CaCl_2$ were quite stimulatory when tested singly. As will be discussed under 'Buffer capacity' phosphate was also stimulatory. Ionic materials which were ineffective (or only slightly stimulatory) include alanine, glutamate, cysteine, glycine, aspartic acid, phthalate and the materials cited above.

Buffer capacity. As the pK of histidine is 6·10, it is possible that it might stimulate the rate of morphogenesis by virtue of its ability to buffer the medium. Clearly, maintenance of pH cannot be an absolute requirement since many substances stimulate while possessing little or no buffering capacity in the physiological pH

range. Furthermore, the pH changes in media containing such materials were much the same as those in the unbuffered control (Table 3).

Figure 3 shows the effect of varying the pH on the stimulation by histidine: essentially, the enhancement by histidine was inversely proportional to the pH.

Table 3. *Environmental* pH *changes during the course of morphogenesis*

The amoebae obtained from the growth on one-half cookie sheet were washed in the manner described under Methods. The final volume of the cell suspension was 4·5 ml. Four-tenths ml. samples of the cell suspension were spread on 2·5 % washed agar (10 ml.: 9 cm. diam.) with additions as specified.

At the times indicated, at least three pH measurements were performed in separate locations on each plate simply by pressing the electrodes of a Beckman Model H pH meter on the surface of the agar. The values given are the averages.

	1 hr.		8·5 hr.		20 hr.		24·5 hr.	
Additions	Stage	pH	Stage	pH	Stage	pH	Stage	pH
None	A*	6·7	Agg.	6·95	S	7·2	PC	6·9
Histidine 0·04 M	A	6·15	Agg.	6·1	F	6·25	F	5·9
KCl 0·03 M	A	6·8	Agg.	6·8	PC	7·2	F	7·3
$MgCl_2$ 0·01 M	A	7·0	Agg.	6·85	PC	7·0	F	6·9
NaCl 0·01 M; KCl 0·01 M; $CaCl_2$ 0·0027 M	A	6·35	Agg.	6·9	PC	7·0	F	6·9
Glucose 0·05 M	A	6·8	Agg.	7·3	C	7·1	F	6·9
NaCl 0·03 M	A	6·6	Agg.	6·6	PC	7·0	F	6·6

* Key to stages of differentiation: A, amoebae; Agg., aggregation; S, migrating pseudoplasmodium; PC, preculmination; C, culmination; F, fruit (or sorocarp).

Figure 4 indicates that phosphate ion was stimulatory at all concentrations tested. However, the combination of phosphate and 0·04 M-histidine was more stimulatory than either compound alone. When the pH of the phosphate was varied the results are those shown in Fig. 5. There was no trend of stimulation of development with changes in pH, in marked contrast to the results obtained with histidine. It should be noted that phosphate was stimulatory regardless of the cationic component (i.e. Na^+ or K^+). Since other sodium salts were not particularly stimulatory (Fig. 2), the phosphate ion itself was clearly stimulatory.

That phosphate was not unique with respect to the lack of a pH effect on development is shown in Fig. 5. Phthalate ion (pK = 5·51) appeared to be a metabolically inert buffer in this system. At every pH tested phthalate was neither stimulatory nor inhibitory regardless of the criteria considered (i.e. total centres, or fruits). In addition, histidine was stimulatory in the usual manner even when the medium was buffered with phthalate.

Chelation. That the rate of morphogenesis was limited by the presence of contaminating metal ions (capable of forming chelates) seems unlikely, since a variety of substances can stimulate the rate of development which would not be expected either to form chelates or relieve the inhibition due to such contaminants. However, histidine is an exception, as it forms very strong chelates with transition metals (Martell & Calvin, 1952, p. 167). The only comparable natural amino acid that does so is cysteine. Experiments were therefore made to ascertain its ability to substitute for histidine (Table 4). Cysteine alone or glycine alone stimulated poorly or not at all at a 0·01 M concentration when compared to 0·04 M-histidine.

It should be noted that, at equimolar concentrations (0·01 M), histidine was quite stimulatory (Fig. 1).

Although other amino acids are not as efficient chelating agents as histidine and cysteine, a variety were tested for their ability to affect the morphogenesis of *Dictyostelium discoideum*. The result is given in Table 6 (see below). The chelating dipeptide, glycylglycine, was also tested, with the results shown in Fig. 6. When combined with 0·04 M-histidine, glycylglycine inhibited sorocarp formation at higher

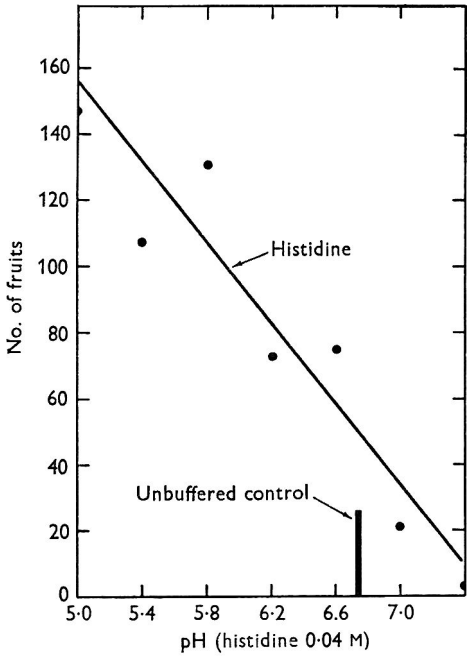

Fig. 3

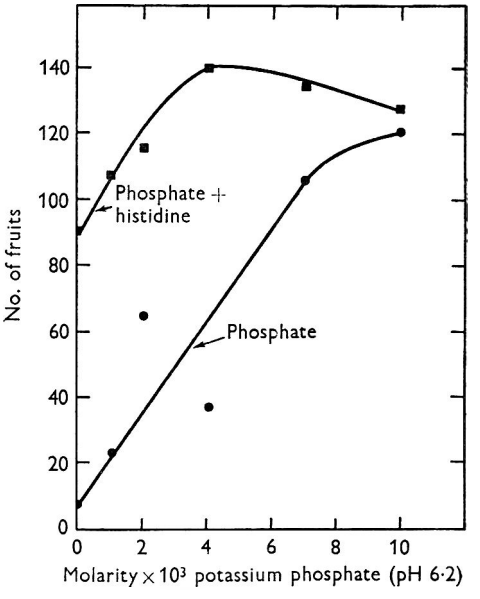

Fig. 4

Fig. 3. Variation of histidine stimulation at different hydrogen ion concentrations. The procedure was that described under Methods except that the pH of the histidine solutions were adjusted to those shown prior to addition of the agar.

Fig. 4. Stimulation of sorocarp formation by potassium phosphate (pH 6·2) with and without added histidine (0·04 M).

Table 4. *Effect of cysteine and/or glycine on differentiation*

See Methods for the experimental procedure.

Additions	Expt. 1	Expt. 2
	No. of fruits	
None	0	10
Cysteine (0·01 M)	0	37
Glycine (0·01 M)	25	12
Histidine (0·04 M)	117	90

concentrations. However, the total number of aggregative centres remained essentially unchanged. In contrast, glycylglycine alone inhibited centre formation in a manner proportional to the concentration.

Protein synthesis. As development proceeds it is evident that new enzymatic activities must be expressed. It is conceivable that the rate-limiting step in morphogenesis is the synthesis of new types of proteins. Additionally, it might be expected that the rate of general protein synthesis could be limited by the availability of one or more amino acids. Krivanek & Krivanek (1959) have shown by paper chromatography that histidine, proline, asparagine and phenylalanine are absent from the free amino acid pool but are present in the proteins of the developing slime mould. Proline was never found to stimulate morphogenesis either alone or in combination with any of the other three amino acids. Phenylalanine appeared to

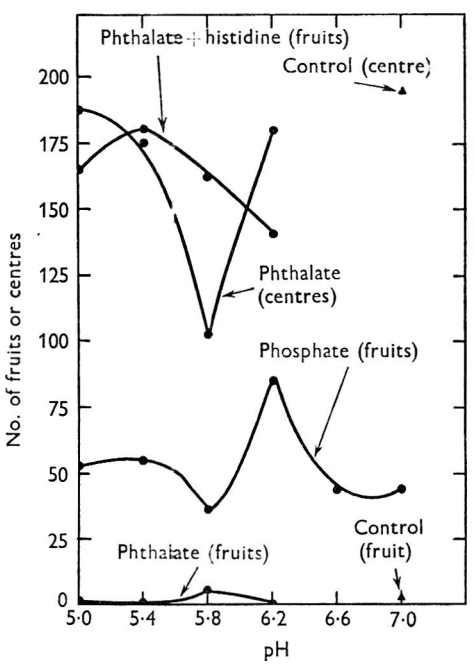

Fig. 5

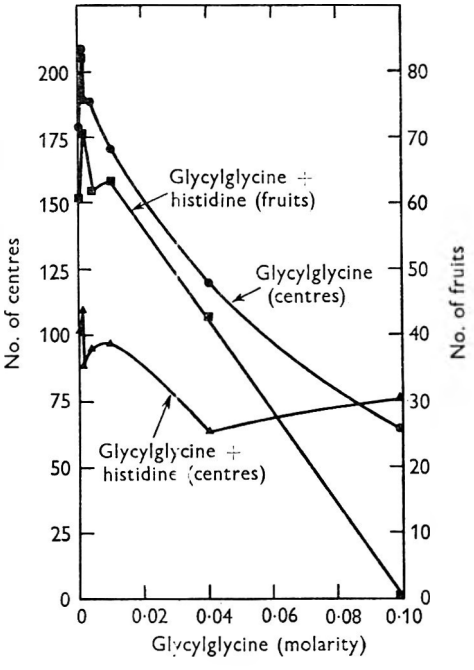

Fig. 6

Fig. 5. Effect of pH variation on morphogenesis. The pH of sodium phosphate and sodium phthalate solutions was adjusted as shown. The final concentration was 0·01 M in each case. The histidine final concentration was 0·04 M.

Fig. 6. Effect of glycylglycine on morphogenesis. Histidine was added in 0·04 M concentration where indicated.

increase aggregative centre formation without affecting the rate of development (with or without added histidine) as did asparagine to a lesser extent. In neither case were the effects consistent. Aspartate, in one trial, had no effect. Therefore, histidine appeared to be unique among the single amino acids tested (i.e. glycine, cysteine, proline, phenylalanine, asparagine, aspartic acid, and glutamic acid) in its ability to stimulate markedly the rate of morphogenesis.

The effect of histidine on protein synthesis at the various stages of development is shown in Fig. 7. It may be seen that, initially, adding histidine inhibited the rate of protein synthesis when the control values were subtracted. The rate of protein synthesis upon the addition of histidine remained constant until preculmination

while the rate in the absence of histidine decreased. A maximum in the rate of protein synthesis due to the presence of histidine was found at preculmination.

The presence of this maximum was confirmed in a more detailed experiment which was carried out over a briefer period with the median time at preculmination. The maximum was quite sharp and occurred at preculmination.

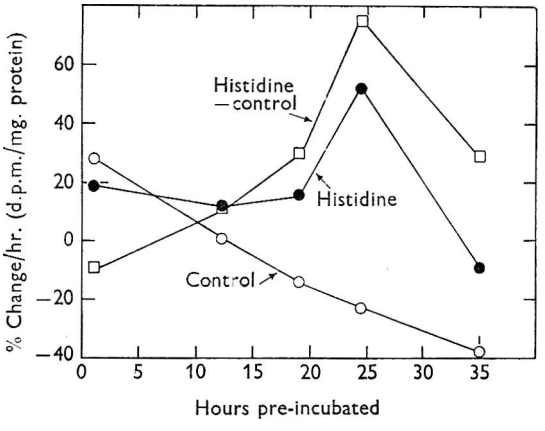

Fig. 7

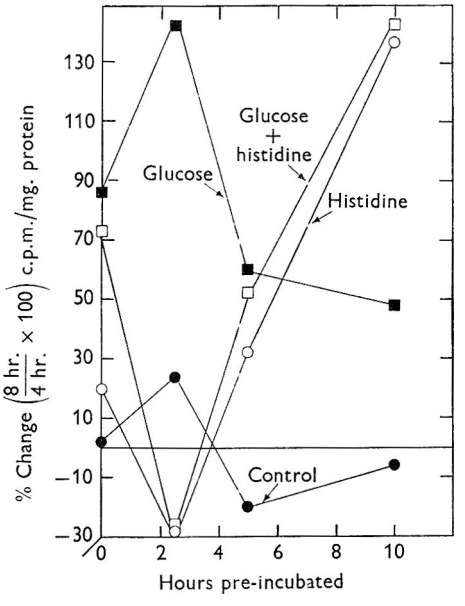

Fig. 8

Fig. 7. Effect of histidine on the rate of protein synthesis at various stages of development. Samples of amoebae were prepared and spread on 2·5 % washed agar as outlined under Methods. The Petri dishes were incubated at 19° until visual inspection showed that morphogenesis had proceeded to the desired stage. Next, to one series, 4·0 ml. of 0·04 M-histidine (pH 6·2) and ^{14}C-amino acids (0·05 μC./sample; 0·65 μg amino acids), dissolved in Bonner's salts, were added to each Petri dish. The histidine was omitted from the solutions added to the second series. The samples were incubated at 22° for either 1 or 2 hr. and trichloroacetic acid added. Protein specific activities were determined as described under Methods, and the % change between the specific activities at one and two hr. calculated. The stages of differentiation at the times indicated were: 1 hr. = amoebae; 12 hr. = early aggregation; 19 hr. = early migrating pseudoplasmodium; 24·5 hr. = preculmination; 35 hr. = fruit.

Fig. 8. Effect of glucose and/or histidine on the rate of protein synthesis. The conditions were those described in Fig. 7, except as follows: Four cookie sheets were harvested and the cell suspension volume (in Bonner's salts) was 130 ml. Four ml. samples were distributed in the Petri dishes and incubated for 0, 2·5, 5 or 10 hr. Then, 2 ml. Bonner salts containing ^{14}C-mixed amino acids with glucose (final concentration = 0·05 M) and histidine (pH 6·2; final concentration = 0·04 M) as indicated were added. Duplicate samples were further incubated for 4 or 8 hr. before the trichloroacetic acid was added. The % change in specific activity of the isolated protein then was determined.

Figure 8 shows that, in contrast to histidine, glucose alone markedly stimulated the rate of protein synthesis at all stages. The interaction between glucose and histidine is complex. Glucose stimulated to a very small extent in the presence of histidine unless pre-incubation was omitted.

Figure 9 shows that, at the preculmination stage, glucose again stimulated the

rate of protein synthesis while the combination of glucose and histidine was mutually exclusive. In fact, the combination of glucose and histidine could inhibit when compared to the rate observed with these compounds added separately. This was in direct contrast to their combined action on morphogenesis.

Energy generation. Since any anabolic process depends on available chemical energy and the morphogenesis of the cellular slime mould involves profound anabolic changes, tests were made of the requirements of the system for such energy limitations. Glucose was highly stimulatory to development regardless of the

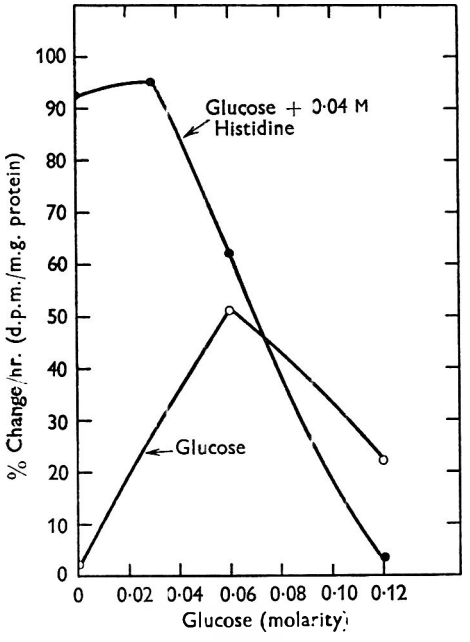

Fig. 9

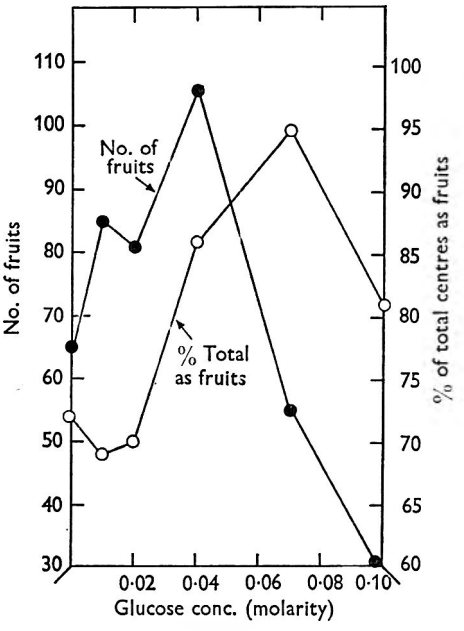

Fig. 10

Fig. 9. Effect of varying glucose concentrations, ± added histidine, on the rate of protein synthesis. The conditions were those described in Fig. 7, except as follows: All incubations were carried out at 22°. No additions were made until the preculmination stage was apparent visually. Glucose was added to the final concentrations indicated. To one series of samples, histidine (pH 6·2) was added to a final concentration of 0·04 M. Note that the initial point in each curve represents controls without added glucose.

Fig. 10. Stimulation of morphogenesis by varying concentrations of glucose.

criterion used (Fig. 10), but the optimum concentration varied with the criterion used. Presumably, at higher concentrations of glucose, the efficiency of response of the individual cells to the aggregative stimulus was enhanced in addition to the rate of morphogenesis. Alternatively, the efficiency of production of the stimulus (i.e. acrasin) may be enhanced. It will be recalled that Mg^{2+} ion showed similar effects (Fig. 2).

The combined action of glucose and histidine is shown in Table 5. Glucose (0·05 M) was stimulatory by itself. When combined with histidine the stimulation by the two was greatly increased. Thus, histidine can stimulate even in the presence of a good energy source such as glucose.

That glucose is an energy source was demonstrated by Liddel & Wright (1961), who found that it stimulated O_2 utilization. A further comparison of the ability of various compounds to stimulate respiration with their ability to stimulate morphogenesis is given in Table 6. There was no correlation between respiration and morphogenetic effects.

Table 5. *Effect of glucose and/or histidine on differentiation*

Additions	Expt. 1	Expt. 2	Expt. 3	Expt. 4
	No. of fruits			
None	1	6	0	15
Histidine (0·04 M)	24	90	62	62
Glucose (0·05 M)	22	54	1	30
Histidine + Glucose	61	119	100	86

See Methods for the experimental procedure.

Table 6. *Comparison of ability to stimulate respiration at the sorocarp stage with ability to stimulate morphogenesis*

Compound	% Stimulation of respiration*	Stimulation of morphogenesis
Glucose	100	Good
Glutamate	30	None
Alanine	30	None
Cysteine	16	Slight
Histidine	Slight	Good
Glycine	0	Slight
Succinate	0	Moderate
Citrate	0	Inhibitory

* Data taken from Liddel & Wright (1961). All values are compared to glucose taken at 100 %.

DISCUSSION

The factors responsible for limiting the rate of morphogenesis in *Dictyostelium discoideum* are unknown at the present time. However, some broad generalizations may be deduced from the data in this paper.

The rate-limiting step or steps presumably plays a central role in the overall metabolism of the organism, since a wide variety of unrelated materials can markedly affect the rate of morphogenesis. In addition, the morphogenetic process itself is markedly resistant to metabolic imbalance, since the addition of high levels of single metabolites affect only the rate and not the course of development.

Ionic strength or tonicity do not appear to play a critical role in the development of *Dictyostelium discoideum*, as shown by the fact that, while many materials stimulate, a number of simple compounds are inert. Therefore, the species of molecule, and not simply the tonicity, affects the rate of morphogenesis.

The demonstration that the positive effect of phosphate and the lack of effect of phthalate on development are not ascribable to change in pH indicates that the environmental pH is unimportant (over the pH range of 5–7). Bradley *et al.* (1956) first observed that the pH of the medium had no effect on aggregation in

unbuffered media. In addition, the histidine stimulation increases with decreasing pH, even to pH 5, where this amino acid has little buffering capacity.

It seems evident that ability to chelate has no bearing on whether a compound will stimulate morphogenesis. Some monovalent metal ions stimulate while others do not. The same is true of chelating amino acids and glycylglycine. According to Gerisch (1961), $2{\cdot}5 \times 10^{-3}$M ethylene-diaminetetracetate profoundly changes the course of development in *Dictyostelium discoideum*. At the level of 5×10^{-3}M, this chelating agent was found to prevent sorocarp formation. No such aberrant morphogenesis was observed with the amino acids even though the concentrations were much higher.

It may be concluded that the rate of general protein synthesis does not control the rate of morphogenesis by limitation of the pool levels of one or more amino acids. If histidine or any of the other amino acids which are low or missing in the pool were limiting for protein synthesis, one would not expect such a variety of materials to stimulate the rate of development as do so.

Whether the synthesis of one or a few specific proteins, at a different rate from the general proteins, can control the rate of morphogenesis is not known, but it seems unlikely because glucose appears to stimulate protein synthesis before histidine does (Fig. 8). If histidine were limiting for the synthesis of even one critical protein, then nothing should substitute for this amino acid unless the substitute were a precursor of the histidine. If glucose addition led to the formation of histidine then it should stimulate no sooner than histidine itself. Also, the combined action of glucose and histidine on protein synthesis is in direct contrast to their action on the rate of development. That is, in the former case, the greater stimulation by the combination on morphogenesis is not observed.

Preliminary experiments on the size of the free histidine pool indicated that the level of intracellular pool is slightly lower with decreasing pH. Previous work indicated that the stimulation by histidine of morphogenesis was higher with decreasing pH. Since it was shown (Tables 1, 2) that histidine lowered the incorporation of extracellular mixed amino acids into the internal free pool, it is conceivable that the action of histidine is at a permeability level.

If the assumption is made that glucose is a good primary energy source in the system, then it would follow that histidine is not. This is because: (*a*) histidine stimulates morphogenesis in the presence of high levels of glucose, (*b*) histidine alone is a more efficient stimulant (on a molar basis) than glucose alone, (*c*) histidine's effect on protein synthesis differs from that of glucose in quality and time of action. In fact, these compounds appear to be mutually antagonistic.

Obviously, none of the above preclude that histidine plays an indirect, but critical, role in energy metabolism. In all probability glucose serves as a primary energy source. Thus, it is possible that both stimulants affect the rate of morphogenesis by affecting the cells' energy metabolism, glucose doing so directly and histidine indirectly. This situation, in turn, may be responsible for their effect on protein synthesis.

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Determination of the Structure and Composition of the 'Sulphur Granules' of *Actinomyces bovis*

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SUMMARY

'Sulphur granules' of *Actinomyces bovis* were isolated from a case of bovine actinomycosis. These were examined in ultrathin sections with the electron microscope, in stained sections with the light microscope or in wet mounts before and after chemical extraction with the light and phase microscope. In addition chemical analyses were done on the granules and on organisms grown *in vitro*. The combined results showed the granule is a mycelial mass of *Actinomyces bovis* cemented together by a polysaccharide+protein complex and containing about 50% calcium phosphate. With the exception of the calcium phosphate, the granule had essentially the same composition as organisms grown *in vitro*. It is concluded that the 'clubs' of the granule represent normal hyphae encapsulated with the same polysaccharide+protein complex, that the basic structure of the granule represents the organism itself or products formed by it and that the mycelial mass is mineralized by calcium phosphate derived from the host as a result of phosphatase activity of the host and organism.

INTRODUCTION

Cohn (1875) first described the microscopic appearance of a strain of Actinomyces in stained preparations. The source of the material was concrements taken from the lacrimal canal of an infected eye. Although these preparations showed the presence of a small branching organism or mycelial masses, it was not until Harz described the organism (Bollinger, 1877; Harz, 1879) in actinomycosis of cows that attention was focused on the very hard grain-like structures formed by the organisms in animals. These structures, because of their colour and consistency, became known as 'sulphur granules'. With the discovery of the disease and its description in man by Israel (1878) and Ponfick (1880) it was soon recognized that the granules were invariably present in the diseased tissue and could be observed and easily isolated from the draining sinuses caused by the infection. Consequently, their presence was soon regarded as a laboratory aid in the diagnosis of the disease. The granule formed by Actinomyces in invasive infections had characteristics which made it readily identifiable. It was hard, gritty, and generally contained a rosette of 'clubs' at its surface. When a crushed granule was stained by the Gram reaction,

a Gram-positive filament was often observed within a club which was, itself, Gram negative. Because of its relationship to the disease and because of the importance of the disease at the time, there was considerable speculation in regard to the structure and composition of the granule and the clubs surrounding it. Knaysi (1951) described the club as a filament of the fungus having a capsule and stated that the club or portion surrounding the mycelial element could not be construed as a part of the organism proper. Emmons (1935) proposed that the club was a result of the host cells depositing on the surface of the filament. Cromartie, Spitznagel & Crawford (1960) showed that an accumulation of a basic protein of high arginine content occurred at the periphery of the sulphur granules but that the protein appeared to be in and around the radially arranged clubs. In an infection of the lacrimal canal in which the organism appeared to be non-invasive the mycelial mass observed was granular in form but was relatively soft and contained no clubs (Pine, Shearin & Gonzales, 1961). Only once have typical sulphur granules been observed to form *in vitro* (Wright, 1905).

To the student of actinomycosis, the question still remains 'What is the sulphur granule?'. Pine & Watson (1959) and Pine, Howell & Watson (1960) described four cases of bovine lumpy jaw and the organisms which caused the infections. Of these cases, three provided large amounts of pus containing the granules which were subsequently frozen for class use. With this material an investigation was undertaken to answer the above question by using the techniques of electron microscopy, classical histochemical examination, and phase microscopy, together with comparative chemical analyses of the *in vitro*-grown isolate and the granules which it formed in the cow. The results show that the sulphur granule is a mycelial mass cemented together by a polysaccharide+protein complex excreted by the organism as a capsule. This material and the mycelial mass in the centre are mineralized with calcium phosphate.

METHODS

Bacteriological methods

Three cow heads with lumpy jaw (cases P 1, P2, P3; Pine & Watson, 1959) were the source of the material used. The large interlocking sinuses were reamed with a semiconical spatula and the total material obtained from each case was frozen in vials and stored separately. In the period of three years all of the vials were subjected several times to thawing so that samples could be withdrawn. It is recognized that such a treatment probably changed the intracellular structures, but the experiments reported here indicate that no major changes occurred which would have altered the basic granule structure. A single direct comparative experiment with Actinomyces P2S grown *in vitro* (Pine *et al.* 1960) frozen once and then thawed, showed no alteration of cellular structures as observed in ultrathin sections examined with the electron microscope.

The culture of *Actinomyces bovis* P2S used for cell-wall analyses was grown in the Casitone medium of Pine & Watson (1959) under an anaerobic seal and supplemented with $NaHCO_3$ during growth (Buchanan, 1962). The culture was grown in a 12 l. Florence flask for 7 days at 37°. The organisms were centrifuged down, washed 3 times with distilled water and dried over P_2O_5 in a vacuum desiccator.

Other cultures of Actinomyces P2S were grown in 5 ml. lots of the same medium for 3–4 days at 37°, the pyrogallol+sodium carbonate seal serving as the source of carbon dioxide.

Electron microscope procedures

Comparative experiments with trypsin or 10% KOH showed that treatment of the granules with KOH was superior for the removal of the pus and debris surrounding the granule and did not appear to alter the granule itself. Consequently, the granules and pus of case P2 were suspended in distilled water, the granules collected by allowing them to settle, and the supernatant fluid removed. The granules were then suspended in 10% KOH for 10 min. at 37° to remove the adhering pus, washed 7 times in distilled water and preserved in 5% formaldehyde until used.

The procedure for fixing the granules for ultrathin sectioning was that of Moore & Chapman (1959). Although initial results were obtained with methacrylate, the use of 'Araldite' (Ciba Ltd) as the embedding medium gave superior preparations. Following fixation, the organisms or granules were passed through 70% (v/v) and 90% (v/v) ethanol in water for 10 min., three times for 10 min. in absolute ethanol, and were then added to 50% (w/v) Araldite in absolute ethanol. The specimens were incubated in the 50% Araldite solution for 2 hr. at 60°, resuspended in 100% Araldite for 1 hr. and placed in fresh Araldite and incubated for 5–8 days before the addition of catalyst. However, later experiments showed that 24 hr. was sufficient to give complete penetration of the Araldite into the granule. Sections about 500 Å thick were cut with a Porter–Blum microtome with glass knives prepared in the laboratory and were mounted on copper grids. Electron microscopy was done by using RCA EML and RCA EMU-3F microscopes.

Light and phase microscopy

The specimens were observed alternately by using the light objective and the medium dark phase attachments of an American Optical Company Microstar microscope having the Polaroid attachments for photography. After each chemical treatment, observations were made with the light and phase objectives. Polaroid photographs were taken by using either no. 53 or no. 55 cut Polaroid film and the negatives of the film developed. The maximum magnification used was ×970; greater magnifications were attained by enlargements from the negatives.

Fixed preparations were prepared and stained by Mr J. P. Pickett (Department of Pathology, Duke University Medical Centre). The Gram reaction, colloidal iron and toluidine blue stains for metachromasia and the alizarin stain for calcium were those given by McManus & Mowry (1960), the periodic acid Schiff stain was that described by Baker (1957). In addition, wet mounts were made to determine the presence of calcium by using the alizarin procedure.

Chemical methods

Phosphate was determined originally by the qualitative benzidine test (Feigl, 1956) while the presence of calcium was observed qualitatively by the alizarin procedure given above. Subsequently, phosphate was determined quantitatively by the procedure of Gomori (1942). Calcium was determined quantitatively by Dr R. E. Thiers (Department of Biochemistry, Duke University Medical Centre);

elementary analyses of the 'sulphur granules' and Actinomyces P2S organisms grown *in vitro* were done by Galbraith Laboratories Inc. (Knoxville, Tennessee). About 10–12 mg. dry weight of 'sulphur granules' or *Actinomyces bovis* organisms were combusted in a porcelain crucible to constant weight to determine the ash content; because of limitation of material, only one sample of each was used. In general, the extraction procedures of Park & Hancock (1960) were used to determine the cellular contents and to prepare the organisms for cell-wall analyses. After the extraction procedure of Park & Hancock (1960) the organisms were digested and then ruptured with a 20 kc. sonifier (Branson Ultrasonic Corp.); cell-wall analyses were done by the procedure of Cummins & Harris (1956). Metachromasia of extracted phosphate was determined by the procedure of Wiame (1949), reducing sugar by the method of Park & Johnson (1949) and glucose by the glucostat reagents of Worthington Biochemical Corp. (Freehold, New Jersey). Methylpentoses were determined qualitatively and quantitatively by the procedure of Dische & Shettles (1948) and quantitatively also by the same procedure as described by Ashwell (1957) with rhamnose as a standard; hexosamine by the procedure of Rondle & Morgan (1955) with glucosamine as standard, and amino acids by the method of Troll & Cannan (1953) with alanine as the standard. Sugars were identified qualitatively by one- and two-dimensional chromatography with phenol + water (70 + 30, by vol.) and butanol + pyridine + water (60 + 40 + 30, by vol.) and were estimated quantitatively by the relative size and colour of the spot obtained with the silver nitrate dip (Trevelyan & Harrison, 1952) and differentiated from amino sugars by the use of the ninhydrin + acetone dip which was allowed to develop overnight at room temperature. The identification of 6-deoxy-L-talose rested solely upon the results of MacLennan (1961) who found this sugar in *Actinomyces bovis* cell walls as the unknown P component of Cummins & Harris (1958). The relative high concentration of methylpentose as determined quantitatively with the presence of relative large concentrations of rhamnose and an unknown sugar having an R_F value greater than that of rhamnose and somewhat greater than that of 2-deoxyribose in chromatograms with known fucose and rhamnose, were taken as evidence for the presence of 6-deoxy-L-talose.

Amino acids were determined qualitatively with one- and two-dimensional chromatograms with the solvents phenol + water (70 + 30, by vol.) and the top layer of a mixture of butanol + acetic acid + water (40 + 10 + 80, by vol.). In some cases lutidine + water (65 + 35, by vol.) was also used to discriminate between monobasic and dibasic amino acids. The hydrolysates were chromatographed alone or with aspartic acid, β-alanine and proline as standards. The latter amino acids were readily determined by their characteristic ninhydrin colours and the relative straight line formed in two-dimensional chromatograms. Identification of the unknown amino acids rested solely on the relative position as determined with these three amino acids, and with other known amino acids, when necessary. The relative quantitative estimation of each amino acid depended on the rate of colour formation, size of spot, and the disappearance of the spots with a decrease in the amount of material applied to the paper.

The effect of lysozyme was determined by the methods of Repaske (1956). Ribonuclease was purchased from Nutritional Biochemical Corp., trypsin and pepsin from Sigma Chemical Co. and lysozyme from Armour & Co.

RESULTS

Morphological aspects

In general the observations of the granules obtained from the above cases of actinomycosis corresponded to those made by other workers (Conant *et al.* 1954). Attempts to section a granule in its entirety generally resulted in its partial destruction; either it shattered or was torn from the paraffin. Consequently, the initial results did not provide an adequate cross-section of a complete granule to determine the relative orientation of the layers observed in the fragments. However, when the granules were first extracted with cold 5% (w/v) trichloroacetic acid sections were obtained which retained the original outline and in which only the central portions were broken (Pl. 1, fig. 1). When observed in section the granule presented a homogeneous series of inner layers (Pl. 1, figs. 1, 2) in which the individual hyphae were not readily observed unless the Gram staining or the periodic acid-Schiff stain were used, and unless one concentrated on those areas in which fragmentation had occurred (Pl. 1, figs. 1, 2; Pl. 4, fig. 8). The outermost layer consisted exclusively of clubs (Pl. 1, fig. 2). Other aspects of granules in which the orientation of clubs and mycelium are clearly depicted have been reported by Emmons (1935), Conant *et al.* (1954), Pine *et al.* (1960), and Pine *et al.* (1961).

As shown by Pl. 5, figs. 11 and 12, a club might measure from 3 to 20 times the thickness of a mycelial element. Large clubs, when observed in a 10% (w/v) KOH wet mount, were glassy in appearance and were readily fragmented or shattered with pressure, although when mounted with care in this medium the preparations were found to last for a year or longer with no apparent change. When such mounts were made in lactophenol (Conant *et al.* 1954) they changed their morphological aspect within several months: the clubs were not readily apparent and the characteristic appearance of the granules and clubs was lost.

Shadowed pseudo-replica preparations of crushed granules observed with the electron microscope showed a very dense central portion in many of the clubs, surrounded by a membranous outer layer (Pl. 2, fig. 3). The dimensions of a club might vary from its tip to its point of attachment with the hypha, but in general there was simply a gradual decrease in diameter until the diameter of the hypha was reached. Although initial attempts to make satisfactory ultrathin sections of clubs failed because of the very hard nature of the granule, several excellent sections were obtained (Pl. 2, figs. 4, 5). In these cases, the club was found to consist of an internal electron-dense hypha surrounded by less dense capsular material. Although the capsule had no definite border, its edge was relatively discrete from the surrounding medium, its internal structure was homogeneous, and structurally it was readily differentiated from the contents of the hypha. Although the clubs themselves were swollen at the tip, the hyphae within them did not show any great enlargement and were normal in their general appearance. The cell walls of the hyphae were readily distinguished from the capsule and internal contents of the cells; in several sections definite cell membranes were observed separated from the cell wall.

In addition, there was observed within many of the cells a very definite crystalline electron-dense material (Pl. 3, figs. 6, 7). The presence of this material was variable and was not found in all cells. Similar material was observed in very large quantities

in the capsules, often completely saturating the cell and the capsule surrounding it (Pl. 2, fig. 4; Pl. 6, fig. 14). In some cases it appeared to originate within the cell (Pl. 3, fig. 6) and could be seen as gradually filling the cell (Pl. 3, figs. 6, 7). As shown in the latter figure, such a cell might exist beside a club which might be totally devoid of the crystalline material. In other cases, the crystalline material appeared to be deposited only on the surface of the cells (Pl. 6, fig. 14). No further analyses of the structural components of the cells or clubs were attempted because of the rigorous treatment given to the material before its fixation.

Histochemical aspects

Because of questions raised by determinations of the chemical composition of the granules, sections were made and stained for metachromasia and calcium. Comparative preparations of trichloroacetic acid extracted tissue were also made. With the metachromatic stains little difference in coloration was observed as between tissue extracted or not extracted with 5% (w/v) trichloroacetic acid. The preparations which were not extracted took the stains badly and were difficult to evaluate because of disintegration of the material. However, Pl. 1 shows the differences in coloration obtained with the colloidal iron stains after extraction with trichloroacetic acid. Plate 1, figs. 1 and 2, in general showed the pink coloration of the granule in the zone of the clubs and the internal layers and the intense blue colour observed in the residual core. Under high magnification however (Pl. 1, fig. 2) many of the clubs also showed a strong blue coloration while others remained pink. Although this result is suggestive of mucopolysaccharide, mucopolysaccharide was not indicated by subsequent chemical analyses. As a consequence of these observations and of results obtained with toluidine blue, it can only be stated that many of the clubs contain polysaccharide with an affinity for iron, but the general composition of the clubs themselves is not clearly revealed by these stains. When stained with the periodic acid-Schiff stain, the granule in general took a light pink stain. Upon examination of the central portion under high power, it was seen that the mycelial elements themselves had stained a deep red and were apparently embedded in a cementing substance similar in colour to that of the clubs (Pl. 4, fig. 8). These results suggested a difference in chemical composition between the capsular material and the cell walls of the organism.

Because of the results obtained by chemical analyses, fixed and wet mounts were prepared and stained for calcium. As shown by Pl. 4, fig. 9, the brilliant red of the alizarin was readily apparent at the surface of the mycelial filaments but it brought out in very strong contrast the large calcium deposits apparent in the clubs themselves (Pl. 4, fig. 10).

Chemical analyses and concomitant changes as observed with light and phase microscope

The basis for much of the preceding and the subsequent analyses rests primarily on several preliminary observations. The very hard gritty nature of the granule itself suggested the possibility of a mineral deposit. A qualitative ashing of several granules on the end of a spatula revealed a very high volume of ash. A benzidine spot test for phosphate showed that the ash contained a very high concentration

of phosphate, much greater than that observed for a similar amount of organism grown *in vitro*. Moreover, it was observed that the granules and the clubs were quite resistant to the effects of 10 % (w/v) KOH but changed and became translucent in the lactophenol mounts. These results suggested that lactophenol was extracting some major components of the granules, while the resistance of the clubs to strong base indicated that the basal structure of the club was polysaccharide in nature. Finally, observation with the phase microscope of granules in semipermanent lactophenol mounts showed with amazing clarity that virtually all the clubs contained an apparently normal internal hypha. In KOH, water or glycerol mounts the use of the phase microscope revealed nothing which was not readily observed with the light microscope. Further investigation showed that extraction with 80 % (v/v) lactic acid, 88 % (w/v) phenol, or 10 % trichloroacetic acid also made the hyphae visible with phase contrast. In addition the latter so altered the clubs, that much of the capsular material could be stripped off with gentle tapping on the coverslip of wet mounts; in some cases, the internal hypha was observed to slip free of the capsule surrounding it.

Consequently, comparative analyses of the granules from actinomycosis case P2 and the *in vitro*-grown organisms of the corresponding isolate (*Actinomyces bovis* P2S) were undertaken by using the extraction procedures of Park & Hancock (1960). Table 1 shows the relative concentration of phosphorus in the four fractions. Of the total phosphorus extracted by cold trichloroacetic acid, 92 % was inorganic phosphate, consequently large amounts of teichoic acids were not present in this fraction. Determination of the index of metachromasia (Wiame, 1949) showed that the trichloroacetic acid-extracted phosphate of the granules was not metachromatic. Comparative elementary chemical analyses are given in Table 2.

Table 1. *Relative amounts of total phosphorus extracted from cells of* Actinomyces bovis *and sulphur granules by different consecutive procedures**

Procedure	*A. bovis* (% of dry wt.)	Sulphur granule (% of dry wt.)
Cold 5 % (w/v) trichloroacetic acid	0·5	10·8†
Aqueous ethanol	0·0	0·0
Hot 5 % (w/v) trichloroacetic acid	0·1	0·0
Trypsin-solubilized	0·6	0·0

* Based upon the procedure of Park & Hancock (1960) the cells and granules (12 mg./ml.) were first extracted with cold trichloroacetic acid for 10 min., washed 3 times in water with centrifugation, extracted with trichloroacetic acid for 6 min. at 90°, washed 3 times with water, and then subjected to trypsin digestion for 6 hr. at 37° as described by these workers.

† The total inorganic phosphorus found in this extract was 10·0 %.

About 9 % phosphorus and 56·6 % ash were found in the granules as compared to 1 and 9·3 % found in the cells, respectively. In the ash of the granule, calcium constituted an average of 29 % (w/w) and could represent as much as 75 % (w/w) $Ca_3(PO_4)_2$; on the basis of calculations the $Ca_3(PO_4)_2$ is estimated between 39·1 and 56·5 % (w/w) of the sulphur granule; sodium and potassium phosphate with other salts most probably account for the remaining ash. Assuming the phosphorus of present as pure calcium phosphate and correcting the analyses of both materials for

the presence of phosphate, the analyses of the granules gave a crude formula of $C_{4\cdot1}H_{8\cdot6}O_{2\cdot0}N_{0\cdot8}$ whereas that of the *in vitro*-grown organisms of *Actinomyces bovis* was $C_{4\cdot1}H_{7\cdot6}O_{2\cdot1}N_{0\cdot7}$. These analyses suggest that there is no major difference in the chemical composition between the *in vitro*-grown organisms and that of the sulphur granules, with the exception of the high percentage of calcium phosphate in the granules.

Table 2. *Elementary analyses of dried organisms of* Actinomyces bovis *and its* in vivo *formed sulphur granules*

Analysis	*Actinomyces bovis* (%)	Sulphur granule (%)
Carbon	45·10	21·22
Hydrogen	6·94	3·72
Nitrogen	8·22	4·98
Phosphorus	1·00	9·07
Sulphur	Trace	Trace
Ash	9·3	56·6
$Ca_3(PO_4)_2$ calculated on the basis of P	nd*	56·5
Calcium of ash	nd*	29·0
$Ca_3(PO_4)_2$ calculated on basis of Ca of ash	nd*	39·1

* nd = not determined.

Table 3. *Quantitative analyses of cell-wall residues of sulphur granules and organisms of* Actinomyces bovis *after sulphuric acid hydrolysis**

Analyses	μmole/100 mg. dry wt. of whole organisms, corrected for the % ash: Sulphur granules	*Actinomyces bovis*
Reducing sugar	26·6	18·4
Glucose	3·1	4·2
Methylpentose	12·4	15·0
Hexosamine	7·5	3·7
Amino acids	32·0	12·4

* Hydrolysis procedure was that of Cummins & Harris (1956). Original samples were 54·4 mg. of sulphur granules, and 40·2 mg. of organisms grown *in vitro*, containing 56·6 and 9·3 % ash, respectively. Amino acids were determined with alanine as a standard.

The cell-wall residues remaining after the extraction procedures of Park & Hancock (1960) were hydrolysed according to the procedure of Cummins & Harris (1956) after additional treatment with pepsin and ribonuclease as described by the latter workers. Experiments were done on cells broken by sonification for 10 min. with a 20 kc. sonifier (Heat Systems Corp., Great Neck, New York). After the preliminary drying of the organisms by the Park & Hancock procedure (1960), the foregoing treatment was adequate to fragment and disrupt completely all the organisms. However, the subsequent extraction procedures and treatment with enzymes were not completely effective in removing protein adherent to the cell walls, as shown by electron microscopic observation.

After hydrolysis of separate samples, the cell wall residues were analysed for

sugars and amino acids. The results of one group of analyses are given in Table 3. The data show that the sulphur granules had a significantly higher amount of hexosamine and amino acids and had approximately 7·5% more reducing sugar not accountable as glucose or hexosamine. The higher concentration of reducing compounds and amino acids in the sulphur granules suggests a major difference in the two materials not indicated by the results of chromatographic analyses. The results of chromatographic separation and gross quantitative estimation of the sugars and amino acids in cell walls of sulphur granules and organisms are given in Table 4.

Table 4. *Comparative qualitative sugar and amino acid analyses of the cell-wall residues of sulphur granules and organisms of* Actinomyces bovis

Analyses	Sulphur granule		*Actinomyces bovis*	
Sugars:				
Glucose	+3		−5	
Galactose	+3		−1	
Fucose	—		Trace	
Rhamnose	+5		−3	
6-Deoxytalose	+4		−3	
Glucosamine	Trace		+1	
Galactosamine	+1		Trace	
	*	†	*	†
Amino acids:				
Alanine	+8	+5	+10	+5
Glutamic	+3	+3	+3	+2
Aspartic acid	+2	+5	+3	+5
Lysine	+2	+2	+2	+2
Valine	+2	+	+2	+
Arginine	+1	−	+1	−
Glycine	+	−	+	−
Serine:				
Serine	+	−	+	−
Threonine	+	−	+	−
Leucine, isoleucine phenylalanine	+3	−	+2	−

* Chromatograms done with phenol + water and lutidine + water and run in two dimensions.
† Chromatograms done with phenol + water and butanol + acetic acid + water and run in two dimensions.

Again there was a close similarity in the qualitative compositions of the products released on hydrolysis. The high concentration of methylpentoses as observed in two-dimensional chromatograms is in accord with the quantitative determinations of Table 3. However, very little hexosamine was observed in the chromatograms, and although the quantitative value is low in Table 3, the amount reported may still be high because of contamination of the walls with cellular protein during hydrolysis (Boas, 1953; Eddy, 1958). Repeated chromatography of amino acids revealed no significant differences, qualitatively or quantitatively, as between organisms and granules. The fact that essentially all the major amino acids were represented by ninhydrin spots is attributed to the contamination of the walls with protein. But it can be seen in Table 4 that alanine, glutamic acid, aspartic acid, lysine and arginine constituted the major amino acids, while leucine, isoleucine and phenylalanine were also present. By decreasing the quantity of material placed on the paper and by using different solvents it was found that the major

amino acids were alanine, glutamic acid, aspartic acid and lysine. On the basis of these results it was concluded that there was little gross difference between the cell wall composition of organisms grown *in vitro* and that of cell walls and capsular material of granules formed *in vivo*.

However, the combined results suggested that there was a difference between the cell wall of mycelium in the granule and the capsular material forming the club. Previous results suggested the possibility of using lysozyme to distinguish between the capsular material and the cell wall. Consequently, about a dozen large granules (0·5–1·0 mm. diameter) were picked and extracted in about 3 ml. of each solvent as given by Park & Hancock (1960). These were then treated with trypsin, ribonuclease and pepsin by the method of Cummins & Harris (1956) but without disruption by sonification. At each step a few granules were removed for observation with the light and phase microscopes, with lactophenol, glycerol or water as mounting medium. The effect of such treatment on the organisms and clubs of the granules is shown in Pl. 5, fig. 11; the clubs remained intact but the cells and the internal filaments changed. In Pl. 5, fig. 11, an extremely large club is seen in which the internal filament is visible in water suspension. No other clubs showing the internal filaments were observed in the water mounts. The granules were then subjected to lysozyme treatment for several hours, with no visible effect; nor did extraction of the granules with 3 ml. 88 % (w/v) phenol in water for 10 min. at 100° render them sensitive to lysozyme. However, results with organisms of *Actinomyces bovis*, *A. naeslundii* and *A. israelii* showed these were not sensitive to lysozyme unless the organisms were extracted with 10 % (w/v) KOH for 10 min. at 100° (Pine, unpublished results). The remaining granules were then subjected to such a treatment and were re-examined with the light and phase microscopes. After the KOH treatment, the capsular material became sufficiently transparent so the internal hypha was now visible in water mounts with the light microscope, although it was much more obvious with the phase microscope (Pl. 5, figs. 12, 13). A ninhydrin spot test on the neutralized KOH extract showed the presence of significant amounts of ninhydrin-reacting material. Because of the relative absence of hexosamines in the cell wall analyses and because of the effect of KOH on carbohydrate protein complexes (Eddy, 1958) these compounds are assumed to be amino acids or degradation products of proteins.

Such KOH-treated cells when subjected to lysozyme became flexible and limp and then disintegrated into fragments within an hour. Essentially the same results were obtained with granules, and the mycelial elements appeared to agglutinate into a limp flexible mass. Although the clubs still remained intact they too became quite flexible with the capsules less sensitive to lysozyme than the cells they contained. It is concluded that the capsule is polysaccharide containing bound protein and having essentially the same basic chemical structure as the cell wall of *Actinomyces bovis*, but probably differing in linkage groups and the presence of unknown amino acids.

Ultrathin sections and the effect of trichloroacetic acid

Although the results presented above suggested some difference between club and capsule, the greatest difference observed was between the phosphate content of the granule and of organisms grown *in vitro*. Therefore the effect of trichloroacetic

acid was investigated by comparing ultrathin sections from extracted and unextracted granules; the results are shown in Pl. 6. The electron-dense material initially observed was absent after extraction (Pl. 6, figs. 14, 15). Observation of Pl. 6, fig. 14, shows that phosphate was exceedingly dense toward the central regions of the granule, that it might completely penetrate the cell and its capsule, or might just coat the surface of some cells. Plate 6, fig. 15, clearly shows the cementing polysaccharide left after extraction, but with many cells in various stages of atrophy or malformation. This abnormal appearance of the cells was consistently observed in the deeper sections of the granule. On the other hand, Pl. 6, fig. 16 clearly shows polysaccharide as a capsule surrounding normal hyphae with normal cell walls. In some cases, however, no internal hypha was evident, and it was assumed that the cell had disintegrated. Observation of the morphological appearance of the cementing substance (Pl. 6, fig. 17) and the capsular material (Pl. 6, fig. 18) suggests they are of similar composition. From Pl. 6, figs. 14–16, it is concluded that the electron-dense material initially observed was calcium phosphate. It is also concluded that the capsular material is present in the granules in sufficiently high concentration to alter grossly the chemical analyses from that of organisms grown *in vitro* if a major difference in chemical composition existed. Consequently, it is concluded that the club and cementing material are of bacterial origin.

DISCUSSION

When first observed, the pseudo-replicas such as shown in Pl. 2, fig. 3, were interpreted as showing a swollen hypha in which the cytoplasm had contracted leaving a border of collapsed cell wall. But such an interpretation was completely inconsistent with the observations made with the phase microscope and made it impossible to interpret the observations made of the initial ultrathin sections. Under the most adverse conditions in culture or within the granule, the maximum diameter of swollen hyphal ends seldom exceeded twice that of the normal hypha. A constant regard to comparative size (observe the hypha of the club of Pl. 2, fig. 4, which is of the same outline but approximately half the size of the club in Pl. 2, fig. 3), the fact that most enlarged filaments were found to contain normal hyphae in capsules, and the fact that no other interpretation was compatible with all the observations indicated that the outer membrane in such pseudo-replicas was capsular material and not the cell wall.

From the above results it is apparent that the sulphur granule and the organism grown *in vitro* have essentially the same qualitative chemical composition with the exception of the high proportion of calcium and phosphorus in the granule. Because of the high concentration of cementing substance and capsule observed with the electron microscope after trichloroacetic acid extraction one would expect a major chemical difference between the granule and the organism grown *in vitro* if such a difference existed. From the chemical analyses, this appeared not to be the case; it is concluded that the club is truly a capsule surrounding the hypha and is not of animal origin. This conclusion was suggested first by Knaysi (1951).

It is recognized that there are a few minor differences in cell wall analyses of *Actinomyces bovis* as reported here and as reported by Cummins & Harris (1958). Glucose was not reported in the cell walls of organisms grown *in vitro* but, as seen

from Table 3, it is a relatively minor reducing sugar. Virtually no fucose was found; the major methyl sugars observed were rhamnose and the P component, the latter being most probably 6-deoxy-L-talose (MacLennan, 1961). Otherwise the sugar and amino acid analyses are in agreement with the results of Cummins & Harris (1956, 1958, 1959) and Cummins (1962) and re-emphasize the probable bacterial origin of the club and granule material.

Concerning the chemical analyses, the high concentration of phosphate found in the granules is of the greatest interest; its source is unknown but it is of interest to relate this observation with the results of several other workers. Howell & Fitzgerald (1953) showed acid phosphatase activity in *Actinomyces israelii* and *A. naeslundii* and Citron (1945) reported on the alkaline phosphatase activity of species of Actinomyces. Rizzo, Martin, Scott & Mergenhagen (1962) found that the dead organisms of Actinomyces and other bacterial species strongly absorbed calcium phosphate of the animal and deposited it in the form of hydroxyapatite. Also, the high concentration of phosphatase in wound tissue or in histocytes and macrophages (Gomori, 1943; Carranza & Cabrini, 1962) probably plays an important role in tissue infected with Actinomyces.

It may be suggested that formation of the granule occurs as follows. Since the capsule is not generally formed *in vitro* it is postulated that it is formed as a consequence of the growth of Actinomyces in the host. As a result of the organism's enzymic activity and the enzymic activity of the host cells, phosphate is released into the area of the mycelial mass. Because of the chemical nature of the cell wall and the capsule, the phosphate is deposited as calcium phosphate on the surface of the cells, sometimes within the cells. As a result of progressive mineralization of the capsule many of the cells became atypical in morphology and die; eventually the central portion of the granule becomes completely impregnated and solidified.

Such an interpretation is supported by the results and photographs of Rizzo *et al.* (1962). These workers observed that calcification seemed to follow the individual outlines of the organisms and that mineralization might occur adjacent to or within the organisms incubated in dialysis tubing implanted in rats. They also observed that dead cells were much more rapid in their mineralization than were viable cells. The results of these workers might have a direct application to the results and interpretations reported here.

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EXPLANATION OF PLATES

PLATE 1. Morphological aspects of the 'sulphur granule'. Colloidal iron stain

Fig. 1. Granule enlarged to show the various layers differentially stained and showing the relatively dark staining layer of clubs. × 100.

Fig. 2. Same granule showing the peripheral arrangement of the clubs. × 430. Sections were prepared from a granule after extraction with 5% (w/v) trichloroacetic acid.

PLATE 2. Morphological aspects of the granules formed by *Actinomyces bovis* as observed with the electron microscope

Fig. 3. Club, chromium shadowed. × 12,200.

Fig. 4. Ultrathin section of clubs, showing capsular material surrounding normal appearing hypha. Note capsules below containing electron dense material. × 20,000.

Fig. 5. Ultrathin section of club showing diffuse nature of capsule surrounding a more electron dense cell-wall of a normal appearing hypha. × 24,000.

PLATE 3. Morphological aspects of the cells within the granules formed by *Actinomyces bovis* as observed in ultrathin sections

Fig. 6. Section showing the crystalline nature of the electron dense material deposited within cells. Upper left, mother cell is completely filled while the bud remains free of the deposit. Second cell (right) appears to be relatively free of the deposit in the central area whereas area bordering the cell wall is saturated. × 35,000.

Fig. 7. Section of the granule showing various aspects of cell morphology. Note single cell heavily impregnated with crystalline material and situated next to an encapsulated cell without crystalline material. × 35,000.

PLATE 4. Histochemical aspects of the 'sulphur granule'

Fig. 8. Periodic acid-Schiff stain of a fragment of the granule showing the pink cementing material of the granule containing the red hyphal elements. Granule was extracted with 5% (w/v) trichloroacetic acid.

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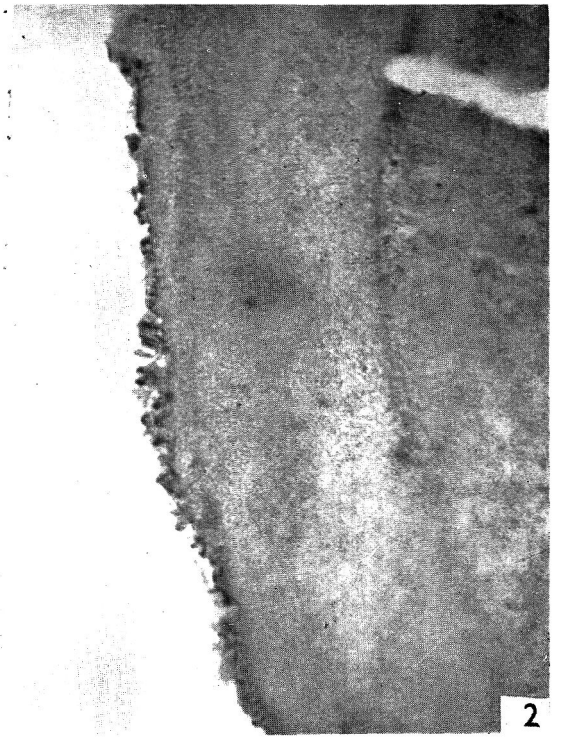

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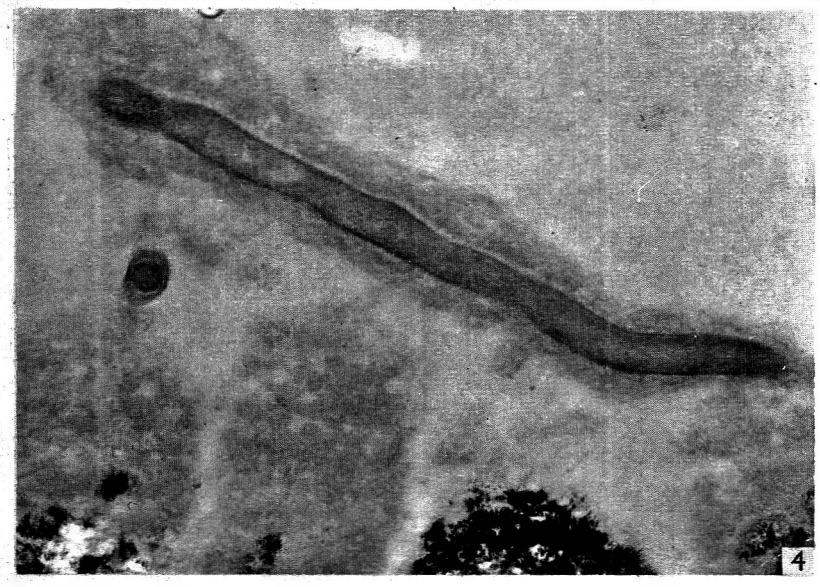
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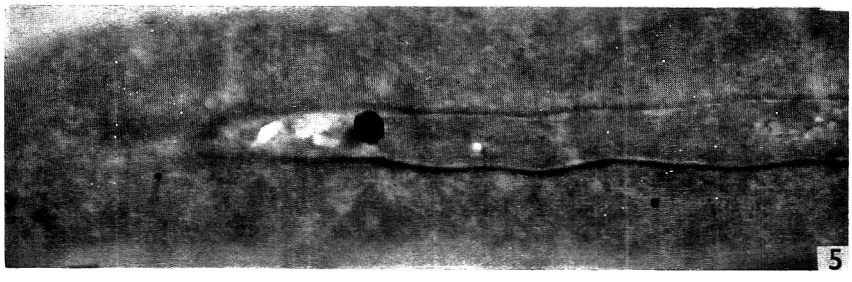
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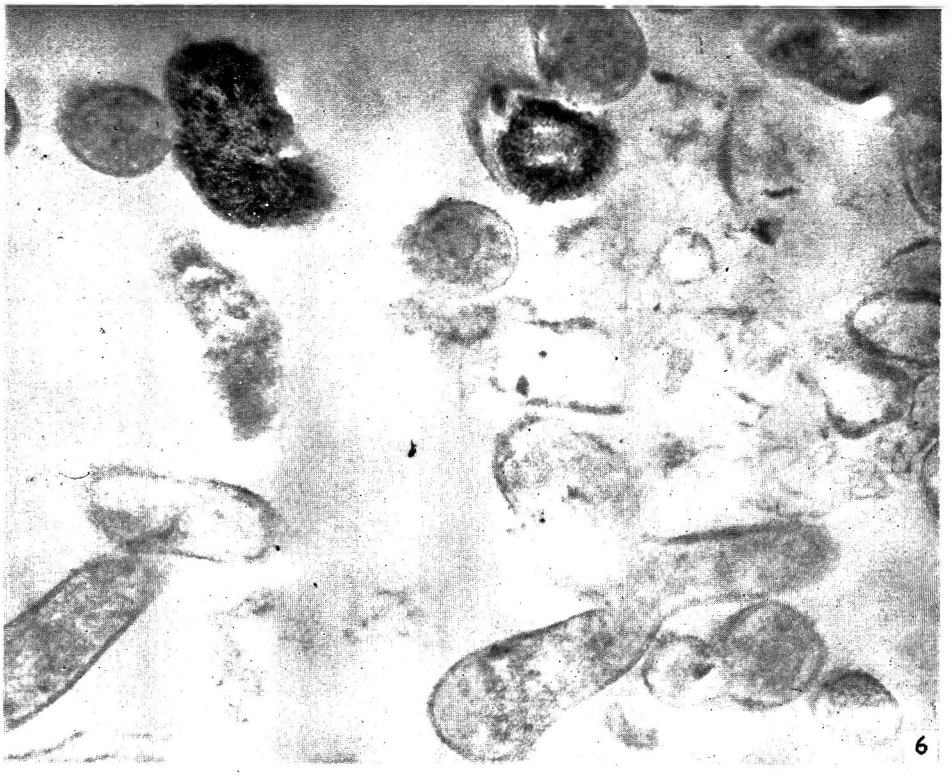
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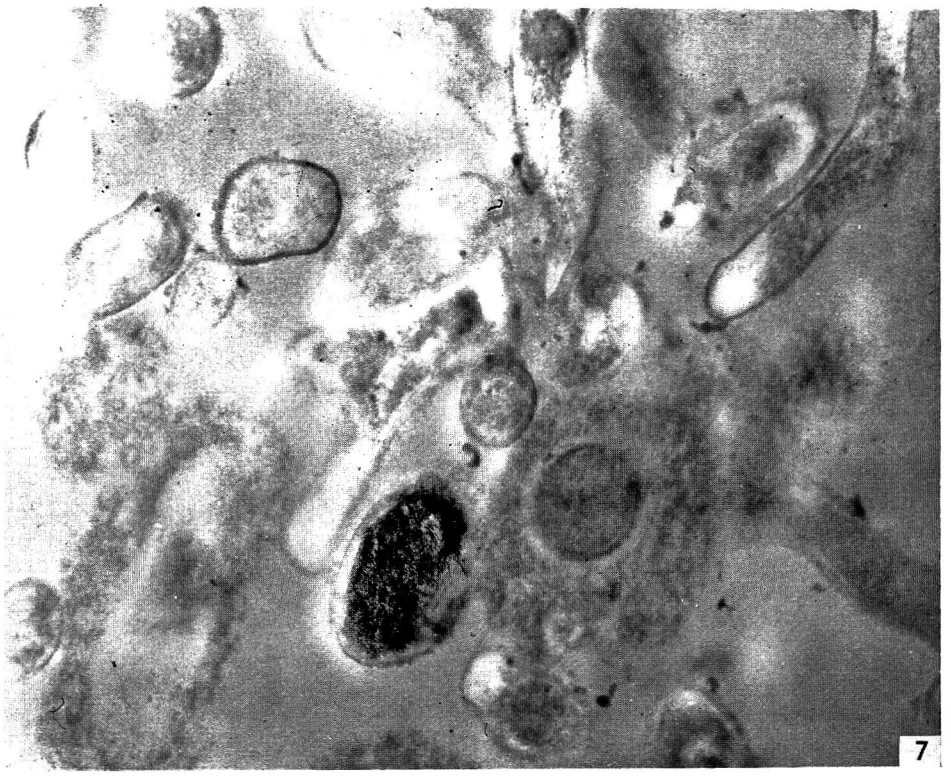
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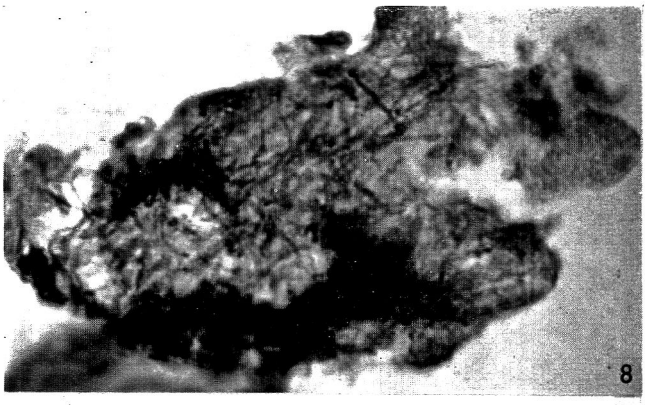
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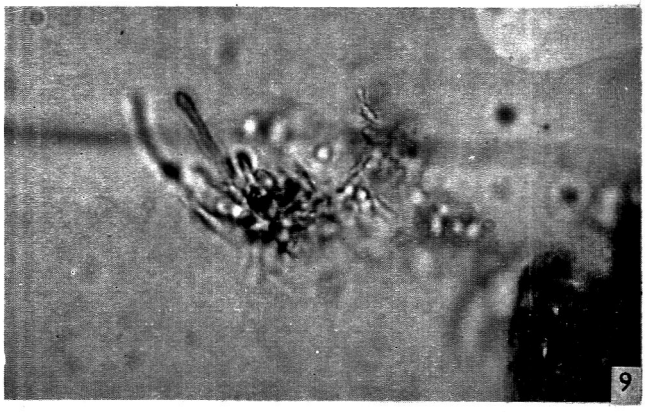
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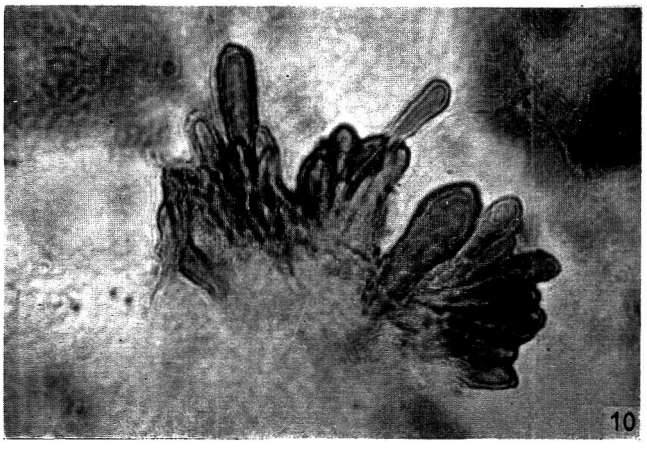
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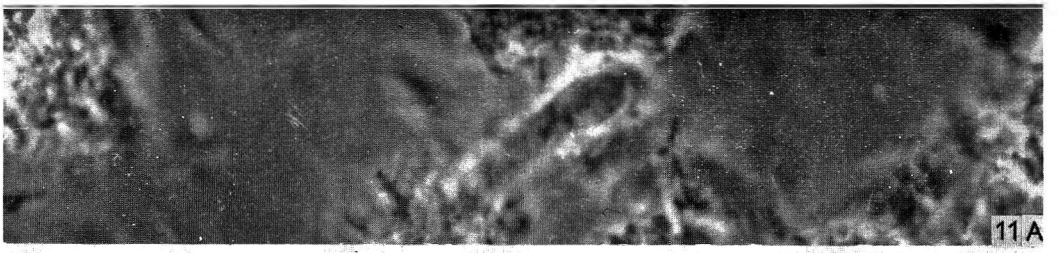
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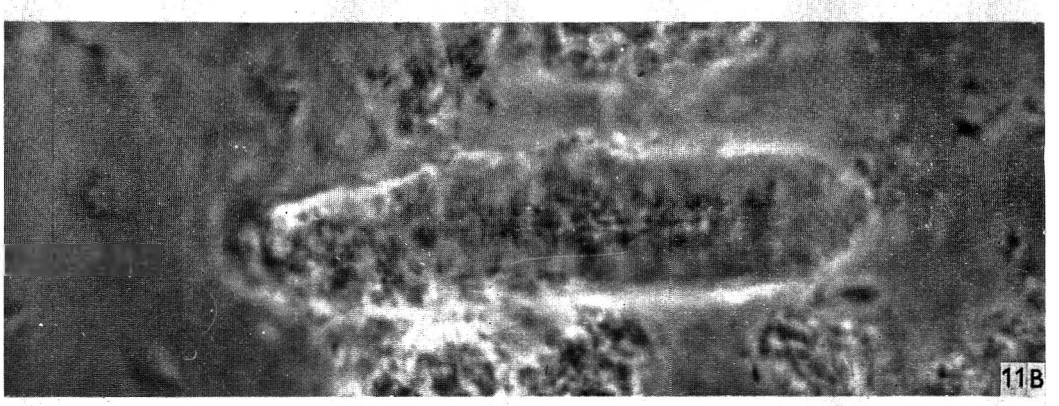
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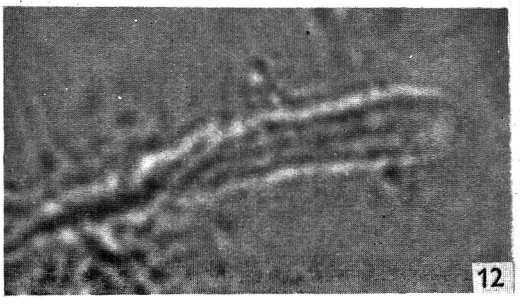
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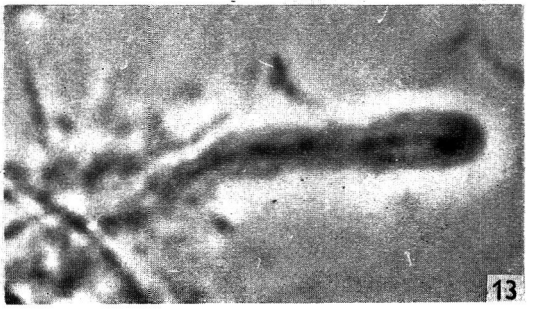
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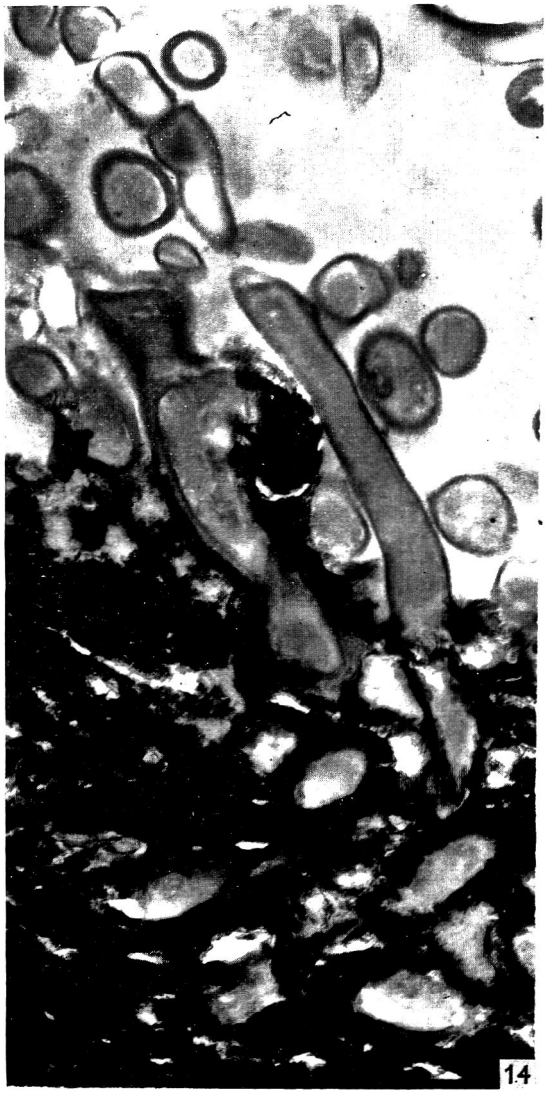
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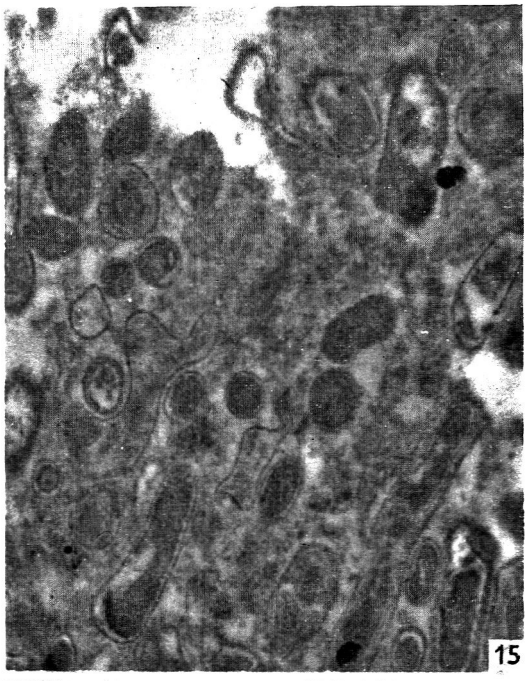
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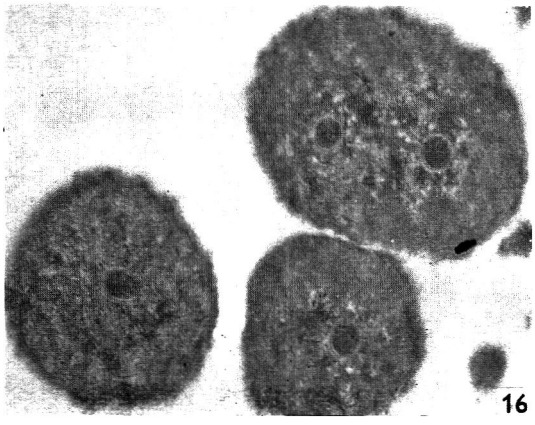
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Fig. 9. Wet mount alizarin red stain of mycelial elements from crushed granule showing the presence of calcium at the surface of the hyphae. × 970.

Fig. 10. Wet mount alizarin red stain of cluster of clubs from a crushed granule showing the heavy calcification of the clubs. × 970.

PLATE 5. Effect of chemical treatment on the cells and clubs of *Actinomyces bovis*

Fig. 11. Crushed preparation of a 'sulphur granule' after extraction with cold trichloroacetic acid, aqueous ethanol, hot trichloroacetic acid of Park & Hancock (1960) and after treatment with trypsin, ribonuclease, and pepsin according to the procedure of Cummins & Harris (1956). Note the difference in sizes of the two clubs in the preparation and the suggestion of the hypha within the larger club. Water mount, medium dark phase contrast. × 1870.

Fig. 12. Club from a granule treated as in fig. 13, but with additional extractions using 88 % phenol (w/v) and hot 10 % (w/v) KOH. Note the internal hypha, now apparent with the light microscope. Water mount. × 2900.

Fig. 13. Same club as in fig. 12 but photographed with medium dark phase contrast, water mount. × 2900.

PLATE 6. Effect of 5 % (w/v) trichloroacetic acid extraction on the 'sulphur granules' of *Actinomyces bovis* as observed with the electron microscope

Fig. 14. Ultrathin section of the granule showing deposit of calcium phosphate on the surface of the cells, within the cells, and saturating the cementing substance. × 11,000.

Fig. 15. Section of a granule after extraction with trichloroacetic acid. Note complete absence of electron dense material and relative high concentration of cementing material containing abnormal cells. × 13,800.

Fig. 16. Cross-section of clubs from a trichloroacetic acid extracted granule showing the relative size of the capsule and normal hyphae with it. × 9100.

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An Immunological Study of Avian, Viral and Bacterial Neuraminidase Based on Specific Inhibition of Enzyme by Antibody

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SUMMARY

Antisera were prepared to several strains of influenza virus (grown in the chick embryo), to purified *Vibrio cholerae* neuraminidase and to partially purified neuraminidase from chick chorioallantois. The ability of the antisera to inhibit the action of each enzyme on substrates of different molecular weight was tested. The substrates used and their molecular weights were sialyl lactose (640), fetuin (48,000) and ovine submaxillary gland mucin, OSM (1×10^6). Antiserum to *V. cholerae* and avian neuraminidase inhibited strongly the action of the homologous enzyme on each substrate. Antiserum to viral neuraminidase inhibited almost completely homologous action on fetuin and OSM but only partially the action on sialyl lactose. LEE influenza virus was grown in embryonated eggs and in cultures of calf kidney cells and from each preparation a soluble neuraminidase was isolated. Antiserum to the egg-grown virus inhibited the enzyme of both viruses not only in the intact virus particle, but also after separation in the form of a soluble, low molecular weight product. There was little or no serological cross-reaction between any enzyme and heterologous antisera, with one exception. Antiserum to avian neuraminidase partially inhibited (fetuin, but not sialyl lactose, as substrate) the soluble enzyme derived from egg-grown LEE virus, but not from virus grown in cultures of calf kidney cells. It was concluded that the soluble enzyme prepared from LEE virus is a virus specific product but also carries some antigenic determinants characteristic of host specificity.

INTRODUCTION

The influenza virus particle displays at least four different biological activities (apart from infectivity), notably two distinct complement-fixing antigens, a haemagglutinin and the enzyme neuraminidase. The particle contains lipid, carbohydrate, ribonucleic acid (RNA) and protein. Terminal amino acid analysis suggests that there may be at least three different protein molecules in the particle (Laver, 1962, 1963*a*). Despite this complexity, this virus contains only about the same amount of RNA per particle as is present in many of the simpler plant and animal viruses, where it seems there is only one protein and this is of low molecular

weight. This discrepancy may mean either that the small viruses use only a small proportion of their RNA to code the protein incorporated in the virus particle, or alternatively that in the influenza virus particle there is some component(s) whose structure is specified by the host cell rather than by the virus genome. We are interested in this latter possibility and the chance to investigate it with respect to one component arose following the demonstration that neuraminidase activity was present in vertebrate cells—the chorioallantois of the chick embryo (Ada & Lind, 1961). If the avian enzyme is present in those cells which support virus growth, there is an obvious possibility that the host enzyme might be incorporated as the functional enzyme of the virus. To confirm or refute this hypothesis it was necessary to compare avian and viral neuraminidase with neuraminidase from other sources, such as bacteria, mammalian tissue, and from influenza virus grown in another host. Since all forms of neuraminidase have not been isolated in the purified form, this limited the ways in which the different enzymes could be compared. However, some properties such as their kinetics, sedimentation characteristics and some aspects of their serological behaviour could be studied with the partially purified preparations which were available. In this paper we present the results of a serological examination of avian, viral and *Vibrio cholerae* neuraminidases, based on the specific inhibition of their action on different substrates by antibody.

METHODS

Substrates. Sialyl lactose, fetuin and ovine submaxillary gland mucin (OSM) were prepared as described elsewhere (Ada, 1963).

Determination of enzyme activity. Enzyme estimations, with either purified or crude preparations, were carried out as described in detail elsewhere (Ada, 1963; Ada, French & Lind, 1961). Enzyme activity is expressed as μmole *N*-acetylneuraminic acid (*N*-ANA) liberated/ml./min. One unit (u.) of enzyme is defined as that amount which catalyses the liberation of 1 μmole *N*-ANA/min. from sialyl lactose at 37° at the pH value of optimum activity; for *Vibrio cholerae* neuraminidase, this was pH 5·6 (in tris + maleate buffer, 10^{-3}M-Ca^{2+}; Ada *et al.* 1961); for virus, pH 5·8 (phosphate buffer; Ada, Cook & Laver, unpublished) and for avian neuraminidase, pH 4·5 (tris + maleate buffer, pH 4·5; Ada, 1963). In view of the instability of the avian enzyme at pH 4·5, estimations were carried out at pH 5·9 (phosphate buffer) and the activity is calculated at this value. Smaller amounts of enzyme are described in terms of milli-units (m-u.).

Preparation of avian neuraminidase (AN). Neuraminidase was purified from extracts of chorioallantoic membrane as described elsewhere (Ada, 1963). The product used contained about 1·2 units enzyme/mg. protein, which is about 50,000-fold purer than a crude extract of membranes from 11-day embryos.

Preparation of mammalian neuraminidase (MN). Mammary gland from a lactating rat (Carubelli, Trucco & Caputto, 1962) was ground into phosphate buffer (0·1 M; pH 5·8) by using a Potter–Elvejehm type homogenizer. The extract was centrifuged (80,000 *g*; 30 min.) and the supernatant fluid (11·6 mg. protein/ml.) used. This contained 1·25 m-u. activity/mg. protein at pH 5·8.

Preparation of viral neuraminidase (VN). Several strains of influenza virus were used at various stages during this work. Results obtained using the B strain LEE

(Francis, 1940) and the A strains, BEL (Burnet, Beveridge, Bull & Clarke, 1942) and PR 8 (Francis, 1934) are reported. Virus was grown in the allantoic cavity of chick embryos according to Beveridge & Burnet (1946). LEE virus was purified by the method of Laver (1962); viruses PR 8 and BEL, concentrated from a red-cell eluate by two cycles of differential centrifugation (sedimentation, 38,000 *g*; 30 min.; clarification, 7000 *g*; 10 min.) were further purified by banding in a potassium tartrate density gradient (McCrea, Epstein & Barry, 1961). LEE virus was also grown in calf kidney cells by the method of Lehmann-Grube (personal communication). Cells grown in a monolayer were inoculated with infected allantoic fluid at a multiplicity of about one. After incubation (35°, 4 days) the released virus was harvested and purified by adsorption to and elution from red cells, followed by differential centrifugation.

Preparation of Vibrio cholerae *neuraminidase* (*VCN*). Neuraminidase from cultures of *Vibrio cholerae* was purified as described previously (Ada *et al.* 1961).

Preparation of antisera

Antiserum to avian neuraminidase (*AN*). The preparation of purified enzyme contained about 2 mg. protein/ml. For the initial inoculation, enzyme solution was emulsified with an equal volume of complete Freund adjuvant (Difco). On day 1, 0·5 ml. was given intraperitoneally to two rabbits, A and B. The rabbits were given 0·5–0·75 ml. of avian neuraminidase intravenously on days 1, 2, 3, 4, 5, 44, 66 and 108. Blood was taken before and after the last three injections. As judged from the degree of enzyme inhibition, the sera obtained, following the last two injections of antigen, were of equal potency. For the work reported in this paper serum from blood taken on days 114, 116 and 119 from rabbit A was used.

Antiserum to viral neuraminidase (*VN*). The virus solution used in each case contained between 5000 and 10,000 agglutinating doses/0·25 ml. Emulsions were made by mixing equal quantities of antigen and adjuvant. On day 1, rabbits received 1 ml. virus emulsion intraperitoneally and 0·25 ml. of virus solution intravenously, and, on day 15, 0·25 ml. intravenously. Blood was taken on days 20, 22 and 24.

Antiserum to Vibrio cholerae *neuraminidase* (*VCN*). The enzyme solution contained 0·2 mg. protein/ml.; water-in-oil emulsions were made as above with Freund adjuvant. The rabbits received 1 ml. emulsion intraperitoneally on day 1 and 0·05 ml. intravenously on days 2, 3, 4 and 5. On day 32, rabbits received 0·5 ml. intravenously and were bled on days 35, 37, 39 and 42.

In all cases, serum was taken from the clotted blood and heated (56°, 30 min.). A fraction rich in γ-globulin was prepared from the serum (Pollock, 1956). The γ-globulin fractions (hereafter referred to as globulin) were stored either frozen or freeze dried. Sera were tested as described below.

Enzyme inhibition tests. These corresponded to constant antigen titrations. The reaction was carried out in total volume of 0·5 ml. The enzyme preparation (0·35 ml.), at a suitable pH value and containing antibiotics when necessary, was added to 0·10 ml. of the globulin preparation and the mixture stood at room temperature for 10 min. The substrate (0·05 ml.) was then added, the contents of the tube mixed and a 0·1 ml. sample taken. The tubes were incubated at 37° and further 0·1 ml. samples taken at appropriate times. The liberated *N*-acetylneuraminic acid was estimated as described previously. Since the globulin preparations themselves

contained substrate for neuraminidase, control solutions containing globulin prepared from preliminary bleed serum were also set up. Where purified preparations of enzyme were used, and sialyl lactose or fetuin as substrate, all dilutions of enzyme (VN, AN) were made in 0·1 M-phosphate buffer (pH 5·8) containing 0·1 % (w/v) bovine plasma albumin (BPA; Armour laboratories) or, for VCN, 0·1 M-tris + maleate buffer (pH 5·6) containing 10^{-3} M-$CaCl_2$ and 0·1 % (w/v) BPA (Ada, 1963). BPA was not included when ovine submaxillary gland mucin was used as substrate.

RESULTS

Effect of normal globulin on neuraminidase activity

Although in most tests controls containing dilutions of normal globulin were set up, it is worth reporting briefly the results obtained with normal globulin prepared from many different sera. With two exceptions, normal globulin at a 1/10 dilution did not inhibit either avian or viral neuraminidase acting on sialyl lactose or fetuin. One preparation (dilution 1/10) inhibited (40 %) BEL virus but this effect was not observed at a 1/40 dilution. Another preparation inhibited avian (25 %) and viral neuraminidase (14 %) at a 1/10 dilution but less than 5 % inhibition was seen at a 1/40 dilution. Some preparations of globulin, when present in high concentration, showed a potentiating effect on enzyme activity. The reason for this is obscure. Globulin from rabbits hyperimmunized against bovine plasma albumin or diphtheria toxin did not inhibit either avian or viral neuraminidase.

Inhibition of neuraminidases by homologous antisera

Inhibition of avian neuraminidase (*AN*). Fig. 1 shows the action of AN on the three substrates in the presence of anti-AN globulin. Action on fetuin and OSM was inhibited by more than 95 % and at least to 80 % in the case of sialyl lactose. It is clear that the antiserum was not very potent however; this may have been due to the small amount of enzyme available for immunization, or to the nature of the enzyme.

Inhibition of Vibrio cholerae *neuraminidase* (*VCN*). Antiserum to VCN inhibited the action of the enzyme on each substrate (Fig. 2); action on fetuin and OSM was inhibited almost completely and a maximum of 90 % inhibition of action on sialyl lactose was observed.

Inhibition of viral neuraminidase (*VN*). Antisera to the three strains of influenza virus LEE, BEL and PR 8 which were grown in the chick embryo were tested for ability to inhibit the enzyme action of the corresponding virus on the three substrates. The results obtained with LEE virus are shown in Fig. 3; almost complete inhibition (> 95 %) of enzyme action on fetuin and OSM occurred, whereas only slight inhibition of action on sialyl lactose was seen. The rate of action of LEE virus on OSM is so low that comparatively large amounts of virus had to be used in this test. Viruses BEL and PR 8 have little action on OSM but, when antisera to these viruses were tested as inhibitors of the action of corresponding virus on sialyl lactose and fetuin, similar patterns were observed: almost complete inhibition of action on fetuin and partial (20–50 %) inhibition of action on sialyl lactose.

Antiserum to LEE virus grown in eggs also inhibited the enzyme action of LEE virus grown in calf kidney cells. When tested in the presence of sialyl lactose or

fetuin, this antiserum was active to the same dilution with virus grown in calf kidney cells as with virus grown in eggs. However, the action of calf kidney cell grown virus on sialyl lactose was inhibited by 40–50 % compared with only 20 % in the case of the egg-grown virus. An extract of cultured uninfected calf kidney cells had a low degree of neuraminidase activity.

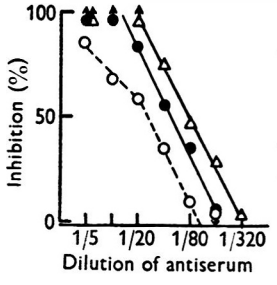

Fig. 1

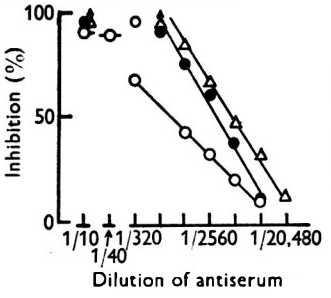

Fig. 2

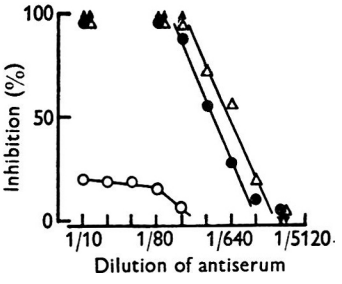

Fig. 3

Fig. 1. Inhibition of avian neuraminidase (AN) acting on sialyl lactose, fetuin or ovine submaxillary mucin (OSM) in the presence of anti-AN globulin. Each solution contained 2·4 m-u. enzyme. Time of incubation 180 min. Sialyl lactose, ○----○; fetuin, ●—●; OSM, △—△.

Fig. 2. Inhibition of *Vibrio cholerae* neuraminidase (VCN) acting on sialyl lactose, fetuin or OSM in the presence of anti-VCN globulin. Each solution contained 2·3 m-u. of enzyme. Times of incubation: for sialyl lactose, 120 min.; for fetuin, 40 min.; for OSM, 30 min. Sialyl lactose, ○—○; fetuin, ●—●; OSM, △—△.

Fig. 3. Inhibition of viral neuraminidase (whole LEE virus) acting on sialyl lactose, fetuin or OSM in the presence of autologous anti-viral globulin. Each solution contained 21 m-u. of enzyme. Times of incubation; for sialyl lactose, 15 min.; for fetuin, 45 min.; for OSM, 90 min. Sialyl lactose, ○—○; fetuin, ●—●; OSM, △—△.

Effect of heterologous antiserum on the activity of different neuraminidases

In these cross-reaction tests, sialyl lactose and fetuin were used as substrates. Irrespective of the substrate, globulin fractions prepared against viral or *Vibrio cholerae* neuraminidase did not inhibit enzyme action of the heterologous antigens (VCN, VN, AN, MN). Globulin prepared against AN did not inhibit VCN or MN but there was slight but variable degree of inhibition of VN of avian origin (LEE, BEL) when fetuin, but not sialyl lactose, was used as substrate.

In the case of CN and MN compared with the other two enzymes, the lack of cross-reaction was expected since cross-reactions between enzymes have been found to depend not upon catalytic but on taxonomic factors (Cinader, 1957). The slight reaction of antiserum against LEE and BEL viruses was suggestive of some relationship between the two enzymes. If the viral and avian enzyme were related, one reason for the slight cross-reaction might be the inaccessability of some groups in the larger and more complex virus particle. Avian neuraminidase is small (S_{20} about 3·3) whereas the intact virus has a sedimentation coefficient of 600–800 S. The solution to the problem lay in finding methods for obtaining from the virus particle an enzymically-aetive fraction of low molecular weight.

Disruption of influenza virus

Treatment with sodium dodecylsulphate. While this work was in progress, one of us (W.G.L.) developed a technique for the disruption of influenza virus by using the detergent sodium dodecylsulphate (SDS). When applied to the LEE strain of virus (with other strains tested, enzyme activity was lost), enzymically active fractions were obtained. These fractions were separated by electrophoresis on cellulose acetate, in a tris + boric acid + EDTA buffer (pH 8·9; Aronsson & Grönwall, 1957) containing 0·4% SDS; at least three bands of protein migrating towards the anode were observed. Most of the protein was in the two faster components (A, B); a third component C of low mobility contained about 6% of the total protein in the case of virus grown in eggs, and about 14% in the case of virus grown in calf kidney cells. Most of the enzyme activity was associated with component C although sometimes a trace was found in the slower of the main bands, B. The soluble enzyme from egg-grown LEE virus was not retarded on a column of Sephadex G-75 and had a sedimentation coefficient of about 9 S (Laver, 1963*b*). We have examined other properties of components C and B and their serological behaviour is reported below.

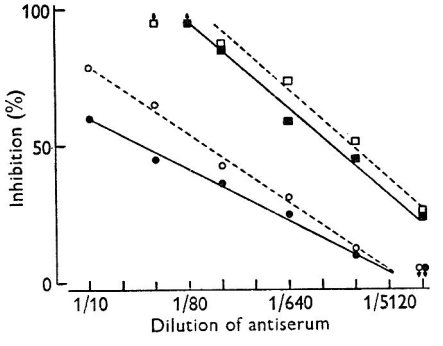

Fig. 4

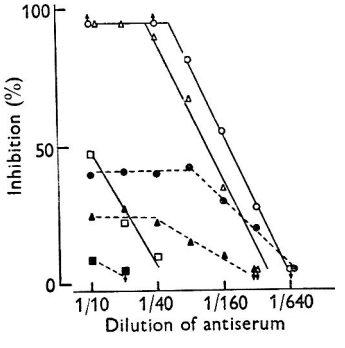

Fig. 5

Fig. 4. Inhibition by anti-LEE globulin of the action of the soluble enzyme from LEE virus grown in eggs and from LEE virus grown in calf kidney cells, on sialyl lactose and fetuin. Each solution contained about 2·3 m-u. of enzyme. Time of incubation 120 min. Soluble enzyme from virus (grown in chick embryo) acting on sialyl lactose ●—●; on fetuin ■—■. Soluble enzyme from LEE virus (grown in calf kidney cells) acting on sialyl lactose ○----○; on fetuin □----□.

Fig. 5. Inhibition by anti-AN globulins of avian neuraminidase (AN) and of the soluble enzyme from egg-grown LEE virus acting on fetuin. The globulins were prepared from serum taken at different times during the immunization of rabbit A. Each solution contained 2·2 m-u. of AN or of the soluble enzyme from LEE virus. Time of incubation 120 min. Enzyme AN, bleed day 119 ○—○; day 270 □—□; day 283 △—△. Soluble enzyme from LEE virus bleed day 119 ●----●; day 270 ■----■; day 283 ▲---▲.

The serological properties of soluble enzyme from LEE virus

Sialyl lactose and fetuin were used as substrates in these tests. Components B and C from egg-grown LEE virus were inhibited to the same extent by anti-LEE globulin; the inhibition curves of component C are shown in Fig. 4. Inhibition of action on fetuin was much the same as found with whole virus at this degree of activity, but there was a very marked increase of inhibition when sialyl lactose was used as the substrate.

Component C from LEE virus grown in calf kidney cells was investigated; the inhibition curves obtained in the presence of anti-LEE globulin are also shown in Fig. 4. With fetuin as substrate, the anti-LEE globulin inhibited to about the same extent the soluble enzymes from LEE virus grown in eggs and from LEE virus grown in calf kidney cells. Inhibition of the action of the soluble enzyme on sialyl lactose was again substantially higher than was the case with the whole virus.

None of these soluble enzymes was inhibited by anti *Vibrio cholerae* neuraminidase globulin. The reactions with anti avian neuraminidase globulin are summarized in Table 1. Only component C from egg-grown LEE virus was appreciably inhibited (and only with fetuin as substrate); the extent of this inhibition is shown in Fig. 5 (curve ●---●). Every preparation of the main enzyme fraction was inhibited by anti-avian neuraminidase globulin to an extent greater than that found with the parent preparation of virus. This inhibition varied from 30 to 50% at a globulin dilution of 1/10 and was not shown at a dilution which varied from 1/80 to 1/640. The usual end point was 1/320, which was also the end point of antibody titration of avian neuraminidase when this enzyme of equal activity (measured on fetuin) was used (see Fig. 5).

Table 1. *Effect of antiserum to avian neuraminidase on the action of soluble enzymes derived from LEE virus on two substrates, sialyl lactose and fetuin*

Soluble enzyme derived from		Substrate	
		Sialyl lactose	Fetuin
LEE virus grown in eggs	Main fraction	No inhibition	Inhibition
	Second fraction	No inhibition	Trace of inhibition
LEE virus grown in calf kidney cells	Main fraction	No inhibition	No inhibition

Globulins prepared from sera taken at different stages of the immunization procedure was tested as inhibitor to soluble enzyme from egg-grown LEE and to avian neuraminidase. Rabbit A was bled on day 270 (previous bleed day 119), then re-injected intravenously with purified avian neuraminidase (9 units), and bled again on day 283. Globulin fractions from each antiserum were tested for their power to inhibit avian neuraminidase and the soluble enzyme from egg-grown LEE virus with fetuin as substrate. The results presented in Fig. 5 show that the ability of the anti-sera to inhibit avian neuraminidase and the soluble enzyme from LEE virus increased in the same order, namely, bleeds on days 270, 282 and 119.

DISCUSSION

Two main points have arisen from this investigation, namely, (1) the effect of size of substrate on the degree of inhibition of enzyme by homologous antibody, and (2) the isolation from the LEE strain of influenza virus of a soluble enzyme, which is inhibited not only by LEE antiserum but also by antiserum to avian neuraminidase. The three substrates used in this work differ in size and linkage. Sialyl lactose has a molecular weight of about 640 and the linkage is between C_2 of *N*-acetyl-neuraminic acid (*N*-ANA) and C_3 of galactose. Fetuin has a molecular weight of about 48,000 and the *N*-ANA is joined probably to galactose but the actual linkage is

unknown. Ovine submaxillary gland mucin (OSM) has a molecular weight of about 1×10^6 (Gottschalk & McKenzie, 1961); the linkage is between the C_2 of *N*-ANA and C_6 of *N*-acetylgalactosamine. Although in many cases the rate of action of enzyme on OSM was very low or negligible, the other results indicate that inhibition studies with either fetuin or OSM as substrate gave similar results, i.e. the different linkages probably played only a minor role in this investigation. The different results obtained when sialyl lactose and fetuin were used as substrates might be due to size differences between the two molecules, since the linkage is to galactose in each case.

In the presence of homologous antibody, action of each enzyme on fetuin (and, when studied, on OSM) was almost completely inhibited. In Figs. 1–3, where the results of study of inhibition of avian, *Vibrio cholerae* and viral (egg-grown LEE virus) neuraminidases in the presence of autologous antisera are shown, the slopes of the inhibition curves are very similar; in each case a 16- to 32-fold decrease in antibody concentration in the mixture caused a change from almost complete to almost no inhibition of enzyme activity. In contrast, the slope of the inhibition curve of the soluble enzyme from egg-grown LEE virus, acting on fetuin in the presence of anti-LEE serum (Fig. 4) is less steep. The reason(s) for this is unknown. Autologous antiserum against the smaller enzymes (avian, *V. cholerae*) largely inhibited action against the small substrate, sialyl lactose, whereas similar action of the viral neuraminidase (Fig. 3) was only slightly inhibited. There are several possible reasons for this but until a more detailed analysis of each action is made, particularly taking into account the extent of enzyme-antibody aggregation, it would be fruitless to speculate.

The second finding, which is more relevant to our thesis, was that the soluble enzyme prepared from LEE virus by treatment with detergent reacted with antiserum to the parent virus particle and with antiserum to avian neuraminidase. The evidence for the specificity of the first reaction was the demonstration that virus grown in the chick embryo and in calf kidney cells, and the soluble enzymes derived from them, have common antigenic groups. The evidence for the specificity of the reaction with antiserum to avian neuraminidase may be summarized as follows. (1) Antiserum to avian neuraminidase does not inhibit *Vibrio cholerae* or mammalian tissue neuraminidases. (2) The ability of globulins prepared from serum taken at different times during the immunization of rabbit A to inhibit the LEE virus soluble enzyme varied in the same order as their ability to inhibit avian neuraminidase. (3) The dilution of antiserum which just failed to inhibit the enzyme action was usually the same for the avian neuraminidase and for the viral soluble enzyme (Fig. 5). (4) The antiserum to avian neuraminidase did not inhibit the activity of the soluble enzyme derived from LEE virus grown in calf kidney cells. (5) No preparation of normal globulin appreciably inhibited the LEE virus soluble enzyme. Preliminary experiments with antiserum prepared against soluble enzyme from LEE virus grown in eggs show that such antiserum inhibits equally well the soluble enzyme isolated from LEE virus grown in either host but does not inhibit purified avian neuraminidase (fetuin as substrate). The evidence thus points strongly to the conclusion that the soluble enzyme in the virus particle is a virus-specific product. An unambiguous interpretation of the reaction of isolated viral neuraminidase with antiserum to avian neuraminidase is not possible. If we take the results at their face value we must conclude that the soluble neuraminidase from egg-

grown LEE virus is associated with some antigenic determinants derived from the host but that these determinants, either because of their low concentration or qualitatively defective antigenicity, are unable to provoke the formation of detectable antibody on injection into rabbits.

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An Electron Microscope Study of Vibrio Flagella

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SUMMARY

Electron microscopic study of vibrio flagella by using the negative staining technique revealed differences in chemical reactivity as between the sheath and core components. The sheath was easily degraded by autolysis and exposure to acid and urea, whereas the core was relatively resistant.

These differences suggest that the sheath and core are of different composition and that, whereas the core consists of the protein 'flagellin', the sheath is probably of cell-wall origin.

INTRODUCTION

Bacterial flagella have been shown by electron microscopy to be uniform sinusoidal filaments. With two organisms, *Bacillus brevis* (De Robertis & Franchi, 1951) and *Vibrio metchnikovii* (van Iterson, 1953), these filaments differ morphologically from all others examined in that they are composed of a core and surrounding sheath whose stability to autolysis (van Iterson) and trypsin treatment (De Robertis & Franchi) has been reported to differ from that of the core. Chemical analyses (Weibull, 1948, 1949, 1950, 1951; Kobayashi, Rinker & Koffler, 1959), and physical analyses (Astbury & Weibull, 1949; Beighton, Porter & Stocker, 1958) of purified non-sheathed flagella have established their protein nature, while physical analysis of the sheathed flagella of *Vibrio metchnikovii* revealed a similarity in basic structure between sheathed and non-sheathed types (Astbury, Beighton & Weibull, 1955). The application of negative staining to electron microscopy (Brenner & Horne, 1959) provided a method complementary to shadow-casting for the study of protein macromolecular organization. This technique has been used in studying the structure of flagella (Kerridge, Horne & Glauert, 1962) and fimbriae (Thornley & Horne, 1962). In the present work (preliminary results were communicated to the Fifth International Congress for Electron Microscopy, Gordon & Follett, 1962) the superiority of negative-staining over shadow-casting in the demonstration of flagellar sheaths is shown, and their response to treatment with various physico-chemical agents assessed. This has established gross differences in stability as between the sheath and core components.

METHODS

Organisms and cultivation. The organisms used were *Vibrio metchnikovii* NCTC 8843 and El Tor vibrio NCTC 5850 grown on nutrient agar at 37° for 12 hr. or at room temperature (about 20°) for 24 hr. The actively motile organisms were harvested as a saline suspension which was used fresh or allowed to autolyse for 48 hr. at 4°; they were fixed by adding 10% formalin to a final volume of 0·5% (v/v); unfixed controls were also examined to exclude the possibility of fixation artifacts. No attempt was made to separate flagella from the bacterial bodies.

Preparation of specimens. Bacteria are known to adhere to collodion film (Houwink & van Iterson, 1950) and when carbon-covered specimen mounts were floated on a suspension of organisms, sufficient bacteria and flagella adhered to the carbon film to provide a suitable specimen for microscopy. This was washed and stained by floating the grid for 10 sec. on the following solutions in series 1% (w/v) potassium iodide, distilled water (twice), 1% (w/v) ammonium molybdate, finally excess stain was removed and the remainder allowed to dry down on the film surface. By this technique, the effect of acid and urea treatment on the flagella was readily studied by interpolating appropriate solutions between the two changes of distilled water. Specimens were floated for various times on N/100 hydrochloric acid adjusted to pH 2 with sodium hydroxide or on a 6 M-urea solution. All electron micrographs were taken with a Siemens Elmiskop 1 with either single or double condenser illumination, at a plate magnification of ×40,000.

RESULTS

In fresh young cultures negative staining revealed the flagella as white sinusoidal ribbons, with little or no evidence of internal structure. In older cultures, the two-component system of core and sheath became apparent, with the core outlined by two lines of stain running parallel to the flagellar axis. That this stain did delineate the core was confirmed on examination of autolysed specimens of *Vibrio metchnikovii* (Pl. 1, fig. 1) and El Tor vibrio (Pl. 1, fig. 2) where the core was observed with and without its surrounding sheath. In both cases the protruding core retained its normal wave form. Numerous specimens were observed with intact flagella demarcated into core and sheath, the degree of differentiation varying from one flagellum to another but being constant for any single flagellum.

Some autolysed specimens (Pl. 1, fig. 3) showed autolytic effects at sites other than the end of the flagellum. As well as completely removing the sheath from some flagella, autolysis merely loosened the sheath from the core from others (Pl. 1, fig. 4). A similar but more pronounced loosening was observed with flagella treated with urea for 5 min., and occasionally this was sufficient to allow a short terminal length of flagellum to escape from its enveloping sheath (Pl. 2, fig. 5). Whereas free cores—whether produced by autolysis, urea or acid treatment—always retained their wave form, empty sheaths showed no inherent rigidity and lay limply in irregular waves except where compressed against neighbouring structures by drying forces. Further urea treatment produced considerable swelling and partial removal of the sheaths (Pl. 2, fig. 6), whereas prolonged treatment completely removed the sheath, leaving an apparently intact core.

Although acid treatment also had more effect on the sheath than the core, the manner of sheath disintegration differed from that observed with autolysis and urea treatment. Instead of the loss of long reaches of sheath, characteristic of autolysis and prolonged urea treatment, exposure to acid for 10 sec. caused widespread comminution of the entire sheath (Pl. 2, fig. 7). Further exposure removed the sheath completely, leaving cores which, as after autolysis and urea treatment, retained their wave form and appeared to be intact.

Measurements of total flagellar width and core width alone were made on all control specimens and on several autolysed specimens. These measurements, a total of 150, fell into two distinct groups. In the major group (78 %) the total width was 350 ± 40 Å, with a core width of 165 ± 15 Å, while in the minor group values were greatly decreased, 270 ± 20 Å for total width and 125 ± 10 Å for core width. These latter measurements were always obtained from specimens which showed little or no demarcation of sheath and core (Pl. 2, fig. 5) and which had been prepared as fresh as possible. Autolysed specimens and suspensions left for several hours gave widths in the major grouping.

DISCUSSION

Negative staining. Negative stain is known to penetrate the hollow channel in rod shaped viruses (Brenner & Horne, 1959) and the clear demarcation of sheath and core in autolysed specimens might therefore be caused by penetration of stain from an autolysed end into a potential space between sheath and core. The observation here of a similar demarcation in intact vibrio flagella, however, indicates that a broken end is unnecessary for demonstration of the core and hence that the sheath must be permeable to the stain. This permeability would permit a thin film of stain to envelop the core with the resulting formation of areas of increased electron density at the edges of the projected image. The core would thus be outlined by two lines of stain along its edges, as was observed.

Flagellar width. The distinct division of the width measurements into two widely separate groups does not necessarily imply the presence of two types of flagella on the same organism. Young fresh organisms gave minimum width measurements and also showed a resistance to the penetration of stain. Organisms harvested and left for several hours, however, permitted ready access of stain through the sheath, indicating a considerable change in the bonding of at least the sheath component; the same flagella also gave much increased width measurements. These greater width values are therefore the result of extraneous factors introduced during preparation, and the true flagellar width is therefore 270 ± 20 Å, with a core width of 125 ± 10 Å.

In addition to the structural changes which occurred in the sheath during preparation the increased core width denotes that similar effects must occur in the core. If the structural modifications were sufficient to relax any inherent rigidity in the cylindrical flagellum and make its shape dependent on the medium surrounding it, then the removal of this medium on drying, combined with the considerable forces exerted on such a small object on drying, would result in compression of the flagellum into an ellipsoid. A much increased width related to the amount of structural relaxation would therefore be observed.

Reactivity. The clear demarcation of core and sheath afforded by negative staining has enabled us to show that the core and sheath constituents of the flagella of *Vibrio metchnikovii* react differently; identical results were obtained with El Tor vibrio. Although there is some evidence to suggest that the mode of attack on the sheath by each of the three reagents may differ, the conclusion reached from all the experiments is the same, namely that the sheath is easily removed, leaving an apparently intact core. This difference in stability indicates that the flagellum consists either of two constituents which differ chemically or of a single constituent in two sufficiently contrasting physical forms.

In the X-ray analyses of Astbury *et al.* (1955) almost identical X-ray diffraction photographs were obtained from the flagella of *Vibrio metchnikovii* and from those of a non-sheathed species, *Proteus vulgaris*. This close similarity is almost certainly produced by the core constituent of vibrios, because a substructure identical in type and periodicity has been observed both in vibrio cores and in the flagella of a paracolon bacillus, another non-sheathed species (Gordon & Follett, to be published). Since physico-chemical analyses have shown that non-sheathed bacterial flagella are composed almost entirely of the protein 'flagellin' these findings strongly suggest that the core of the vibrio flagella is also composed of the same contractile fibrous protein. Furthermore, the vibrio species of bacteria has a single flagellar antigen and thus, if their surrounding sheath is composed of flagellar protein, it must be identical antigenically with the core protein and yet have markedly differing physical properties from it. What seems more probable is that the sheath differs in chemical composition and may be of cell-wall origin.

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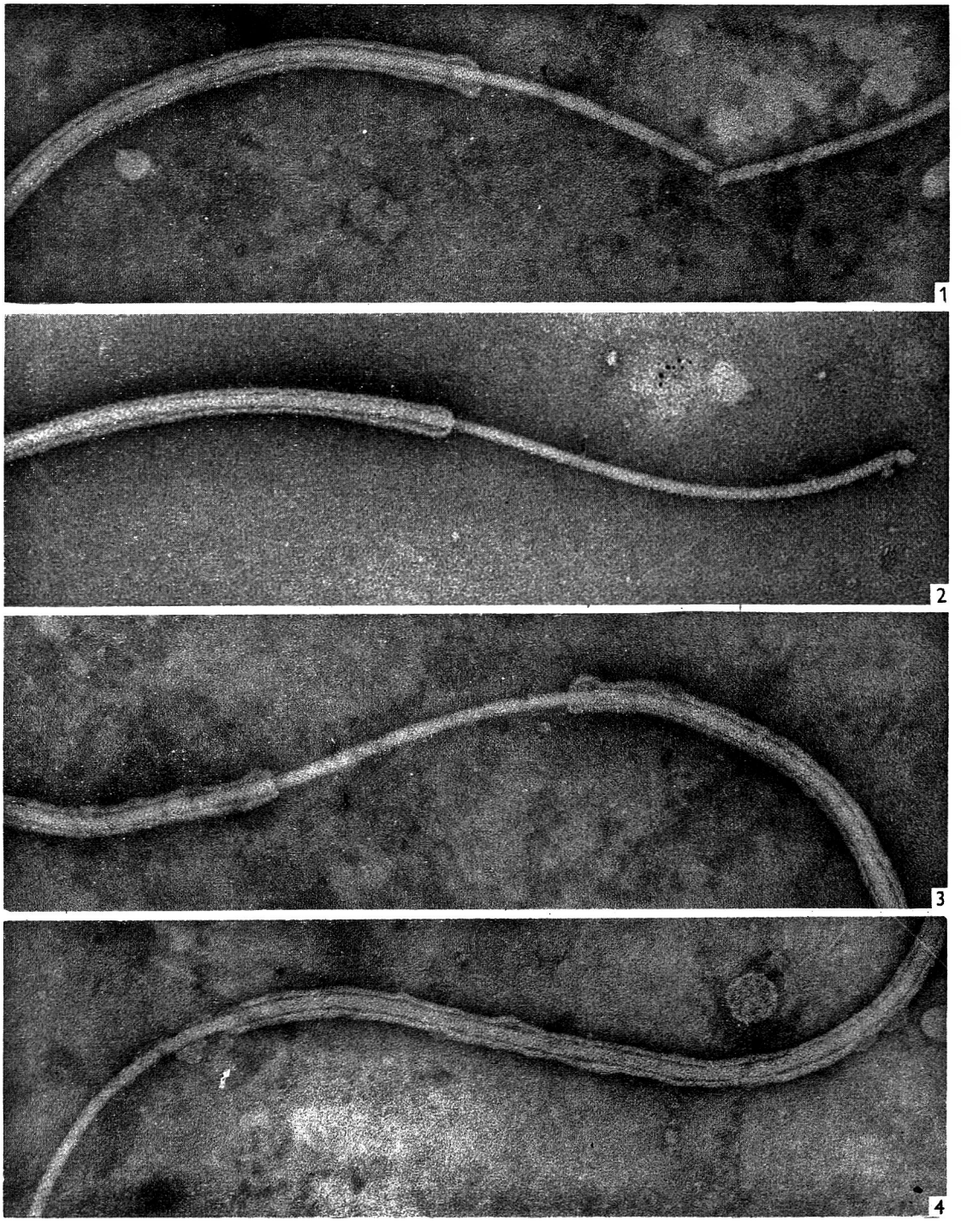

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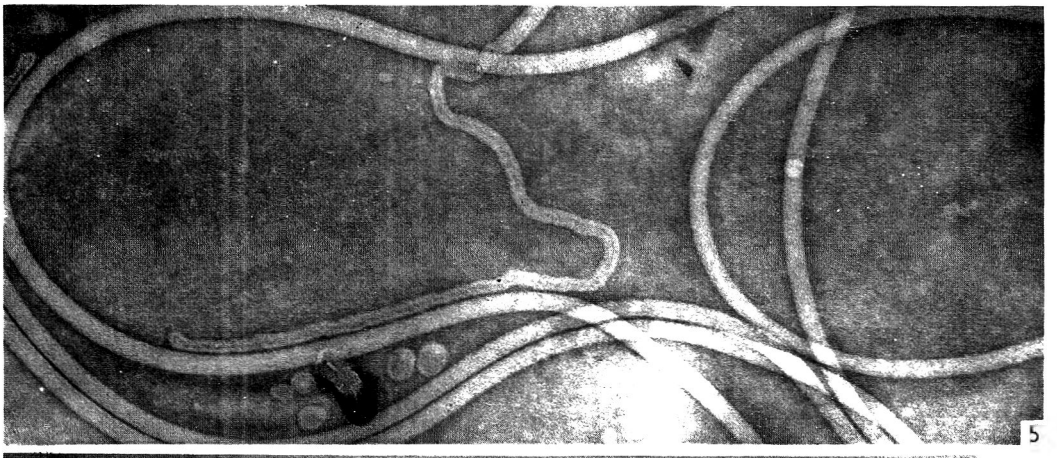

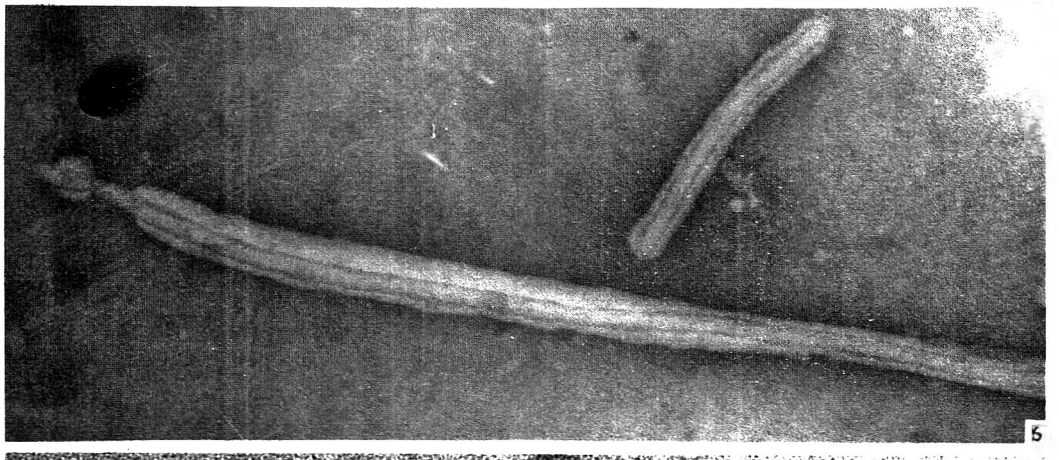

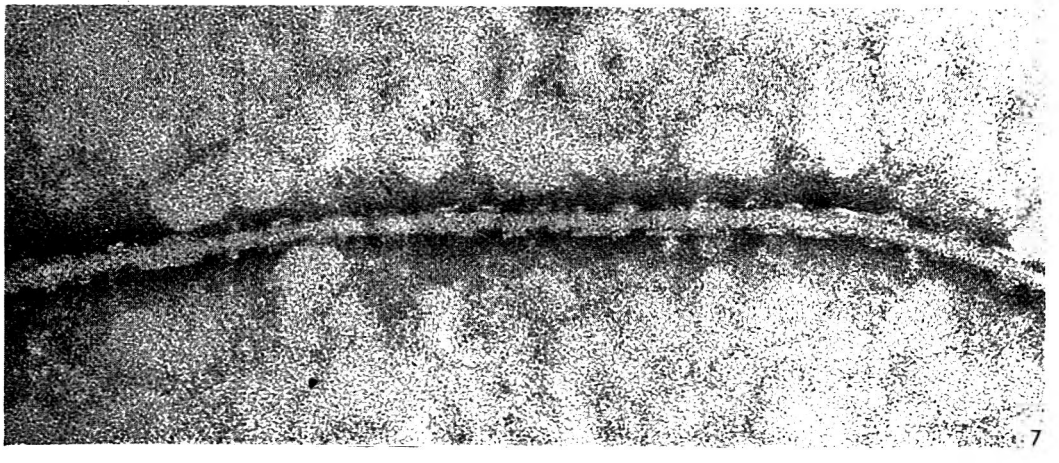

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. Autolysed flagellum of *Vibrio metchnikovii*. × 125,000.

Fig. 2. Autolysed flagellum of El Tor vibrio. × 135,000.

Fig. 3. Flagellum of *V. metchnikovii* showing autolytic effects in non-terminal section. × 128,000.

Fig. 4. Loosening of sheath produced in flagellum of *V. metchnikovii* by autolysis. × 128,000.

PLATE 2

Fig. 5. Flagella of *Vibrio metchnikovii* treated with urea for 5 min. × 125,000.

Fig. 6. Flagellum of *V. metchnikovii* treated with urea for 15 min. × 120,000.

Fig. 7. Flagellum of *V. metchnikovii* treated with acid for 5 min. × 170,000.

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A Comparative Study of Virus Haemagglutinins. The Stability of Haemagglutinins and Red Cell Receptors to Certain Physical and Chemical Treatments

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SUMMARY

A study of 22 haemagglutinating viruses was made to see whether by treating the viruses and the receptors on the red cells with a variety of physical and chemical agents, a convenient means could be devised for identifying and classifying these viruses, while throwing some light on the chemical basis of the haemagglutination reaction. The viruses were submitted to 13 different treatments; acid, urea, *p*-chloromercuribenzoic acid, deoxycholate and possibly bisulphite might be useful for the classification of unknown agents since they gave similar results with all members of a biological group. Other treatments (e.g. formaldehyde) gave results which varied from strain to strain in such a group of viruses and might be useful for genetic studies. The red cells were treated in nine different ways; formalin, papain, chymotrypsin, periodate, receptor-destroying enzyme, (RDE) and swine influenza virus each prevented agglutination by one or two viruses (apparently by inactivating cell receptors). These results were complementary to those with the virus haemagglutinins. The importance of standardized conditions of test are emphasized.

INTRODUCTION

It is known that viruses belonging to many biological groups can agglutinate red cells, and that this is usually a property of the virus particle. The haemagglutinins of vaccinia and the psittacosis group can be separated from the particle itself, but in the work reported here only viruses in which the haemagglutinin and virus particle are closely associated were used. We wished to obtain by simple *in vitro* tests some basic information on the constitution of various viruses. The inactivation of receptors by neuraminidase provided a useful criterion for identifying members of the myxovirus group (Andrewes, Bang & Burnet, 1955). It seemed likely that if a variety of agents were used on the haemagglutinins and the red cell receptors of a variety of viruses the results would fall into a pattern which might provide a rapid method of classifying and identifying haemagglutinating viruses.

METHODS

Table 1 shows the viruses that were used and outlines their preparation in chick embryos, mice and tissue culture. In tests on the viral haemagglutinin it was necessary to prepare the viruses in a non-inhibitory suspension since, for instance, allantoic fluid contains a strong trypsin inhibitor. Therefore, myxoviruses in allantoic

fluid were purified by adsorption on to, and elution from, red cells, or were grown in media containing lactalbumin hydrolysate (LAH) and yeast extract (YE) but no serum; the latter medium was used for ECHO and REO viruses also.

Table 1. *Viruses used, their preparation and the species of red cells used in haemagglutination tests*

All culture media were based on Hanks' saline and contained 0·1 % sodium bicarbonate and 100 units penicillin/ml., 50 units mycostatin/ml. and 100 μg. streptomycin/ml. 0·5 % red cells used with GD VII; otherwise 1 %. Test at 4° with EMC group and GD VII; otherwise at room temperature.

Virus group	Type and strain	Host	Culture media and purification, if any	Red cells
Myxovirus	Influenza A WS	Calf kidney	0·1 % yeast extract, 0·5 % LAH* or	Human
	Influenza A Mel	Calf kidney	5 % horse serum, 0·25 % LAH	Human
	Influenza A2 A/Eng/59	Chick embryo allantois	—	Human and sheep
	Influenza A2 Iksha	Chick embryo allantois	—	Human and sheep
	Influenza B Lee	Calf kidney	As for WS and Mel	Human
	Parainfluenza I Sendai	Calf kidney: chick embryo allantois	As for WS and Mel	Human and sheep
	Mumps	Chick embryo amnion and allantois	—	Human and sheep
REO virus	Type 1	Monkey kidney	199 (Parker), also 0·1 % yeast extract 0·5 % LAH	Human
	Type 2	Monkey kidney	1 % or 2 % calf serum, 0·25 % LAH	Human
ECHO virus	Type 7	Human embryo lung	2 % calf serum, 0·25 % LAH or 0·5 % LAH, 0·1 % yeast extract	Human
	Type 11 prototype	Monkey kidney		Human
	Type 11 U	Monkey kidney		Human
	Type 12	Monkey kidney		Human
Theiler's mouse encephalitis virus	GD VII	Mouse	Arcton treated brain	Human
Encephalomyocarditis virus	EMC	Mouse ascites tumour cells	Supernatant fluid†	Sheep
	Columbia SK		Arcton-treated brain	Sheep
Adenovirus	Adeno 7 prototype	HeLa cells	5 % rabbit serum, 0·25 % LAH	Rhesus monkey
	Adeno 9 prototype	HeLa cells	5 % rabbit serum, 0·25 % LAH	Human
	SV 17	Mouse kidney	199 (Parker) or 2 % calf serum, 0·25 % LAH	Human
Arthropod-borne virus	A. Semliki Forest	Suckling mouse	Arcton-treated brain	Goose
	B. West Nile	Suckling mouse	Arcton-treated brain	Goose
Pneumonia virus of mice	PVM	Mouse	Arcton-treated lung	Mouse

* Lactalbumin hydrolysate. † Martin, Malec, Sved & Work (1961)

Adenovirus suspensions were prepared from the cells only and purified by extraction with trifluorotrichloroethane ('Arcton' 113: Imperial Chemical Industries Ltd.) All these virus suspensions had no inhibitory action on proteolysis of gelatin by trypsin.

In tests on the red cell receptors untreated culture fluids were used except in the case of REO and adenoviruses where the cells were disintegrated in the medium by a mechanical blendor.

Saline. Except where stated otherwise, phosphate buffered saline (PBS) was used, i.e. 0·14 M-sodium chloride + 0·01 M-phosphate buffer at pH 7·2.

Red cells. These were taken with sterile precautions, stored in glucose + gelatin + veronal solution (Clarke & Casals, 1958). Human group O cells from one donor were used but rhesus monkey cells were used for adenovirus type 7, sheep cells for encephalomyocarditis virus (EMC), goose cells for arthropod-borne viruses, and mouse cells for pneumonia virus of mice (PVM).

Treatment of viruses

Urea. Dilutions from 1 to 8 M were mixed with equal volumes of virus and incubated for 1 hr. at 37° and dialysed at 4° against PBS for 3 hr.

pH *values.* M-buffers were made with a range from pH 2 to pH 10; pH 2, 3 and 4 were citrate, pH 5 acetate, pH 6, 7 and 8 phosphate, pH 9 and 10 glycine buffers. Equal volumes of virus and buffer were mixed for 1 hr. at room temperature, then dialysed against PBS for 3 hr. at 4°.

Heat. Separate samples were kept in 56° water bath for 20, 40, 60 min.; in the 37° bath for 1, 3 and 16 hr.

Ether. Four volumes of virus + 1 volume of ether were held at 4° overnight. The ether layer was pipetted off.

Formalin (formaldehyde solution 40 %, w/v). Virus was mixed with an equal volume of 20 % formalin in PBS, left at 4° overnight and then dialysed for 2 hr. at 4°.

Periodate. Equal volumes of virus suspension and 0·046 M-sodium metaperiodate were mixed and held at room temperature for 1 hr. and then dialysed against PBS for 3 hr.

Bisulphite. As for periodate, but with 0·104 M-$Na_2S_2O_5$ and subsequently with 0·052 M, 0·026 M and 0·013 M solutions.

Enzymes. Trypsin and chymotrypsin were prepared by dissolving 0·1 g. crystalline material in 10 ml. 0·01 N-HCl. This stock solution was kept frozen and diluted 1/100 with 2-amino-2-hydroxymethylpropane-1,3-diol (tris) buffer (pH 7·6) before use. Equal parts of enzyme solution and of virus were held for 2 hr. at 37°. The control in tris buffer was similarly treated.

Papain. This was prepared according to the method of Muschel & Piper (1959); 2 g. crude papain in 10 ml. 0·05 N-HCl was diluted in tris buffer with ethylene diamine tetra-acetic acid (EDTA; 0·001 M) and cysteine 0·001 M (pH 7·1) to 1 % papain.

Thiol reagents: see Buckland (1960).

Sodium deoxycholate. Virus was mixed with an equal volume of 0·0048 M-sodium deoxycholate, incubated at 37° for 1 hr. and then dialysed overnight against 0·01 M-PBS.

Treatment and method of use of red cells

The red cells were usually suspended in the reagent dissolved in saline and shaken by hand at intervals. Thereafter the cells were centrifuged (500 *g*. for 5 min.), resuspended in saline and used immediately or after a short time at 4°. Table 2 shows the concentrations of reagents and red cells used and other details of the treatments. The mouse red cells used for PVM did not withstand these treatments; consequently only the haemagglutinin was investigated.

Table 2. *Treatment of red cells*

Proteolytic enzymes were assayed frequently Two-fold dilutions were made in Hanks' saline containing 5 % sodium bicarbonate. To 1·25 ml. of each dilution 0·5 ml. of 7·5 % gelatine were added. They were incubated at 37° for 1 hr. and failure to set on cooling was taken as the end-point.

Reagent	Quantities used	Temperature and time of treatment
Formalin (formaldehyde solution 40 %, w/v)	1 vol. packed cells and 3·4 vol. of 1 % formalin	37°, overnight
Sodium metaperiodate	*(*a*) 1 vol. of 10 % cell suspension and 9 vol. 0·0058 M-$NaIO_4$ (*b*) 1 vol. of 10 % red cells, 1 vol. of $NaIO_4$ at dilution ranges 0·0016 M to 0·0008 M	Room temperature; 1 hr. 4°; 1 hr.
Sodium metabisulphite	1 vol. of 10 % cell suspension and 9 vol. of 0·026 M-$Na_2S_2O_5$	Room temperature; 1 hr.
Papain (BDH) titre 13,000	Stock prepared according to Muschel & Piper (1959), 5 vol. of 2 % papain and 1 vol. packed cells	37°; 30 min.
Trypsin (crystalline, Difco) titre 8000–64,000	Stock 0·1 % in 0·01 N-HCl, 1 vol. of 0·01 % trypsin and 1 vol. packed cells	37°; overnight
Chymotrypsin (Armour) titre 4000	As for trypsin	As for trypsin
p-Chloromercuribenzoic acid	10^{-3} M or 10^{-4} M dilution mixed with equal volume 10 % cells	Room temperature; 1 hr.
Iodoacetamide	10^{-3} M or 10^{-4} M dilution mixed with equal volume 10 % cells	Room temperature; 1 hr;
Cholera filtrate (N.V. Philips–Roxane (Duphar))	Reconstituted: diluted in saline. Serial dilutions mixed with 5 % packed cells	37°; 2–5 hr.

* In method (*a*) with ambient temperature 15–20° the experiment was carried out on the bench. In method (*b*) with ambient temperature 21–26° to avoid haemolysis the treatment of red cells was carried out at 4°, the test set up on the bench, but subsequently allowed to settle at 4°.

Haemagglutination tests

Haemagglutination tests were usually carried out at room temperature in plastic plates. Serial two-fold dilutions of virus were made in saline and an equal volume (0·25 ml.) of 1 % red cell suspension was added to each cup. The test was read by observing the pattern of sedimentation, and partial agglutination was taken as the end-point. Glass test tubes were used in titrations of GD VII virus as, otherwise,

clear patterns were not obtained. Other variations in technique are given in Table 1. It was found that non-specific inhibitors for SV 17 and arthropod-borne viruses, derived from tissues, were removed by treatment in a mechanical blendor with Arcton 113.

Assessment of results

An attempt was made to obtain quantitative results with each reagent. Viruses or red cells were treated with various dilutions. The difference in titre from that of the controls was called the 'titre reduction' and plotted or tabulated. A reduction of four-fold was taken as significant in a single test; an average reduction of less than four-fold was sometimes significant in repeated tests (see Table 4). When a difference was not precisely known because an end-point was not reached it was assumed for purposes of calculation that the end-point lay at the next step in the dilution series, e.g. a titre reduction from 64 to < 4 was treated as a reduction of 64 to 2. The concentrations of reagents tested usually ranged from the maximum that could be used without producing obvious signs of damage to the red cells, (e.g. haemolysis or spontaneous agglutination), to the lowest concentration which produced any effect with the most sensitive virus. In most instances, this was a narrow range, but with periodate and cholera filtrate various dilutions were studied. All the results reported here are based on tests repeated several times, usually with different batches of virus, red cells and reagents.

RESULTS

Treatment of haemagglutinins

The haemagglutinins of most viruses were impaired or destroyed by a variety of reagents, but there were significant differences in the types of reagents which were active and the concentrations at which they were effective on different viruses.

pH *range.* Some typical results are shown in Fig. 1; it may be seen that the haemagglutinins were mostly stable in alkaline conditions and that there was a sharply defined transition from no effect to a marked one over a pH range of 1 to 2 units. These results held good for other viruses in the same biological group.

PVM and ECHO viruses were extremely stable throughout the range, the adenoviruses stable from pH 10 to 3, the myxoviruses from pH 10 to about pH 5; the arthropod-borne viruses were stable only in very alkaline conditions.

Urea. Urea was tested because it is known to split hydrogen bonds and at concentrations between 0·5 and 4 M a wide variety of responses was found. Some representative results are shown in Fig. 2. PVM virus and GD VII were unaffected by even the highest concentration though PVM was readily inactivated by acid. Myxoviruses were inactivated by urea concentrations of 3 M or more; adenoviruses and enteroviruses by approximately half this concentration.

Bisulphite. The effect of a mild reducing agent was next studied. Sodium metabisulphite at 0·104 M was convenient for a screening test; the myxoviruses, GD VII virus and the arthropod-borne viruses were sensitive, but the others were not. The most sensitive of the myxoviruses was influenza strain WS which was inactivated by one-eighth of the screening concentration whereas Sendai virus was affected by the screening concentration only.

Periodate. Treatment with periodate had an effect on all the viruses tested, but

closely related viruses reacted differently; for example, ECHO 7 and SV 17 were little affected, but ECHO 12 and Adeno 7 were inactivated.

Sodium deoxycholate. Theiler (1957) showed that the infectivity of arthropod-borne viruses was readily abolished by deoxycholate. We found complete inactivation of haemagglutinin in our tests with Semliki Forest and West Nile viruses, representatives of groups A and B, respectively. No other haemagglutinin showed any change in titre after treatment.

Ether. Ether treatment also affected only the two arthropod-borne viruses, but the effect on Semliki Forest virus was weak and barely significant. Cheng (1961) found that treatment for a shorter time with two volumes of ether for two periods of 10 min. at 3° destroyed almost all the infectivity, but made no significant difference to the haemagglutinin of Semliki Forest virus.

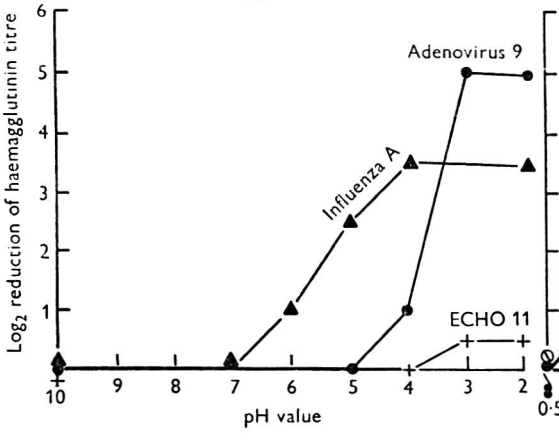


Fig. 1

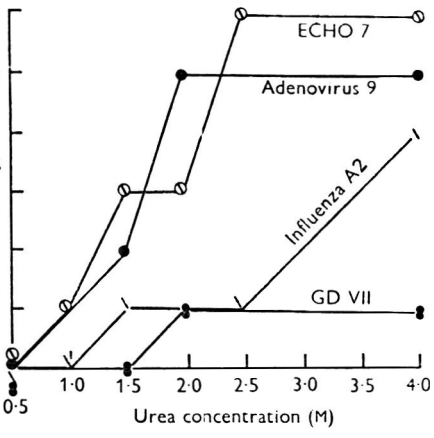


Fig. 2

Fig. 1. The effect on the haemagglutinin titre of exposure of certain viruses to various pH values. —▲—, influenza A; —●—, adenovirus 9; —+—, ECHO 11.

Fig. 2. The effect on haemagglutinin titre of exposure to urea at various molarities —⊘—, ECHO 7; —●—, adenovirus 9; —╲—, influenza A2; —■—, GD VII.

Proteolytic enzymes. Trypsin and chymotrypsin at 0·1 mg./ml. inactivated adenovirus type 9 only. Recently, however, Gresser & Enders (1961), who used a higher concentration of trypsin (0·5 mg./ml.) and sampled periodically up to 48 hr., found that of the myxoviruses, mumps haemagglutinin was highly susceptible, Sendai relatively so and influenza A insusceptible. They considered that these findings might be of value in the classification of the group.

Papain attacked more viruses than did trypsin, but individual viruses were found to be susceptible or resistant in a way that bore no relationship to their broad biological groupings. REO virus type 2, Iksha and the Lee viruses were inactivated by 0·1% papain, but WS, Mel and mumps were unaffected by 1%.

Formalin (formaldehyde solution 40%, w/v). Formalin is widely used at dilutions of less than 1% for inactivating virus infectivity, but the effect on the haemagglutinin of the high concentration used in these experiments varied from strain to strain. Some members of all virus groups were susceptible and myxoviruses were in general inactivated, but WS and Sendai were relatively resistant. Similarly,

REO type 1 was susceptible although REO type 2 was not. Underwood & Weed (1961) reported that glyoxal inactivated the infectivity of several viruses at a concentration that left that of poliomyelitis unimpaired. When we tested this compound on the haemagglutinin of some representative viruses it behaved like formalin, but was less active on a molecular basis.

Heat. All the viruses were heated at 56° for 20, 40 and 60 min. as well as at 37° for 1, 3 and 16 hr. The pH value was kept at pH 9 for arthropod-borne viruses, at pH 7·2 for other viruses; the viruses were suspended in serum-free medium or in PBS. All haemagglutinins but those of PVM and some myxoviruses were completely destroyed at the higher temperature in 20 min., while at lower temperatures only arthropod-borne viruses, ECHO 11 and EMC, were decreased in titre. The effects of the higher temperature, together with that of periodate, bisulphite, papain and formalin are shown in Table 3.

Thiol reagents. The effect of *p*-chloromercuribenzoic acid on haemagglutinins has already been reported from this laboratory (Buckland, 1960) and by Philipson & Choppin (1960). Enteroviruses, REO, EMC and adenovirus type 7 were highly susceptible while the remainder were unaffected.

Table 3. *Log_2 reduction in haemagglutinin (HA) titre produced by indicated treatment of viruses*

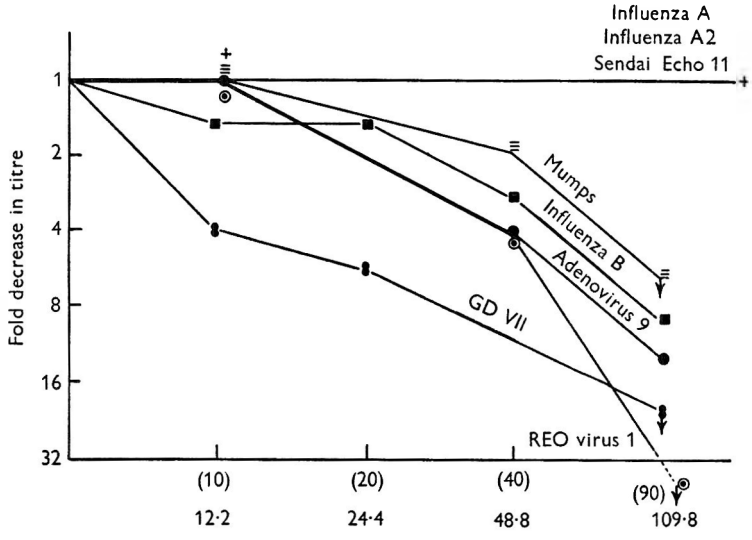
Virus	Treatment: Papain (1 %)	formalin (20 %)	bisulphite (0·104 M)	periodate (0·046 M)	heat 56° for 20 min.
	log_2 reduction in HA titre				
WS	0·5	1·75	6	4·5	3
Mel	0	5	6	4·0	1
A/Eng/59	3	6	3·5	2·5	2
Iksha	5	3	4	4·5	4
Lee	5	2·3	4	4·0	2
Sendai	2	2	5	6·0	5
Mumps	0	4	2	2·5	2
REO 1	3	3·5	1	4	4
REO 2	3	0	1	3	5
ECHO 7	1·5	3·6	0	1·5	7
ECHO 11	0	3	0	2·5	5
ECHO 12	0	1	0	4·6	9
GD VII	0	6	2	5	3
Adeno 7	0	6·5	0	5·3	7
Adeno 9	2	1	0	2·25	7
SV 17	0·8	1	1	1·0	5
EMC	2	4	0	5	5
Columbia SK	0·5	4·5	1	4·3	6
Semliki Forest	—	4	4	4·5	5
West Nile	—	5	6	6·0	5
PVM	2	—	0	2·75	0

Effect of various agents on red cell receptors

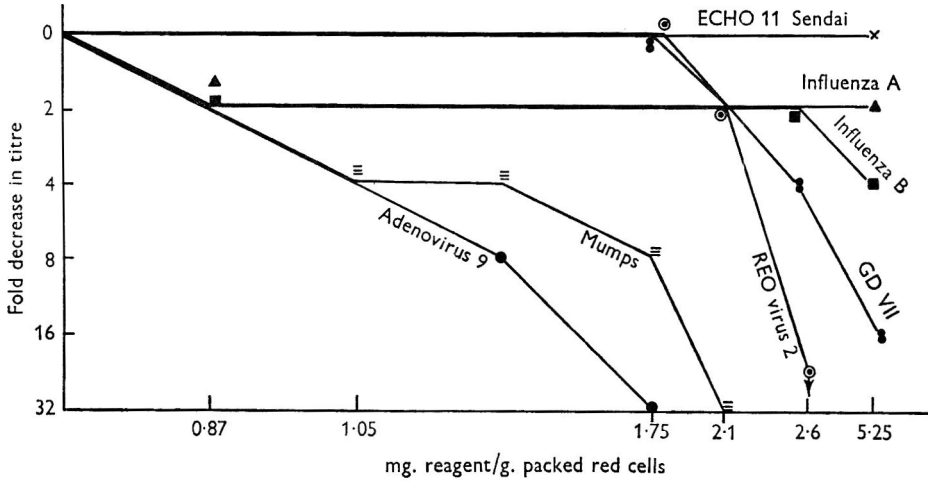
Formalin. Treatment of red cells with less than 1 % formalin produced little effect. At this concentration, however, there was no decrease or a slight increase of agglutinability with myxoviruses and arthropod-borne viruses, but a marked

decrease on testing with ECHO, REO and adenoviruses; the decrease was somewhat less using EMC and Columbia SK. Some of these results have already been reported (Buckland, 1959).

Proteolytic enzymes. As it seemed that formalin might be acting on the amino groups of proteins, the effect of proteolytic enzymes was next tested. Philipson (1959) showed that chymotrypsin destroyed receptors for ECHO 11 virus; this was



(*a*)



(*b*)

Fig. 3(*a*). Effect of increasing volumes of 0·0058 M-metaperiodate at ambient temperature 15° to 20°. —+—, influenza A, A2 Sendai, ECHO 11; —≡—, mumps; —■—, influenza B; —●—, adenovirus 9; —◘—, GD VII; —⊡—, REO virus 1. 3(*b*). Effect of increasing concentration of reagent at ambient temperature of about 23° (21–26°). —×—, ECHO 11, Sendai; —▲—, influenza A; —■—, influenza B; —◘—, GD VII; —⊙—, REO virus 2; —≡—, mumps; —●—, adenovirus 9.

confirmed, but it was found that, to a lesser extent, it also rendered cells inagglutinable by ECHO viruses types 7 and 12, REO viruses types 1 and 2 and adenovirus type 7 (Tables 3 and 5). Papain treatment prevented agglutination by more viruses than did treatment with chymotrypsin; agglutination by adenovirus type 9 and EMC virus was inhibited; agglutination by influenza A viruses was slightly decreased, but other myxoviruses and REO viruses were unaffected. The effectiveness of papain may be correlated with its powerful and relatively non-specific proteolytic activity. The slight effect of trypsin on agglutination by influenza A (Hirst, 1948) was not observed, but different red cells and different virus strains were used.

Receptor destroying enzyme (neuraminidase): RDE. The receptor destroying enzyme (RDE) of cholera filtrate (Burnet & Stone, 1947) has long been known to destroy receptors for influenza viruses and other myxoviruses (Andrewes *et al.* 1955). Our experiments confirmed this and also showed that the two A 2 strains we used behaved as '+' viruses, i.e. the receptors were not all destroyed by cholera filtrate (Takátsy & Barb, 1959; Choppin & Tamm, 1960). The receptors for adenoviruses were destroyed by RDE as was also observed by Kasel, Rowe & Nemes, 1960. We confirmed that receptors for ECHO viruses are not inactivated by RDE (Goldfield, Srihongse & Fox, 1957). As our experiments were done with a crude cholera filtrate preparation parallel tests were performed with an allantoic fluid containing swine influenza virus. The similarity in results suggested that the effect was due to RDE and not to any possible contaminating enzyme.

Oxidizing and reducing agents. Bisulphite at the concentration used had no effect on receptors. The destruction of receptors for influenza virus by periodate was reported by Hirst (1948) and Fazekas de St Groth (1949). A difficulty in extending these tests was the haemolytic effect of the higher concentrations of periodate used by these workers, but we found that this could be circumvented if the cells were treated with the same amount of periodate in a greater volume—the highest concentration used (0·0058 M) lay at the lower end of the range of concentrations which caused destruction of receptors for Mel; raising the concentration further in attempts to destroy all receptors resulted in haemolysis. The degree of receptor destruction was markedly affected by the exact conditions under which the test was carried out as can be seen by inspection of Figs. 3*a* and 3*b* and Table 4. However, the general order of sensitivity was more or less the same whatever technique was used. The receptors for myxoviruses varied greatly in their sensitivity; Sendai virus receptors were completely resistant, while those of mumps were very sensitive. Adenovirus, GD VII and REO virus receptors were also very sensitive, but ECHO virus receptors were unaffected.

Agents which combine with -SH groups. Since *p*-chloromercuribenzoic acid had been found to inactivate certain virus haemagglutinins very effectively (Buckland, 1960) it was thought that this substance or iodoacetamide might act on red cell receptors. However, no inactivation was observed under the conditions used.

Experiments with sheep, rhesus monkey and goose red cells. Since certain viruses will agglutinate not only human red cells but those of some animals and birds, a limited number of experiments was carried out to see whether similar results were obtained when those cells were used instead of human ones. The reactions of treated human and sheep cells were similar (Table 5). The receptors for EMC and

Table 4. *Summary of the effect of six treatments on the agglutinability of human red cells by the viruses used*

The figures given are averages of from two to eleven results. Those greater than 1 are statistically significant; $P < 0.05$ by the t test. When red cells were agglutinated after treatment with undiluted reagent (i.e. 2·5 % cholera filtrate or swine influenza virus in saline) the result is recorded as 0.

	Treatment of red cells						
				Periodate			
Virus	Formalin (1 %)	Papain (2 %)	Chymotrypsin (0·01 %)	109 mg./g. cells at 17°	5·23 mg./g. cells at 23°	Cholera filtrate	Swine influenza
	Log_2 geometric mean titre reduction					Log_2 of highest dilution of reagent able to render cells inagglutinable by 8 units of the virus used	
Myxoviruses							
Influenza A WS	0	1·4	0	0	1	5	9
Influenza A Mel	0	1·4	0	0	1	5	3
Influenza A2 A/Eng/59	0	1·0	0	0	—	0·5	—
Influenza A2 Iksha	0	1·0	0	0	—	0·5	—
Influenza B Lee	0	0·25	0	2·9	2	5	2
Parainfluenza I Sendai	0	0	0	0	0	0·25	3
Mumps	0	0	0	4·6	4	2·5	—
REO virus Type 1	4	0·7	1·5	4	—	0	0
REO virus Type 2	7	0	1·3	2·3	4	0	—
Enteroviruses							
ECHO Type 7	4	1	1	0	—	0	—
ECHO Type 11	4·8	2·8	2·5	0	0	0	—
ECHO Type 11 (U virus)	6·6	2	3·7	0	—	0	0
ECHO Type 12	2·3	1·5	1·5	0	—	0	—
GD VII	6·3	0	0·8	4·6	4	0	—
Adenoviruses							
Type 9	2·5	5·5	0·5	3·1	4	4	5
SV 17	4·6	1·3	0·5	4·3	—	1·25	—

— = not tested.

Table 5. *Summary of the effect of five treatments on the agglutinability of sheep, rhesus or goose red cells by certain viruses*

Virus	Red cells	Treatment of red cells: Formalin (1 %)	Papain (2 %)	Chymotrypsin (0·01 %)	Periodate	Cholera filtrate $\log_2$ of highest dilution of reagent able to render cells inagglutinable by 8 units of the virus used
		$\log_2$ geometric mean titre reduction				
Myxoviruses						
Influenza A 2 A/Eng/59	Sheep	0	0	0	0	1
Influenza A 2 Iksha	Sheep	0·5	0	0	0·5	1
Parainfluenza I Sendai	Sheep	1	0	0	1	3
Mumps	Sheep	2	2	1	2	2
EMC group						
EMC	Sheep	1·75	5	0	3	7
Col SK	Sheep	2·25	4	0	3	9
Adenovirus Type 7	Rhesus	7·3	8·5	—	1·0	0
Arthropod-borne viruses						
Semliki Forest	Goose	0	—†	0	0	0
West Nile	Goose	0	—	0	0	0

* Treatment—9 vol. of 0·0058 M periodate.

† Cells destroyed by papain.

Columbia SK viruses were more readily destroyed than those for myxoviruses. On the other hand, the reaction of adenovirus 7 with treated rhesus red cells was slightly different from that of adenovirus 9 with human cells; adenovirus type 7 was able to agglutinate cells treated with periodate and cholera filtrate. This may have been due to differences in the virus or in the cells (see Table 5). It was also found that patas virus (SV 17) receptors on ox cells were not inactivated by formalin while receptors on human cells were inactivated. Treated goose cells, which reacted with influenza viruses like treated human cells, were agglutinated to full titre by the arthropod-borne viruses used. It was concluded that generally speaking, the results of treatment of red cells were not influenced by the species of red cell used.

Adsorption experiments. Red cells were treated with formalin, periodate, chymotrypsin and papain. The cell suspensions were then mixed with virus and the amount of haemagglutinin left in the supernatant fluid measured. In this way the capacity of treated red cells to absorb influenza WS and LEE, adenovirus type 9, REO virus types 1 and 2, ECHO viruses types 7, 11 and 12 and GD VII was measured. The results can be summarized by saying that treated cells which were agglutinable by the virus with which they were mixed could also absorb the virus; cells which were inagglutinable did not absorb virus. The only exception was that papain treated cells failed to absorb REO virus. It can, therefore, be concluded that in general the treatments which rendered red cells inagglutinable by specific viruses destroyed the receptors for that virus, just as in the classical work with RDE.

DISCUSSION

When the experimental work described in the paper was completed (1960) relatively little had been published on the *in vitro* behaviour or the haemagglutinins and receptors of viruses such as the adenoviruses and enteroviruses. Much more work has been published since then which confirms or goes beyond the facts reported here. However, this is the first publication of a systematic comparison of stability of a range of haemagglutinins on treatment with urea, different pH values, deoxycholate and bisulphite, of comparison of the effects on different virus receptors of proteolytic enzymes and periodate and the lack of effect of bisulphite on receptors of adeno, REO and ECHO viruses. A systematic comparison seemed desirable because differences in the source of viruses, the concentration of reagents or the time and temperature of the reaction may affect the results significantly. From the tables it can be seen that when the same techniques are used, the haemagglutinins of influenza WS and mumps were inactivated by periodate, the receptors for influenza WS were unaffected while those for mumps were destroyed. In contrast the haemagglutinin of REO virus type 1 was destroyed by formalin whereas that of REO virus type 2 was not, although the red cell receptors for both were destroyed.

The findings of these experiments suggest that there are chemical differences between the haemagglutinins in the main groups of viruses and in the red cell receptors for them and that certain subdivisions within a virus group based on a serological difference such as that found between influenza A and B have also a chemical difference which can be demonstrated by the use of papain. When an unknown virus isolated from Erythrocebus patas monkeys was investigated on

these lines it was found to have a haemagglutinin resembling that of adenoviruses before it was shown to possess the adenovirus complement-fixing antigen (Tyrrell, Buckland, Lancaster & Valentine, 1960) and it was finally identified as SV 17.

We are indebted to Dr H. G. Pereira for adenovirus type 7 suspensions, Drs T. H. Work and E. M. Martin for EMC preparations, Dr J. S. Porterfield for the arthropod-borne viruses, Dr A. C. Allison for the *p*-chloromercuribenzoic acid and iodoacetamide, and especially to Mrs P. K. Brown for technical assistance.

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The Nature of Inhibitory Activity by *Staphylococcus aureus* Type 71

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SUMMARY

Antibiotic production in liquid media by *Staphylococcus aureus* strains of bacteriophage 'type 71' was poor as measured by cup assays against susceptible corynebacteria. Aerobic incubation in freshly prepared tryptic-digest broth containing a fermentable carbohydrate and adjusted to pH 7·8 gave the greatest yields. The antibiotic material was not obtained in a pure state, but was concentrated by evaporation of crude solutions. These were prepared by adding trichloroacetic acid to broth cultures and centrifuging to remove the organisms. The antibiotic was stable and relatively heat-resistant under acid conditions, but was rapidly destroyed when alkaline; it diffused slowly through cellophan, was adsorbed by charcoal, and was inactivated by trypsin but not by pepsin. These properties suggest that it may be protein or polypeptide in nature. Inhibitory activity by type 71 cocci was shown in mixed broth cultures against other non-inhibitory strains of *Staphylococcus aureus*, and was similar to that previously observed on solid media.

INTRODUCTION

Staphylococcus aureus strains of bacteriophage 'type 71' are frequently isolated from superficial infections of the skin (Barrow, 1955; Parker, Tomlinson & Williams, 1955). They produce sharply defined inhibition zones in direct antagonism tests against corynebacteria on solid media (Parker, 1958). In deferred antagonism experiments, Parker & Simmons (1959) also observed weak activity against other strains of *Staphylococcus aureus*, but failure to obtain active bacteria-free preparations from cultures grown either in liquid or on solid media restricted their investigation of the antagonistic agent. Later, type 71 organisms were found to be strongly and directly active on solid media against most other *Staphylococcus aureus* strains, as well as against many other Gram-positive species (Barrow, 1963). The present paper concerns further observations on the nature and properties of the inhibitory agent produced by type 71 staphylococci in liquid media.

METHODS

Organisms. These were described previously (Barrow, 1963). They included: two indicator corynebacteria (an unnamed diphtheroid organism 'Bradford' and an avirulent strain of *Corynebacterium diphtheriae* type *mitis* 'Manchester') which are susceptible to inhibition by active (DI+) staphylococci; seven DI+ strains of *Staphylococcus aureus* type 71, which give sharply defined inhibition zones with the

corynebacteria; two DI+ *aureus* strains from the National Collection of Type Cultures (NCTC 6507, NCTC 8004) which produce hazy zones of inhibition with the Manchester corynebacterium. The two indicator corynebacteria were used in parallel; one of the type 71 strains was used extensively, but confirmatory tests were made with the others. Strains of *Staphylococcus aureus* not active against corynebacteria (DI−) were also employed in some experiments, and as indicator organisms in assays of type 71 material.

Medium. Suitable broth was important for detectable antibiotic production by type 71 staphylococci. Horse-flesh trypsin-digest broth, with the addition of 0·002% (w/v) cystine hydrochloride, was usually satisfactory for this purpose. Incubation was aerobic at 37° overnight, unless otherwise stated.

Assay methods. The methods used to obtain active bacteria-free material are described later. These preparations were assayed for inhibitory activity against corynebacteria and DI − staphylococci as follows.

(i) *Agar-cup method.* Blood agar medium in Petri dishes was seeded with broth suspensions of indicator organisms. When dry, cups 8·0 mm. in diameter were punched out, and without sealing, were filled with twofold dilutions of the assay material made in quarter-strength Ringer solution. After incubation, the width of any inhibition zone was recorded. This method was sufficiently accurate for comparative purposes.

(ii) *Dilution method.* Dilutions, ranging from 10^{-1} to 10^{-6}, were made from a broth culture of a staphylococcal strain known to be inhibited on solid media by type 71 organisms. Equal quantities (usually 1·0 ml.) of normal broth and of the material to be assayed were distributed in two sets of sterile tubes. A standard volume (0·02 ml.) of each culture dilution was then dropped into one tube of each set. Readings were made after incubation at 37° for 24 and 48 hr. The end-point was taken as the largest inoculum which gave no visible growth in the assay material, but visible growth in the corresponding control tube.

RESULTS

Production of antibiotic in liquid media

Antibiotic material. Preliminary attempts to detect activity against corynebacteria in cup assays of type 71 broth culture preparations were equivocal, partly because of low titres and partly because of difficulty in obtaining suitable material. Broth cultures passed through Seitz or sintered glass filters were inactive, and repeated centrifugation seldom yielded a sterile supernatant fluid. The following methods were found useful.

Cellophan-sac cultures. A piece of washed cellophan (Visking) tubing was tied at the ends to inlet and outlet tubes in the stopper of a conical filtration flask, the loop of tubing so formed was covered with broth, and the flask autoclaved. When cool, the cellophan loop was filled with sterile broth through the inlet tube and then inoculated with type 71 organisms. On incubation, growth occurred inside the tubing; the broth outside remained sterile and was found to have inhibitory activity against corynebacteria. Controls, obtained similarly after growth of non-inhibitory staphylococci, were inactive.

Heat treatment. The inhibitory agent in broth cultures withstood a considerable

degree of heating without much loss of inhibitory activity. Usually cultures were placed in boiling water for a few minutes, sufficient to kill all the organisms, and then cooled rapidly by immersion in cold water. After centrifugation the supernatant fluid was removed and was active in cup assays against corynebacteria.

Acid treatment. The inhibitory agent was retained in solution after precipitation of the organisms and proteins in broth cultures by the addition of an equal volume of 10% (w/v) trichloroacetic acid. After centrifugation, the supernatant fluid was carefully neutralized with 5 N-NaOH (phenol red as indicator) and showed antibiotic activity, whereas cultures of non-inhibitory staphylococci, treated in the same way, gave inactive fluids. Although diluted twofold, this material usually gave inhibition titres in cup assays against corynebacteria similar to those of the undiluted preparations obtained from cellophan-sac or heated cultures. Material prepared by acid treatment was therefore mainly used.

Conditions for antibiotic production. Preliminary work suggested that broths varied considerably in their ability to allow detectable antibiotic production by type 71 staphylococci. Different broths, including meat infusions and digests, Lemco broth, casein and lactalbumin hydrolysate media, and several commercial products, were therefore compared. Cup assays of the crude solutions against corynebacteria indicated that broth freshly prepared by tryptic digestion of meat usually gave the greatest yields; many of the others showed no inhibitory activity at all. Variation was, however, observed between different batches of digest broth, and deterioration also occurred during prolonged storage. The addition of glucose or mannitol, and an initial pH of about 7·8, appreciably increased the antibiotic titre. This was highest after aerobic incubation at 37°, and was directly related to the amount of growth. Maximum activity was reached within 24 hr., after which the titre slowly fell. Under these conditions, the highest titre obtained in cup assays against corynebacteria was 1 in 128. Decreased O_2 or increased CO_2 tension made little difference; anaerobic incubation was unsatisfactory. Shallow and deep cultures, with and without intermittent shaking, all gave similar results. Screw-capped containers filled with digest broth were therefore used for growth of type 71 organisms.

Concentration of the antibiotic agent. Extraction, or filtration, of the inhibitory substance from crude broth preparations was unsuccessful. The addition of alkali, HCl, glacial acetic acid and concentrated trichloroacetic acid in the cold resulted in loss of activity. Separation was not obtained with organic solvents, including methanol, ethanol, chloroform, ether, pyridine. Adsorption with activated charcoal removed the activity from solution, but all attempts at subsequent elution failed. Doubtful activity was found in the precipitate given by the addition of an equal volume of saturated $(NH_4)_2SO_4$ solution. Considerable activity was, however, present in the neutralized supernatant fluid obtained after precipitation of organisms and proteins from broth cultures with an equal volume of 10% (w/v) trichloroacetic acid. No activity was detected in the residue from this culture supernatant fluid after evaporation to dryness, nor did re-inoculation of the supernatant fluid with type 71 organisms enhance the inhibitory activity. The antibiotic material was concentrated by evaporating the neutralized trichloroacetic acid fluid supernatant in Visking tubing in a current of warm air to about one-eighth of its volume. This gave a copious inactive deposit which was removed by centrifugation. After

dialysis, the residual fluid contained about four times the activity of the original culture preparation against corynebacteria, and in cup assays it also produced weak inhibition of DI− staphylococci.

Properties of the antibiotic material

Stability. Concentrated antibiotic preparations, adjusted to pH 3·0, 7·0 and 8·5, were assayed against corynebacteria after heating at (*a*) 60° for 1 hr., (*b*) 100° for 15 min., (*c*) 120° for 15 min. in the autoclave. Under neutral and acid conditions, the inhibitory activity remained substantially the same after heating at 60°, slight loss occurred at 100°, and complete loss occurred after autoclaving. In alkaline solution the activity was rapidly destroyed by any form of heating; gradual loss occurred at room temperature (about 18°) and at 4°, or after long periods at −40°. Care was therefore necessary when neutralizing crude material. Under acid conditions, the antibiotic activity was stable at low temperatures.

Dialysis. Crude antibiotic material from heated cultures was placed in cellophan bags made from washed Visking tubing. These were immersed in an equal volume of water or saline and kept at 4°. Material from inside and outside some of the bags was assayed each day for several days, but no evidence of diffusion was observed. However, when unheated concentrated trichloroacetic acid preparations were similarly tested, slow passage of the antibiotic through the cellophan was found.

Susceptibility to enzyme digestion. Equal amounts of sterile enzyme solutions (1 %, w/v, in physiological saline) were added to concentrated antibiotic material in tubes, and adjusted to pH 3·0 for pepsin (British Drug Houses 1:2500) and pH 8·0 for trypsin (Difco 1:250). The tubes were incubated at 37° in a water bath for 1 hr., the solutions neutralized and assayed in cups for activity against corynebacteria. Controls similarly tested included the same antibiotic material (*a*) with the addition of saline instead of enzymes, (*b*) with the addition of heat-denatured enzymes, (*c*) without pH adjustment. The results showed that inhibitory activity was completely destroyed in 1 hr. by trypsin, but was only slightly decreased by pepsin.

Antibiotic activity in mixed broth cultures

Although inhibitory activity against corynebacteria was readily detected in cup assays of type 71 digest broth preparations, little was found against DI− staphylococci. Dilution assays were therefore used to try to show such activity. With crude antibiotic preparations, the growth of the corynebacteria was completely suppressed, no matter how large the inoculum, but difficulties were experienced with staphylococci because of inhibition of some type 71 control inocula in the antibiotic material, and the frequent failure of small staphylococcal inocula to grow in normal broth. Some satisfactory results were, however, obtained (Table 1), indicating partial inhibition of DI− staphylococci after overnight culture, but growth usually occurred on further incubation.

Because of these difficulties with dilution assays, mixed broth cultures were examined for inhibitory activity against DI− staphylococci. For this purpose, a DI− strain of *Staphylococcus aureus*, which produced characteristic colonies readily distinguishable from type 71 staphylococci, was used. Colony counts of each organism, grown alone as well as together in digest broths, were made by the method of Miles & Misra (1938) on nutrient agar medium. An average of two samples

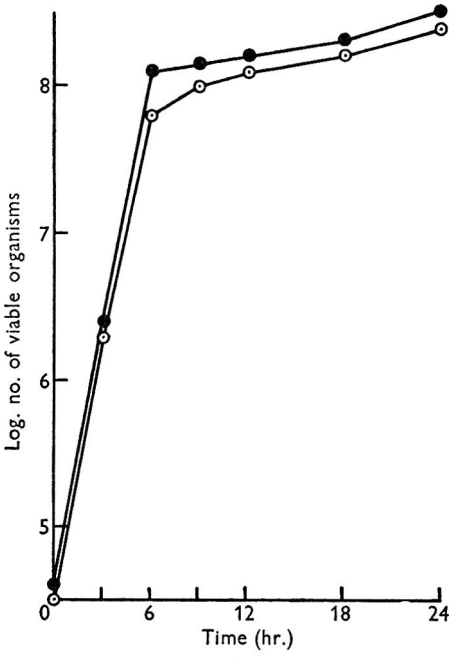

Fig. 1

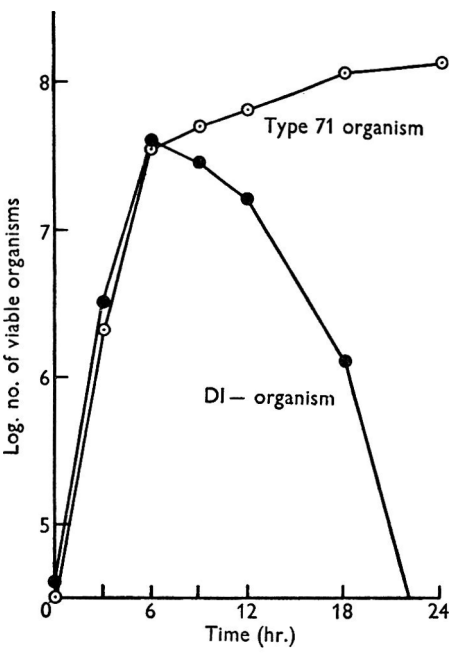

Fig. 2

Fig. 1. Growth of control cultures of non-inhibitory (DI−) and of active (DI+) type 71 strains of *Staphylococcus aureus* alone in digest broth. Inoculum 0·02 ml. × 10^{-1} of an overnight broth culture. —⊙—, Type 71 organisms; —●—, DI− organisms.
Fig. 2. Growth of non-inhibitory (DI−) and active (DI+) type 71 *Staphylococcus aureus* strains together in digest broth. Inoculum 0·02 ml. × 10^{-1} of an overnight broth culture of each strain. —⊙—, Type 71 organisms; —●— DI− organisms.

Table 1. *Inhibition of growth of DI− staphylococci in bacteria-free type 71 antibiotic material by the dilution assay technique*

Material dispensed in 1·0 ml. amounts	*Staph. aureus* inoculated	Period of incubation (hr.)	Growth from 0·02 ml. inocula of broth culture dilutions 10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Normal broth	Type 71	24	++++	++++	++++	++++	++++	++++
		48	++++	++++	++++	++++	++++	++++
	DI−	24	++++	++++	++++	++++	++++	++++
		48	++++	++++	++++	++++	++++	++++
Type 71 culture supernatant	Type 71	24	++++	++++	+++	+++	++	+
		48	++++	++++	++++	++++	++++	+++
	DI−	24	++	−	−	−	−	−
		48	++++	+++	++	+	±	−
DI− culture supernatant	Type 71	24	++++	++++	++++	++++	+++	++
		48	++++	++++	++++	++++	++++	++++
	DI−	24	++++	++++	++++	++++	+++	++
		48	++++	++++	++++	++++	++++	++++

++++, +++, ++, +, ± = arbitrary visual assessment of growth; − = no visible growth.

was taken for estimation of the numbers of viable organisms. Samples were withdrawn at 3 hr. intervals, with as little disturbance of the cultures as possible. Duplicate counts, determined by inhibition of corynebacteria seeded on blood agar medium, were substantially similar.

The results obtained, starting with approximately equal inocula, are shown as growth curves in Figs. 1 and 2. In the mixed culture, each organism multiplied normally during the log phase of growth. Thereafter the numbers of surviving DI – staphylococci decreased rapidly as compared with the control culture, whereas growth of the type 71 organisms was unimpaired. In further experiments with different proportions of organisms in the inoculum, the growth of the DI – staphylococci was largely suppressed when they were outnumbered initially by type 71 organisms in a ratio of more than 10:1. Conversely, when DI – organisms were in similar initial excess, their growth was not suppressed by type 71 staphylococci. In no instance was growth of type 71 organisms impaired.

DISCUSSION

In the present work, different liquid media were found, after growth of type 71 staphylococci, to vary considerably in their ability to yield preparations active in cup assays against indicator corynebacteria. Broth freshly prepared by tryptic digestion of meat was usually satisfactory for this purpose, although deterioration occurred on storage. The inhibitory agent was not, however, extracted from this broth. It remained in solution after the addition of trichloroacetic acid to broth cultures, and some of its properties were investigated after concentrating this material by evaporation. It was stable and relatively heat resistant under acid conditions, inactivated by trypsin but not by pepsin, was readily destroyed when alkaline, and was slowly dialysable. Resistance to heat and rapid passage of the inhibitory agent through similar Visking cellophan was also observed with type 71 cultures on solid media (Barrow, 1963). These properties suggest that it may be a small protein or a polypeptide (Waksman, 1947).

Although inhibitory to corynebacteria, the concentrated broth preparations showed only doubtful activity in cup assays against other staphylococcal strains. Inhibitory activity by type 71 strains, however, was detected in mixed broth cultures against non-inhibitory staphylococci, and was similar to that previously observed on solid media (Barrow, 1963). The type 71 inhibitory substance differs in activity from the agents produced by the two antagonistic NCTC 'aureus' strains; one of these was investigated by Gardner (1949) and the inhibitory agent thought to be a protein. These organisms were used for comparison in the present work, but active bacteria-free preparations were not obtained by the methods described for type 71 organisms. Similar antibiotic substances, thought to be proteins or polypeptides, were obtained in a crude state from staphylococcal cultures by Loeb, Moyer & Murray (1950) and by Halbert, Swick & Sonn (1953). Many of these agents differed only in their heat stability or in their specificity of action, and were thus analogous to the colicines produced by enterobacteria (Fredericq, 1957). Numerous bacterial species are now known to produce active metabolites of this kind, but their role in the initiation or in the severity of infections is still uncertain.

I am grateful to Dr H. G. Smith for advice and encouragement during this work.

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The Requirement of Fatty Acids by *Pityrosporum ovale*

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SUMMARY

Fatty acids are required for the growth of *Pityrosporum ovale* (Bizzozero) Castellani et Chalmers; myristate or palmitate satisfies this requirement. Oleate increases the crop of organism in a medium containing limiting concentrations of myristate or palmitate. When [1-^{14}C]-myristate was added to the medium the cells of *P. ovale* contained myristate, palmitate, stearate, oleate and linoleate with approximately the same molar specific radioactivity as myristate. Thus *P. ovale* can synthesize both saturated and unsaturated fatty acids of higher molecular weight from myristate.

INTRODUCTION

Pityrosporum ovale does not grow in conventional complex media without added fats. Ota & Huang (1933) supplemented their medium with butter or lecithin to obtain growth of this organism. Benham (1939) found that ether extracts of lanolin or butter supported good growth of *P. ovale*, and later (1941) showed that the organism would not grow in a defined medium without added fat; addition to the medium of 'oleic acid' of undefined purity permitted growth.

METHODS

Organism. The strain of *Pityrosporum ovale* (Bizzozero) Castellani et Chalmers used in our work was obtained from the Centraalbureau voor Schimmelcultures (Delft); it had been originally isolated by Benham (New York) from scales of human scalp. The description and historical survey of this yeast were given by Lodder & Kreger van Rij (1952).

Cultivation. The stock culture of *Pityrosporum ovale* was maintained on slopes of the following composition (all quantities/100 ml.): yeast autolysate (Albimi), 1 g.; Proteose-Peptone (Difco), 1 g.; agar (Difco), 2 g.; oleic acid (C.P., Fisher Sci. Co.), 0·1 g., and D-glucose, 1 g. A defined medium (Table 1) was used to determine the requirement for fatty acids. The medium was adjusted to pH 6·5, and 5·0 ml. portions were dispensed in 25 ml. micro-Fernbach flasks. The flasks were inoculated with 0·05 ml. of a suspension in saline (optical density, see below, about 0·5) of cells from a slant. The cultures were incubated in a humidified chamber at 37° (Hutner, Cury & Baker, 1958). After incubation for 6 days the optical density of the culture was measured with a spectrophotometer (Bausch & Lomb Spectronic 20) at 640 mμ.

Fatty acids. Caproic, caprylic, capric, myristic, palmitic, stearic, oleic, linoleic, and linolenic acids were purchased from California Corporation for Biochemical Research (Los Angeles); lauric acid was obtained from the Eastman Kodak Co.

(Rochester); all were estimated > 95% pure by gas-liquid chromatography. Oleic acid, C.P. (referred to in this paper as crude oleic acid) was bought from the Fisher Scientific Co. (Chicago). Radioactive myristic and stearic acids were bought from New England Nuclear (Boston).

Table 1. *Basal medium for the growth of* Pityrosporum ovale

Compound	(g./100 ml.)	Compound	(μg./100 ml.)
$NH_4H_2PO_4$	0·2	H_3BO_3	50
$MgSO_4.7H_2O$	0·05	$CuSO_4.5H_2O$	4
KH_2PO_4	0·1	KI	10
L-Asparagine	0·25	$FeCl_3.6H_2O$	20
D-Glucose	1·0	$MnSO_4.H_2O$	40
		$(NH_4)_2MoO_4.2H_2O$	20
		$ZnSO_4.2H_2O$	40

Recovery of fatty acids from cells of Pityrosporum ovale. The organism was grown in 2·5 l. Fernbach flasks containing 500 ml. basal medium supplemented with appropriate fatty acids. After incubation for 2 weeks the organisms were harvested by centrifugation, washed by centrifugation three times from 0·01 M-$NaHCO_3$ and three times from distilled water, and were then lyophilized. The powder was resuspended in 2 N-HCl and hydrolysed by heating in a sealed glass ampoule for 1 hr. at 120°. The fatty acids were extracted twice with two volumes of diethyl ether, the pooled extract washed with water and dried with anhydrous Na_2SO_4.

Esterification. Fatty acids were esterified at 65° for 1 hr. in 5 ml. anhydrous methanol containing 0·1 N-HCl. The methanol was evaporated with a stream of nitrogen to about 1 ml., and the solution diluted with 2–3 ml. water. The methyl esters were extracted twice with 5 ml. portions of light petroleum (b.p. 60–70°), the pooled extracts dried with anhydrous Na_2SO_4, the solvent evaporated and the residue dissolved in benzene for analysis by gas-liquid chromatography.

Gas-liquid chromatography. The methyl esters of fatty acids were separated by chromatography at 189° on an 8 ft × 0·25 in. column of 25% diethyleneglycol-succinate polyester on firebrick (Wilkins Instruments, Walnut Creek, California) with helium as carrier gas. The effluent was monitored with a 4-filament katharometer, the electrical output of which was recorded with a 1 mV. recorder. The amount of each ester was estimated from the area under the recorded peak. The esters were identified by comparison of their retention volumes with the retention volumes of known methyl esters. The identity of esters of unsaturated fatty acids was confirmed by catalytic hydrogenation; the esters were hydrogenated in anhydrous methanol with a catalyst of 5% Pt on charcoal (Matheson, Coleman and Bell Co., Norwood, Ohio) at room temperature and at one atmosphere pressure of H_2. Individual esters were recovered by passing the appropriate portion of the effluent gas through glass wool moistened with toluene.

Assay of radioactivity. Radioactivity was estimated by scintillation counting. Samples were dissolved in 15 ml. counting fluid (toluene, 380 ml.; absolute ethanol, 250 ml.; dioxane, 380 ml.; naphthalene, 50 g.; 2,5-diphenyloxazole, 5 g.; 1,4-bis-2-(5-phenyloxazolyl)-benzene, 0·1 g.) and placed in 25 ml. glass vials. The samples were counted with a scintillation spectrometer (Packard Instrument Co., La Grange, Illinois).

Peroxidation of oleic acid. Oleic acid was oxidized to dihydroxystearic acid with performic acid according to the method of Swern, Billen, Findley & Scanlan (1945). A sample containing 100 mg. or less of oleic acid was allowed to react with 1 ml. formic acid and 0·1 ml. of 30 % H_2O_2 at 40° for 2 hr. with occasional shaking. The reaction mixture was diluted with 5 ml. water, extracted three times with 5 ml. portions of diethyl ether, the extracts pooled and the ether evaporated off. The product (hydroxyformoxystearic acid) was saponified in 2 ml. 3 N-NaOH at 100° for 30 min. After the alkaline solution had cooled, it was acidified with 4 ml. 3 N-HCl and extracted three times with 5 ml. portions of diethyl ether. The ether extract contained dihydroxystearic acid which was identified by a comparison of the infrared spectrum of the product with the spectrum of authentic 9,10-dihydroxystearic acid.

Chromatographic separation of dihydroxystearic acid. In the experiments designed to determine the conversion of radioactive stearic acid to oleic acid it was necessary to separate oleic acid from the radioactive stearic acid. Resolution of methyl stearate and methyl oleate by gas-liquid chromatography was inadequate. The problem was solved by the prior conversion of oleic acid to dihydroxystearic acid, followed by the separation of dihydroxystearic acid from the non-polar fatty acids by partition chromatography. A sample of the mixed fatty acids was peroxidized and the products were transferred to a 3 g. column of silicic acid (Mallinkrodt). The non-polar fatty acids were eluted with 30 ml. 10 % (v/v) diethyl ether in light petroleum. The column was washed with 30 ml. diethyl ether to remove residual non-polar acids. The dihydroxy acid was eluted with 30 ml. methanol.

The efficiency of the method was tested by separating a mixture of 50 mg. each of stearic and oleic acid, to which mixture was added a small amount of [1-^{14}C]-stearic acid. The mixture was peroxidized and chromatographed. The first fraction (stearic acid) contained 526 c.p.m.; the second fraction contained 5 c.p.m., and the third fraction (dihydroxystearic acid) 5 c.p.m.

RESULTS

Response to oleate

Pityrosporum ovale did not grow in the basal medium supplemented with pure oleic acid but did grow in basal medium supplemented with crude oleic acid or with catalytically hydrogenated crude oleic acid.

Table 2. *Fatty acid composition of crude oleic acid*

Fatty acid	% (w/w)	Fatty acid	% (w/w)
Myristic	3·61	Hexadecenoic	13·20
Tetradecenoic	2·73	Oleic	75·48
Palmitic	4·98	Linoleic	Trace

The composition of a sample of crude oleic acid was determined by gas-liquid chromatography (Table 2); oleic acid was the major constitutent and the sample also contained considerable amounts of myristic and palmitic acids and the corresponding mono-unsaturated fatty acids, tetradecenoic and hexadecenoic acids.

Response to saturated fatty acids

Since the response to crude oleate was not affected by hydrogenation, it appeared that the saturated fatty acids, myristic and palmitic, were responsible for the growth of *Pityrosporum ovale* in basal medium supplemented with crude oleic acid. This possibility of a requirement for saturated fatty acids was tested by addition to the basal medium of single saturated fatty acids with an even number of carbon atoms (Table 3). Saturated fatty acids from C_4 to C_{10} and stearic acid did not permit growth; lauric acid (C_{12}) permitted some growth; myristic (C_{14}) and palmitic (C_{16}) gave good growth.

Table 3. *Effect of additions of single fatty acids to basal medium on the growth of* Pityrosporum ovale

	Concentration of added fatty acid (mg./100 ml.)		
	1	5	10
Fatty acid added	Optical density reading (640 mμ)		
Butyric	0	0	0
Caproic	0	0	0
Caprylic	0	0	0
Capric	0	0	0
Lauric	0·04	0·12	0·15
Myristic	0·12	0·31	0·40
Palmitic	0·15	0·30	0·40
Stearic	0·03	0·05	0·08

Sparing effect of oleic acid

The efficacy of palmitic and myristic acids in permitting growth of *Pityrosporum ovale* when added to the basal medium explained in part the response of the organism to the crude oleic acid, which contained both myristic and palmitic acids. However, the response to the crude oleic acid was greater than expected from its content of myristic and palmitic acids. Various mixtures of myristic and palmitic acids added to the basal medium did not give greater than an additive increase in the yield of *P. ovale*. It appeared that oleic acid might be sparing the requirement for saturated fatty acids. Figure 1 shows the effect of adding pure oleic acid (5 mg./100 ml.) to basal medium containing different concentrations of myristic acid. Oleic acid significantly increased the crop of *P. ovale* in basal medium containing limiting concentrations of myristic acid. A similar increase in crop was observed when oleic acid was added to basal medium containing limiting concentrations of palmitic acid.

If stearic acid is an effective precursor of oleic acid, stearic acid, like oleic acid, should spare the requirement for myristic acid. Supplementation with stearic acid of a medium containing a limiting concentration of myristic acid gave equivocal results; in some tests stearic acid did not spare the requirement for myristic acid, while in other tests stearic acid increased the yield of organism. Linoleic acid did not spare the requirement for myristic acid.

Fatty acid composition of the lipids of Pityrosporum ovale

Pityrosporum ovale harvested from basal medium supplemented with myristic or palmitic acids was analysed for fatty acids by gas-liquid chromatography. The organisms grown in basal medium supplemented with myristic acid contained

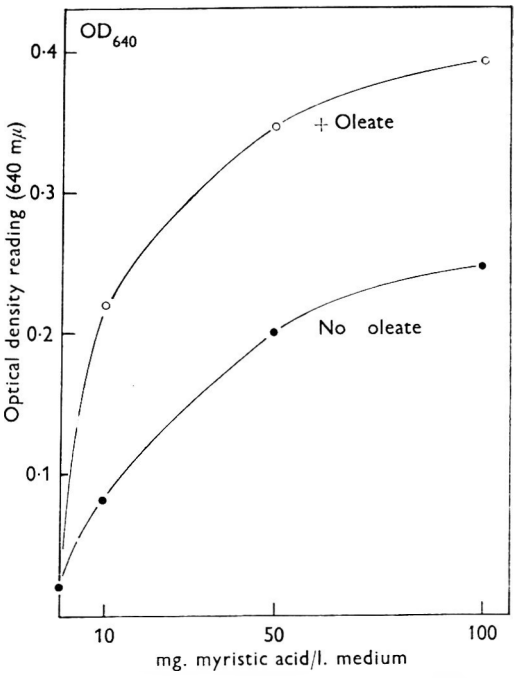

Fig. 1. The effect of oleic acid (5 mg./100 ml.) on growth of *Pityrosporum ovale* in basal medium containing different concentrations of myristic acid.

Table 4. *Composition of fatty acids from* Pityrosporum ovale *grown in basal medium supplemented with myristic acid or palmitic acid*

	Organism grown on basal medium + supplement	
	Myristate	Palmitate
Fatty acid from organism	Fatty acids from organisms (% by wt. of total fatty acids)	
Lauric	None	7·5
Myristic	6·7	None
Palmitic	15·7	73·5
Hexadecenoic	2·3	Trace
Stearic	7·3	1·7
Oleic	56·9	13·1
Linoleic	11·1	3·9

myristic, palmitic, hexadecenoic, stearic, octadecenoic (oleic), and octadecadienoic (linoleic) acids (Table 4), of which oleic acid was the principal one. The organisms grown in basal medium supplemented with palmitic acid differed in that they did

not contain myristic acid but did contain a considerable amount of lauric acid. Palmitic acid rather than oleic acid appeared to be the principal fatty acid in these organisms. The variation in proportion of different fatty acids from organisms grown with the same supplement was not sufficient to change the order of abundance of different fatty acids. Thus the change in composition of fatty acids from organisms grown with different supplements is significant.

Table 5. *Radioactivity of fatty acids of* Pityrosporum ovale *grown in basal medium supplemented with* [1-^{14}C] *myristic acid*

Fatty acid	c.p.m./μmole	Percentage of the specific activity of added myristic acid
Myristic	13,200	72
Palmitic and tetradecenoic	11,000	60
Stearic	15,600	85
Oleic	8,200	45
Linoleic	12,200	68

Synthesis of higher fatty acids from myristic acid

The results of the nutritional experiments together with the analyses of the organisms for fatty acids suggested that *Pityrosporum ovale* is unable to synthesize myristic acid or higher fatty acids. The possibility that higher fatty acids are synthesized from myristic acid was tested by using radioactive myristic acid. *P. ovale* was grown in basal medium supplemented with [1-^{14}C]-myristic acid and with sufficient acetate to dilute the label of any radioactive acetic acid produced from the metabolism of myristic acid. After hydrolysis of the organisms, the fatty acids were extracted, esterified, and separated by gas-liquid chromatography. The molar radioactivities of the fatty acids (Table 5) are computed on the assumption of complete recovery of each ester from the effluent. Since recovery was not complete the molar specific activities of all of the fatty acids except oleic acid must be considered as essentially the same as the molar specific activity of the [1-^{14}C]-myristic acid added to the basal medium. Perhaps oleic acid is formed by a separate route as well as from myristic acid. Thus, most if not all of the higher fatty acids are synthesized from myristic acid.

Conversion of stearic acid to oleic acid

Since stearic acid gave a slight but significant sparing of the requirement of *Pityrosporum ovale* for lower fatty acids it appeared likely that stearic acid could serve as a precursor of oleic acid as it does in Saccharomyces (Bloomfield & Bloch, 1960). This was tested by supplying a culture of *P. ovale* with radioactive stearic acid and measuring the radioactivity in oleic acid recovered from the lipids. *P. ovale* was grown in basal medium supplemented with myristic acid (5 mg./100 ml.) and 2 μc of [1-^{14}C]-stearic acid (0·74 mg./100 ml.). The organisms were harvested, washed, hydrolysed, and the fatty acids extracted from the hydrolysate. One

portion of the hydrolysate was esterified and the methyl esters analysed by gas-liquid chromatography (Table 6). Another portion was peroxidized, the non-polar fatty acids in the peroxidized sample separated from dihydroxystearic acid by chromatography on silicic acid and the radioactivities of the fractions eluted from the column were determined (Table 6). It was assumed that all of the radioactivity in the fraction containing non-polar acids was contained in the stearic acid.

The results in Table 6 show a significant difference in the composition of fatty acids when stearic acid was added to the medium (compare with the first column in Table 5). Stearic acid appeared to be the principal C_{18} fatty acid; oleic acid was no longer a predominant fatty acid. Another difference in the composition was the presence of two unidentified components not detected in previous analyses (compare with Table 4).

Table 6. *Composition and radioactivity of fatty acids from* Pityrosporum ovale *grown in basal medium* + [1-^{14}C]-*stearic acid*

Fatty acid in organism	Percentage by weight of total fatty acids	Radioactivity (c.p.m.)	Relative specific activity
Lauric	14·7	—	—
Myristic	23·1	—	—
Palmitic	15·7	—	—
Stearic	30·0	10,800	121
Oleic	3·7	2,800*	254
Linoleic	Trace	—	—
Unidentified I†	6·7	—	—
Unidentified II‡	6·1	—	—

* The sample of dihydroxystearic acid obtained by peroxidation was purified twice by chromatography before assay for radioactivity.

† Retention volume relative to methyl palmitate was 0·80.

‡ Retention volume relative to methyl palmitate was 1·32.

The results are in accord with a conversion of stearic acid to oleic acid. At the time of harvest the specific radioactivity of stearic acid was lower than the specific radioactivity of oleic acid. This may have resulted from a continuous dilution of the radioactive stearic acid resulting from synthesis of stearic acid from myristic acid during growth of the culture.

DISCUSSION

Our results do not confirm that *Pityrosporum ovale* requires added oleic acid for growth (Benham, 1941). Addition of oleic acid alone under our conditions did not permit growth in a defined medium; however, oleic acid did increase the crop of organisms in a medium containing limiting concentrations of either myristic or palmitic acid. Perhaps the results of Benham can be attributed to impurities of saturated fatty acids in the sample of oleic acid used, comparable to the impurities we found in a commercial sample of oleic acid. Both nutritional and tracer experiments suggest that the requirement for fatty acids in *P. ovale* results from an inability to synthesize myristic acid. When *P. ovale* was grown on basal medium with [1-^{14}C]-myristic acid, the myristic acid of the organisms had essentially the same specific radioactivity as the myristic acid added to the medium. Furthermore,

the higher fatty acids isolated from the organisms (palmitic, stearic, oleic, linoleic acids) also had nearly the same molar radioactivity as had the myristic acid. Thus, the higher fatty acids appear to have been synthesized by elongation of the carbon chain of myristic acid. Myristic acid itself is not an essential nutrient for *P. ovale*. Organisms grown in basal medium + palmitic acid did not contain detectable amounts of myristic acid. Organisms grown with palmitic acid contained rather large amounts of lauric acid which was not present in organisms grown with myristic acid.

Stearic acid either did not spare the requirement for lower molecular weight saturated fatty acids or was less effective than oleic acid in sparing this requirement. From these results it appeared unlikely that the oxidation of stearic acid was a major pathway contributing to the synthesis of oleic acid. However, the high specific radioactivity of oleic acid from organisms grown in a medium containing radioactive stearic acid is in agreement with the formation of oleic acid from the oxidation of stearic acid, a major pathway in Saccharomyces (Bloomfield & Bloch, 1960). The analysis of the fatty acids from *Pityrosporum ovale* grown in medium containing stearic acid + myristic acid showed an abnormally low content of oleic acid and the presence of substantial amounts of previously undetected fatty acids. This suggests an explanation for the failure of stearic acid to spare as effectively as did oleic acid the requirement for myristic acid. The supplementation of the medium with stearic acid apparently inhibited the synthesis of oleic acid and perhaps perturbed the synthesis of other fatty acids, possibly by interfering with the normal mechanism of control.

Pityrosporum ovale is so far unique among micro-organisms in its requirement of C_{14} or C_{16} saturated fatty acids, which requirement results from a block in the synthesis of myristic acid. All of the higher saturated fatty acids can be formed by chain-elongation, and oleic acid is formed, presumably, by oxidation of stearic acid. The inability of *P. ovale* to synthesize higher fatty acids from acetate permits a study of the conversion of the higher fatty acids without the complication of *de novo* synthesis.

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The Distribution of Teichoic Acids in Staphylococci

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SUMMARY

Wall and intracellular teichoic acids were prepared from several strains of staphylococci. Intracellular glycerol teichoic acids were found in all the cases examined but their detailed chemical composition was not determined. The presence and structure of wall teichoic acids is characteristic of the different species; thus, strains of *Staphylococcus aureus* all possess a teichoic acid containing glucosamine in their walls; this was shown previously to be indistinguishable from the group-specific antigen. Similarly, *S. saprophyticus* strains contain in their walls a glycerol teichoic acid with glucosyl substituents probably identical to the group-specific precipitinogen, polysaccharide B. The species *S. lactis* is heterogeneous: three groups are distinguishable, one without wall teichoic acid and the others with a teichoic acid containing glucosamine or galactosamine.

INTRODUCTION

The teichoic acids are widely distributed in Gram-positive bacteria (Armstrong *et al.* 1958, 1959; Baddiley, 1961). An investigation of these glycerol phosphate or ribitol phosphate polymers containing alanine ester and sugar residues in Lactobacillus species indicated that those in the cell walls are of taxonomic significance (Baddiley & Davison, 1961). Moreover, the presence and structure of teichoic acids have been correlated with the serological behaviour of lactobacilli, and it has been shown that these compounds are indistinguishable from the group antigens in many cases (Baddiley & Davison, 1961; and unpublished work with Dr M. Elizabeth Sharpe). Similarly, in group D streptococci the group antigen is serologically identical with the intracellular teichoic acid, and differences in the serological behaviour of group D antigen preparations from several strains of organism have been attributed to small differences in their chemical structure (Wicken, Elliott & Baddiley, 1963).

In staphylococci the wall teichoic acids have also been associated with serological reactions. That in the walls of *Staphylococcus aureus* strain H is a ribitol phosphate polymer to which are attached D-alanine ester and *N*-acetylglucosaminyl residues, the latter being in predominantly β linkage (Baddiley, Buchanan, RajBhandary & Sanderson, 1962*a*; Baddiley, Buchanan, Martin & RajBhandary, 1962*b*). This teichoic acid is serologically identical with the group-specific polysaccharide A of this organism (Haukenes, Ellwood, Baddiley & Oeding, 1961; Haukenes, 1962). *S. aureus* strain Copenhagen possesses a chemically similar wall teichoic acid which contains a higher proportion of α glycosidic linkages, and serological differences between these compounds may be related to the chemical differences (Natherson & Strominger, 1962). Although serological specificity is associated with the glycerol

teichoic acid from the walls of *S. lactis* (*S. albus*) NCTC 7944 (Haukenes *et al.* 1961), in which the sugar is *N*-acetylgalactosamine (Ellwood, Kelemen & Baddiley, 1963), no teichoic acid has been found in certain other staphylococci, e.g. *S. afermentans* (*Micrococcus lysodeikticus*) and *S. roseus* (Salton & Pavlik, 1960; Armstrong, unpublished work). The present work was undertaken in an attempt to correlate the taxonomy of staphylococci with the presence and composition of teichoic acids in their walls, and as a preliminary study connected with their serological classification.

METHODS

The sources of bacteria, other than those from the National Collection, are given in Table 1. Freshly isolated strains were classified according to the method of Shaw, Stitt & Cowan (1951), and strains of *Staphylococcus aureus* were classified by the bacteriophage-type procedure in the Department of Bacteriology, Royal Victoria Infirmary, Newcastle upon Tyne.

Bacteria were grown in batches (15 l.) with forced aeration at 37° for 16 hr. in a liquid medium of the following composition: nutrient broth no. 2 (Oxoid), 25 g.; glucose, 10 g.; yeast extract (Oxoid), 5 g.; dipotassium hydrogen phosphate, 3 g.; olive oil, 0·3 ml.; water (demineralized), 1000 ml. Cocci were harvested in a Sharples refrigerated centrifuge and washed with cold 0·85% (w/v) sodium chloride solution. Strains 15379, 15499 and 17261, which had been isolated from patients with staphylococcal infections, were grown in screw-capped bottles on solid medium of the same composition + agar for 16 hr. at 37°, killed with formol-saline and the cocci collected in a closed refrigerated centrifuge.

Coccal walls were prepared by mechanical disruption with glass beads (Dawson, 1949; Salton & Horne, 1951); cocci were suspended in cold water (18–20 g. wet wt. in 100 ml. water) and shaken with no. 11 Ballotini beads in an International centrifuge with shaker head (Shockmann, Kolb & Toennies, 1957) at 1250 rev./min. for 45 min. at −8°. Beads were removed by filtering with a no. 1 sintered glass funnel, and the filtrate was centrifuged at 25,000*g* for 30 min. The cloudy supernatant fluid was separated from sedimented walls and a few unbroken cocci, and then centrifuged at 100,000*g* in a Spinco model L ultracentrifuge for 1 hr. The sedimented gel contained ribosomal material and intracellular teichoic acid; it was preserved by freeze-drying. The wall fraction was washed by centrifugation six times with 5 vol. of cold M-phosphate buffer (pH 7·0) and six times with cold water; material was recovered each time at 25,000*g* for 30 min., the wall fraction being carefully separated from any lower layer of unbroken cocci. Walls recovered by freeze-drying were homogeneous and of clean appearance under the electron microscope.

Teichoic acid was extracted from walls (0·5 g.) with 10% (w/v) trichloroacetic acid (30 ml.) for 4 days at 2°. After centrifugation, the material was precipitated from the solution with 5 vol. cold ethanol, collected after 24 hr. at 2°, redissolved in 10% trichloroacetic acid solution and precipitated as before. The precipitate was washed with acetone, ethanol and ether, then dried *in vacuo* (yield 10–15% of the dry wt. of the walls.) A similar isolation procedure was adopted for the teichoic acid in the ribosomal gel (cf. Baddiley & Davison, 1961).

The chemical composition of teichoic acids was determined by hydrolysis with 2N-hydrochloric acid or 2N-sodium hydroxide solution for 3 hr. at 100° and exami-

nation of hydrolysates by several paper-chromatographic procedures described in detail by Armstrong *et al.* (1958, 1959), Baddiley *et al.* (1962*a*) and Ellwood *et al.* (1963); sugars and amino sugars were separated using a mixture of pyridine + ethyl acetate + aq. acetic acid (Fischer & Nebel, 1956). Glucosylglycerol was characterized by hydrolysis with a β-glucosidase (see Armstrong *et al.* 1959), glucosaminylribitol by dephosphorylation with intestinal phosphomonoesterase and chromatographic comparison of the product with authentic glucosaminylribitol (Baddiley *et al.* 1962, *a*, *b*), and the saccharinic acid by its similarity on paper chromatograms with that obtained by heating 3-*O*-methylglucose (provided by D. A. Applegarth) in alkali under comparable conditions. Alanine ester residues were detected by reaction with aqueous ammonia and chromatography of the resulting alanine and its amide.

RESULTS

The composition of teichoic acids from walls and cell contents of different staphylococci is given in Table 1. Intracellular teichoic acids were examined with respect to glycerol and its phosphates but not sugars. The hospital strains of *Staphylococcus aureus* were not examined for the presence of intracellular compounds and alkali hydrolysis of their wall teichoic acids was not attempted because of shortage of material.

Table 1. *Intracellular and wall teichoic acids in staphylococci*

		Teichoic acid		Composition of wall teichoic acid	
	Origin, phage type, group	Intracellular	Wall	Sugar	Hydrolysis product
Staphylococcus aureus					
H	Dr J. T. Park, 52/52A/79/8C, group 1	G	R	Glucosamine	Glucosaminylribitol
I 5379	Hospital isolate, 3B/55/71, group 2	—	R	Glucosamine	—
I 5499	Hospital isolate, 75/77, group 3	—	R	Glucosamine	—
A 1	Animal strain, 42D, group 4	G	R	Glucosamine	Glucosaminylribitol
Staphylococcus saprophyticus					
NCTC 7292	—	G	R	Glucosamine	Glucosaminylribitol
NCTC 7291	—	G	G	Glucose	Glucosylglycerol
C 1	Professor M. R. J. Salton (received as *S. citreus*)	G	G	Glucose	Glucosylglycerol
I 2	Fresh isolate from animal gland	G	G	Glucose	Glucosylglycerol
I 7261	Fresh isolate from hospital (pathogenic)	G	G	Glucose	—
NCTC 7617	—	G	G*	Galactosamine	Galactosaminylglycerol
I 3	Fresh isolate from animal gland	G	G†	Glucosamine	Saccharinic acid
I 4	Fresh isolate from milk	G	G	Galactosamine	Galactosaminylglycerol
NCTC 7564	—	G	—	—	—
NCTC 7567	—	G	—	—	—

* No alanine detected; † no glycerol on acid or alkali hydrolysis; G = glycerol teichoic acid; R = ribitol teichoic acid.

DISCUSSION

The taxonomic and serological significance of the intracellular teichoic acids in staphylococci is not known. Like all others found in underlying regions of bacterial cells, they are glycerol phosphate polymeric derivatives, but in only one case has detailed structural work been carried out; that from *Staphylococcus aureus* strain H is a 1,3-polymer of glycerol phosphate with D-alanine ester residues at position 2 on most glycerol units and a small number of gentiobiosyl (6-*O*-β-D-glucopyranosyl-D-glucosyl) and *N*-acetylglucosaminyl residues situated at position 2 (RajBhandary & Baddiley, 1963). All the coagulase-positive and coagulase-negative staphylococci contained intracellular teichoic acid, again illustrating the widespread occurrence of these compounds in Gram-positive bacteria (Baddiley & Davison, 1961; Baddiley, 1961). All accompanied the ribosomal RNA on centrifugation at 100,000*g*, a property which has been utilized in the isolation of these substances from many different bacteria (Baddiley & Davison, 1961; Critchley *et al.* 1962; Wicken & Baddiley, 1963).

The composition of teichoic acids in the walls of staphylococci may be used in the classification of these bacteria, a problem which often presents difficulty (see Hill, 1959). Shaw *et al.* (1951) assigned all coagulase-positive members to the species *Staphylococcus aureus*, but separated fermentative coagulase-negative members (*S. albus*) into two species *S. saprophyticus* and *S. lactis*, according to their behaviour in the Voges-Proskauer test. The composition of wall teichoic acids in staphylococci (see Table 1) supports this general classification into three species, but indicates that the species *S. lactis* is heterogeneous. Yellow staphylococci (micrococci) of Gibson's groups 3A and 3B (Abd-el-Malek & Gibson, 1948) which contain no teichoic acid in their walls could be distinguished from those strains of *S. lactis* with a wall teichoic acid. Moreover, two distinct types of *S. lactis* containing wall teichoic acids were observed: strains isolated from milk (14, NCTC 7617) had a glycerol teichoic acid containing galactosamine, whereas a strain from an infected animal gland, which probably corresponded to *Gaffkya tetragena*, had a glycerol teichoic acid containing glucosamine. This last teichoic acid probably differs structurally from the others, since it yields a compound ressembling a saccharinic acid on alkali hydrolysis and no glycerol is produced under these conditions.

Strains of *Staphylococcus saprophyticus* were clearly distinguishable from those of *S. aureus* and *S. lactis* since they possess a glycerol wall teichoic acid containing glucose. The chemical similarity between this teichoic acid and an unidentified phosphoric ester containing glucose (polysaccharide B) isolated by Wieghard & Julianelle (1935) from a strain of *S. albus* suggests that the group-specific precipitinogen, polysaccharide B, is the wall teichoic acid; serological studies are in progress to examine this point. In this connexion, Morse (1962) has differentiated the wall teichoic acids of *S. aureus* and *S. albus* by haemagglutination, and has shown that the wall teichoic acid from a strain of *S. albus* is a glycerol phosphate polymer containing glucose.

The type strain of *Staphylococcus saprophyticus* (NCTC 7292) proposed by Shaw *et al.* (1951) possesses a ribitol teichoic acid containing glucosamine, apparently identical or very similar to that from the walls of species of *S. aureus* representing the four main bacteriophage groups. We conclude that strain 7292 is atypical.

whereas the freshly isolated pathogenic strain of *S. saprophyticus* (17261) contained the characteristic glycerol wall teichoic acid with glucosyl substituents as found in other members of the species. Thus, knowledge, of the composition of wall teichoic acids in staphylococci is valuable for the accurate classification of this important group of organisms.

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The Role of Interferon in Persistent Infection with Foot-and-Mouth Disease Virus

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SUMMARY

Calf kidney cell cultures persistently infected with foot-and-mouth disease virus (FMDV) resist challenge with related and unrelated viruses. Attachment of challenge virus to persistently infected cells is not impaired. A challenge with viral ribonucleic acid (RNA) will not overcome the resistance of persistently infected cells. The initiation of persistence is correlated with the amount of interferon produced in the cells. It is concluded that interferon plays a major role in initiation and maintenance of the carrier state of the cells.

INTRODUCTION

Interferon, a soluble product of virus-infected cells capable of interfering with replication of a wide range of viruses (Isaacs, 1961), plays a role in the initiation and maintenance of chronic virus infections *in vitro* (Ho & Enders, 1959; Henle, Henle, Deinhardt & Bergs, 1959; Chany, 1961; Glasgow & Habel, 1962). Baron & Isaacs (1961) recently reviewed the evidence, which suggests that interferon may be important in natural recovery from viral infections. Foot-and-mouth disease virus (FMDV), which induces marked degeneration in calf kidney cells, gives rise to a persistent infection when the early cycles of multiplication are suppressed by the presence of viral antibodies (Dinter, Philipson & Wesslén, 1959). The antibodies can subsequently be removed and most cells will remain intact. The cultures produce active virus for at least 20 days. Prolonged treatment with viral antibodies cures the cultures from the carrier state (Dinter *et al.* 1959). An interferon-like inhibitor from calf kidney cells infected with FMDV was recently described (Dinter & Philipson, 1962). This formed the basis for investigating the role of interferon in the initiation and maintenance of the carrier state in cells persistently infected with FMDV. The present study demonstrates the resistance of such cells to related and unrelated viruses and the role of interferon in this process.

METHODS

Viruses. The Lindholm O_2-strain of FMDV (FMDV O_2), the Brussels A_5 strain of FMDV (FMDV A_5), a strain of parainfluenza virus type 3 (PI-3), a virulent strain of poliovirus type 1 (E 206) and the Z strain of pseudo-rabies virus were used. Poliovirus stocks were prepared in monkey kidney cultures, the others in calf kidney cultures. FMDV and poliovirus were stored at $+4°$ and the other viruses at $-60°$.

Cell cultures and virus assay. The preparation of cell cultures, the plaque technique,

and the methods for virus assay have been described previously (Dinter & Philipson, 1962). Virus infectivity is expressed as the logarithm of the 50% endpoint (log TCD50)/ml. when tube titrations were used or as plaque-forming units (p.f.u.)/ml. when plaque assay was performed. When two viruses were present in samples to be tested, one of them was neutralized by antiserum before assay. Hemagglutinating activity of PI-3 virus was expressed as the $\log_2$ of the reciprocal of the greatest dilution of the sample giving complete agglutination in tube tests.

Persistently infected cultures. Flask cultures of calf kidney were exposed to about 10^4 TCD50 of FMDV O_2. After incubation for 90 min. at 37°, 50 ml. of Hanks's salt solution + 0·5% (w/v) lactalbumin hydrolysate and antibiotics (H + Lah) + 0·5% (v/v) immune serum were added. After incubation for about 42 hr. at 37° the medium was changed to H + Lah + 2% (v/v) normal calf serum. After a further 18 hr. at 37° the cells were trypsinized and subcultured in tubes or Petri dishes. H + Lah + 2% (v/v) normal calf serum was used as medium for these cultures. Control cultures from the same calf kidneys were treated the same way in each experiment except for virus inoculation and are designated normal cultures.

Cured cultures. Antiviral serum at a final concentration of 1% (v/v) was added to the medium of persistently infected cells in Petri dishes. The cultures were then incubated for a period of 6 days and the medium containing antiserum was replaced by H + Lah + 2% (v/v) normal calf serum. On each of the following 3 days the medium of these cultures was assayed for virus infectivity. If no virus was found the cultures were considered 'cured'.

Antiserum. FMDV antiserum was obtained from cattle and guinea pigs hyperimmunized against the respective virus.

Production and assay of interferon. Interferon was produced in calf kidney cells and assayed as previously described (Dinter & Philipson, 1962). Interferon titre was expressed as the reciprocal of the final dilution resisting a challenge of 100 TCD50 of pseudo-rabies virus.

Extraction of viral RNA. Viruses were concentrated by aqueous polymer phase systems (Philipson, Albertsson & Frick, 1960) and treated with phenol saturated with 0·02M-phosphate buffer (pH 7·4) containing 10^{-4}M-ethylenediaminetetraacetate (EDTA) at 4° according to Gierer & Schramm (1956). The water phase was collected and shaken six times with ether, which was subsequently removed by nitrogen. This material was assayed for infectivity with and without treatment for 10 min. at 37° with ribonuclease (recrystallized three times, Worthington Inc., New Jersey, U.S.A.) at a final concentration of 10 μg./ml.

Assay of viral RNA. The nucleic acid preparations were diluted and adsorbed for 10 min. at 18° in different buffers and tested for infectivity by the plaque method. Dilution and adsorption in 2M-$MgSO_4$ according to Holland, Hoyer, McLaren & Syverton (1960) gave 20-fold higher titres than other methods tested (Table 1). The nucleic acid infectivity was completely inactivated by treatment with ribonuclease which did not affect the titre of intact virus. The procedure adopted therefore included both dilution of the virus-RNA preparation and adsorption for 10 min. at 18° in 2M-$MgSO_4$. Cultures were subsequently washed twice with phosphate buffered saline (PBS) before application of the agar overlay.

Staining with fluorescent antibody. The indirect method was used. Coverslips with a uniform cell sheet were washed three times in PBS, fixed in acetone for 5 min. and

dried in air. Viral antiserum was subsequently applied for 30 min. at room temperature, the slides washed three times in PBS and overlayed with goat antirabbit globulin conjugated with fluorescein isothiocyanate (Microbiological Associates, Bethesda, Maryland, U.S.A.). The sera were adsorbed with acetone powder from calf kidney.

Table 1. *Infectivity of foot-and-mouth disease virus (FMDV) RNA under different experimental conditions*

	p.f.u./ml.	
Diluent and adsorption medium	RNA	Intact virus
2M-$MgSO_4$	$1{\cdot}9 \times 10^5$	n.d.
0·9M-KCl 0·05M-Tris (pH 8·0)	9×10^3	n.d.
PBS	$3{\cdot}5 \times 10^3$	$1{\cdot}3 \times 10^{10}$

n.d., Not done; PBS, phosphate buffered saline.

RESULTS

Resistance of persistently infected cultures to different viruses. The resistance of cells persistently infected with FMDV O_2 to a challenge with related and unrelated viruses was investigated by following virus multiplication in persistently infected cultures of calf kidney, in such cultures cured by excess antibody, and in normal

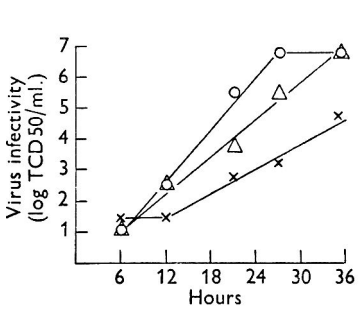

Fig. 1

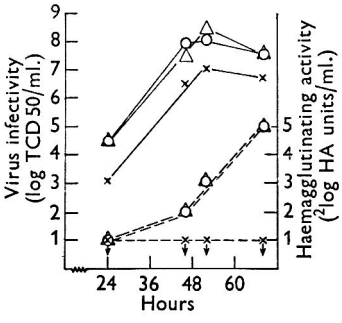

Fig. 2

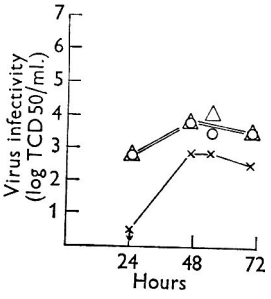

Fig. 3

Fig. 1. Growth curves for foot-and-mouth disease virus (FMDV) type A_5 in calf kidney cultures; ×, persistently infected with FMDV type O_2; △, persistently infected with FMDV type O_2 and subsequently cured with excess anti-O_2 antibody; ○, normal cultures.

Fig. 2. Growth curves for parainfluenza type 3 virus in calf kidney cultures; ×, persistently infected with foot-and-mouth disease virus (FMDV) type O_2; △, persistently infected with FMDV type O_2 and subsequently cured with excess anti-O_2 antibody; ○, normal cultures. —, Virus infectivity; – –, haemagglutinating activity.

Fig. 3. Growth curves for pseudo-rabies virus in calf kidney cultures ×, persistently infected with foot-and-mouth disease virus (FMDV) type O_2; △, persistently infected with FMDV type O_2 and subsequently cured with excess anti-O_2 antibody; ○, normal cultures.

cultures. These cultures were challenged with 100 TCD50 of FMDV A_5, parainfluenza-3 virus (PI-3) and pseudo-rabies virus. After different periods of incubation at 37° samples were removed from the supernatant fluid and assayed for infectivity.

When PI-3 challenge was used titres of haemagglutinin were also determined. Multiplication of FMDV A_5 was suppressed in persistently infected cultures. The multiplication in cured cultures was only slightly inhibited compared with the controls (Fig. 1). Inhibition of multiplication of PI-3 and pseudo-rabies viruses was also evident only in persistently infected cultures and not in cured cultures (Figs. 2 and 3).

Cytopathic effect in persistently infected cultures challenged with different viruses. The cytopathic effect of a virus challenge on persistently infected, cured and normal calf cultures was followed in cultures infected with 100 TCD50 of pseudo-rabies virus, FMDV A_5, and RNA from the latter virus. Four plates were infected in each group. The cytopathic effect was graded from 0–4. Cured and normal cultures were completely degenerated 2 or 3 days after infection, irrespective of the type of inoculum used for superinfection; the persistently infected cells scored between only 0·5–1·5 on day 4 after infection (Table 2).

Table 2. *Cytopathic effect of different challenge viruses on persistently infected foot-and-mouth disease virus (FMDV) cultures*

	Cytopathic effect											
	Persistently infected cells				Cured cells				Normal cells			
Challenge virus Days after infection...	1	2	3	4	1	2	3	4	1	2	3	4
FMDV type A_5 (intact virus)	0	0	1	1·5	1	3	4	4	2	4	4	4
FMDV type A_5 (RNA)	0	1·3	1·3	1·5	1	3	3	3	1	4	4	4
Pseudo-rabies virus	0	0·5	0·5	0·5	1	2	4	4	1	2	4	4

Cytopathic effect was graded from 0–4. The figures represent the mean for 3 cultures. The viruses were inoculated at day 0 in amounts of 100 TCD50 per culture.

Table 3. *Percentage fluorescent cells in normal calf kidney cultures at different multiplicities and in persistently infected cultures*

	Fluorescent cells* (%)			
	Normal cultures			Persistently infected cultures
Virus multiplicity (p.f.u./cell)...	1000	100	10	
Virus				
FMDV type A_5	98·9	24·2	2·1	n.d.
FMDV type O^2	100	33·7	0·42	0·2

* Mean of 1000 counted cells from 4 slides; n.d., not done.

Staining of persistently infected cells with fluorescent antibodies. Calf kidney cultures persistently infected with FMDV O_2 synthesize virus in cycles. The titres in these cultures are 100- to 1000-fold lower than those in normal cultures. The viral yield implies either infection of one of 200–600 cells and a normal yield from this cell or else suppressed virus multiplication in all cells (Dinter *et al.* 1959). To differentiate between these two alternatives, cultures persistently infected with FMDV type O_2 on coverslips were stained with fluorescent antibodies. As controls,

normal calf kidney cultures on coverslips were exposed to FMDV A_5 or FMDV O_2 at different multiplicities for 30 min. at 37°. Six hr. after infection the cultures were fixed and stained with fluorescent antibody. Cells exhibiting fluorescence in persistently infected cultures show the same intensity of fluorescence as normal cells infected with FMDV O_2 (Pl. 1). On the average one in 500 cells contains viral antigen in persistently infected cultures. Depending upon the multiplicity of infection, normal calf kidney cultures contain viral antigen in one in 50–200 cells at multiplicities of 10 and in every cell at multiplicities of 1000 (Table 3). The results suggest that the resistance of persistently infected cells to a challenge virus might be ascribed to a non-viral factor protecting the majority of the cells.

Attachment of challenge virus to persistently infected cultures. Persistently infected cultures and normal cultures were washed twice with PBS and inoculated with 300–500 p.f.u. of FMDV A_5 in 0·2 ml. At intervals, 0·02 ml. was removed from each culture in the two groups, diluted tenfold in PBS and assayed for remaining infectivity on normal calf kidney cultures. The rate of attachment of FMDV A_5 was the same in persistently infected and normal cultures (Fig. 4), indicating that blockage of attachment sites was not a major factor in maintenance of the persistent infection.

Challenge with viral RNA in persistently infected and interferon treated cells. The experiments reported above suggested that a step in virus multiplication beyond attachment was blocked in the majority of the persistently infected cells. This block interfered alike with the synthesis of related and unrelated viruses. A similar effect has been ascribed to interferon which also inhibits virus multiplication when viral RNA is used as inoculum (Ho, 1961). To investigate this aspect properly, however, a cell system must be used which does not propagate the intact virus particle but gives only a single cycle of replication in response to the introduced RNA. Therefore calf kidney cultures, both persistently infected with FMDV O_2 and normal, were infected with 100 p.f.u. of RNA from poliovirus type 1 or FMDV A_5. At

Table 4. *Yield of poliovirus type* 1 *in persistently infected calf kidney cultures of different ages with viral RNA as inoculum*

Days after induction of persistence	Yield in p.f.u./ml. at 18 hr. of incubation (% of control)
2	< 0·5
7	< 0·5
11	4·0
14	10·0

intervals the cultures were frozen and thawed and the total yield of intact virus assayed in normal monkey and calf kidney cultures respectively. To study the role of interferon in maintenance of the carrier state, these experiments were also performed with persistently infected cultures of different ages. Intact poliovirus type 1 does not normally multiply in calf kidney cultures (McLaren, Holland & Syverton, 1959). The yield of poliovirus type 1 in persistently infected cultures when viral RNA was used as inoculum was much decreased regardless of whether persistence had been established 2 or 14 days earlier (Table 4). The multiplication of FMDV

A_5 in persistently infected cells was also inhibited when viral RNA was used as inoculum (Fig. 5). This latter finding alone does not permit the conclusion that in persistently infected cells, virus multiplication is blocked beyond penetration, since viral synthesis induced by intact FMDV A_5 particles formed in the first cycle may be inhibited. With regard to the results with poliovirus, which cannot use calf kidney cells as a natural host, it is likely, however, that in both cases viral synthesis is blocked intracellularly.

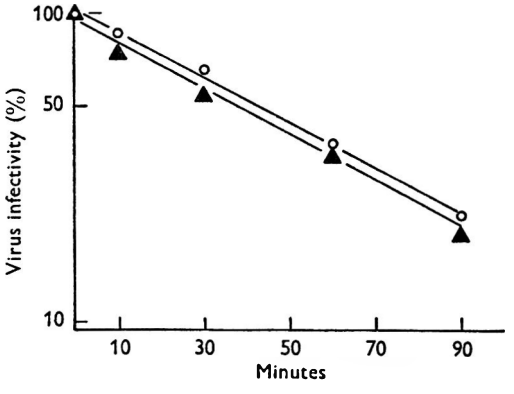

Fig. 4

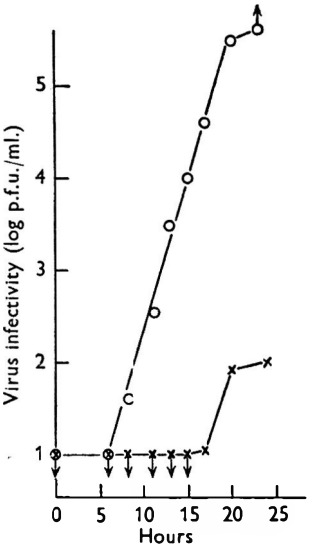

Fig. 5

Fig. 4. Attachment of foot-and-mouth disease virus (FMDV) A_5 to calf kidney cultures persistently infected with FMDV O_2 (○) and to normal cultures (▲). Virus infectivity is expressed as the % p.f.u. remaining in the supernatant fluid after different periods of adsorption.

Fig. 5. Reproduction of foot-and-mouth disease virus (FMDV) in calf kidney cultures persistently infected with FMDV type O_2 (×) and in normal cultures (○) after inoculation with RNA of FMDV type A_5.

The suppressed multiplication of challenge virus in persistently infected cultures was compared with the inhibition of viral synthesis by interferon in normal cultures. Calf and monkey kidney cultures were treated with interferon for 24 hr. and subsequently inoculated with 100 TCD50 RNA from poliovirus type 1 and FMDV A_5. The preparation of interferon was described by Dinter & Philipson (1962). After incubation for 18 hr. at 37°, control and interferon-treated cultures were frozen at −60°, thawed and subsequently assayed for infectivity of intact virus in normal monkey and calf kidney cultures, respectively. The yield of the two viruses was suppressed in interferon-treated cells when viral RNA was used as inoculum. This effect was observed both in the natural host cells, monkey kidney for poliovirus, and in the calf kidney cells insusceptible to this virus (Table 5). Intact FMDV A_5 however, multiplied in both calf and monkey kidney cultures.

Correlation between production of interferon and initiation of persistent infection. Persistently infected cultures were produced in tubes as described under methods.

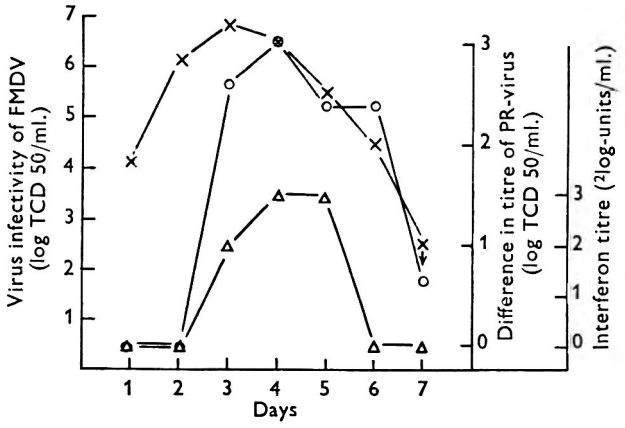

Fig. 6. Correlation between formation of infectious virus and the production of interferon in calf kidney cultures persistently infected with foot-and-mouth disease virus (FMDV). ×, Virus titre of FMDV type O_2 in the medium; ○, difference in titre of pseudo-rabies virus in normal cultures and in persistently infected cultures; △, interferon titre of medium according to methods.

Table 5. *Yield of foot-and-mouth disease virus (FMDV) type A_5 and poliovirus type* 1 *in interferon-treated calf and monkey kidney cultures with viral RNA as inoculum*

Challenge RNA 100 p.f.u./culture	Yield in p.f.u./ml. at 18 hr. of incubation (% of control)	
	Monkey cells	Calf cells
FMDV type A_5	< 0·4	0·18
Polio type 1	2·7	< 0·03

At daily intervals, the medium of 5 tubes was removed and pooled. The infectivity of FMDV O_2 was assayed and subsequently the interferon titre was determined in these pools after inactivating the virus by adjustment to pH 2·5 with citrate buffer and back to pH 7 with 0·1 M-NaOH. At daily intervals pseudo-rabies virus was also titrated in persistently infected and in control tube cultures. The difference in titres between these two titrations was considered an estimate of the resistance of the persistently infected cultures to the challenge virus. There was a rise in FMDV O_2 titre with a maximum 3 days after the antiserum was removed from the persistently infected cultures. Interferon production and resistance to challenge virus reached a maximum at the 4th day after induction of persistence. The resistance to challenge virus was correlated in time with the interferon production in these cultures (Fig. 6). In this experiment the persistent infection was cured spontaneously at 10 days after induction; in other experiments, however, in which the persistent infection lasted for longer periods, the interferon titres were too low to be demonstrated 10–15 days after induction.

DISCUSSION

The studies presented demonstrate a correlation between interferon production and persistence of FMDV O_2 in calf kidney cultures during the early stages of the persistent infection. In persistently infected cells kept for 20–40 days, the presence

of interferon could not be correlated with the resistance of these cells to challenge with another virus. Several theories can be put forward to explain this phenomenon. First, the interferon present might be bound intracellularly and more cells might be needed to demonstrate the presence of interferon. This is the case with MCN cultures persistently infected with myxoviruses (Henle *et al.* 1959). Secondly the interferon, initially present in the majority of the cells, might induce an alteration of the cells as reported by Gresser (1961). Evidence in favour of this theory has been obtained since the persistently infected cells show differences in morphology and nuclear size compared with controls (unpublished). Finally, since a heterogeneous cellular population was used, a selection of resistant cells might occur with gradual loss of cells susceptible to virus infection. This could also tally with the observed difference in cellular morphology of persistently infected cells. In a previous communication (Dinter & Philipson, 1962) the possibility was discussed that the resistance to challenge virus could be due to a block of receptor sites on the cells by inactive virus. Such cells would produce virus normally if infectious RNA was used as inoculum as demonstrated by Crowell & Syverton (1961). The present investigation demonstrates that the virus synthesis is inhibited when infectious RNA is used for challenge and that attachment of the challenge virus to the persistently infected cells is unaffected. All this favours the hypothesis that virus synthesis is blocked intracellularly.

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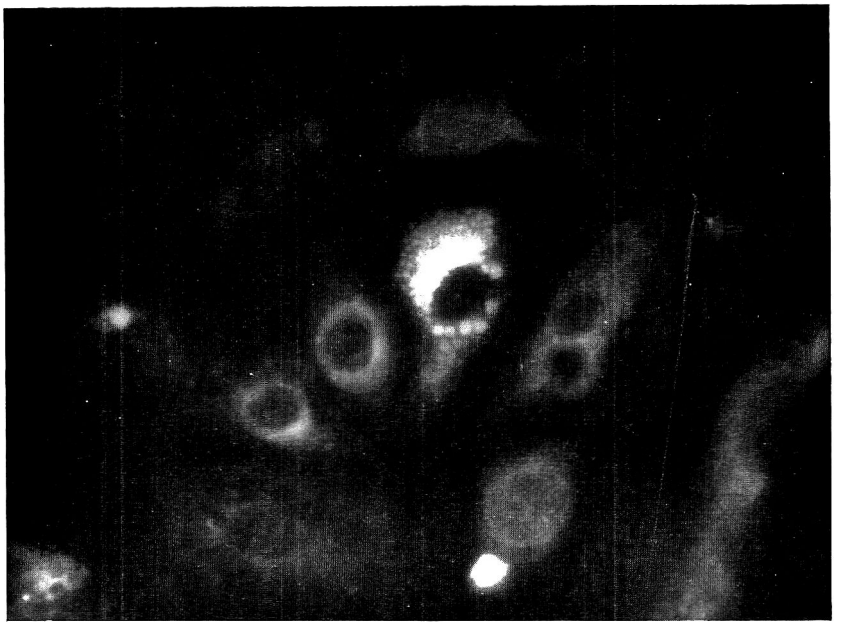

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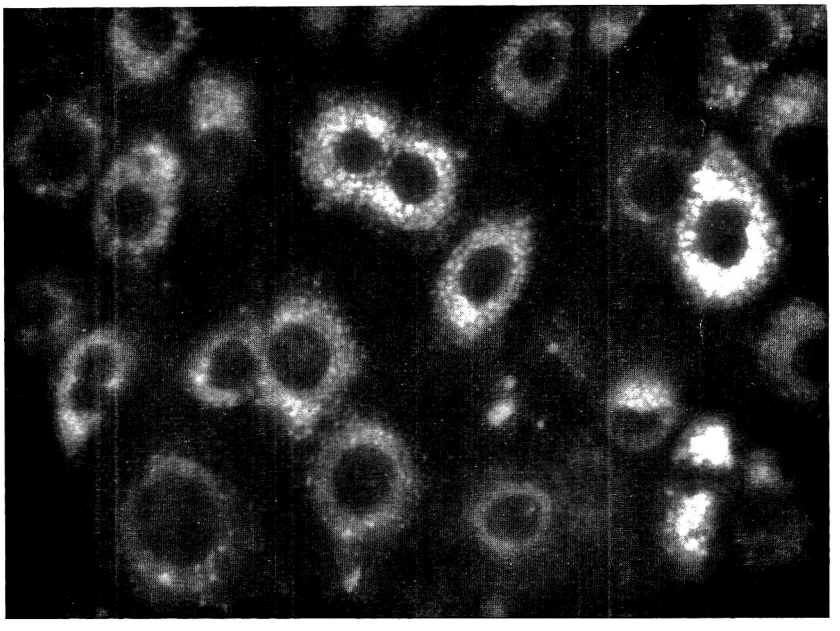

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EXPLANATION OF PLATE

Immunofluorescence of cells persistently infected with foot-and-mouth disease virus (FMDV) type O_2; magnification × 800 (*a*). Immunofluorescence of normal calf kidney cells infected with FMDV type O_2 at a multiplicity of 50 at 6 hr. after infection; magnification × 800 (*b*).

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Effect of Metal Ions and Polyamines on the Development of Bacteriophage ϕR

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SUMMARY

One-step growth experiments with bacteriophage ϕR showed that the yield of phage increased with increasing Mg^{2+} concentration. The function of Mg^{2+} was not primarily that of a lysis cofactor since the titre of intracellular phage was also dependent upon metal ion concentration. Phage development could be promoted by addition of Mg^{2+} after the end of the normal latent period. This stimulatory action was not appreciably inhibited by chloramphenicol or respiratory poisons, though it was abolished at low temperature and gradually became less as the time between infection and Mg^{2+} addition was increased. Qualitatively similar effects on phage growth were obtained with other divalent metal ions and with some polyamines. It is concluded that all these substances act at a late stage in the intracellular development of phage ϕR, possibly at some step involving the neutralization of nucleic acid. Whether or not Mg^{2+} ions are also required for the release of phage particles cannot be determined from the present experiments, though some of the results suggest that they may be.

INTRODUCTION

Previous observations on the role of Mg^{2+} in the growth of bacteriophage ϕR (phage anti-R) have shown that the metal is required at the end of the growth cycle (Tucker, 1961). In the present investigation an attempt was made to determine the nature of this late step, in particular to find out whether it involves an increase in the total number of infectious particles or merely an increased liberation of phage.

METHODS

Host organism. Escherichia coli strain c (kindly supplied by Dr K. Burton, Department of Biochemistry, University of Oxford) was used throughout the present work. This organism was chosen in preference to *Salmonella typhi* which had previously been used as the phage host (Tucker, 1961) since it gave better yields of phage.

Phage. Phage ϕR (Kay, 1962), which has also been called phage anti-R (Fildes, 1954; Kay, 1955; Tucker, 1961), is one of a group of small coliphages containing single-stranded DNA. Lysates of phage ϕR were prepared in glycerol + casein hydrolysate medium (Fraser & Jerrel, 1953) from which $CaCl_2$ and gelatin were omitted and to which 4×10^{-3}M-$MgSO_4$ was added after autoclaving (121°, 10 min.). The crude lysates, containing $1–2 \times 10^{11}$ plaque-forming units (pfu)/ml. were purified by a method essentially that of Tromans & Horne (1961). Phage was assayed by the

double-layer technique (Adams, 1950) with L-broth (Lennox, 1955) $+2{\cdot}5 \times 10^{-3}$M-$CaCl_2$ $+5 \times 10^{-3}$M-glucose solidified with 2 and 0·7% agar (Difco) in the bottom and top layers, respectively.

Media. The chemically defined medium, which will be referred to as 'minimal medium' was that of Kay & Fildes (1950) used at half strength and supplemented with 0·2% (w/v) lactose. The low Mg^{2+} concentration of this minimal medium (2×10^{-5}M) permits only about half the total amount of growth of *Escherichia coli* c which is possible when Mg^{2+} is in excess (greater than 5×10^{-5}M) but it does not affect the rate of growth.

The buffer used for diluting organisms and phage consisted of minimal medium without Mg^{2+} and without lactose.

One-step growth experiments. Escherichia coli c was grown in minimal medium with aeration at 37° until the viable count reached 4×10^8 organisms/ml. Five ml. of the culture were centrifuged and the pellet resuspended in 5 ml. solution containing 1% (w/v) peptone (Evans), 4×10^{-3}M-$MgSO_4$ and $1{\cdot}5 \times 10^{-4}$M-chloramphenicol (Parke Davis), and phage ϕR was added to give a cell:phage ratio of unity. After incubation at 37° for 10 min. the unadsorbed phage was inactivated by addition of 0·1 ml. phage antiserum and incubation for a further 5 min. at 37°. The culture was then diluted in buffer and a suitable volume was transferred to a ⊥-shaped tube containing minimal medium (growth tube) which was incubated at 37° with rocking. When necessary, samples were diluted in buffer before plating.

In the experiments that follow the plaques obtained when samples were plated without further treatment, i.e. the plaques from phage particles which were free at the time of plating as well as those from infected bacteria, will be called 'infective centres'. 'Total phage' refers to the plaque count obtained after the release of intracellular phage by artificial lysis. The method of Kohn & Szybalski (1959) was adapted to give a highly convenient procedure for liberating phage ϕR. Samples (2·5 ml.) were removed from the growth tube and transferred to 110×15 mm. lusteroid centrifuge tubes containing 50 μg. lysozyme (Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex) and rapidly frozen by plunging into liquid air. After thawing and incubation at 37° for 5 min. the samples were plated as usual. Control experiments showed that the method gave complete lysis of the organisms as judged by the release of β-galactosidase, and that it neither destroyed free phage ϕR nor reactivated phage which had been treated with antiserum.

Materials. Where possible, solutions of metal salts were prepared from Analytical Reagent Grade chemicals. Polyamines were from the following sources: agmatine sulphate, spermidine phosphate, spermine hydrochloride and putrescine hydrochloride from Roche Products Ltd. (Welwyn Garden City, Herts); 1,3-diaminopropane from Light and Co. Ltd. (Colnbrook, Bucks); 1,2-diaminopropane from British Drug Houses Ltd. (Poole, Dorset).

RESULTS

One step growth experiments with Escherichia coli *strain c*

Cultures of *Escherichia coli* c, which had been infected with phage in the presence of 4×10^{-3}M-$MgSO_4$ and then diluted into minimal medium, showed only a small increase in the number of infective centres after 60 min. incubation at 37°. Under

these conditions the intracellular phage count showed a somewhat greater increase, but good phage development, measured either as the increase in the infective centre count or in the intracellular phage count after artificial lysis, required added Mg^{2+} (Fig. 1). These results are qualitatively the same as those reported (Tucker, 1961) for another bacterial host, *Salmonella typhi*, the main difference being the greater intracellular phage development in *E. coli* c in minimal medium without extra Mg^{2+}.

Figure 2 shows the phage production at 60 min. in a series of one-step growth experiments with different Mg^{2+} concentrations. The increase in the total phage count with increasing amounts of Mg^{2+} means that this cation is concerned in the

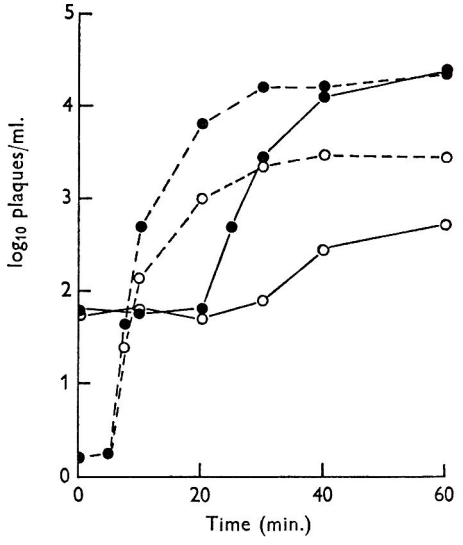

Fig. 1

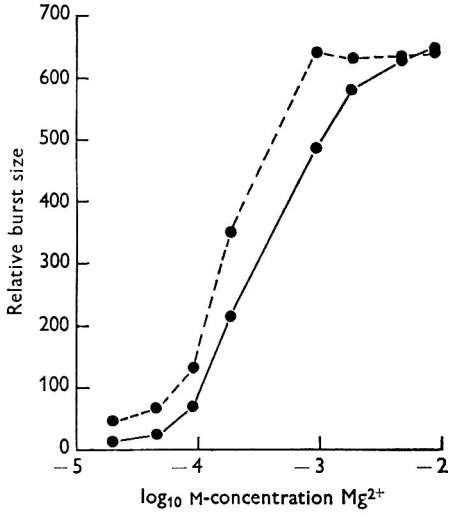

Fig. 2

Fig. 1. Effect of Mg^{2+} in one-step growth experiments. *Escherichia coli* c was infected with phage ϕR in the presence of 4×10^{-3}M-$MgSO_4$ and $1{\cdot}5 \times 10^{-4}$M-chloramphenicol. After inactivation of free phage with antiserum the culture was diluted $1{\cdot}5 \times 10^6$ into minimal medium, without (○—○) and with (●—●) added $MgSO_4$ (4×10^{-3}M). Phage present after artificial lysis is denoted by (○---○) and (●---●) respectively.

Fig. 2. Effect of Mg^{2+} concentration on phage yield in one-step growth experiments. *Escherichia coli* c was infected with phage ϕR in the presence of 4×10^{-3}M-$MgSO_4$ and $1{\cdot}5 \times 10^{-4}$M-chloramphenicol. Free phage was inactivated with antiserum and the culture was diluted $1{\cdot}5 \times 10^6$ into growth tubes of minimal medium containing varying concentrations of $MgSO_4$. The tubes were assayed at 60 min. for infective centres (●—●) and total phage (●---●).

formation of infectious particles. However, except at the highest concentrations of Mg^{2+} used the number of infective centres was less than that of total phage, i.e. there was incomplete liberation of phage at 60 min. In experiments in which the phage was assayed at 180 min. after infection a similar dependence of yield of phage upon Mg^{2+} concentration was found, though the discrepancy between the number of infective centres and the total phage was less marked.

Effect of delaying the addition of Mg^{2+} *to infected organisms*

The increase in plaque count caused by the late addition of Mg^{2+} which had previously been noted with *Salmonella typhi* as host was also observed with *Escherichia coli* C. Thus when a phage-infected culture which had been diluted into minimal medium was supplemented with 4×10^{-3}M-Mg^{2+} at 40 min. (i.e. 20 min. after the expected end of the latent period) there was a sharp increase in plaque count (Fig. 3). In this experiment the cation produced a fourfold increase in total phage after 5 min., which supports the view that Mg^{2+} is required for actual phage formation and not merely for the release of preformed infectious particles. Adding Mg^{2+} late also increased the plaque count of directly-plated samples as well as of those treated with lysozyme, but there was always a lag in phage liberation. The ability of Mg^{2+} to stimulate phage production was gradually lost on prolonged incubation of infected organisms in minimal medium (Table 1).

Table 1. *Effect of time of addition of* Mg^{2+} *on phage* ϕR *development in* Escherichia coli *strain* C

Escherichia coli C was infected with phage ϕR in the presence of 4×10^{-3}M-$MgSO_4$ + $1{\cdot}5 \times 10^{-4}$M-chloramphenicol. After inactivation of free phage with antiserum the culture was diluted $1{\cdot}5 \times 10^6$ in minimal medium and assayed periodically for numbers of infective centres and total phage. At 0, 30, 90, 150 and 180 min. after dilution, portions of the culture were transferred to growth tubes containing 4×10^{-3}M-$MgSO_4$ and assayed for infective centres and total phage after 40 min. incubation at 37°.

	At time of sampling		After 40 min. with 4×10^{-3}M-$MgSO_4$	
Time (min.)	Infective centres	Total phage	Infective centres	Total phage
	Plaque counts			
0	65	0	39,500	37,000
30	170	1700	38,400	38,100
60	310	1350	—	—
90	460	1350	7,100	12,700
120	720	2330	—	—
150	530	2330	2,780	5,500
180	730	1740	980	2,730

The increase in the plaque count brought about by Mg^{2+} was not due to the extracellular activation of non-infective phage material. A phage-infected culture of *Escherichia coli* C was washed with buffer then resuspended in minimal medium and incubated at 37°. After assaying the culture for infective centres at 50 min. two volumes were taken and the bacteria in one of them were removed by centrifugation. Magnesium sulphate (to 4×10^{-3}M) was then added to the supernatant fluid of one sample and to the other uncentrifuged sample. After incubating for 10 min. at 37° only the sample containing bacteria showed an increase in the number of infective centres.

Effect of metabolic inhibitors on the action of Mg^{2+}

In attempts to determine the mechanism of action of Mg^{2+} in stimulating phage production, the action of chloramphenicol and of respiratory poisons was investigated. Chloramphenicol ($1{\cdot}5 \times 10^{-4}$M), potassium cyanide (10^{-2}M) or sodium azide (2×10^{-2}M) added to a phage-infected culture of *Escherichia coli* C immediately after transfer to minimal medium containing 4×10^{-3}M-Mg^{2+} completely inhibited

Table 2. *Effect of chloramphenicol on stimulatory effect of Mg^{2+} on phage ϕR production*

Phage-infected organisms were diluted in minimal medium at 37°. Forty min. after dilution, portions of the culture were transferred to tubes containing 4×10^{-3}M-$MgSO_4$ or 4×10^{-3}M-$MgSO_4 + 1{\cdot}5 \times 10^{-4}$M-chloramphenicol (CAP) and incubated at 37°. At the times indicated the growth tubes were assayed for infective centres and total phage.

			4×10^{-3}M-Mg^{2+} added at 40 min.			
	No added Mg^{2+}		−CAP		+CAP	
Time (min.)	Infective centres	Total phage	Infective centres	Total phage	Infective centres	Total phage
	Plaque counts					
0	112	10	—	—	—	—
40	440	1900	—	—	—	—
55	—	—	38,400	66,500	4,700	59,300
70	—	—	64,500	74,200	66,100	67,100
85	995	4400	83,500	83,000	63,800	60,200

phage growth. However, none of these compounds was able to prevent rapid phage development when added with Mg^{2+} at later times after infection. The results of an experiment with chloramphenicol are given in Table 2; both azide and cyanide were very similar in their action though it was consistently noticed that phage release was slower in their presence (Fig. 4). These results imply that the synthesis of protein necessary for phage formation proceeded in the absence of added Mg^{2+}, and that the acquisition of infectivity brought about by the divalent metal was not dependent upon respiratory energy. In contrast to the failure of the above inhibitors to abolish the Mg^{2+} effect, cooling the culture in an ice bath before adding Mg^{2+} prevented the increase of infective centres and of intracellular phage.

Effect of other metal ions and amines on phage development

Magnesium ions are not unique in stimulating phage formation in the minimal medium; several other divalent metal ions and polyamines also caused an increase in total phage count when added late to phage-infected organisms. The efficiency of different compounds was compared by adding them to infected cultures 40 min. after dilution into minimal medium; chloramphenicol ($1{\cdot}5 \times 10^{-4}$M) was also added to eliminate the possible complication of continued protein synthesis. After 20 min. incubation in test tubes at 37° the total number of phage was determined after the addition of lysozyme and freezing. Because of the day-to-day variation in the absolute increase in plaque count with a given substance, a control with $CaCl_2$ (4×10^{-4}M) was included routinely, the results being expressed as a percentage of

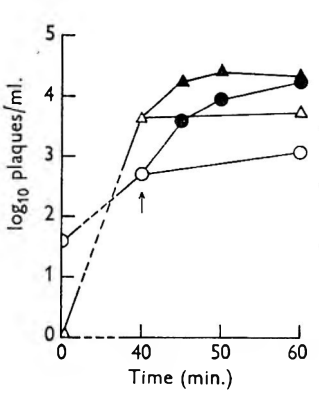

Fig. 3

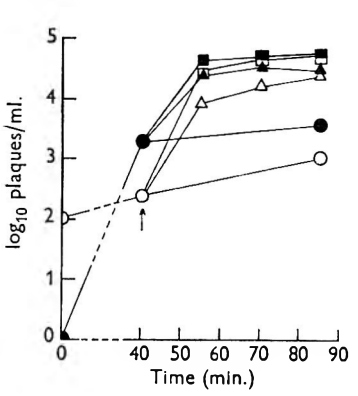

Fig. 4

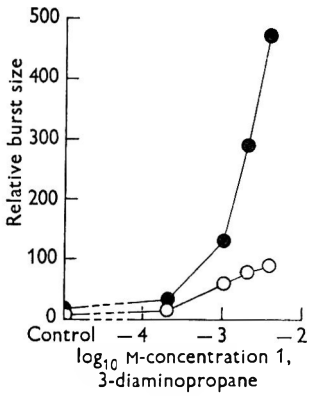

Fig. 5

Fig. 3. Effect of delaying the addition of Mg^{2+} on phage development. *Escherichia coli* C was infected with phage ϕR in the presence of 4×10^{-3}M-$MgSO_4$ and $1{\cdot}5 \times 10^{-4}$M-chloramphenicol. Free phage was inactivated with antiserum and the culture was diluted into minimal medium (infective centres, ○—○; total phage, △—△). At 40 min. a portion of the culture was transferred to another growth tube containing 4×10^{-3}M-$MgSO_4$ (infective centres, ●—●; total phage, ▲—▲).

Fig. 4. Effect of sodium azide on stimulatory effect of Mg^{2+}. *Escherichia coli* C was infected with phage ϕR in the presence of 4×10^{-3}M-$MgSO_4$ and $1{\cdot}5 \times 10^{-4}$M-chloramphenicol, and after inactivation of free phage with antiserum the culture was diluted into minimal medium (infective centres, ○—○; total phage, ●—●). At 40 min. portions of this culture were transferred to two growth tubes containing 4×10^{-3}M-$MgSO_4$ (infective centres, □—□; total phage, ■—■) and 4×10^{-3}M-$MgSO_4 + 2 \times 10^{-2}$M-sodium azide (infective centres, △—△; total phage, ▲—▲).

Fig. 5. Effect of 1,3-diaminopropane concentration on phage yield in one-step growth experiments. *Escherichia coli* C was infected with phage ϕR in the presence of 4×10^{-3}M-$MgSO_4$ and $1{\cdot}5 \times 10^{-4}$M-chloramphenicol. Free phage was inactivated with antiserum and the culture was diluted into minimal medium containing various amounts of 1,3-diaminopropane. The growth tubes were assayed at 60 min. for infective centres (○—○) and total phage (●—●).

Table 3. *Effect of late addition of metal ions and polyamines on phage ϕR development in* Escherichia coli *strain* C

Phage-infected *E. coli* C was diluted in minimal medium and incubated at 37°. After 40 min., samples were transferred to tubes containing the substance under investigation $+ 1{\cdot}5 \times 10^{-4}$M-chloramphenicol. After incubation at 37° for 20 min. the total phage in the tubes was assayed after artificial lysis. Metal salts at 4×10^{-4}M; polyamines at 4×10^{-3}M.

Substance	Titre × 100 / Titre with 4×10^{-4}M-Ca^{2+}	Substance	Titre × 100 / Titre with 4×10^{-4}M-Ca^{2+}
None	12	Agmatine	99
$CaCl_2$	100	1,3-Diaminopropane	99
$SrCl_2$	88	Spermidine	72
$MgSO_4$	84	1,2-Diaminopropane	57
$BaCl_2$	79	Spermine	56
$MnCl_2$	74	Putrescine	34
$CdCl_2$	71	$CaCl_2 + MgSO_4$	106
$CoSO_4$	70	$CaCl_2$ + agmatine	90
$NiCl_2$	54	$CaCl_2$ + 1,3-diaminopropane	110
$BeCl_2$	41		

the titre obtained after 20 min. exposure to Ca^{2+}. Data from a number of experiments are given in Table 3. No appreciable increase in phage titre was obtained with NaCl or LiCl over the range $7{\cdot}5 \times 10^{-2}$M to 3×10^{-1}M, with zinc acetate (4×10^{-4}M) or with ferrous sulphate (4×10^{-4}M).

The substances tested in Table 3 also increased the plaque count of samples plated without artificial lysis though they were not equally active in this respect. 1,3-Diaminopropane in particular caused only a small increase in the number of infective centres in spite of its being one of the most active of the amines in stimulating phage development. One-step growth experiments with organisms infected in the presence of Mg^{2+} + chloramphenicol and subsequently diluted into minimal medium containing various concentrations of 1,3-diaminopropane also showed that there was relatively poor phage release with this compound (Fig. 5). The addition of Mg^{2+} at 40 min. to similar cultures in 4×10^{-3}M-1,3-diaminopropane resulted in an increase in the number of infective centres (Table 4) which may indicate that phage release has a specific requirement for the metal ion.

Table 4. *Effect of adding Mg^{2+} to phage ϕR-infected* Escherichia coli *strain* c *in medium containing* 1,3-*diaminopropane*

A culture of *Escherichia coli* c was infected with phage ϕR in the presence of 4×10^{-3}M-$MgSO_4$ + $1{\cdot}5 \times 10^{-4}$M-chloramphenicol. The culture was diluted $1/5 \times 10^6$ in minimal medium containing 4×10^{-3}M-1,3-diaminopropane and incubated at 37°. After 40 min. a portion of the culture was supplemented with 4×10^{-3}M-$MgSO_4$ and assayed for number of infective centres and total phage after a further 20 min. incubation.

	No addition		4×10^{-3}M-Mg^{2+} at 40 min.	
Time (min.)	Infective centres	Total phage	Infective centres	Total phage
	Plaque counts			
0	44	0	—	—
40	3,200	19,700	—	—
60	6,300	24,800	16,900	20,700

DISCUSSION

The dependence of the intracellular phage ϕR concentration upon Mg^{2+} concentration, and the increase in intracellular phage titre when Mg^{2+} was added late to infected *Escherichia coli* c show clearly that Mg^{2+} is needed for some step in the development of the phage. Since neither chloramphenicol nor respiratory inhibitors appreciably affected the stimulatory action of the late addition of Mg^{2+}, it is concluded that synthesis of components of phage ϕR can proceed in the absence of added Mg^{2+} and that higher concentrations of Mg^{2+} are needed for some terminal event in the growth of the phage leading to an increase in the number of infectious particles. Without knowledge about the immediate precursor of mature phage ϕR the nature of this step must remain unknown, though the qualitatively similar effects of other divalent metals and of polyamines suggest that neutralization of nucleic acid charge may be involved. These substances can neutralize and stabilize nucleic acids *in vitro* (Felsenfeld & Huang, 1960; Tabor, Tabor & Rosenthal, 1961; Eichhorn, 1962) and it is believed that they function similarly in some phages (Ames, Dubin & Rosenthal, 1958; Kay, 1959; Ames & Dubin, 1960). The idea that the

cations diffuse into and neutralize the nucleic acid of structurally completed phage ϕR particles is an attractive explanation of the observed results. However, if this be so, some further change in the particles has to be postulated to account for the failure to obtain the effect of the metals once the cells have been lysed and for the fact that free phage is stable in the presence of chelating agents.

The present experiments do not permit a decision about whether or not the cations are also concerned in phage liberation. In one-step growth experiments with 4×10^{-3}M-Mg^{2+} present from the beginning of infection lysis appeared to be complete by 60 min.; the ability of Mg^{2+} to stimulate phage production when added as late as 150 min. after infection might therefore be explained if phage release was slower or incomplete in the whole culture without added cations. Some support for this comes from experiments where Mg^{2+} increased the number of infective centres of cultures incubated with 1,3-diaminopropane (Table 4), and from the observation (Tucker, 1961) that the number of infective centres did not increase when Mg^{2+} was removed by chelation during the rising period of phage growth.

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An Electron Microscope Study of Maturation and Germination of Sporangiospores of Two Species of *Rhizopus*

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(*Received* 18 *February* 1963)

SUMMARY

Immature sporangiospores of *Rhizopus nigricans* and *R. sexualis* were relatively thin-walled, although thickened longitudinal ridges were already discernible. The position of the nuclei suggested that they had recently divided; the mitochondria were globose and similar to those of vegetative hyphae; the protoplast was surrounded by a thin plasmalemma and contained numerous food globules. The endoplasmic reticulum was sparse. Mature spores had a thick wall of reticulate structure and contained large contorted mitochondria. The first visible stage in germination was the formation of an inner cell wall of tangential elements resembling that of the vegetative hyphae and contrasting with the original spore wall. The mitochondria increased in number, probably by division of the large contorted ones of the dormant spore, and were again regularly globose. The original spore wall became considerably stretched and finally ruptured to allow the germ tube enveloped in the newly formed elastic inner wall to emerge.

INTRODUCTION

Electron-micrographs of sporangiospores of *Rhizopus sexualis* (Smith) Callen and *R. nigricans* Ehrenb. revealed hitherto undetected changes in fine structure during maturation and germination.

METHODS

The material was fixed with Luft's permanganate (Luft, 1956) or with permanganate followed by osmium tetroxide as previously described (Hawker & Abbott, 1963). The fixed material was embedded in araldite, sectioned with a glass knife on a Huxley ultra-microtome (Cambridge Instrutment Co.) and examined with a Philips EM 200 electron microscope, as in earlier work.

RESULTS

Maturation of sporangiospores

Immature spores (Pl. 1, fig. 1), presumably released by rupture of the sporangial wall during fixation, were often slightly flattened at one side, probably where they had been in contact with other spores. The spore wall was relatively thin and consisted of a single layer, but already showed the thickening which would later have become the characteristic longitudinal ridges of the mature spore. These thickenings were blackish in osmic-fixed material, indicating the presence of lipid material. The plasmalemma was thin and in close contact with the wall. The endoplasmic reti-

culum was sparse with a few scattered cisternae, thus resembling that of the vegetative hyphae (Hawker & Abbott, 1963). Typical nuclei, mitochondria, and numerous reserve food globules, many of which were of a fatty nature, were present. The nuclei in these immature spores were more or less centrally placed in a group of two or four, suggesting recent division. The nuclear membrane and the nucleolus were similar to those of young vegetative hyphae. The mitochondria were globose or ovoid with a regular outline and the inner membrane was infolded to give groups of roughly parallel plate-like cristae, as in young vegetative hyphae.

During maturation (Pl. 1, fig. 2) the spore outline became symmetrical, finally becoming more or less lemon-shaped and slightly flattened longitudinally. The longitudinally ridged wall became very thick (0·3–0·6 μ) and consisted of apparently regularly arranged elements enveloped in an amorphous mass of fatty material (staining black with osmium tetroxide; Pl. 2, fig. 4). At this stage there were no tangentially arranged elongated elements such as those which make up the wall of the vegetative hypha. The plasmalemma remained thin, closely lining the wall. The nuclei had moved apart. Complete sets of serial sections were not obtained for any one spore but a study of a large number of sections suggested that the usual number of nuclei was four and that these were arranged equatorially. The number, size and distribution of storage granules and the scattered cisternae showed no significant change. The most striking change, apart from the thickening of the wall, was in the shape and size of the mitochondria (Pl. 2, figs. 5, 6). These had enlarged, had become convoluted and now contained a complex system of closely packed cristae. The contorted shape of the mitochondria was not due to shrinkage during fixation since their actual size had increased in comparison with those of immature spores. Moreover, the nuclei and plasmalemma were neither shrunken nor contorted, indicating satisfactory fixation.

Germination of sporangiospores

Spores were suspended in water; they germinated after 3 hr. or more and were fixed after 5–6 hr. The first visible structural change was the laying down of a thin inner wall (Pl. 2, figs. 3, 4). This consisted of tangentially arranged elements and thus resembled the wall of the vegetative hypha (Hawker & Abbott, 1963) but was strikingly different from the original spore wall. During these early stages of germination the spore enlarged and the wall became stretched, as indicated by the increasing distance between the wall ridges. The mitochondria became more numerous, smaller and regular in outline (Pl. 2, fig. 7). It must be assumed that the large irregularly-shaped mitochondria of the mature spore had undergone fission to give an increased number of these organelles in the germinating spore. In many spores, the nuclei and mitochondria were more densely congregated at one side, presumably at the point where the germ tube was about to emerge.

When germination took place the original spore wall, now much stretched, broke and the germ tube emerged, enclosed by the newly-formed elastic inner wall (Pl. 3, figs. 8, 9, 10). The contents of the spore flowed into the germ tube, both nuclei and mitochondria becoming elongated and orientated in the direction of flow. Flow lines, or perhaps thin membranes, could be traced in the endoplasm. Just behind the tip of the germ tube these resembled the membranes already reported in vegetative hyphae (Hawker & Abbott, 1963). The extreme tips of the germ tubes contained

mitochondria; the nuclei, as in vegetative hyphae, were some distance behind the tip (Pl. 3, fig. 11). Vacuoles developed in the part of the spore distant from the germ tube.

DISCUSSION

The most striking new facts about the maturation and germination of Rhizopus spores shown by the present work are the rapid formation of an inner wall, of a type radically different from the original spore wall, immediately after the spores are suspended in water, and the changes in the mitochondria. The tangential arrangement of the elements of the new wall is likely to give greater elasticity in a direction parallel to the surface than would the reticulate structure of the old wall. Nevertheless the latter does stretch considerably before it becomes ruptured to allow the emergence of the germ tube. During this stretching the original wall becomes correspondingly thinner and the ridges less marked. The shape of the spore becomes altered owing to the slight plasticity of the original wall, so that there is no constriction of the emerging germ tube. Under the light microscope the spore wall appears to be entire and to continue around the germ tube. The electron microscope shows conclusively that this is not so and reveals the rapid formation of an elastic inner wall as one of the first stages in germination. At the point of emergence of the germ tube the original cell wall shows a clean break.

Multiplication of mitochondria by division of old ones rather than by *de novo* formation was shown by Manton (1961) to occur in the liverwort *Anthoceros laevis* and in the Chrysophycean flagellate *Chrysochromulina brevifilum*. Hashimoto, Conti & Naylor (1958), however, claimed that the mitochondria in ascospores of *Saccharomyces cerevisiae* disappeared during dormancy and that on germination they were formed anew. An examination of their photographs, however, suggests that the material was incompletely fixed, since few details of internal structure can be seen. We had considerable difficulty in securing proper fixation of the thick walled Rhizopus spores and concluded that fixation was best with slightly damaged spores. Only spores which showed well-fixed contents were considered in the present study. Mitochondria could not be distinguished in poorly fixed spores.

Thanks are due to the Department of Physics, University of Bristol, for allowing us to use the electron microscope. We are also indebted to the D.S.I.R. for a grant in aid of this investigation.

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EXPLANATION OF PLATES

PLATE 1. *Rhizopus nigricans*

Fig. 1. Approximately transverse section of an immature sporangiospore, showing relatively thin, ridged spore wall (*SW*); plasmalemma (*P*); four nuclei (*N*) the relative positions of which suggest that they originated from a single nucleus and had just completed a second division; mitochondria (*M*) with entire outlines and well-defined cristae; miscellaneous food globules (*F*); and sparse endoplasmic reticulum (*R*).

Fig. 2. Oblique section through mature sporangiospore; showing thick spore wall surrounded by black amorphous layer (*B*); plasmalemma; three nuclei; large complex lobed mitochondria; food globules; and endoplasmic reticulum. Other lettering as in fig. 1.

PLATE 2. *R. nigricans.* Lettering as in Pl. 1

Fig. 3. Approximately transverse section through sporangiospore soaked in water for 5 hr.; showing development of inner wall (*IW*) and apparent fission of lobed mitochondria (e.g. M_1 and M_2) to form more numerous, smaller ones of more regular shape.

Fig. 4. Section through part of wall of a spore at same stage as that shown in fig. 3. Note reticulate arrangement of elements in original spore wall and presence of newly formed inner wall consisting of tangentially arranged elements.

Fig. 5. Mitochondria from young spore (note thin spore wall on left) showing incipient lobing.

Fig. 6. Two complex lobed mitochondria and part of a third from a mature spore. Enlarged from area indicated by arrow in fig. 2. Note complex groups of cristae.

Fig. 7. Mitochondria from germinating spore. Note relatively small size, regular outline and groups of parallel cristae.

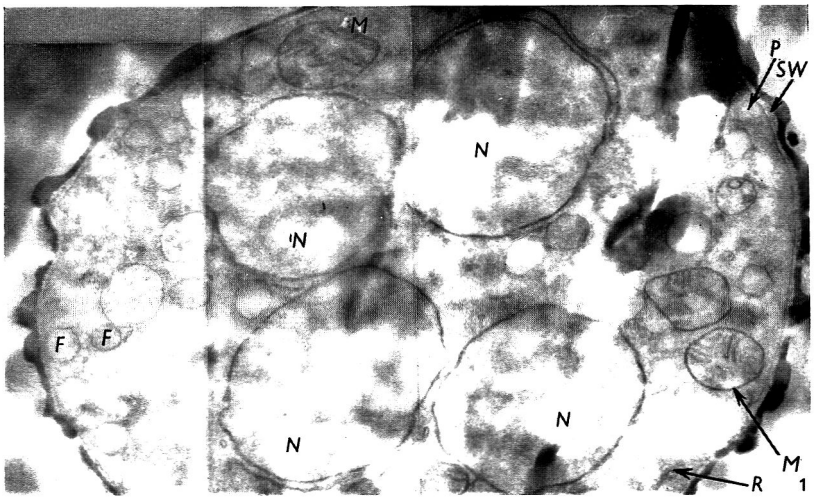
PLATE 3. *Rhizopus sexualis.* Lettering as in Pls. 1 and 2

Fig. 8. Oblique section through germinating sporangiospore (soaked in water for 5 hr. before fixation); showing germ tube (*GT*), enclosed by wall continuous with inner wall of spore, emerging through clean break (*X*) in outer space wall. Note enlongated mitochondria and nuclei flowing towards and into germ tube, and lines of flow, or possibly 'cortical' membranes, (*C*) in endoplasm near base of germ tube.

Fig. 9. Part of fig. 8 enlarged to show broken spore wall at points of emergence of germ tube.

Fig. 10. Approximately median section through emerging germ tube of another spore at same stage as that shown in figs. 8 and 9; showing clean break of original spore wall. Part of spore concealed by grid.

Fig. 11. Longitudinal section of germ tube at a slightly older stage than those of figs. 8–10. Spore concealed by grid. Note presence of mitochondria and absence of nuclei at extreme tip, presence of nuclei further back, and flow lines or 'cortical' membranes in endoplasm.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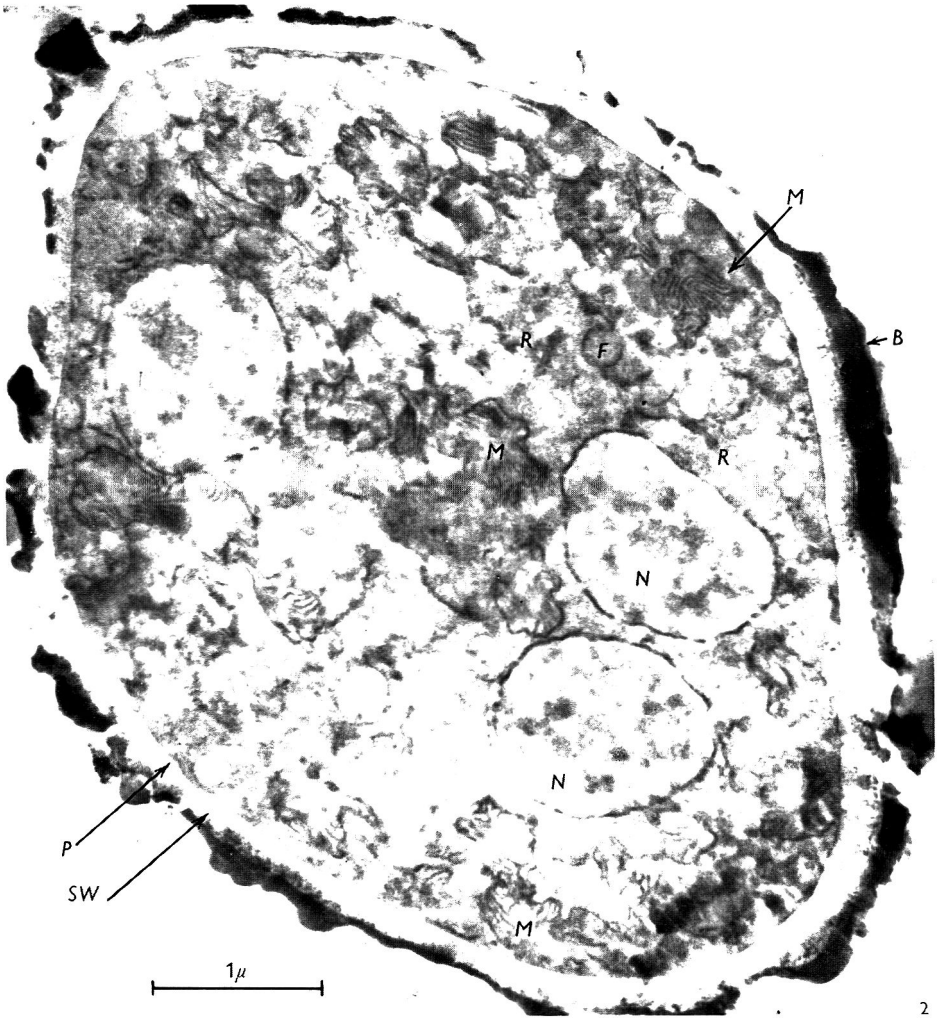
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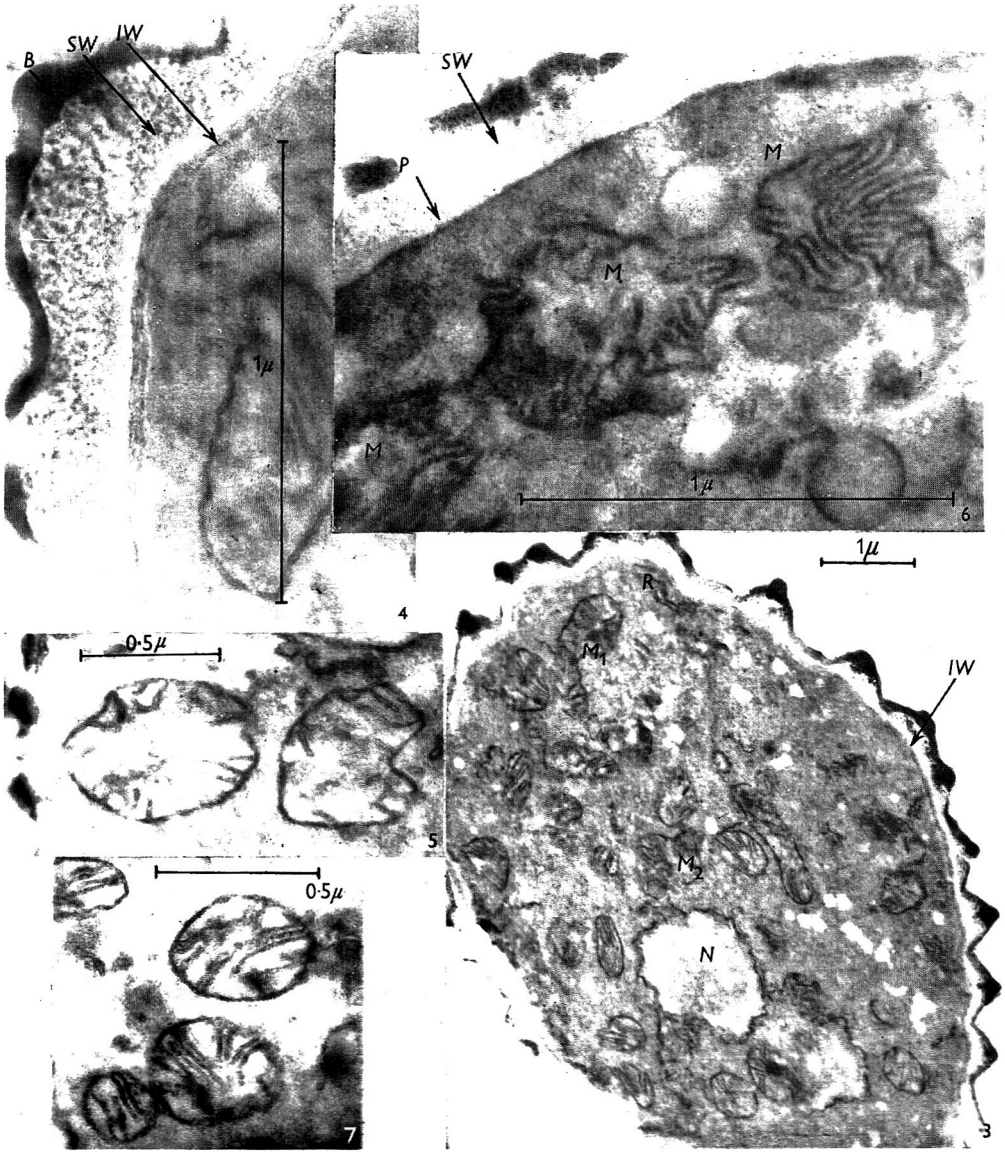
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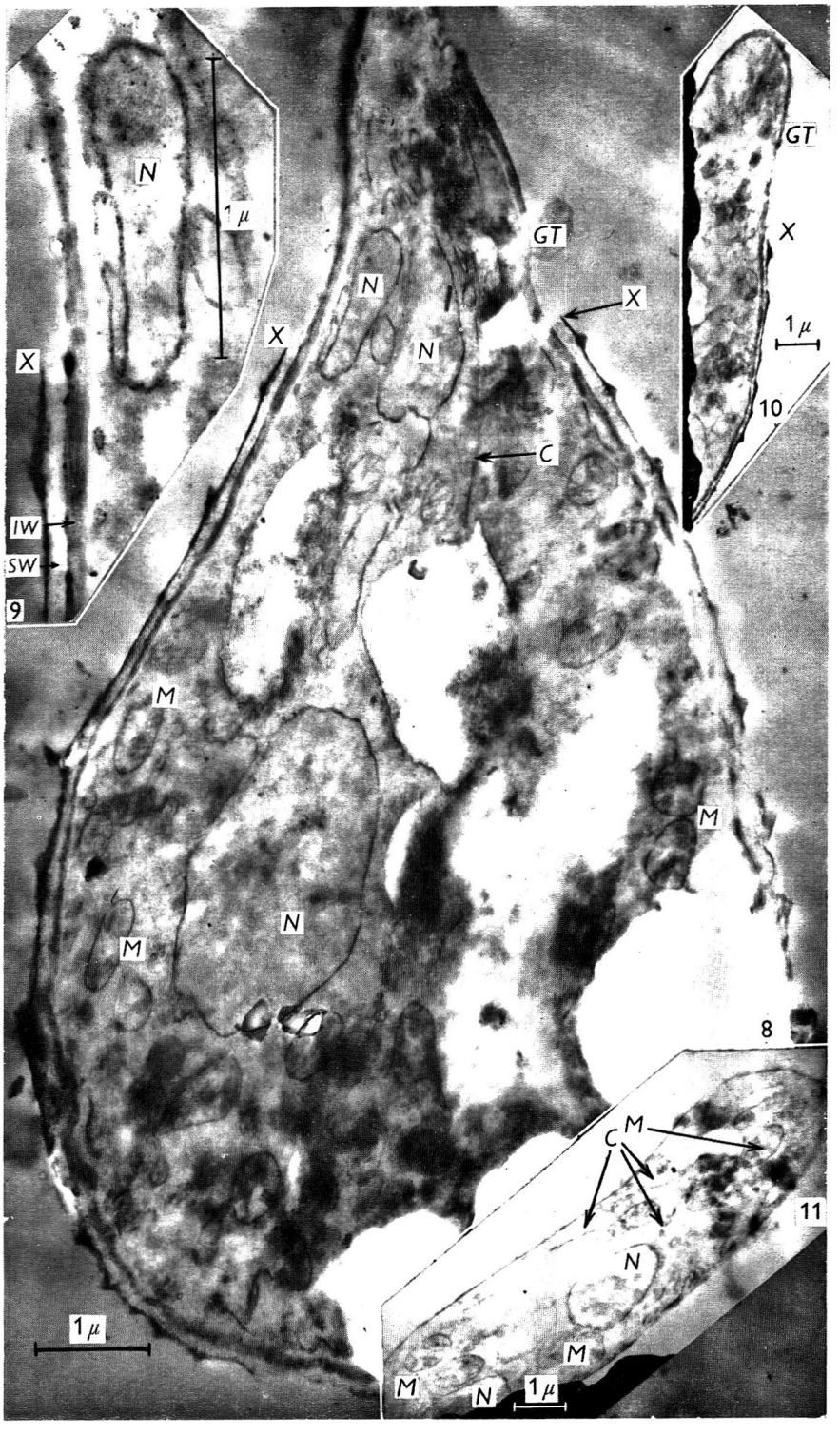
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Ribonucleic Acid of Chloramphenicol-Treated *Shigella flexneri*

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SUMMARY

Resting organisms of *Shigella flexneri* serotype 3 are able to synthesize soluble and ribosomal ribonucleic acid (RNA) in the presence of chloramphenicol. The antibiotic stimulates the synthesis of soluble RNA but has no apparent effect on ribosomal RNA production. In contrast, chlortetracycline, which also suppresses formation of protein, stimulates soluble RNA synthesis and inhibits ribosomal RNA synthesis. The soluble RNA of the chloramphenicol-treated organisms possesses amino acid accepting activity comparable to that of the soluble RNA of untreated organisms. The findings indicate that chloramphenicol does not promote the synthesis of biologically inactive soluble RNA. The stimulation of soluble RNA synthesis appears to be the result, rather than the cause, of the inhibition of protein production by the antibiotic.

INTRODUCTION

Protein synthesis in bacteria is inhibited markedly by chloramphenicol; in contrast, ribonucleic acid (RNA) synthesis is not affected and may even be stimulated (Gale & Folkes, 1953; Wisseman, Smadel, Hahn & Hopps, 1954). Since RNA plays a major role in the production of protein (Chantrenne, 1961), it has been postulated that the inhibitory action of chloramphenicol is the result of the formation of non-functional RNA (Gale, 1958; Ramsey, 1958). The results of some of the studies of the RNA of chloramphenicol-treated *Escherichia coli* suggest that this nucleic acid is 'abnormal' and perhaps physiologically inactive. Neidhardt & Gros (1957) showed that the RNA produced in the presence of chloramphenicol, 'chloramphenicol-RNA', was unstable, being degraded when the treated organisms were placed in chloramphenicol-free medium. Other workers have shown that chloramphenicol-RNA was present in ribonucleoprotein particles of sizes smaller than those present in untreated organisms (Dagley & Sykes, 1959; Nomura & Watson, 1959). Horiuchi, Horiuchi & Mizuno (1959) observed that, after being washed and resuspended in drug-free medium, chloramphenicol-treated *Escherichia coli* showed a lag period before beginning to synthesize protein. The authors conclude that chloramphenicol-RNA does not participate in protein synthesis.

Evidence that chloramphenicol-RNA is similar to the RNA of untreated organisms has been reported; Pardee & Prestidge (1956) found that the base composition of both types of RNA was the same. Aronson & Spiegelman (1961) showed that chloramphenicol-RNA was converted to a stable form when chloramphenicol-treated *Escherichia coli* was resuspended in a medium which permitted rapid protein formation. They also presented data which demonstrated that organisms con-

taining chloramphenicol-RNA produced protein without any detectable nucleic acid synthesis. The authors have proposed that chloramphenicol-RNA is not abnormal but is a normal unstable intermediate of ribonucleoprotein and that chloramphenicol blocks the conversion of the intermediate to a stable form.

We have made experiments designed to characterize further the synthesis of RNA in the presence of chloramphenicol and the biological activity of some of the RNA. A previous study showed that chloramphenicol stimulated RNA production in resting *Shigella flexneri* serotype 3 (Yee, Pan & Gezon, 1962). The present paper presents evidence which indicates that the soluble RNA synthesis and not the ribosomal RNA synthesis in this organism is stimulated by chloramphenicol and that the soluble RNA can bind amino acids. A preliminary report of some of the findings was published elsewhere (Yee, Gezon & McElligott, 1962).

METHODS

Cultivation and starvation of organisms. *Shigella flexneri* serotype 3 strain B-1003 (Pan, Yee & Gezon, 1957) was used. The previously described methods for growing, starving and harvesting the organisms in phosphate buffered saline (pH 6·9) to prepare resting suspensions (Yee, Pan & Gezon, 1962) were modified as follows, mainly to accommodate the large volume of organisms required: (1) a sufficient quantity of organism was obtained by growing them in flasks containing 3·5 l. defined medium; (2) each flask was seeded with 5 ml. of an 18 hr. starter culture which had been inoculated with a loopful of growth from a defined-medium slope culture; (3) cultures were aerated on a rotary shaker; (4) the defined medium with glucose as carbon and energy source and ammonium monobasic phosphate as nitrogen source did not contain nicotinamide since this was not needed for optimum growth of the organism (Pan, Yee & Gezon, 1962).

Treatment with chloramphenicol. Defined medium containing L-aspartic acid as sole nitrogen source was used to obtain maximum RNA synthesis. The starved organisms were resuspended in the medium described before (Yee, Pan & Gezon, 1962). Equal volumes of suspension were added to each of three flasks. One flask serving as a 0 hr., or unincubated, control was centrifuged immediately in a refrigerated Sharples supercentrifuge. Chloramphenicol was added to the second flask to final concentration 2 μg./ml.; the third flask was used as a chloramphenicol-free control. The latter two flasks were incubated at 37° on a rotary shaker for 4 hr. and the organisms then collected on a Sharples supercentrifuge and stored at −20°.

Separation of soluble and ribosomal RNA. A procedure based on the method of Tissières, Watson, Schlessinger & Hollingworth (1959) was used. The organisms were disrupted by grinding in a mortar with alumina A301 and extracted with 0·01 M-Tris(hydroxymethyl)aminomethane buffer (pH 7·4) containing 0·01 M-magnesium acetate to minimize degradation of ribosomal particles. Alumina, whole organisms and cell debris were removed by centrifugation at 6000g at 0°. The resulting crude extract was centrifuged at 100,000g for 120 min. to sediment ribosomal RNA. The upper third of the supernatant fluid was removed for analysis of soluble RNA; RNA and protein were precipitated with cold 5% (w/v) trichloroacetic acid. The RNA was extracted from the precipitates with hot trichloroacetic acid by the method of Gale & Folkes (1953) and measured by the orcinol reaction (Schneider, 1957) with adenosine-5′-monophosphate as standard.

Isolation of soluble RNA. Soluble RNA was isolated by phenol treatment of supernatant fluids of crude extracts obtained after centrifugation at 100,000*g* (Tissières, 1959). Polysaccharide was removed from the RNA preparations by treatment with 2-methoxyethanol (Kirby, 1956). The RNA was stored at −20°.

Amino acid accepting activity. The total amino acid accepting activity of the soluble RNA of untreated and of chloramphenicol-treated organisms was measured by the assay system of Berg & Ofengand (1958) using a ^{14}C algal protein hydrolysate as a source of labelled amino acids. After incubation the soluble RNA was re-isolated (Ofengand, Dieckmann & Berg, 1961) and its radioactivity measured in a Nuclear-Chicago counter equipped with a Micromil window. The ability of the soluble RNA preparations to accept individual amino acids was determined by the method of Brown (1960). The amino acid + RNA complexes were removed from the reaction system by charcoal (Norite A) adsorption and re-isolated by phenol treatment of the charcoal. The amino acids were released from the soluble RNA at pH 10 and separated by two-dimensional paper chromatography. Butanol + water + glacial acetic acid (60 + 25 + 15, by vol.), and water-saturated phenol containing 0·002 % 8-hydroxyquinoline in an atmosphere of ammonia, were used as solvents. Development in each solvent was for 24 hr. The amino acid spots were located with ninhydrin, cut out, eluted with 10 % (v/v) isopropanol in water and the radioactivity of the solutions measured.

RESULTS

Effect of chloramphenicol on the synthesis of soluble and ribosomal RNA. Experiments were made to determine whether soluble and/or ribosomal RNA synthesis was stimulated by chloramphenicol. Initially attempts were made to extract soluble RNA from dried organisms of untreated and chloramphenicol-treated *Shigella flexneri* with hot sodium dodecylsulphate by the method of Ofengand *et al.* (1961). However most of the RNA of the organisms was removed, indicating that ribosomal RNA also was present in the preparations. Fractionation of lysates of spheroplasts also was unsuccessful. The bulk of the cellular RNA was found in the supernatant fluids of the lysates after centrifugation at 100,000*g*.

A procedure which used alumina grinding to disrupt the organisms was found to be suitable. The organisms were able to synthesize both soluble and ribosomal RNA in the presence of chloramphenicol (Table 1). This finding is consistent with those reported for *Escherichia coli* (Nomura & Watson, 1959; Aronson & Spiegelman, 1961). In addition, soluble RNA production in resting *Shigella flexneri* was stimulated by chloramphenicol whereas ribosomal RNA production appeared to be unaffected. As a result, the chloramphenicol-treated organisms possessed low ribosomal RNA: soluble RNA ratios. It should be pointed out that the degree of RNA synthesis varied with different preparations; however, the ratios were relatively constant and reproducible.

The cell-wall and membrane fractions of the disrupted organisms were also examined. The RNA content of the fractions was less than 0·5 % of the total cellular RNA and no detectable increase occurred during the incubation of untreated or chloramphenicol-treated organisms.

Effect of chlortetracycline on RNA synthesis. Chlortetracycline also inhibits protein synthesis (Gale & Folkes, 1953). The action of this antibiotic on RNA synthesis

was compared to that of chloramphenicol. The results indicated that the observed effect of chloramphenicol on RNA synthesis was not a non-specific one, i.e. antibiotics which inhibited protein synthesis might not be similar in their action on

Table 1. *Effect of chloramphenicol on RNA synthesis by resting* Shigella flexneri *serotype* 3

Resting organisms were prepared by washing and starving freshly harvested organisms in phosphate buffered saline (pH 6·9). The organisms were harvested by centrifugation and resuspended in a solution of KH_2PO_4, Na_2HPO_4 and NaCl; the organism concentration was adjusted so that the suspension gave 10% light transmittance at 425 mμ with a light path of 18 mm. in a Coleman model 6B spectrophotometer. Two volumes of the suspension were mixed with one volume of a solution containing L-aspartic acid as sole nitrogen source, D-glucose and $MgSO_4.7H_2O$. The final concentration of all compounds was the same as that in the defined medium used for growth of the organisms. This suspension containing *c.* $2·5 \times 10^8$ resting organisms/ml. was incubated on rotary shaker at 37°.

		Expt. 1			Expt. 2		
Organism preparation	Cell fraction	RNA content*	Net increase	Ribosomal:soluble	RNA content	Net increase	Ribosomal:soluble
0 hr. (before incubation)	Ribosomal†	104	—	2·9:1	75	—	2·4:1
	Soluble	36	—		31	—	
	Total	140	—		106	—	
Incubated 4 hr. without chloramphenicol	Ribosomal	127	23	3·2:1	106	31	3·2:1
	Soluble	39	3		33	2	
	Total	166	26		139	33	
Incubated 4 hr. with 2 μg. chloramphenicol/ml.	Ribosomal	119	15	1·8:1	121	46	2·0:1
	Soluble	65	29		60	31	
	Total	184	44		181	77	

* Expressed as mμ mole RNA nucleotide/ml. organism suspension.

† Calculated value (total minus soluble). Total and soluble RNA were determined by chemical analysis.

Table 2. *Effect of antibiotics on protein and RNA synthesis by* Shigella flexneri *serotype* 3

Antibiotic concentration (μg./ml.)	Change in amount of synthesis (%)	
	Protein	RNA
Chloramphenicol		
0·5	−24*	+18*
1	−41	+36
2	−76	+68
Chlortetracycline		
0·1	−15	+92
0·25	−71	+54
0·5	−94	+12

* −, Decrease; +, increase.

RNA synthesis. The effect of chlortetracycline on protein and RNA synthesis is shown in Table 2; the action of chloramphenicol is shown for comparison. Concentrations of chlortetracycline less than the minimum required for complete inhibition of protein synthesis and cell multiplication stimulated RNA synthesis. As the chlortetracycline concentration was increased the degree of stimulation

decreased, contrary to the finding with chloramphenicol. Also, chlortetracycline not only stimulated soluble RNA synthesis but also decreased the production of ribosomal RNA (Table 3).

Table 3. *Effect of chlortetracycline on RNA synthesis by resting* Shigella flexneri *serotype* 3

Organism preparation	Cell fraction	Net increase, mμ mole RNA nucleotide/ml. suspension
Incubated 4 hr. without antibiotic	Ribosomal	105
	Soluble	17
	Total	122
		(3·8:1)*
Incubated 4 hr. with antibiotic	Ribosomal	55
	Soluble	50
	Total	105
		(1·8:1)

* Numbers in parentheses represent ratio of ribosomal RNA to soluble RNA.

Organism suspension and incubation same as in Table 1. Chlortetracycline concentration, 0·5 μg./ml.

Table 4. *Amino acid accepting ability of soluble RNA of untreated and chloramphenicol-treated* Shigella flexneri *serotype* 3

	RNA preparations		
Source of soluble RNA	I	II	III
Untreated organisms	1877*	2400	1833
Chloramphenicol-treated organisms	2163	2300	1900

* Counts/min./μmole RNA nucleotide.

Table 5. *Amino acid accepting ability*

	Source of soluble RNA	
Amino acid	Untreated organisms	Chloramphenicol-treated organisms
Leucine, Isoleucine, Phenylalanine	41·2*	39·0
Tyrosine	19·3	20·6
Arginine	8·0	8·6
Valine	3·9	5·0
Glutamic	5·0	5·8
Aspartic	4·7	4·6
Histidine	3·9	3·5
Alanine	4·0	4·4
Glycine	2·5	2·0
Serine	2·0	1·8
Proline	2·0	1·9
Threonine	1·8	1·5
Lysine	1·7	1·5

* Percentage of total count.

Amino acid accepting ability. Soluble RNA mediates protein synthesis by binding amino acids and transporting them to the ribosomes where peptide bonding occurs (Chantrenne, 1961). The production of non-functional RNA may cause diminution of protein synthesis. Eaton & Caffrey (1961) reported that the soluble RNA formed by *Escherichia coli* in the presence of dihydrostreptomycin was unable to accept amino acids. It seemed feasible to test the soluble RNA produced by chloramphenicol-treated *Shigella flexneri* (soluble chloramphenicol-RNA). As a working hypothesis it was assumed that the stimulation of soluble RNA synthesis by chloramphenicol might be an indication that the nucleic acid was 'abnormal' and physiologically inactive. As shown in Table 4, the soluble chloramphenicol-RNA had a total amino acid accepting ability which was comparable to that of the soluble RNA of untreated organisms. Similar results were obtained when the accepting ability for individual amino acids was determined (Table 5). These findings indicate that chloramphenicol does not induce the formation of nonfunctional soluble RNA in *S. flexneri* serotype 3.

DISCUSSION

The conclusion that chloramphenicol stimulates soluble RNA synthesis is based on the observation that resting chloramphenicol-treated *Shigella flexneri* had a higher soluble RNA content than untreated resting organisms. Alternative explanations which might be given for this finding can be excluded. The resting suspensions were prepared by washing and starving the organisms in phosphate buffer saline. This solution was used in an earlier study in which the stimulation of total RNA synthesis in *S. flexneri* by chloramphenicol was shown (Yee *et al.* 1962*a*). The starvation of the organisms in a magnesium-free medium may have resulted in a lower ribosomal RNA:soluble RNA ratio, indicating that degradation of ribosomes may occur. This did not affect the results. Chloramphenicol-treated organisms which had been washed and starved in a magnesium-containing solution to maintain the integrity of the ribosomes also had a higher soluble RNA content than untreated organisms (Yee *et al.* 1962*b*).

The use of alumina grinding to disrupt the organisms may have introduced an error. Aronson & Spiegelman (1961) reported that the use of this method to disrupt *Escherichia coli* resulted in the release of some of the ribosomal chloramphenicol-RNA into the soluble fraction. In addition, the observed increase in the soluble RNA of chloramphenicol-treated *Shigella flexneri* may be attributed to degradation of the ribosomal RNA in the organisms as a result of exposure to the antibiotic, or to the inability of the treated organisms to assemble newly formed ribosomal RNA subunits. However, the amino acid accepting activities of soluble chloramphenicol-RNA and soluble RNA of untreated organisms are similar. The above alternative explanations would require the unlikely assumption that functional soluble RNA is a degradation product or a precursor of ribosomal RNA. Lacks & Gros (1959) were unable to show any conversion of soluble RNA to ribosomal RNA. On the basis of this finding and the difference in the base composition of the two types of RNA, they concluded that soluble RNA is not a precursor of ribosomal RNA. The difference in base composition also casts doubt on functional soluble RNA as being a degradation product of ribosomal nucleic acid. It thus seems likely that chloramphenicol stimulates soluble RNA synthesis in resting *S. flexneri* serotype 3.

The demonstrated activity of soluble chloramphenicol-RNA suggests that the stimulatory action is the result, rather than the cause, of the blockage of protein synthesis by the drug. A suppression of protein production may result in the channelling of available nitrogen into RNA (Fraenkel & Neidhardt, 1961). The results thus indicate it is doubtful that chloramphenicol inhibits protein synthesis by inducing the production of physiologically inactive 'abnormal' RNA or by altering the activity of RNA.

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