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THE JOURNAL OF GENERAL MICROBIOLOGY

The *Journal* will publish accounts of original research in general microbiology, i.e. the study of bacteria, microfungi, microscopic algae, protozoa, and viruses in their biological activities and, more particularly, the fundamental aspects of the study of these forms, including structure, development, physiology, genetics, cytology, systematics and ecology. Writers of papers on a specialized aspect of their subject should describe their work so that its relevance to their own science and to microbiology in general will be apparent to readers who may be unfamiliar with the particular aspect.

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'Easy reading's curst hard writing.'—*The Editors, J. gen. Microbiol.*

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References. References in the text are cited thus: Brewer & Stewer (1942), (Brewer & Stewer, 1942). Where a paper to be cited has more than two authors, the names of all the authors should be given when reference is first made in the text, e.g. (Brewer, Stewer & Gurney, 1944), and subsequently as (Brewer *et al.* 1944); but papers with more than four authors may be cited, e.g. (Cobley *et al.* 1940) in the first instance. Where more than one paper by the same author(s) has appeared in one year the references should be distinguished in the text and the bibliography by the letters *a*, *b*, etc. following the citation of the year (e.g. 1914*a*, 1914*b*, or 1914*a*, *b*).

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Description of Solutions The concentrations of solutions are preferably defined in terms of normality (N) or molarity (M). The term '%' must be used in correct sense, i.e. g./100 g. of solution. For 'per cent of volume', i.e. ml./100 ml., the term '% (v/v)' should be used, and for weight of a substance in 100 ml. of solution, the term '% (w/v)'.

Proprietary Substances and Materials. At first mention, the correct designation of a manufacturer should be given in the text, with address to enable other workers to obtain the product mentioned.

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Nomenclature and Descriptions of Micro-organisms. Binomial Latin names of micro-organisms, the generic name only with a capital, must be used in accordance with International Rules of Nomenclature; in full at the first mention in each paragraph and in the Summary but in subsequent mention with the generic name abbreviated. Single initial letter abbreviations are used where they are not ambiguous. Binomials should be underlined in the typescript. Scientific epithets or trivial names are not underlined and should be without capitals.

Descriptions of new species of cultivable microbes should not be submitted unless an authentic specimen of a living culture has been deposited in a recognized culture collection.

The word 'generation' should not be used synonymously with 'subculture'. For an agreed use of terms like strain, type, variant, phase, etc., see the International Bacteriological Code of Nomenclature, Section 1, Rules 7 and 8.

Except for good reasons, micro-organisms should be designated by the names used in the works listed below. When other authorities are followed, they should be cited whenever obscurity might result from their use.

MICROFUNGI. *Ainsworth & Bisby's Dictionary of the Fungi*, 1961, 5th ed. (Kew: Commonwealth Mycological Institute.)

PLANT PATHOGENIC FUNGI AND PLANT DISEASES. *List of Common British Plant Diseases*, 1944. (Cambridge University Press.)

PLANT VIRUSES AND VIRUS DISEASES (1957). *Rev. appl. Mycol.* **35**, Suppl. 1–78.

BACTERIA. Author's references in naming are at present accepted provided that the designation is unambiguous and conforms with the International Bacteriological Code of Nomenclature (1949; *J. gen. Microbiol.* **3**, 444) and the Opinions issued by the International Committee on Bacteriological Nomenclature. If desired, a synonym may be added in brackets when a name is first mentioned.

CORRIGENDA

The Cellular Location of Antigens in Streptococci of Groups D, N and Q.
By D. G. Smith and the late P. M. Frances Shattock (1964, **34**, 165–175).

Page 165, last sentence of paragraph 3 of Introduction should read:

Because of some difficulties in preparing true protoplasts by using lysozyme a phage-associated cell-wall lysin (Bleiweis & Zimmerman, 1961) was also used in the present work.

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The Discovery, Isolation, and Classification of Various α-Haemolytic Micrococci which Resemble Aerococci

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(*Received* 10 *June* 1963)

SUMMARY

This work describes the identification and classification of a hitherto unknown group of α-haemolytic micrococci isolated from bottles containing dregs of fluid medicaments. The majority of the strains were both catalase-positive and nitratase-positive. In view of several similarities between these bacteria and the aerococci described by Shaw, Stitt & Cowan (1951), and later by Williams, Hirch & Cowan (1953), it is proposed that both groups should be incorporated in a new bacterial family, Aerococcaceae, despite the fact that the aerococci are catalase-negative and nitratase-negative. The suggestion that a new family should be established is prompted by the many dissimilarities between both the aforementioned groups and the representatives of the families to which they are closest according to the Bergey system. The new family ought to include one genus, *Aerococcus*, and two species, one of which, *A. viridans*, should comprise catalase-negative and nitratase-negative strains, and the other, *A. catalasicus*, strains which are obligate catalase-positive and may also be nitratase-positive.

INTRODUCTION

In the course of investigations made into the microbial flora found in the dregs of fluid medicaments in bottles handed in at three Oslo pharmacies in exchange for new medicines, I isolated α-haemolytic micrococcus strains which, in their haemolytic, cultural and morphological properties, appear to be akin to the α-haemolytic micrococci described by Shaw *et al.* (1951). It was proposed by Williams *et al.* (1953) that these bacteria, which are generally present in air and dust, should form a new genus, *Aerococcus*, with one species, *A. viridans*. Williams *et al.* thought that the genus *Aerococcus* was more closely related to the streptococci than to the micrococci, and that it should be placed in the same family as the streptococci. They referred to the similarity between aerococci and pediococci, but contended that the difference between these two groups was so marked that they should not be put in the same genus.

Deibel & Niven (1960) found *Aerococcus viridans* to be identical, or very similar, to *Gaffkya homari* and proposed that both these micro-organisms should be included in a single species in the genus *Pediococcus*, since there are several characteristics common to these two species and the pediococci.

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Tjeltveit & Clausen (1954) have recently shown *inter alia* that the strains of α-haemolytic micrococci and a strain of *Aerococcus viridans* are homofermentative and produce lactic acid when a sufficient number of active bacterial cells are present during conversion of the glucose. The specific rotation of the lactic acid formed was not investigated.

Cultural and morphological characteristics of the α-haemolytic micrococcus strains

Because the growth of these bacterial strains on solid media, such as ordinary agar and blood agar medium, resembles that of enterococci, they were at first considered to be α-haemolytic species of *Streptococcus faecalis*. This assumption was borne out by their capacity for growth on 40 % ox bile blood agar, in 6·5 % sodium chloride beef-infusion peptone phosphate broth, and in the same broth with a normal content of sodium chloride (0·3 %) but with a pH value of 9·6. However, microscopic examination of ordinary broth cultures failed to reveal any true chain formation. The cocci were comparatively large, being approximately 1–2 μ in diameter, spherical or slightly irregular in shape, though not elongated; they occurred singly, in pairs, and in micrococcus-like formations. They were Gram-positive. Eleven culturally similar α-haemolytic micrococcus strains were isolated from the medicine bottles and these strains were subjected to detailed study.

The α-haemolytic strains develop at 22°, 30°, and 37°; growth is poorest at 22°, best at 37°. On 5 % citrated horse blood agar the size of the colonies ranges from 0·5 to 2 mm. diam. after 18–24 hr of incubation at 37°. They are convex in shape and vary in colour from grey to white. On one occasion the colonies were clear, transparent, and formed 'craters' when they aged, as do pneumococcus colonies. The α-haemolytic micrococci formed colonies with green zones on 'chocolate' agar. Aerobic cultivation for 24 hr at 37° in beef-infusion peptone phosphate broth and in the same broth with 1 % glucose resulted in sparse growth and relatively meagre sediment. When grown in broth with a petroleum jelly seal, all strains showed slighter growth with the formation of a little sediment after 24 hr incubation at 37° than when cultured aerobically. All strains grew well in modified HS-medium with 10 % normal horse serum (Clausen, 1956), but the best growth occurred in the upper third of the medium. When a comparison was carried out using the type strain *Aerococcus viridans* NCTC 8251, it was found that this—compared with the eleven α-haemolytic micrococcus strains—produced relatively weaker growth and a granular sediment in the aforementioned media.

The α-haemolytic micrococci were examined according to the methods described below.

METHODS

Formulae are not given for media the composition of which must be assumed to be generally known. Unless otherwise stated, incubation was carried out at 37°. All cultures were incubated aerobically.

Optimum temperature for growth. Blood agar plates were prepared by addition of 5 % citrated horse blood to a medium consisting of 1 % peptone (Danish, Orthana Bacteriological Brand), 0·3 % NaCl, 0·2 % Na_2HPO_4, and 1·8 % agar (Japanese, quality Kobe I) in aqueous beef-infusion (pH = 7·4). Plates were poured to a

constant depth, about 4 mm. Each individual strain was subcultured to three blood agar plates; one was incubated at 22°, the others at 30°, and 37°. Growth was recorded after 24 and 48 hr.

Morphology. Unstained wet preparations of 24 hr cultures of beef-infusion peptone phosphate broth with and without 1 % glucose were examined. Gram-preparations of the broth and blood agar cultures were examined.

Haemolysis was tested on 5 % citrated horse blood agar after 24 and 48 hr incubation.

Ox bile resistance was studied by inoculation on to 40 % ox bile blood agar (40 % autoclaved ox bile, 8 % defibrinated horse blood, and 52 % agar medium with 2·6 % agar, quality Kobe I) and incubation for 20 hr.

Tellurite resistance was investigated by inoculating the strains on to McLeod's blood agar containing 0·04 % potassium tellurite. Growth or lack of growth was recorded after 20 hr incubation.

Thermoresistance was tested by heating a 20 hr beef-infusion peptone phosphate broth culture at 60° (in a water bath) for 30 min., cooling, and subculturing to new broth and blood agar. The media were incubated for 48 hr before the results were recorded. Each broth culture to be tested was drawn sufficiently far up a Pasteur pipette to enable the ends of the pipette to be sealed without heating the cultures. The pipette was then plunged so deeply into a water bath at 60° that the entire section moistened with culture was submerged.

Growth tests were carried out in beef-infusion peptone phosphate broth with 6·5 % NaCl or with a pH of 9·6; incubation period, 72 hr.

Methylene-blue reductase was tested in milk culture to which had been added 0·1 % methylene blue. The reduction or lack of reduction was recorded after 20 and 48 hr and finally after 5 days' incubation.

Reduction prior to coagulation of litmus milk was observed after 12 and 24 hr, and daily for 5 days.

Utilization and acid formation of certain sugars, alcohols and glycosides. 0·5 % (aesculin 0·2 %) of the following sugars, etc., were added to peptone water + 5 % normal horse serum + 1 % Andrade's indicator: lactose, glucose, maltose, sucrose, raffinose, galactose, xylose, mannitol, dulcitol, glycerol, starch, dextrin, salicin, aesculin. Incubation period: 14 days.

Proteolytic activity: ability to liquefy gelatin was tested in stab cultures incubated at 22° for 30 days.

The ability to liquefy inspissated ox serum was determined by incubation at 22° for up to 14 days.

Formation of catalase. 10 % H_2O_2 was added to 24 hr agar slant cultures and observed for up to 5 min. to determine whether gas was formed. Control tests were performed on non-inoculated agar slant media after 24 hr incubation.

Formation of nitratase. Four-day cultures in nitrate broth were tested by adding Griess–Ilosvay reagent. Negative tests were checked by adding powdered zinc to prove that there was nitrate left in the culture medium. A surplus of nitrite in a culture can 'conceal' a positive reaction by decolorizing the red stain; for this reason the test was performed daily for up to 4 days (Shaw *et al.* 1951).

Formation of plasmacoagulase. Tests for this enzyme were performed by mixing equal parts of 24 hr broth culture and a 1/10 dilution of citrated human plasma in

sterile saline solution, followed by incubation in a water bath at 37° for 4 hr and storage overnight at room temperature.

Formation of H_2S. Stab cultures in lead acetate agar were incubated for 14 days.

Hydrolysis of arginin in broth accompanied by formation of NH_3 was demonstrated by mixing equal parts of Nessler's reagent and 48 hr arginine-containing broth culture.

Hydrolysis of sodium hippurate (1 %) in broth into sodium benzoate after 5 days' cultivation was determined by means of 12 % $FeCl_3$ solution with 0·2 % concentrated HCl (Roemer, 1948).

Hydrolysis of urea. Christensen (1946) urea medium with indicator was used to demonstrate formation of NH_3 by urease activity. Incubation period: 14 days.

Methyl red (MR) and Voges-Proskauer (VP) tests were carried out with the following incubation periods and temperatures: MR test: 4, 5 and 7 days at 30°. VP test: 3 and 4 days at 30°.

Determination of final pH in 1 % glucose beef-infusion peptone phosphate broth (pH = 7·4) after 14 days. The pH was measured with a Beckman pH meter.

The following bacteria were used in certain tests as reference species: *Aerococcus viridans* NCTC 8251, *Pediococcus cerevisiae* NCTC 8066 and *P. acidilactici* NCIB 6990.

RESULTS

Most of the results of these investigations are given in Table 1. Comments on the results not tabulated are given below.

The test for tellurite resistance gave varying results, as one or two of the strains were tellurite-resistant, but the majority were not, or showed only traces of growth on tellurite medium. This test would appear to be of no significance in classifying these bacteria.

Growth in broth with 6·5 % NaCl was investigated with a positive result on all micrococcus strains as well as on *Aerococcus viridans.*

The test for hydrolysis of sodium hippurate gave positive results in the case of eight of the eleven strains examined, and two showed traces of hydrolysis. The test was not performed on aerococci.

In tests not recorded in the tables, the α-haemolytic micrococcus strains did not form soluble haemolysin, they were non-pathogenic when injected intraperitoneally into white mice, and did not precipitate with streptococcus antisera of group D (Clausen, 1961).

The α-haemolytic micrococci proved biochemically different from *Aerococcus viridans* in the following ways. (The biochemical properties of the aerococcus strains were determined by Williams *et al.* 1953, and Shaw *et al.* 1951).

(1) *Reduction prior to coagulation of litmus milk.* Five of eleven strains of α-haemolytic micrococci reduced litmus in milk prior to coagulation, whereas none of 29 aerococcus strains did so.

(2) *Acid production from raffinose.* All α-haemolytic micrococci utilized this carbohydrate, whereas only four of twelve aerococci did so.

(3) *Acid production from mannitol.* Only one of eleven α-haemolytic micrococcus strains utilized this alcohol, whereas five out of twelve aerococci are reported to have done so.

(4) *Liquefaction of inspissated ox serum.* Nine out of eleven α-haemolytic micrococcus strains liquefied inspissated ox serum, whereas none of 29 aerococcus strains did so.

(5) *Formation of catalase.* Eight out of eleven α-haemolytic micrococcus strains were catalase-positive, the majority being relatively strong formers of catalase.

Table 1. *Cultural and biochemical properties of a group of α-haemolytic micrococci compared with various strains of Aerococcus viridans*

\+ denotes positive reaction: 5 or more positive strains out of 11 or 12 tested. − denotes negative reaction: up to 4 positive strains out of 11 or 12 tested.

	α-Haemolytic micrococci	No. positive out of 11 strains tested	*A. viridans*	No. positive out of 12 strains tested*
Optimum temperature for growth 37°	+	11	+	66 %†
α-Haemolytic on blood agar	+	11‡	+	12
Growth on 40 % ox bile agar	+	11	−	12
Resist 60° for 30 min.	+	11	−	12
Growth in broth at pH 9·6	+	11	−	11
Reduction of 0·1 % methylene blue in milk	−	1 (2)§	−	0
Reduction prior to coagulation of litmus milk	+	5‖	−	0†
Utilization of				
Lactose	+	9	−	10
Glucose	+	11	+	12
Maltose	+	11	+	12
Saccharose	+	11	+	12
Raffinose	+	11	−	4
Galactose	+	11	+	97 %†
Xylose	−	0	+	41 %†
Mannitol	−	1	+	5
Dulcitol	−	0	−	0†
Glycerol	+	9	+	97 %†
Starch	−	4	−	0¶
Dextrin	−	4	−	38 %†
Salicin	+	8	+	83 %†
Aesculin	+	8	+	12¶
Gelatin liquefaction	−	0	−	0
Liquefaction of inspissated ox serum	+	9	−	0†
Catalase formation	+	8	−	0
Nitratase formation	+	6 (7)§	−	0†
Plasma coagulase formation	−	0	−	0
H_2S formation	−	0	−	0
Hydrolysis of arginine	−	0	−	0
Hydrolysis of urea	−	0	−	0
MR positive	−	0**	+	93 %†
VP positive	−	0	−	0
Final pH	5·2–5·6	11	5·5–5·8	12

* Most of these data are from Williams *et al.* (1953).

† According to Shaw *et al.* (1951): the percentage positive out of 29 strains tested.

‡ From distinctly α-haemolytic to faintly α′-haemolytic.

§ The figures in parentheses include also strains which show weak positive reactions.

‖ Slow reduction.

¶ Examined in solid media.

** The negative MR results all showed weak to very weak pink, but they were distinctly different from a positive reaction.

Only one of the catalase-forming strains may be described as weakly positive, and one as a moderate to weak catalase former. These two strains did not reduce nitrate to nitrite. The aerococci are reported not to form catalase.

(6) *Formation of nitrite from nitrate.* Six (+1 uncertain) out of the eleven α-haemolytic micrococcus strains formed nitrite from nitrate, whereas none of 29 aerococcus strains did so.

Differences between the α-haemolytic micrococci, Aerococcus viridans and the pediococci

The α-haemolytic micrococci and *Aerococcus viridans* have been compared with *Pediococcus cerevisiae* and *P. acidilactici* with the aid of various growth and resistance tests, which gave the following results: the α-haemolytic micrococci and *A. viridans* did not grow at 37° (14 days) or 25° (30 days) in the pediococcal media, unhopped wort, yeast-water with autolysed yeast, or Bettge & Heller's beer with starch (Jörgensen, 1956*a*), the last two media containing 2·5 % (v/v) alcohol, whereas both pediococci grew well in all three media. The α-haemolytic micrococci and the aerococcus were also MR and VP negative, whereas the pediococci were highly MR positive. On the other hand, only *P. cerevisiae* (not *P. acidilactici*) produced acetyl methyl carbinol. Moreover, the α-haemolytic micrococci and the aerococcus grew in 6·5 % NaCl broth and in broth with a pH of 9·6. Neither of the pediococci developed in these two media; this applied both when incubated at 37° for 5 days and when incubated at 25° for 15 days. Furthermore, the pediococci were not α-haemolytic when grown on blood agar (*P. cerevisiae* may at the most produce a very weak greening reaction), they have a much lower final pH value (cf. the strong MR positive reaction) than the α-haemolytic micrococci and the aerococci (Williams *et al.* 1953), and they form more typical microaerophilic colonies when grown under aerobic conditions on ordinary agar medium and blood agar. One species, *P. cerevisiae*, had a growth-optimum at a comparatively low temperature between 18 and 25° (Jörgensen, 1956*b*).

DISCUSSION

While it has been possible to demonstrate appreciable differences in the biochemical properties of the α-haemolytic micrococci investigated and the aerococci described by Shaw *et al.* (1951) and Williams *et al.* (1953), it has also been shown that there is a great similarity between them, culturally and morphologically, and in regard to many biochemical criteria, especially to the tolerance tests: growth in broth with a pH of 9·6, growth on 40 % ox bile blood agar, and resistance to 60° for 30 min. On the other hand the aerococci, unlike the α-haemolytic micrococci, are reported to be negative with regard to several other properties.

The formation of catalase is probably the most important difference between the α-haemolytic micrococci and the aerococci, and eight out of eleven of the former strains are catalase formers, whereas the aerococci are reported to be catalase negative. *Aerococcus viridans* (NCTC 8251) showed a negative or at the most a slight trace of a positive reaction. As the formation of catalase is a property characteristic of micrococci, but invariably absent from streptococci, a distinction should be made between catalase-positive and catalase-negative strains of the α-haemolytic micro-

cocci. The three catalase-negative strains investigated differed very little in other biochemical properties from the rest of the strains. However, they were nitrite-negative, as is *A. viridans*. A minor difference between them and the one aerococcus strain with which they have been compared (*A. viridans* NCTC 8251) was their formation of denser white colonies on blood agar; the α-haemolysis was weaker in the case of two of the strains, and they also appeared by microscopic examination to be somewhat larger in fluid medium. Moreover, *A. viridans*, unlike the α-haemolytic micrococcus strains, formed granular sediment in broth cultures.

Viewed in the light of these properties, the micrococcus strains investigated may be said to be closely akin to the aerococci, but the catalase-positive and nitratase-positive strains were not identical with the aerococcus species *Aerococcus viridans* proposed by Williams *et al.* (1953).

All the α-haemolytic micrococci investigated and also the *Aerococcus viridans* strain examined were homofermentative lactic-acid formers (Tjeltveit & Clausen, 1964), but a reference staphylococcus strain, *Staphylococcus epidermidis*, was also found to have the same property. It may be mentioned that Orla-Jensen (1919) found that *S. aureus* formed optically inactive or laevorotatory, and *Micrococcus caseolyticus* dextrorotatory lactic acid from D-glucose, so that the fermentative powers of the strains cannot be regarded as decisive for their classification, either. Morphologically the bacterial strains were not streptococci, and it is obvious that the aerococci and the other catalase-negative micrococci lack the conclusive criteria for classification within the enterococcus group, the only group they really can be compared with in the family Lactobacillaceae. The catalase-positive strains cannot be classified in any genus under Tribe I, Streptococceae, *Bergey's Manual* (1957) as all the genera within the Tribe are catalase-negative. From these comparative studies of the α-haemolytic micrococci and of *A. viridans* on the one hand and of the pediococci on the other, it was found that the difference between the two groups is so great that there is no justification for including the first group in the genus *Pediococcus*. Nor, without further study, can it be decided whether *Gaffkya homari*, which is of marine origin, is identical with the air and dust bacterium *A. viridans*, as proposed by Deibel & Niven (1960).

The α-haemolytic micrococci can be classified neither in the genus *Leuconostoc*, which includes only heterofermentative species, nor in the genus *Peptostreptococcus*, which includes only anaerobes. These α-haemolytic micrococci differ, however, to such an extent from the representatives of the genera in the family Micrococcaceae, that they do not appear to belong here either. The genera of this family do not include α-haemolytic species. Deibel & Niven (1960) found that *Gaffkya homari* produced a strong greening reaction on sheep blood agar, whereas *Bergey's Manual* (1957) describes this bacterium as β-haemolytic on human blood agar. The only genus which might be considered, viz. *Micrococcus*, comprises only species which differ markedly from the bacteria being considered.

It is proposed to establish a new family in the Order Eubacteriales, because of the great specificity of the bacterial group in question. It is now suggested that the species *Aerococcus viridans*, which is catalase-negative, should be supplemented by a new, catalase-positive species. As the generic name of *Aerococcus* has formerly been used, this name should be retained, and the same designation should be incorporated in the new family name.

In the light of the foregoing data it is proposed that the following family, genus, and species names should be given to the bacteria:

Family	Aerococcaceae fam.nov.
Genus I	*Aerococcus* Williams *et al.*
Species 1	*Aerococcus viridans* Williams *et al.* (Catalase-negative and nitratase-negative)
Species 2	*Aerococcus catalasicus* sp.nov. (Catalase-positive and possibly also nitratase-positive)

Further details of the family, generic, and specific properties will be evident from the foregoing.

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An Investigation into the Ability of Certain α-Haemolytic Micrococci to Form Lactic Acid from D-Glucose

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SUMMARY

The ability of some α-haemolytic micrococci described by Clausen (1964) to form lactic acid from glucose has been determined by means of 'resting' bacterial cells. The total quantity of lactic acid was measured in each individual test. Reference organisms used were a strain of *Aerococcus viridans*, 2 enterococcus strains, and 1 strain of *Staphylococcus epidermidis*. Glucose conversion was found to be in large measure dependent upon the concentration of active bacterial cells in the suspension. All the α-haemolytic micrococcus strains tested, together with the reference strains, proved to be homofermentative or near homofermentative formers of lactic acid, provided that the number of active organisms was brought to a sufficiently high level in the suspension when converting the glucose.

INTRODUCTION

This work constitutes a supplement to an investigation by Clausen (1964) into localization, identification, and classification of eleven strains of α-haemolytic micrococci and deals with their formation of lactic acid. Culturally and morphologically these bacterial strains appear to be closely akin to the α-haemolytic micrococci described by Shaw, Stitt & Cowan (1951), and, subsequently, by Williams, Hirch & Cowan (1953), who named them aerococci. In regard to important biochemical properties, however, most of Clausen's strains differ from these aerococci.

This paper includes a description of the method employed to determine the formation of lactic acid from glucose by the micrococci and aerococci and various reference bacteria. Conversion of glucose was carried out by means of 'resting' bacterial cells (Quastel & Whetham, 1924). The total quantity of lactic acid was measured in each experiment, but further investigation into the specific rotation of the acid was not made.

METHODS

Bacteria. The eleven α-haemolytic micrococci strains and the following reference strains: *Aerococcus viridans* (NCTC 8251), *Streptococcus faecalis*, *S. faecalis* var. *zymogenes*, and *Staphylococcus epidermidis*.

Media, culture, and pre-treatment

One solid medium was used, 5% citrated horse blood agar, which consisted of 1% peptone (Danish, Orthana Bacteriological Brand), 0·3% NaCl, 0·2% Na_2HPO_4

and 1·8% agar (Japanese, quality Kobe I) in ordinary aqueous beef infusion (pH = 7·4).

The broth used was Pope trypsin broth prepared according to Graudal (1955).

All cultures were incubated aerobically at 37°.

Method a

Before use the bacterial strains were subcultured daily for 3 days on blood agar. A small inoculum (platinum loop) of strains 1–7 inclusive and the reference bacteria *Aerococcus viridans*, *Streptococcus faecalis*, and *S. faecalis* var. *zymogenes* (see Table 1) all of which grew well in Pope trypsin broth, was added to 50 ml. of trypsin broth: after 12 hr 45 ml. of the culture were centrifuged (Graudal, 1955), the sediment washed three times in 15 ml. sterile saline, and the bacteria resuspended in 3 ml. of the same liquid. Strains 8–11 were also examined by this method.

The results are set out in Table 1 and in the lines marked 'a' in Table 2.

Table 1. *Transformation of glucose into lactic acid by the α-haemolytic micrococcus strains which grew well in trypsin broth*

Strains	mg. glucose transformed from 15 mg.	% glucose transformed	mg. lactic acid formed	mg. lactic acid which can be formed theoretically	Lactic acid formed as % theoretical quantity
No. 1	13·7	91	11·0	13·7	80
No. 2	13·9	93	10·8	13·9	78
No. 3	14·7	98	12·6	14·7	86
No. 4	13·9	93	11·3	13·9	81
No. 5	14·1	94	11·5	14·1	82
No. 6	7·0	47	6·6	7·0	94
No. 7	12·6	84	10·9	12·6	86
Aerococcus viridans	14·3	95	12·0	14·3	84
Streptococcus faecalis	14·9	99	14·6	14·9	98
S. faecalis var. *zymogenes*	14·9	99	12·1	14·9	81

Method b

Strains 8–11 and the reference strain *Staphylococcus epidermidis* (see Table 2), all of which showed poor growth in Pope trypsin broth, were adapted to this by daily subculture for 1 week. The results of subsequent investigations are tabulated in the lines marked b1 and b2 in Table 2, examination being carried out with bacterial suspensions obtained by the following methods:

Method b1: a small inoculum of the adapted bacterium was transferred to 50 ml. trypsin broth, which was incubated for 48 hr; then 45 ml. of the culture was centrifuged and the sediment washed and suspended in sterile saline solution as described under method a.

Method b2: 0·9 ml. trypsin broth culture at the final stage of the adaptation series was added to 90 ml. trypsin broth. After 15 hr of incubation the culture was centrifuged and the sediment washed and suspended in sterile saline solution as described under method a.

Glucose conversion

1 ml. of bacterial suspensions was transferred to each of two 10 ml. volumetric flasks, both of which contained 5 ml. phosphate buffer (Graudal, 1955), and one of

which contained 0·3 % glucose. The other flask served as a control. After gentle shaking to ensure thorough mixing, conversion proceeded in an incubator at 37° over a period of 20 hr.

Chemical analysis

The solutions containing converted glucose were diluted to 10 ml. with distilled water and analysed. The glucose assay was performed according to the Somogyi–Nelson method (Somogyi, 1952). The lactic acid content was determined by the Barker and Summerson method (Barker & Summerson, 1941).

RESULTS

The results of the analysis are set out in Tables 1 and 2. (Somogyi (1952) and Barker & Summerson (1941) estimate the accuracy of the analytical methods used in this work as being about ± 5 %. The results of the investigations described are well within these limits.)

Table 2. *Transformation of glucose into lactic acid by the α-haemolytic micrococcus strains which showed poor growth in trypsin broth*

Strains	Method of growth	mg. glucose transformed from 15·0 mg.	% glucose trans-formed	mg. lactic acid formed	mg. lactic acid which can be formed theoretically	Lactic acid formed as % theoretical quantity
No. 8	a	5·0	33	3·2	5·0	64
	b2	14·6	97	13·8	14·6	95
No. 9	a	2·3	15	0·5	2·3	—
	b2	14·3	95	13·7	14·3	96
No. 10	a	0·9	6	0·3	0·9	—
	b1	0·3	2	0·2	0·3	—
	b2	14·2	95	14·0	14·2	99
No. 11	a	0·6	4	0·5	0·6	—
	b1	0·2	1	0·15	0·2	—
	b2	14·2	95	12·7	14·2	89
Staphylococcus epidermidis	a	0·7	5	0·6	0·7	—
	b1	5·5	37	4·5	5·5	82

DISCUSSION

All the strains included in Table 1 caused high glucose conversion according to method a. The strains entered in Table 2, on the other hand, caused low glucose conversion both by method a and method b1; an exception was *Staphylococcus epidermidis*, which according to method b1 converted 37 % of the glucose. The failure of method a and b1 is thought to arise from the fact that the suspension of active organisms was too weak to bring about sufficiently rapid conversion of the glucose. In the b1 method the suspension was concentrated enough, but it is probable that it contained a relatively large number of inactive organisms as a consequence of the prolonged period of incubation. Method b2 yielded abundant and active bacteria which caused extensive splitting of the glucose.

Lactic acid formation by these strains was found to range from 78 to 99 % of the

theoretically possible. Graudal (1955) characterizes a strain as homofermentative when the lactic acid formation is 80–100 % of the theoretically possible.

With the α-haemolytic micrococcus strains, the glucose conversion appears in large measure to depend on the number of active bacteria in the suspension. A sufficient number of such organisms cannot always be obtained by the commonly used methods of cultivation for investigation of lactic acid formation by enterococci.

All the bacterial strains examined in the course of this work proved to be formers of lactic acid, and must be regarded as being homofermentative or near homofermentative.

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Hydrogen Peroxide Formation and Catalase Activity in the Lactic Acid Bacteria

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SUMMARY

Some lactic acid bacteria formed detectable H_2O_2 and some did not, regardless of their preference or requirement for aerobic or anaerobic conditions. Whether or not H_2O_2 was formed depended in some instances on the substrate used as energy source. Two H_2O_2-splitting activities were encountered though never in the same organism. One, named pseudo-catalase activity, was insensitive to 0·01 M-azide or 0·01 M-cyanide and appeared to be the action of an acid-sensitive non-haem-containing enzyme detectable in some leuconostocs and pediococci when grown in media containing a low concentration of glucose. The second, named catalase activity, was detected in a number of lactobacilli, leuconostocs, streptococci and pediococci grown on media containing haematin or heated blood; presumably these organisms are able to synthesize the apoenzyme but not the prosthetic group of catalase. This activity was inhibited by 0·01 M-azide or 0·01 M-cyanide; it was not acid-sensitive. There was little correlation between H_2O_2-splitting activity and the preference or requirement of the organisms for aerobic or anaerobic conditions, or between H_2O_2-splitting activity and H_2O_2 formation. Of a few organisms examined, some appeared capable of forming cytochromes when grown in media containing heated blood. One showed traces of a cytochrome whether grown in the presence or absence of heated blood.

INTRODUCTION

Evidence has accumulated to show that oxygen can be of benefit in the utilization of sugars and polyhydroxy alcohols by certain lactic acid bacteria. Gunsalus & Sherman (1943) found that a strain of *Streptococcus faecalis* (which would now be classified as *S. faecium*) and other lactic acid bacteria utilized glycerol only in presence of oxygen. Gunsalus & Umbreit (1945), with the same strain of *S. faecalis*, recorded that one mole lactate and one mole hydrogen peroxide were formed per mole glycerol and mole oxygen used. Such a reaction indicates that oxygen serves as an exogenous hydrogen acceptor, accepting two atoms of hydrogen from glycerol and thereby allowing the balanced fermentation of glycerol to proceed. Dolin (1955) showed that oxygen could be used advantageously in the utilization of glucose by a strain of *S. faecalis*. Under anaerobic conditions, the pyruvate normally formed acted as a hydrogen acceptor in the balanced fermentation of glucose to lactic acid. In presence of oxygen, however, pyruvate was relieved of its role as oxidant and was further utilized to form an additional mole of adenosine triphosphate/mole triose. Theoretically, therefore, glucose might yield aerobically twice as much energy as anaerobically. Dobrogosz & Stone (1959) reported a strict requirement for oxygen

by certain pediococci which utilized glycerol; Whittenbury (1963) gave examples where oxygen was of benefit or was required in the utilization of certain substrates by certain lactic acid bacteria. The present paper gives the results of studies about hydrogen peroxide formation and hydrogen peroxide splitting by lactic acid bacteria and some preliminary findings about cytochrome formation by some of these organisms. A note on the catalase-like activity has already been published (Whittenbury, 1960).

METHODS

Organisms. Lactobacilli, streptococci, leuconostocs, pediococci and aerococci obtained from sources described previously (Whittenbury, 1963) were examined. The pediococci were classified according to Nakagawa & Kitahara (1959).

Media. Basal medium agar was meat extract (Lab Lemco), 0·5 g.; yeast extract (Difco), 0·5 g.; Tween 80, 0·05 ml.; $MnSO_4.4H_2O$, 0·01 g.; agar (Davis), 1·5 g.; made up to 100 ml. with tap water. The pH value was adjusted as required and the medium autoclaved at 121° for 15 min.

The 'inoculum medium' was similar to the basal medium agar with the exceptions that agar was omitted and glucose (0·5 %, w/v) added and the medium adjusted to pH 6·5.

Soft agar medium, which was used for showing oxygen relationships, was prepared and inoculated as described previously (Whittenbury, 1963) with the addition of $MnSO_4$, 0·01 % (w/v).

Manganese dioxide (MDO) agar was basal medium agar adjusted to pH 6·5 and containing 1 % (w/v) of a separately sterilized sugar or polyhydroxy alcohol; this was poured in a plate and then a very thin layer of the same medium (1–2 ml.) to which had been added 4 % (w/v) manganese dioxide (black tech. Harrington Bros. Ltd.) was poured on top. This medium is a variation of the pyrolusite agar described by Kneteman (1947). Clearing of the manganese dioxide under and around the bacterial growth indicates H_2O_2 formation.

Heated blood *o*-dianisidine (HBD) agar was made as follows. Basal medium agar (90 ml. adjusted to pH 6·5) was melted and 5 ml. of a 1 + 1 mixture of defibrinated ox blood and water added and the whole heated at 100° for 15 min. *o*-Dianisidine (0·1 g. in 5 ml. sterile water heated at 100° for 15 min.) was transferred whilst still hot by a wide-mouthed pipette to the melted agar. The medium, cooled to 48°, was completed by adding a separately sterilized sugar or polyhydroxy alcohol solution (final concentration 1 %, w/v) and poured in plates. This medium was a variation of the media used by Penfold (1922), Berger (1953) and Kraus, Nickerson, Perry & Walker (1957). Benzidine was replaced by *o*-dianisidine because of the carcinogenic property of the benzidine. Heated blood was used because many organisms produced a dark brown growth on media containing unheated blood, irrespective of the presence of *o*-dianisidine or the formation of H_2O_2. The production of H_2O_2 was indicated by the growth and the surrounding medium becoming dark brown or black, haem compounds having a peroxidase-like reaction in the oxidation of *o*-dianisidine by peroxide.

Heated blood (HB) agar was basal medium agar (95 ml.; pH 6·8–7) containing 1 % (w/v) glucose autoclaved at 121° for 15 min. After adding to the molten medium 5 ml. of a 1 + 1 mixture of defibrinated ox blood + water, the complete medium was heated at 100° for 15 min.

Haematin agar was basal medium agar (pH 6·8–7) with 1 % (w/v) glucose autoclaved at 121° for 15 min. Haematin (50 μg./ml.) was then added from a stock solution (50 mg. haematin in 10 ml. water and sufficient 0·1 N-NaOH to dissolve the haematin) which had been heated at 100° for 15 min.

Carbohydrates and polyhydroxy alcohols when not autoclaved in the media were used as distilled water solutions sterilized by Seitz-filtration.

Detection of hydrogen peroxide formation. Plates of MDO agar and HBD agar were inoculated by streaking with a capillary pipette containing an 18 hr old inoculum. Cultures were examined daily for 7 days.

Detection of hydrogen peroxide-splitting activity. Four media were used: HB agar, haematin agar and two basal medium agars (pH 6·8–7), one containing 0·05 % (w/v) glucose and the other 1 % (w/v) glucose. The first two media were prepared as plates, the last two as slopes. All were inoculated by streaking with a capillary pipette containing an 18 hr old culture. Activity was recognized visually by effervescence on the addition of hydrogen peroxide ('10 vol.') to heaped growth. Negative results were checked by placing heaped growth into H_2O_2. The media themselves showed no effervescence on adding H_2O_2.

The action of bacterial suspensions on hydrogen peroxide. Bacteria harvested from nutrient agar cultures in flat bottles which had been inoculated aerobically for 15–18 hr were washed twice with sterile tap water and resuspended in 0·07 M-phosphate buffer (pH 6·5). Bacterial suspensions (0·5 ml. equiv. 10–15 mg. dry wt.) were distributed in Durham tubes which were placed in a water bath at 30°. When the effect of haematin or protoporphyrin IX was being tested, 0·1 ml. of a stock solution or a distilled water dilution of a stock solution of these substances was added to a tube. Stock solutions were prepared by dissolving 50 mg. haematin or protoporphyrin IX in a solution of 9 ml. distilled water + 1 ml. triethanolamine. Distilled water solutions of sodium azide and potassium cyanide were added (0·1 ml.) to bacterial suspensions to provide the required concentrations. The ability to split H_2O_2 was recognized by effervescence on adding hydrogen peroxide ('10 vol.').

Determination of the rate of hydrogen peroxide-splitting activity. Bacterial suspensions were prepared as described above and the method of Herbert (1955) used to determine their hydrogen peroxide-splitting activity. This involved adding bacterial suspensions to a solution of H_2O_2 in test-tubes, the termination of enzyme activity by adding H_2SO_4, the addition of KI and catalytic amounts of ammonium molybdate, and the titration of the free iodine released by the action of residual peroxide by standard $Na_2S_2O_3$ with starch as indicator. When determining the effect of haematin, protoporphyrin IX, sodium azide or potassium cyanide on H_2O_2-splitting activity, 0·1 ml. amounts of solutions prepared as described above were added to the bacterial suspensions before these were placed in the H_2O_2 solution. A stock solution of H_2O_2 was prepared by diluting hydrogen peroxide ('20 vol.') with 0·07 M-phosphate buffer (pH 6·5) to give about 120 μmole H_2O_2/5 ml. stock solution.

Oxygen uptake by bacterial suspensions containing glucose at 30°. This was measured by conventional Warburg procedures with air as gas phase. Bacteria harvested from a 15–18 hr aerobic glucose nutrient agar culture were washed twice with sterile tap water, suspended in 0·07 M-phosphate buffer (pH 6·8) and 2 ml. (equiv. 5–10 mg. dry wt. bacteria) were placed in the main compartment. After temperature

equilibration 1 ml. of glucose solution was added from the side arm to give a final glucose concentration of 0·1 M in 3 ml. fluid volume. The centre well contained 0·2 ml. 2 N-KOH and a filter-paper wick.

Incubation. All the cultures were grown at 30° and all the experiments with bacterial suspensions were carried out at 30°.

RESULTS

Hydrogen peroxide formation

Tests were carried out with MDO and HBD agars containing different substrates as energy sources, and in some instances with the basal (unsupplemented with sugar or polyol) HBD agar. The results in Table 1 support four main conclusions: (1) not all the lactic acid bacteria tested formed detectable amounts of H_2O_2; (2) H_2O_2 might accumulate during the utilization of one substrate but not of another; (3) a preference or a requirement for aerobic or anaerobic conditions, as judged by growth in soft agar, was not necessarily related to H_2O_2 accumulation; (4) H_2O_2 accumulation might be a useful taxonomic characteristic (this latter aspect will be considered subsequently).

When inocula were grown anaerobically or were taken from static deep cultures incubated aerobically, traces of H_2O_2 sometimes appeared in the first aerobic subculture on MDO or HBD media but not on serial transfer on these media. This behaviour was not observed with inocula from nutrient agar slope cultures grown aerobically; these were consistent and either produced or did not produce H_2O_2 on serial aerobic subculture.

Representative organisms which formed H_2O_2 (11 strains) and others which did not do so (9 strains) were examined manometrically for oxygen uptake with glucose as substrate. All were active: in 12 of 20 cases the Q_{O_2} was in the range 20–40. Bacteria harvested at 15–18 hr were much more active than those harvested at 24–36 hr; this was especially marked with some of the H_2O_2-forming cultures. Oxygen uptake with and without the formation of detectable amounts of H_2O_2, together with the evidence of the presence of flavoprotein oxidases and peroxidases in lactic acid bacteria (Dolin, 1953, 1955, 1957; Niederpruem & Hackett, 1958; Strittmatter, 1959), suggests that the significant difference between these strains, with respect to H_2O_2 production, is that organisms which form H_2O_2 possess flavoprotein oxidases whereas the others possess also, or form adaptively, flavoprotein peroxidases. Since all our strains were not tested for oxygen uptake, it is possible that some did not form peroxide because they were unable to utilize oxygen, for example, as reported for a strain of *Leuconostoc mesenteroides* by Johnson & McCleskey (1957).

Hydrogen peroxide-splitting activity

Since H_2O_2 may be toxic it is obviously an advantage to an organism to be able to destroy any which it may form. Several lactic acid bacteria do so peroxidatively (Greison & Gunsalus, 1943; Douglas, 1947; Seeley & Vandemark, 1951; Johnson & McCleskey, 1958). Some of the organisms possess flavoprotein peroxidases (Dolin, 1953, 1955, 1957; Niederpruem & Hackett, 1958; Strittmatter, 1959). In the present work two catalytic activities were observed.

The first type of activity was detected in eleven strains of *Pediococcus pentosaceus* and six of *Leuconostoc mesenteroides*, and in two strains of *Lactobacillus plantarum* (NCDO 963, 965) not recorded in Table 1 which had been shown to have a weak H_2O_2-splitting activity by Dacre & Sharpe (1956). This type of activity was most obvious when the organisms were grown aerobically on the basal medium or on

Table 1. *Hydrogen peroxide formation, H_2O_2-splitting activity and manner of growth in soft agar of some lactic acid bacteria*

Organism and no. of strains		Growth on media: Basal medium	Glucose	Glycerol	Mannitol	Sorbitol	Type of H_2O_2-splitting activity; no. of strains positive: Pseudo-Catalase	Catalase (haem-requiring)
Genus *Streptococcus*								
S. faecalis	19	−f	−f	−f	−f	−f	0	15
S. faecium	1	+m	−f	+a	+f	+f	0	0
S. faecium	3	+m	+f	+a	+f	.	0	0
S. faecium	10	+m	−f	+a	+f	.	0	0
S. durans	2	+m	−f	+a	.	.	0	0
Genus *Pediococcus*								
P. pentosaceus	13	+m	−f	+a	.		11	0
P. acidilactici	4	+m	−f	+a	.		0	3
P. halophilus	1	−f	−f	−f	.		0	0
P. urinae-equi	1	+a	+pa	+a	+a	+a	0	0
P. cerevisiae	2	−m	−f	.	.	.	0	0
Genus *Aerococcus*								
A. viridans	1	+a	+pa	+a	+a	+a	0	0
Genus *Leuconostoc*		Glucose	Fructose	Sucrose	Arabinose			
L. mesenteroides (dextran formers)	72	−f	−f	−f	−f		6	0
L. mesenteroides (non-dextran formers)	10	+f	+f	+f	+f		0	0
L. mesenteroides (non-dextran formers)	8	+pa	+pa	+pa	+pa		0	8
L. dextranicum	2	−f	−f	−f	.		0	0
L. citrovorum	4	−f	−f	.	.		0	0
Genus *Lactobacillus*		Glucose						
L. brevis	22	+pa					0	22
L. brevis	8	−pa					0	8
L. brevis-like	2	+pa*					0	2
L. buchneri	25	+pa					0	0
L. viridescens	2	+pa					0	2
L. cellobiosus	2	+pan					0	0
L. fermenti	14	−f					0	0
L. plantarum	15	+f					0	14
L. plantarum	8	−f					0	8
L. casei	5	−f					0	1
L. casei	3	+f					0	0

H_2O_2 formation: + = detected, − = not detected. Type of growth in soft agar: f = facultative, = aerobic, pa = preferentially aerobic, pan = preferentially anaerobic, m = microaerophilic, . = not utilized.

* Positive reaction on glucose MDO agar but negative on glucose HBD agar; attributed to strong catalase activity of these organisms when grown on HBD media.

0·05 % (w/v) glucose nutrient agar; effervescence then began within 1 min. of adding H_2O_2. The activity was not observed in any organism grown on 1·0 % (w/v) glucose nutrient agar or in any leuconostoc when grown anaerobically on any medium. When the initial pH value (7·0) of 0·05 % (w/v) glucose agar was lowered the activity of all strains diminished; at pH 6·0 all were relatively active, at pH 5·0 they were less active and at pH 4·5 only some of the pediococci split H_2O_2. These pediococci were the only organisms which showed H_2O_2-splitting activity on 0·5 % (w/v) glucose agar (initially pH 6·8). The addition of haematin to the growth medium did not obviously affect the H_2O_2-splitting activity of any of these organisms.

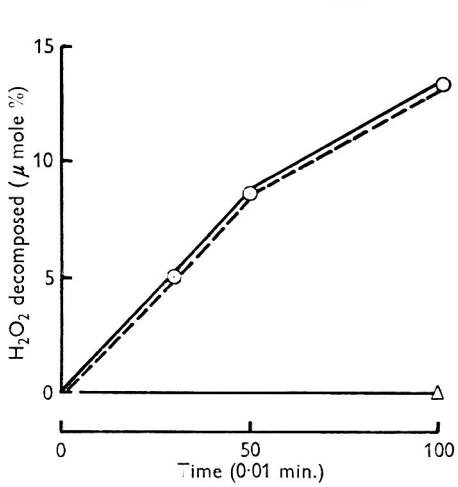

Fig. 1

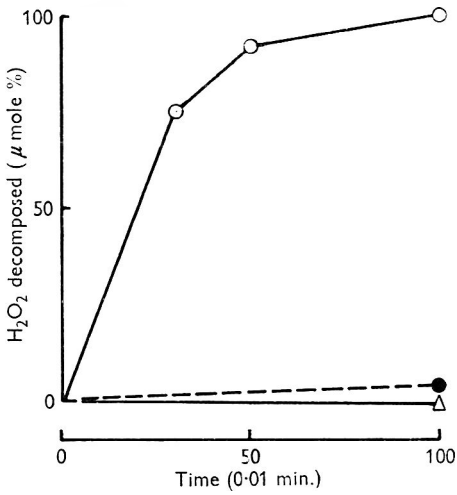

Fig. 2

Fig. 1. Pseudocatalase activity of suspensions of *Leuconostoc citrovorum* NCIB 7837 (*Pediococcus pentosaceus*). Each test-tube contained 120 μmole H_2O_2 and bacteria equivalent to 3·7 mg. dry wt. in 6 ml. 0·07 M-phosphate buffer (pH 6·5). Incubation at 30°. Residual H_2O_2 determined by method of Herbert (1955). ○—○, Untreated bacteria; - ○ - - ○ -, bacteria treated with 0·01 M-sodium azide; △—△, bacteria heated at 100° for 2 min.

Fig. 2. Haematin-requiring catalase activity of suspensions of *Pediococcus acidilactici* RW7. Each test-tube contained 120 μmole H_2O_2 and bacteria equivalent to 3·3 mg. dry wt. in 6 ml. 0·07 M-phosphate buffer (pH 6·5). Incubation at 30°. Residual H_2O_2 determined by method of Herbert (1955). ○—○ = bacteria after 2 hr incubation with 5 μg. haematin/ml.; - ● - - ● - = bacteria treated with 0·001 M-sodium azide after incubation with 5 μg. haematin/ml. for 2 hr; △—△ = untreated bacteria.

The activity of suspensions of whole organisms and of water extracts of broken bacteria was examined. H_2O_2-splitting activity was completely eliminated by heating at 100° for 2 min. but was not noticeably inhibited by sodium azide or potassium cyanide added to a final concentration of 0·01 M 5 min. before the addition of H_2O_2. The reaction rates of a suspension of a pediococcus is shown in Fig. 1. Neither protoporphyrin IX nor haematin affected H_2O_2-splitting rates.

The insensitivity to cyanide and azide suggested that the enzyme was an atypical catalase, possibly not a haem derivative. This H_2O_2-splitting activity appears to be identical with that described by Delwiche (1961) and by Johnston & Delwiche (1962). Delwiche (1961) examined a partially purified enzyme preparation obtained from

a pediococcus and found it to be insensitive to 0·01 M-azide or 0·01 M-cyanide; no haem-pigment or flavin-coenzyme peaks were observed spectrophotometrically; acriflavin did not inhibit activity. These observations suggest that the enzyme was neither a haemoprotein nor a flavoprotein. Johnston & Delwiche (1962) found a similar H_2O_2-splitting activity in some lactobacilli, leuconostocs, streptococci and additional pediococci. Earlier reports on weak catalase activity in pediococci (Felton, Evans & Niven, 1953; Jensen & Seeley, 1954), in strains of *Lactobacillus plantarum* (Dacre & Sharpe, 1956), in strains of *Leuconostoc mesenteroides* (Langston & Bouma, 1960) and in strains of *Streptococcus faecalis* (Langston & Bouma, 1960) are probably similar to or have been shown by Johnston & Delwiche (1962) to be similar to the type described by Delwiche (1961). To avoid confusion with a second type of activity demonstrable in bacteria grown in the presence of heated blood or haematin (Whittenbury, 1960) the first type will be referred to as pseudocatalase and the second as catalase.

The discovery of a haematin-requiring catalase activity in lactic acid bacteria originated from the observation that some heterofermentative lactobacilli formed H_2O_2 on MDO agar but not on HBD agar. Added H_2O_2 was split by cultures on HBD agar or HB agar but not by those on MDO agar or basal agar + 0·05 % (w/v) glucose. A survey was made of organisms grown on HB agar and haematin agar. Table 1 shows the strains in which catalase activity was detected. *Streptococcus faecalis* and some of the lactobacilli had a weak action; they produced a vigorous effervescence only when heaped growth was placed into H_2O_2 solution. Pseudocatalase was not demonstrated in any strain which produced catalase.

Catalase formation was not prevented by altering the concentration of glucose in HB agar from 0·05 to 2·0 % (w/v) nor by altering the initial pH value of HB agar containing 1 % (w/v) glucose from 7·0 to 4·5. Some strains of *Lactobacillus brevis* which were active when grown aerobically were inactive or only weakly positive when grown anaerobically. Conversely, one strain of *L. viridescens* was active when grown anaerobically but inactive when grown aerobically. Replacement of haematin or heated blood by protoporphyrin IX (50 μg./ml. medium) and/or by various iron salts did not result in the development of detectable H_2O_2-splitting activity. It appears, therefore, that some lactic acid bacteria are able to form the apoenzyme of catalase and are able to synthesize catalase when provided with the preformed prosthetic group haem, but not with its immediate precursor protoporphyrin IX.

Washed suspensions of several strains, harvested from 1·0 % (w/v) glucose agar, were tested for ability to form catalase when provided with haematin (5 μg./ml.). The strains which were catalase-positive when grown on HB agar or on haematin agar developed H_2O_2-splitting activity within 2 hr at 30° with the exception of *Streptococcus faecalis* H 69 D 5, which did not develop H_2O_2-splitting activity; in most instances the activity was detectable within 5 min. Strains which were catalase-negative when grown on HB agar showed no activity. Suspensions heated before or after addition of haematin, or pre-incubated for 20 min. with haematin and a further 2 min. with azide or cyanide (at 0·001 M) did not split added H_2O_2, judged visually.

The H_2O_2-destroying rates found with haematin-treated organisms of *Pediococcus acidilactici* RW 7 in the presence and absence of azide are shown in Fig. 2. In contrast to pseudocatalase, the catalase of this organism was almost completely inhibited

by 0·001 M-sodium azide. The activity of suspensions of this pediococcus and of *Lactobacillus plantarum* NCIB 5914 after treatment with haematin and/or protoporphyrin IX was examined; the results are shown in Table 2. These suggest that the apoenzyme of catalase can combine with protoporphyrin IX or haematin and that, at least in the case of the pediococcus, neither appears to exchange with the other once the combination has been effected.

Table 2. *The effect of different treatments on H_2O_2-splitting activities of suspensions of Lactobacillus plantarum* NCIB *5914 and Pediococcus acidilactici* RW *7*

Each test-tube contained 120 μmole H_2O_2 and bacteria equivalent to 3·3 mg. dry wt. pediococcus or 4·3 mg. dry wt. lactobacillus in 6 ml. of 0·07 M-phosphate buffer (pH 6·5). Organisms during treatment and testing were at 30°. Residual H_2O_2 was determined by method of Herbert (1955).

Previous treatment of bacterial suspensions	Lactobacillus H_2O_2 split in 30 sec. (μmole)	Pediococcus H_2O_2 split in 30 sec. (μmole)
Haematin (5 μg./ml. for 2 hr)	62·9	74·0
Haematin (5 μg./ml. for 2 hr) then heated at 100° for 2 min.	1·8	1·8
Haematin (50 μg./ml. for 2 hr)	21·3	74·0
Untreated	0	0
Protoporphyrin IX (5 μg./ml. for 2 hr)	3·7	2·8
Haematin (5 μg./ml. for 15 min. then added protoporphyrin IX, 5 μg./ml. for 2 hr)	9·3	75·8
Protoporphyrin IX (5 μg./ml. for 15 min. then added haematin, 5 μg./ml. for 2 hr)	9·3	7·4
Haematin (5 μg./ml. + protoporphyrin IX 5 μg./ml. simultaneously for 2 hr)	13·0	13·0

Table 3. *Distinguishing features of pseudocatalase-forming and haematin-requiring catalase-positive cultures of lactic acid bacteria*

	Pseudocatalase	Haematin-requiring catalase
H_2O_2 split by cultures grown on 0·05 % (w/v) glucose nutrient agar	+	−
H_2O_2 split by cultures grown on 1·0 % (w/v) glucose nutrient agar containing haematin or heated blood	−	+
H_2O_2-splitting activity inhibited by treating bacteria with 0·01 M-azide	−	+
H_2O_2-splitting activity inhibited by treating bacteria with protoporphyrin IX before treatment with haematin	−	+

The foregoing observations show that two distinguishable forms of H_2O_2-splitting activity occur in some lactic acid bacteria; so far no organism has been found to possess both activities. The characteristics which separate the two activities are summarized in Table 3.

A comparison of the H_2O_2-splitting activity of some lactic acid bacteria and of some organisms which normally form catalase is given in Table 4. The pseudocatalase-positive lactic acid bacteria, *Lactobacillus plantarum* NCDO 965 and *Leuco-*

nostoc citrovorum NCIB 7837 (*Pediococcus pentosaceus*), showed the slowest reaction rates. This result is like that of Johnston & Delwiche (1962), who found that lactic acid bacteria which had a non-haem catalase were much less active than a strain of *Escherichia coli*; the catalase-positive lactic acid bacteria, on the other hand, showed an activity comparable with that of bacteria which normally produce catalase.

Table 4. *A comparison of H_2O_2-splitting activities of various organisms*

Each tube contained 120 μmole H_2O_2 and bacteria of equivalent dry wt. 2·5–5·0 mg. in 6 ml. 0·07 M-phosphate buffer (pH 6·5). Incubation at 30°. Residual H_2O_2 determined by method of Herbert (1955). A, grown on nutrient agar lacking added sugar; B, pseudocatalase-positive; grown on 0·05 % (w/v) glucose agar; C, grown on 1·0 % (w/v) glucose agar; washed bacteria incubated for 2 hr with haematin (5 μg./ml.).

	H_2O_2 decomposed	
	In 30 sec.	In 1 min. after treatment with azide (0·001 M)
Organism	μmole H_2O_2 decomp./mg. dry wt. bacteria	
A		
Pseudomonas fluorescens CF 21	20·9	2·2
P. fragi NCIB 8542	5·7	1·9
P. fermentans L 417 (*Aeromonas liquefaciens*)	13·2	3·5
Alkaligenes faecalis G 21/3	31·4	8·1
Proteus vulgaris G 22	16·3	6·2
B		
Lactobacillus plantarum NCDO 965	1·6	3·2
Leuconostoc citrovorum NCIB 7837 (*Pediococcus pentosaceus*)	2·9	6·0
C		
Lactobacillus plantarum NCIB 5914	12·2	1·3
Pediococcus acidilactici RW 7	27·4	2·2

Dr J. R. Norris (personal communication) compared the H_2O_2-splitting enzymes of some of the present organisms, sent to him as acetone-dried preparations, with the catalases of various aerobic bacteria. After starch-gel electrophoresis the enzymes were detected by treating the gel with a preparation of KI and H_2O_2. Dr Norris found that: (*a*) the catalase of lactic acid bacteria grown on media containing heated blood or haematin travelled at the same rate as the catalase of bacteria which normally form that enzyme; (*b*) no catalase was detectable in the same lactic acid bacteria when these were grown in absence of heated blood or haematin; (*c*) the pseudocatalase of lactic acid bacteria moved at a rate different from that of bacterial catalases. These findings indicate that the catalase of lactic acid bacteria is very similar to that of various aerobic organisms and that pseudocatalase is another type of enzyme. In the past, absence of catalase has been a useful diagnostic feature of the lactic acid bacteria. Recent findings have not diminished the value of this criterion, provided that the conditions of cultivation are appropriate. No organism among those examined was catalase-positive or pseudocatalase-positive when grown on 1 % (w/v) glucose agar without added haematin.

The relationship between hydrogen peroxide accumulation and the hydrogen peroxide-splitting activities

Table 1 indicates that in the lactic acid bacteria examined there was little correlation between H_2O_2 production and ability to form catalase or pseudocatalase. Nevertheless, catalase formation was in certain instances clearly beneficial to the organisms. An outstanding example was provided by the eight leuconostocs which in glucose soft agar showed a strong preference for aerobic conditions. On the surface of solid glucose (1 %, w/v) nutrient agar these organisms produced little growth and were not viable after 1–2 days. The addition to that medium of 10 μg. haematin/ml. was sufficient to enable these organisms to form catalase, to produce good growth and to remain viable for a prolonged period; glucose HBD agar gave the same results. This further showed that, despite the formation of catalase, these organisms continued to produce H_2O_2. The pediococci which formed pseudocatalase likewise produced H_2O_2 on glycerol HBD agar. These observations suggest that catalase remains in and protects the cytoplasm of the organism while the oxidases occur in the plasma membrane, from which H_2O_2 can diffuse outwards. The H_2O_2-sensitive organisms showed excellent growth and viability on MDO agar. The insolubility of MnO_2, which was protective, suggests that in this case the destruction of H_2O_2 occurred outside the bacterial cell.

Plant juices as media for the organisms

For lactic acid bacteria the ability to destroy H_2O_2 may be a significant survival factor in some natural environments. Organisms which produced catalase or pseudocatalase have been isolated from herbage where, if they proliferate, they will utilize substances liberated by the living or dead tissues. To test the suitability of plant juices as nutrients for these organisms, cultures were grown on the surface of solid media consisting of water agar to which had been added filter-sterilized or autoclaved extracts of the leaves of rye-grasses, cocksfoot or clovers, or extracts of potato tubers or turnips. The final concentrations of the plant juices were about 25–50 % (v/v). Organisms which formed catalase in the presence of haematin split added H_2O_2 when grown on the leaf extracts of grasses or clovers, but did not do so on potato or turnip extracts. Pseudocatalase-forming strains split added H_2O_2 when grown on any of the media; their pseudocatalase activity was eliminated by adding 1 % (w/v) glucose to the media. Strains which on artificial media did not split H_2O_2 were inactive when grown on the plant extracts. It seems reasonable to suppose that catalase may be formed from an iron porphyrin in leaf juices, thus enabling certain organisms to proliferate in a herbage environment without succumbing to H_2O_2 poisoning.

The formation of cytochromes

Jensen (1957) described a streptomycin-resistant mutant of *Micrococcus pyogenes* var. *aureus* (*Staphylococcus aureus*) which did not synthesize haem but was able to form catalase and cytochromes when provided with haem. Since some lactic acid bacteria appeared similar in that they were able to form catalase when provided with haematin, their ability to form cytochromes as well was examined. Physiological tests gave negative results. Dense suspensions of organisms grown in a

succinate medium containing a trace of glucose did not reduce 2,3,5-triphenyltetrazolium chloride in the presence of succinate; none oxidized *p*-phenylenediamine (Nadi reagent). Organisms which were grown aerobically with haematin and nitrate in the medium and then incubated anaerobically did not form nitrite (or, if they did so, no more vigorously than when grown in absence of haematin). The fact that certain strains formed H_2O_2 on glucose HBD agar suggests that cytochromes, if formed, were not significantly functioning.

Table 5. *Cytochrome content of some lactic acid bacteria*

When cytochromes were not observed, presence or absence of porphyrin compounds was checked by treatment with pyridine.

H_2O_2 – splitting characteristic and organism	*Medium from which bacteria were harvested	Cytochromes detected after adding $Na_2S_2O_4$	Formation of pyridine haemochromogen
Catalase +, pseudocatalase –			
Lactobacillus plantarum NCIB 5914	A	None	Trace
	B	a_2 b_1	—
L. brevis NCIB 947	A	None	None
	B	b_1	—
Leuconostoc mesenteroides NCIB 8018 (*Pediococcus acidilactici*)	A	None	None
	B	None	Trace
L. mesenteroides RW 1 (non-dextran forming)	A	None	None
	B	a_2 b_1 (very weak bands)	—
Streptococcus faecalis H 69 D 5	A	None	None
	B	a_1 a_2 b_1	—
Catalase –, pseudocatalase –			
Lactobacillus plantarum NCIB 6105	A	b_1 Very weak bands	—
	B	b_1 Very weak bands	—
Catalase –, pseudocatalase +			
Leuconostoc citrovorum NCIB 7837 (*Pediococcus pentosaceus*)	C	None	None
L. mesenteroides RW 66	C	None	None

* A = 1·0 % (w/v) glucose agar, B = 1·0 (w/v) glucose agar containing heated blood, C = 0·05 % (w/v) glucose agar.

Suspensions of a few strains of lactic acid bacteria were sent to Dr E. F. Hartree (Molteno Institute, Cambridge), who examined them for cytochrome content by the low temperature method (Keilin & Hartree, 1949, 1950); the results are given in Table 5. Four of the organisms which formed catalase possessed cytochromes when grown in the presence of heated blood but not in its absence. Perhaps heavier suspensions might have revealed that all four strains possessed the *a* and *b* types of cytochromes found in *Streptococcus faecalis* H 69 D 5. Only one organism was tested which was unable to attack H_2O_2; it showed a weak cytochrome *b*1 band whether grown in the presence or absence of heated blood. The two pseudocatalase-positive organisms grown on 0·05 % (w/v) glucose agar formed no cytochromes and contained no haem demonstrable as pyridine-haemochromogen. It thus appears

possible that cytochromes may be formed by some lactic acid bacteria under certain conditions of cultivation. Whether these cytochromes are functional, vestigial, or artifacts resulting from the cultural conditions, remains to be determined.

DISCUSSION

Enzymic and growth studies are revealing the ability of many lactic acid bacteria to utilize oxygen with benefit to themselves and to dispose of the H_2O_2 which may be formed. Dolin (1961) in discussing this question suggested that clostridia and lactic acid bacteria represent different stages in physiological development (evolution) and that, although both types of organisms use similar respiratory systems, the lactic acid bacteria are more successfully adapted to aerobic conditions. Streptococci, he considered, may be related to the more aerobic bacteria through the pediococci, presumably because of the finding (Dolin, 1955) that *Streptococcus faecalis* possesses a cytochrome *c* reductase but no demonstrable cytochromes, whilst pediococci appear to possess a cytochrome *c*–cytochrome oxidase activity, since they oxidize *p*-phenylenediamine (Jensen & Seeley, 1954). This argument, however, is questionable. The possession by *S. faecalis* of a cytochrome *c* reductase does not necessarily imply that reduction of cytochrome *c* is or was the true physiological role of such an enzyme. Also, the ability of pediococci to oxidize *p*-phenylenediamine is only indirect evidence of the possible presence of cytochrome *c* and cytochrome oxidase. No oxidation of *p*-phenylenediamine by similar pediococci was observed in the present work. Clearly it is necessary that direct evidence of the presence or absence of cytochromes should be provided The suspensions of two strains of pediococci examined in the present work did not contain detectable cytochromes (Table 5). However, the available evidence, though fragmentary, suggests that some lactic acid bacteria do possess the rudiments of a respiratory system. The ability of some to form haemoproteins when provided with a source of iron porphyrin suggests that, rather than adapting to aerobic conditions as implied by Dolin (1961), they have evolved retrogressively from facultatively aerobic haemoprotein-forming organisms. Their survival in nature could be related to their specialization for life in distinctly acid, sugar-containing environments from which oxygen is frequently absent. A study of iron porphyrin-less mutants of haemoprotein-forming facultative aerobes might reveal some close relationships with lactic acid bacteria. It may prove difficult, for instance, to distinguish the haematin-less mutant of *Staphylococcus aureus* (Jensen, 1957) from pediococci or aerococci.

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The Fixation of Complement by Virus-Antibody Complexes: Equivalence and Inhibition in the Reactions of the Viruses of Tomato Bushy Stunt and Foot-and-Mouth Disease with Rabbit and Guinea-Pig Antisera

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SUMMARY

A sensitive and simply calculated complement-fixation test is described in which viral antigen and antibody react to completion for up to 90 min. at 37° before the addition of one 70 % haemolysis unit of complement. The test has been applied to reactions between the components of the virus system of foot-and-mouth disease, tomato bushy stunt virus, bovine plasma albumin and their rabbit or guinea-pig hyperimmune sera. In the restricted region of proportional fixation each μl. of complement represents about 3×10^3 virus particles and 6×10^{11} molecules of albumin. In suspensions of foot-and-mouth disease virus of highest infectivity (10^{10} mouse ID50/ml.), probably less than 1 in 30 of the characteristic 25 mμ particles are infective.

Quantitative data define the regions of antibody excess, equivalence and antigen excess, and show that complement is bound as a secondary process to that of antigen/antibody complex formation, that complement is bound only by relatively massive complexes and that the independent formation of unrelated complexes in the same system may be sensitively detected. The location of the optimum reaction and the confirmation of equivalence allow fixation data to be related closely to parallel data on the neutralization of infectivity. Since the concentrations of antigen and of antibody for optimal fixation are almost independent, it is concluded that the maintenance of equivalence in terms of a constant antigen/antibody ratio is not a valid principle for the interpretation of such data.

INTRODUCTION

Recent comparative studies by neutralization of infectivity and by fixation of complement of the foot-and-mouth disease virus system (Bradish & Farley, 1960; Bradish, Farley & Ferrier, 1962) have shown a close quantitative correspondence between these distinct manifestations of a common antigen/antibody interaction. The definition of the optimum reaction is of particular significance in this correlation. Most studies of neutralization and complement fixation in animal virus systems have been restricted to reactions in the region of antibody excess; few data have been related to block-assays covering the critical regions of optimum reaction in which certain features are most apparent. The present work parallels that on the neutralization of infectivity and investigates, through complement fixation, the antibody-combining properties of the viruses of foot-and-mouth disease and tomato bushy stunt, particularly in and beyond the regions of antigen/antibody equiva-

lence. Concurrent experiments with bovine serum albumin as antigen illustrate the generality of the pattern of reactions described. It is shown that particular conditions with respect to the relative concentrations of reactants must be satisfied before a meaningful titration of antigen or of antibody can be completed, and before the properties of the antigen/antibody system can be resolved from those of the complement assay system. Reference may be made to Kabat & Mayer (1961) and Rice (1959*b*, *c*, 1960) for discussions of earlier studies.

METHODS

Preparation of antigens. The 25 mμ infective component and 7 mμ non-infective component of the virus system of foot-and-mouth disease were separated from guinea-pig pad vesicular fluid or epithelium suspension by procedures described elsewhere (Bradish, Brooksby, Dillon & Norambuena, 1952; Bradish, Henderson & Kirkham, 1960). The concentrates of the 25 mμ infective component contained up to 10^{10} mouse ID50/ml.

Tomato bushy stunt virus was available as a purified concentrate through the courtesy of Drs C. A. Knight and G. Rushizky (Virus Laboratory, Berkeley, California, U.S.A.). This preparation was standardized by electron microscopy and by observation of ultraviolet (u.v.) absorption at 260 mμ and it was diluted to the concentrations required in the appropriate buffer solutions. Bovine plasma albumin fraction V as a dry powder was obtained from the Armour Pharmaceutical Company Ltd.

Estimation of virus infectivity and concentration. The infectivity of suspensions of foot-and-mouth disease virus was determined by the intraperitoneal inoculation of serial 10-fold dilutions into unweaned white mice of the Pirbright strain (Skinner, 1951). The volume of inoculum was 0·03 ml. and five or six mice from randomized litters were inoculated at each dilution; the scores of dead and paralysed mice were counted up to the third day. The concentration of 50 % infective units (ID50/ml. of undiluted sample) was calculated by the method of Reed & Muench (1938).

The concentration of characteristic particles in suspensions of each virus was determined by direct counting, by means of electron microscopy (RCA type EMU-2B), of the number of such particles in at least 10 microdrops. The mixture of virus and the 88 mμ polystyrene marker was sprayed on to the mounting films by means of a special low-pressure totally enclosed glass gun. The procedures generally followed those already described (Backus & Williams, 1950; Breese & Trautman, 1960).

Preparation of antisera in guinea-pigs and rabbits. Guinea-pigs were hyperimmunized against foot-and-mouth disease virus and their antisera collected and stored in the manner described by Brooksby (1952). Rabbits (1·7–2·3 kg. initial weight) were immunized by the inoculation of the fractions described below into the marginal vein of the left ear. Daily inoculations of 1 ml. virus suspension in phosphate buffer (0·04 M, pH 7·6) continued for 10 days. After a rest for 11 days and starvation for 24 hr, about 30 ml. blood were collected from the right ear of each animal. Following separation of clot, the antisera were inactivated for 30 min. at 56° and then stored at −20° in 1 ml. samples. The courses of immunization were repeated on the same basis after a rest period of at least 6 weeks. These procedures in rabbits follow those described by Matthews (1957).

The inocula for immunization of rabbits were as follows. Foot-and-mouth disease

virus: preparations of the 25 mμ infective component of appropriate virus type isolated as defined in the previous section; each ml. of inoculum contained about 10^7 mouse ID 50 and 10^{10} characteristic particles. Thus, in 10 days, each rabbit received about 1 μg. of virus.

Tomato bushy stunt virus: rabbits received daily 4 mg. for 10 days.

Bovine plasma albumin: 4 mg. in 1 ml. daily for 10 days.

Complement-fixation test. The reagents used in this test were generally those described by Brooksby (1952). Pooled normal guinea-pig serum was the source of complement (*C′*) and this was freeze-dried or stored frozen at −20°. All dilutions and controls were made in veronal buffer (0·005 M, pH 7·6) with added calcium and magnesium (Mayer, Osler, Bier & Heidelberger, 1946). The haemolytic indicator was a suspension of sheep erythrocytes maximally sensitized by 4 minimal haemolytic units of haemolysin. Haemolysin and cell suspension were thoroughly mixed and incubated for 30 min. at 37° immediately before addition to the antigen − antibody + complement mixture, also at 37°. The haemolytic indicator was adjusted to contain 45×10^6 cells/ml. by preliminary dilution of the red cell suspension. The final mixture of 1 ml. indicator + 1·8 ml. reaction (antigen + antibody + complement) mixture then gave 50 % haemolysis for 50–60 % light transmission at 520 mμ in the matched 1 cm. diameter tubes of the Coleman Junior Spectrophotometer. A maximum sensitivity of observation of percentage haemolysis was thus obtained in the subsequent test.

The complement-fixation test used was initially the '50 % haemolysis procedure' of Wadsworth, Maltaner & Maltaner (1938) as described by Brooksby (1952) and used in previous studies of components of the foot-and-mouth disease virus system (Bradish, Brooksby & Tsubahara, 1960; Bradish & Brooksby, 1960). However, the need to analyse many antigen/antibody mixtures covering wide ranges of reactant concentrations demanded a diminution in the quantities of reactants required per tube and in the number of tubes to be set up with each reaction mixture. Studies of reactions in antigen excess were particularly restricted by the available quantities of virus fractions since only about 10 μg./week of the separated 25 mμ component of the virus system of foot-and-mouth disease was available.

The following 'haemolysis-depression' procedure was therefore developed; this is an extension of the procedure proposed by Stein & van Ngu (1950). Each reaction mixture was prepared with a constant volume of complement rather than with the 5 or more graded volumes of complement used in the earlier 50 % haemolysis procedures. The constant volume of complement used in these studies was that required to produce 70 % haemolysis (65–75 %) of the red cells in 1 ml. of standardized indicator system defined above. The 70 % haemolysis point was selected as being conveniently high on the central linear region of the haemolysis sigmoid curve, yet below the region of sharp curvature above 80 % haemolysis. The 70 % haemolysis dose of complement was determined for each experiment by a preliminary standardization (made as in Fig. 1) of a 1/50 or 1/100 dilution of pooled normal guinea-pig serum. An appropriate further dilution containing one 70 % haemolysis dose/ml. was then prepared for use in the main experiment. The main experiment always included a re-standardization of the final dilution of complement. Since, in the range from 20 % to 80 % haemolysis, the percentage haemolysis increases essentially linearly with the volume of available complement, it follows that any 'fixation' of

Table 1. *Distribution of reactants in titration of antigen in presence of optimal* concentration of homologous antibody*

	Antigen titration and controls						Antiserum control	Complement control
Tube number and contents	1	2	3	4	5	6	7	8
Compensating volume of veronal buffer (ml.)	0	0·4	0	0·4	0	0·4	0·4	0·8
Hyperimmune serum at optimal* dilution (ml.)	0·4	0	0·4	0	0·4	0	0·4	0
Complement standardized† for 70 % haemolysis in control tubes (ml.)	1·0	1·0	1·0	1·0	1·0	1·0	1·0	1·0
Volume (ml.) and dilution* of antigen taken	0·4 at 1/50		0·4 at 1/100		0·4 at 1/200		None	
Equivalent volume of undiluted antigen taken (μl.)	8		4		2		0	
Standardized haemolytic indicator (ml.)	1·0		1·0		1·0		1·0	
Optical density† of clarified supernatant fluid (o.d.)	*A*	*A* 70	*B*	*B* 70	*C*	*C* 70	*D*	*D* 70
Depression of optical density (Δ o.d.)	(*A* 70 − *A*)		(*B* 70 − *B*)		(*C* 70 − *C*)		(*D* 70 − *D*)	

* (1) The appropriate dilution of antiserum for optimal fixation of complement is determined by preliminary tests in which tables of this type are extended to include a range of antiserum dilutions from 1/50 to about 1/500 or beyond. The appropriate dilutions of viral antigens usually lie between 1/10 and 1/300.

† (2) The standardized complement produces 70 % haemolysis in the 2·8 ml. total volume if no fixation occurs. This corresponds with the optimal density *D* 70 for the complement control tube no. 8. If the antiserum is anti-complementary the optical density for tube no. 7 is less than *D* 70 although at the dilutions of antiserum usually required no significant difference is detected. Similarly, if the antigen is anti-complementary at the dilutions tested, the optical densities *A* 70, *B* 70, *C* 70 for the control tubes no. 2, 4, 6 are less than *D* 70. Thus, the optical density depressions (Δ o.d.) shown in the table include corrections for any anti-complementariness of the corresponding antigen dilutions.

(3) The mean optical density depression due to fixation of complement in the proportional region is obtained from these data as

$$\Delta \text{ o.d. per } \mu\text{l. of undiluted antigen} = \frac{(A\,70 - A) + (B\,70 - B) + (C\,70 - C)}{8 \quad + \quad 4 \quad + \quad 2}.$$

(4) In pre-incubation procedures the complement is added to the systems after completion of the primary reaction between antigen and antibody. The titration and calculations are otherwise unchanged.

complement by antigen/antibody complexes will cause the percentage haemolysis now observed (x on line B in Fig. 1) to be below 70 % (s on line B) by an amount proportional to the volume of complement fixed ($\Delta C'$). The operational basis of this procedure is developed in the next section.

Table 1 illustrates the scheme of mixtures and controls required for an antigen titration by the haemolysis-depression procedure. In this case a constant and 'optimal' concentration of serum antibody is shown. All reactants were kept in iced water during the preparation of the mixtures and were added in the order shown. The reaction mixtures and controls of 1·8 ml. total volume (1·0 ml. complement + 0·4 ml. each of antibody and antigen dilutions) were prepared in duplicate in the matched 1 cm. diam. tubes of the spectrophotometer. In the '30 min. test' the reaction mixture was incubated at 37° for 30 min. before addition of 1 ml. haemolytic indicator system, also at 37°. After further incubation for 30 min. at 37° the samples were centrifuged to remove intact red cells and the optical densities of the clear supernatant fluids observed at 520 mμ. The final observation of the optical density of the supernatant fluid was reproducible to better than $\pm$ 5 % at the central point. The 'pre-incubation' procedure is defined in a later section.

Since the antigens, and occasionally the antisera, were slightly anticomplementary, the corrected depression of optical density for a given reaction tube was obtained by subtracting its optical density value from that of the appropriate 'antigen-only', or 'antibody-only', control. The method for calculation of results is shown in Table 1; it is equivalent to that followed in previous investigations (Bradish, Brooksby & Tsubahara, 1960) using the earlier procedure of '50 % haemolysis'.

Interpretation of complement-fixation data in terms of haemolysis depression. Consider a series of reaction mixtures of constant volume V (2·8 ml.) containing virus + antiserum + complement with 1 ml. standardized haemolytic indicator. The haemolytic indicator contains N red cells/ml. After incubation and centrifugation the supernatant fluids show the optical densities D 100, D 70, D, D 0, corresponding with the percentage haemolysis, 100, 70, H, 0.

We may now write
$$N = K(D\,100 - D\,0).\,V, \tag{1}$$
where the constant K defines the number of red cells in 1 ml. which gives an optical density of 1·0 at 100 % haemolysis. The value $K = 225 \times 10^6$ red cells/ml. was obtained when dilutions of suspensions of washed sheep red cells were counted on a Fuchs–Rosenthal slide before haemolysis in distilled water and observation of optical density.

Control tubes without virus or antiserum show 70, 50, H % haemolysis when the volumes of complement $C'70$, $C'50$, C' ml. are included (Fig. 1). The empirical equation of von Krogh (Kabat & Mayer, 1961) now relates the volume of complement to the percentage haemolysis through the expression
$$C' = C'50\left(\frac{H}{100-H}\right)^{\frac{1}{n}},$$
where n is a constant of value near 4. Over the experimentally significant central region from 20 to 80 % haemolysis, this relationship differs by less than $\pm$ 2 % from the linear form
$$C' = C'50\left(1+\frac{H-50}{25.n}\right). \tag{2}$$

This equation is illustrated by the data of the typical complement standardizations shown in Fig. 1. All such complement standardization lines cut the axis of haemolysis at the value $(50-25n)\,\%$. In 40 standardizations of the type shown in Fig. 1, the mean value of n was $3{\cdot}60 \pm 5\,\%$. The volume of complement for 50 % haemolysis ($C'50$) in this standardized system varied in different batches of guinea-pig serum over the range 2 to 10 μl.; the median value was about 6 μl. If now $C'70$ indicates the initial complement volume contained by every tube (point s on line B in Fig. 1) and C' the residual complement volume (point x) observed after fixation of the volume $\Delta C'$ by antigen/antibody complexes, then we may write, using equation (2),

$$\Delta C' = C'70 - C' = \frac{C'50}{25.n}(70-H). \tag{3}$$

or, in terms of the actually observed optical densities,

$$\Delta C' = \frac{4C'50}{n}\,\frac{D\,70-D}{D\,100-D\,0}. \tag{4}$$

The N red cells in the reaction mixture are 50 % haemolysed by the complement volume $C'50$. It follows that, under these reaction conditions, the volume of complement fixed by antigen/antibody complexes is equivalent to that required for the 50 % haemolysis of F red cells, according to the equation

$$F = \frac{\Delta C}{C'50}N. \tag{5}$$

Combination of expressions (1), (4) and (5) now gives

$$F = \frac{4\,K.V}{n}(D\,70-D). \tag{6}$$

This number of red cells which, at 50 % haemolysis, bind as much complement as that bound by the specific complexes, is thus obtained directly from the depression of optical density $(D\,70-D)$ by multiplication by the method constant $4\,K.V/n$. Using the numerical data quoted above, this constant has the value 7×10^8, and is essentially independent of the concentration of complement and red cells in the reaction mixture.

In the present report, later fixation data are given in terms of the directly observed depression of optical density ΔO.D. $= (D\,70-D)$. Previous reports from this laboratory have quoted experimental data in terms of units of complement fixed but this is less absolute than the present statement of F or of optical density depression. The simple numerical equivalence between these methods of presentation is defined by equation (4). The simplicity of this treatment of the sensitive linear region of the sigmoid response curve will be noted.

RESULTS

Quantitative aspects of antigen titration

Foot-and-mouth disease virus. The titration of antigen in the presence of excess antibody is a major application of the complement-fixation reaction. The proportionality between the volume or mass of reacting antigen and the volume of complement fixed has been shown for many systems and provides the basis for the

quantitative assessment of such titration data (Rice, 1946, 1947, 1959*a*, *b*, *c*, 1960). This proportionality appears as an initial linearity in the plot of optical density depression against volume or mass of antigen (Fig. 10). For the systems investigated here, however, this simple linear relationship is valid over only a relatively narrow range of antigen/antibody ratio. Outside this range, as discussed in following sections, the extent of fixation is limited or inhibited by either antigen or antibody.

When attention was confined to the titration of antigen in the presence of a nearly optimal content of antibody, data of the type shown in Fig. 2 were obtained. Such data define the maximum fixation of complement for a given mass of antigen.

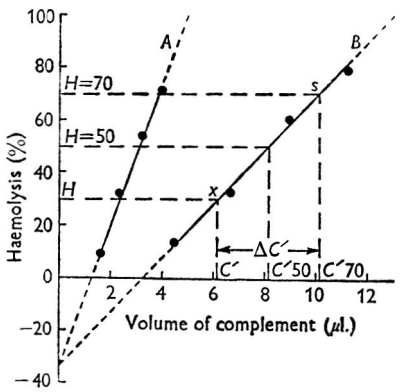

Fig. 1

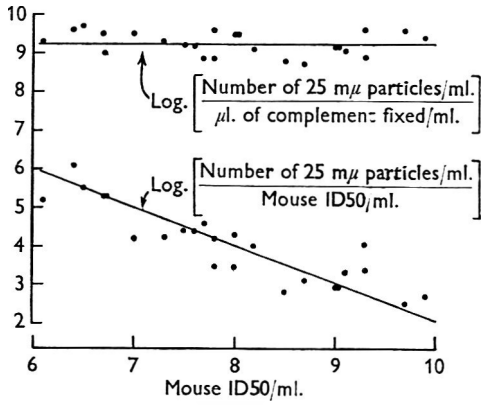

Fig. 2

Fig. 1. Standardization of two normal guinea-pig sera (A and B) as sources of complement. The indicated volumes of complement (1 ml. of normal serum diluted 1/50 ≡ 20 μl.) were made up to 1·8 ml. with veronal buffer and incubated 30 min. at 37° with 1 ml. standardized haemolytic indicator. The supernatant fluids were then clarified and their optical densities observed. Percentages of haemolysis were calculated from the mean optical density for two tubes. Point *s* on line *B* indicates that one 70 % haemolysis unit of complement is contained by 1 ml. of the normal serum at a dilution of about 1/100. At a dilution of 1/250 the serum A contains one 70 % haemolysis unit of complement in 1 ml. If 1 ml. of a standardized complement yields 70 % haemolysis in control tubes but only *H* % haemolysis when incubated with an antigen/antibody mixture (Table 1), then the corresponding points *s* and *x* on line *B* indicate that the volume of complement $C'70 - C' = \Delta C'$ has been fixed.

Fig. 2. Relationship between infectivity, complement-fixing activity and absolute number of 25 mμ virus particles in 24 separations from guinea-pig pad vesicular fluid and epithelium.

The antigen used in the experiments was the infective 25 mμ component of the foot-and-mouth disease virus system (type O/VI) isolated from guinea-pig pad vesicular fluid and epithelium. Each 1 ml. of antigen suspension represented the virus yield from the lesions on the ten plantar pads of five guinea pigs. The homologous guinea-pig antisera, at dilutions from 1/30 to 1/300, were mixed with complement and antigen and incubated for 30 min. at 37°. The data for 24 virus suspensions of extreme infectivity are shown in Fig. 2 in terms of the number of 25 mμ particles required for each ml. of complement fixed (upper line) and for each mouse infective unit (lower line). The number of 25 mμ particles for each ml. of complement fixed was essentially constant at $10^{9\cdot3\pm0\cdot1}$, regardless of the wide range of initial infectivity from 10^6 to 10^{10} ID50/ml. A linear histogram of the same data is given in Fig. 3. The

complement-fixation procedure may evidently be used to provide an estimate of the absolute count of 25 mμ particles on the basis of 2×10^9 particles/μl. complement fixed.

In contrast to the complement-fixation data, the number of 25 mμ particles for each mouse ID 50 (Fig. 2) decreased steadily as the infectivity of the virus suspension increased. Preparations of highest infectivity showed about 300 25 mμ particles/mouse ID 50. These data relate only to the intraperitoneal route of inoculation in unweaned mice (Pirbright strain), which was shown to be up to 1 log. unit less sensitive than the intramuscular route (Heatley, Skinner & Subak-Sharpe, 1960). It follows that, in the most infective suspensions, one mouse ID 50 by the intramuscular route might represent as few as 30 virus particles or even less, for infectivity assays of even higher efficiency. Bachrach & Breese (1958) observed particle/plaque ratios of 30–1600 in their studies of virus concentrates derived from cultures of bovine kidney cells. Such data are unrelated to the 'purity' of the preparations.

The slope of the lower line of Fig. 2 indicates that the virus suspensions contained about 10^{12} particles/ml. regardless of the initial infectivity. This implies that the epithelium from each guinea-pig pad yielded 10^{11} characteristic 25 mμ particles, of which, if every infective particle initiated infection, any proportion up to 1 in 30 might be infective. Thus, even in the most infective preparations of 10^{10}–10^{11} ID 50/ml., at least 97 % of the specific antigenic mass due to 25 mμ particles was non-infective or failed to initiate infection, and could be detected only by observation of complement fixation or other *in vitro* activity. Similar observations have been made for poliomyelitis virus (Mayer *et al.* 1957). Although the accountable infective component cannot contribute significantly to the observed fixation of complement, a coincidental correlation between infectivity and degree of complement fixation might be observed if, in the range of samples investigated, the concentrations of the infective component happened to be about proportional to the concentrations of the non-infective antibody-combining components.

Tomato bushy stunt virus and bovine plasma albumin. Titrations by complement fixation of these antigens were made under the same conditions as those defined in the previous section for the 25 mμ component of foot-and-mouth disease virus. The specific rabbit antisera were present at nearly optimal concentrations and incubation of the reaction mixture with complement continued for 30 min. at 37° (short test).

The complement-fixation data obtained are summarized in Table 2. Each entry relates to maximal fixation of complement by a given mass of antigen in the presence of nearly optimal antibody concentration. The mean maximal fixation extended only from 14 to 50 μl. complement/μg. antigen despite the use of many preparations of different antigens and antisera. Thus, a single conversion factor of 30 μl. complement/μg. antigen or 3 Δ O.D./μg. antigen summarizes to within a factor of 2 the data for the three systems entered in Table 2.

For the small 'spherical' particles of foot-and-mouth disease and bushy stunt viruses, each μl. normal guinea-pig serum as complement (Δ O.D. $\equiv$ 0·10) was equivalent to about 3×10^9 particles. At 50 % haemolysis each red cell in the haemolytic indicator system presents a complement-binding capacity equivalent to that of about 40 virus particles or 9000 molecules of bovine plasma albumin. Since the present procedure is sensitive to the fixation of less than 0·3 μl. complement

Table 2. *Maximal fixation of complement by three antigens in presence of optimal concentration of homologous antibody*

Antigen and molecular weight	Antiserum and dilution	Maximal fixation $\equiv \frac{\Delta \text{ O.D.}}{\mu\text{g. antigen}}$	Mean maximal fixation activity $\equiv \frac{\mu\text{l.}C'\text{ fixed}}{\mu\text{g. antigen}}$	$\frac{\text{Molecules antigen}}{\mu\text{l. } C' \text{ fixed}}$	Molecules of antigen equivalent to one red cell at 50 % haemolysis (equation 6)
25 mμ component of foot-and-mouth disease; $\sim 7 \times 10^6$	Guinea pig at 1/30 to 1/300	See data of Figs. 2 and 3	50	$1\text{–}5 \times 10^9$	14–70
Tomato bushy stunt virus: 9×10^6	Rabbit at 1/100 to 1/3000	1·00 1·32 1·46 1·58 1·58 1·62 1·66 1·97 2·27 2·30 2·67	18	$3{\cdot}8 \times 10^9$	54
Bovine plasma albumin: 7×10^4	Rabbit at 1/300	0·86 1·01 1·37 1·55 2·12	14	$6{\cdot}2 \times 10^{11}$	9000

($\pm 0{\cdot}03$ *C*′50 unit or $= 0{\cdot}03$ in optical density), it follows that 0·01 μg. antigen may be detected under appropriate conditions of maximal fixation. The fixation data published by Mayer and colleagues (1957) for purified preparations of poliomyelitis virus showed that about 10^{10} physical particles corresponded to one *C*′ 50 unit, or about 2×10^9 particles/μl. complement. This conforms closely to the data of Table 2, despite great differences in procedure.

Time of incubation of reaction mixtures

In previous studies from this laboratory, the mixtures of complement + antibody + antigen (Table 1) were placed together in the reaction tube and incubated for 30 min. at 37° before addition of the haemolytic indicator and further incubation for 30 min. at 37°. However, preliminary tests on the rate of complement uptake

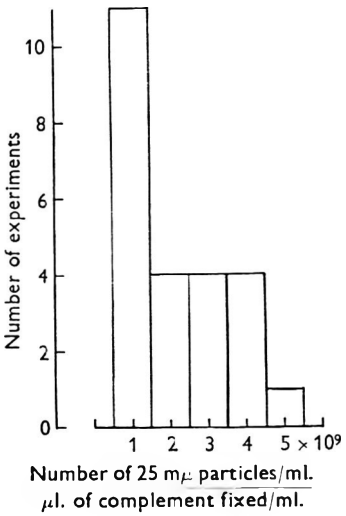

Fig. 3

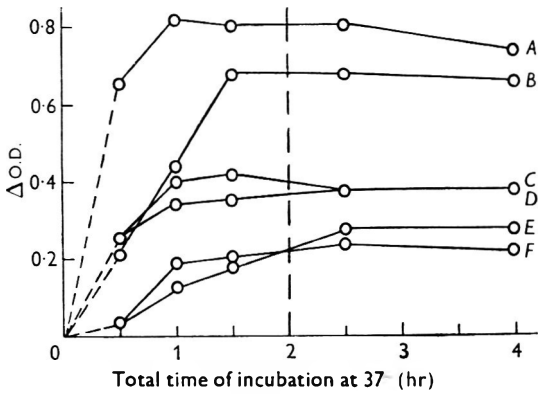

Fig. 4

Fig. 3. Histogram showing distribution of number of 25 mμ particles required to fix 1 μl. complement. This figure summarizes the data of the 24 experiments in Fig. 2.

Fig. 4. Relationship between fixation of complement as depression of optical density (Δo.d.) and total time of incubation at 37° for reaction mixtures of constant volume (0·8 ml.) and near-optimal proportions. The 70 % haemolysis unit of complement in 1 ml. was added only for the last 30 min. of incubation.

A and *B*: 1 and 4 μg. of bovine plasma albumin in 0·4 ml. with 0·4 ml. of rabbit hyperimmune serum diluted 1/200.

C to *F*: Dilutions of fractions of foot-and-mouth disease virus system (type C/GC) with equal volumes of guinea-pig hyperimmune serum diluted 1/150. In *C* and *D* 0·8 ml. and in *E* and *F* 0·4 ml. of the reaction mixture was tested. In *C* and *F* a 1/2 dilution of a fraction of the non-infective 7 mμ component was used and in *D* and *E* a 1/32 dilution of a fraction of the infective 25 mμ component.

by preformed complexes showed that, although complement was absorbed completely within 20–30 min. at 37° (Barbaro & Becker, 1962), the actual formation of the antigen/antibody complexes responsible for fixation was not always complete at this time. Figure 4 illustrates experiments in which antigen and antibody were allowed to react for periods of up to 3·5 hr at 37° before addition of standardized complement and further incubation for 30 min. at 37°. The addition of the stan-

dardized haemolytic indicator and observation of the optical density of the supernatant fluid then followed, as already described. The data of Fig. 4 show that the fixation of complement, or depression of optical density (Δ O.D.), was not complete in the then standard 30 min. incubation period, but that a pre-incubation of antigen and antibody for at least 1·5 hr at 37° was often necessary if a maximal reaction in terms of complement fixation was to be assured. Under conditions of lower antigen content, the terminal fixation was then 3 to 4 times greater than that observed in the '30 min. test'.

These experiments extend the previous observations on this system (Bradish *et al.* 1960) that the fixation of complement is secondary to that of complex formation, and that essentially as much complement is fixed by pre-incubated complexes to which complement is added later as is fixed by the same reactants when these are incubated with complement from the start. In equilibrium experiments like those of Fig. 4 it is, in any case, undesirable to incubate complement for the whole period with the other reactants since haemolytic activity will decay appreciably during the necessarily longer periods of incubation. For this reason, the standardized complement at 37° was added to all reaction systems only for the last 30 min. of incubation. This also served to minimize further the anti-complementariness of the reactants, which increased with time of incubation with complement.

A 'pre-incubation' test in which mixtures of antigen + antibody were incubated for up to 1·5 hr at 37° before addition of the '70 % haemolysis' unit of complement for a further 30 min. at 37° was adopted in the following experiments; the preparation and composition of reaction mixtures otherwise followed the scheme of Table 1. The use of a 'pre-incubation' procedure in place of the '30 min. test' ensured that maximal fixation of complement by equilibrated complexes was observed under conditions of extreme antigen or antibody excess.

The observation through complement fixation that under many conditions the formation of antigen/antibody complexes only reached completion in about 1·5 hr at 37° contrasted sharply with parallel observations through neutralization of infectivity (Bradish *et al.* 1962), which indicated that complex formation was essentially complete within 10 min. at 37°. This disparity suggested that the neutralization of infectivity was complete when only small early complexes of antigen + antibody had been formed. At this stage, the specific complex was still too small to show significant fixation of complement; the larger complexes required for maximal fixation of complement only appeared much later.

Inhibition of fixation by antigen excess

It was shown in a recent report concerning complement fixation and neutralization of infectivity (Bradish & Farley, 1960) that the fixation of complement was inhibited completely when a constant dilution of guinea-pig or rabbit hyperimmune serum reacted with the 25 mμ component of foot-and-mouth disease virus at a sufficiently high concentration. Similar inhibitions of complement fixation occurred when the antigen was the 7 mμ component of the virus system. The complexes formed in the virus/antibody reactions thus showed fixation properties which were similar to those observed for many non-viral systems. The present comparative data for the viruses of foot-and-mouth disease (Fig. 5) and tomato bushy stunt (Fig. 6) in combination with constant antibody show that, in the initial region of low antigen

content, the fixation of complement increased with mass of available antigen. This is the titration region of proportional fixation discussed in an earlier section. Reaction mixtures of higher antigen content showed a fixation of complement which was below that required by the initial linear relationship, through partial inhibition by slight antigen excess. With yet higher concentrations of antigen (Figs. 5, 6), the extent of fixation passed through a maximum value and was then depressed to a negligible value. The value of complement fixation at the maximum is defined by the antibody content of the system and occurs at a definite equivalent concentration of antigen. In general, the degree of fixation is 50 % of the maximum value at an antigen concentration of 2 to 3 equivalents, and is negligible at an antigen concentration of 5 to 10 equivalents.

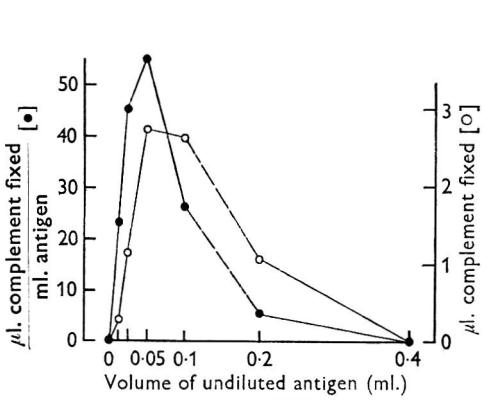

Fig. 5

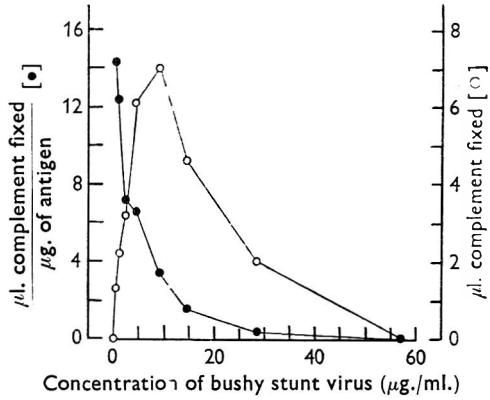

Fig. 6

Figs. 5 and 6. The relationship between level of complement-fixation (○) and specific-fixation activity (●) as a function of the mass of antigen reacting with constant antiserum for 30 min. at 37° before addition of complement for 30 min. at 37°.

Fig. 5. Guinea-pig hyperimmune serum (0·4 ml.) at dilution 1/200 with equal volume of dilutions of fraction of 7 mμ non-infective component of foot-and-mouth disease virus (type O/VI).

Fig. 6. Rabbit hyperimmune serum (0·4 ml.) at 1/1000 dilution with equal volume of dilutions of a suspension of bushy stunt virus.

The maximal fixation of complement/unit mass antigen occurred only in the initial proportional region where antibody was in adequate excess with respect to antigen. The maximal fixation of complement for unit mass of antibody occurred at the peak of fixation (Figs. 5, 6) where antigen was in slight critical excess with respect to antibody. Thus, antigen and antibody cannot be present simultaneously under conditions appropriate to their individual maximal fixation activities. This is illustrated by the second curves (filled circles) of Figs. 5 and 6, which show that the maximal fixation of complement with respect to antigen occurred for significantly lower concentration of antigen than that for the maximum of total fixation (open circles).

The confirmation of antigen excess

Direct tests of the actual availability of free antigen or of antibody-combining sites in reaction mixtures of higher antigen content than that which gave maximal fixation of complement were set up as shown in Fig. 7. Dilutions of the separated

7 mμ (curves *A* and *B*) and 25 mμ (curves *C* and *D*) components of foot-and-mouth disease virus (type A/GB) were mixed with homologous guinea-pig antiserum at dilutions 1/150 or 1/300. After incubation for 30 min. at 37°, the 70 % haemolysis unit of complement was added with and without a further portion of antiserum. The fixation test continued in the standard manner after a second incubation for 30 min. at 37°. The data of Fig. 7 for the control mixtures without the second addition of antiserum (curves *B* and *D*) showed the usual maxima of fixation with later inhibition by excess antigen. When second additions of antiserum were made (curves *A* and *C*) the degrees of fixation were unchanged at antigen concentrations below the optimum, but were significantly increased in the regions to the right of the fixation maxima. It therefore appeared that only in such regions was antigen in excess and free to combine with later additions of antibody and to fix more complement. In regions to the left of the fixation optima (Fig. 7) the addition of complement and more antiserum did not increase the degree of fixation. Thus antibody was already in effective excess in this region. It seems probable that, in the region of 'antigen excess' to the right of the fixation maximum, the antigen was present as small complexes with antibody which were able to bind more antibody and thereby fix more complement. An increasing proportion of free uncombined antigen became available only at higher excess of antigen. Identical conclusions follow from parallel studies of the reactions of other foot-and-mouth disease viruses, tomato bushy stunt virus and bovine plasma albumin with their specific rabbit antisera.

Cross-reactions in regions of antigen excess

Since the present studies are concerned with complement fixation by specific antigen/antibody complexes, it was necessary to confirm that fixation by one antigen/antibody system was uninfluenced by reactions in a second system present in the same medium (Hoyle, 1945–46; Fulton, 1958; Rice, 1959*a*, *b*, *c*). If specific cross-reactions between two systems are to be detected by complement fixation, these reactions must occur because the antigens and antibodies involved are related and not because hybrid complexes are formed through the non-specific association or entanglement of unrelated complexes.

The independent formation of unrelated complexes was tested through the reactions of a mixture of two rabbit hyperimmune sera with a mixture of homologous antigens. The mixture of rabbit anti-sera contained anti-bushy stunt serum at 1/64,000 and anti-foot-and-mouth disease virus serum at 1/32. These dilutions were established by preliminary titrations as being suitable for the separation of the anticipated fixation peaks. Samples of the mixture of antisera were incubated with dilutions of a suspension of bushy stunt virus, dilutions of a suspension of the 25 mμ component of foot-and-mouth disease virus (type O/VI) and dilutions of a mixed suspension containing these two antigens at the same concentrations. After 30 min. at 37°, the 70 % haemolysis dose of complement was added and the test continued in the standard manner.

The results of this experiment are shown in Fig. 8. The reaction with foot-and-mouth disease virus (*F*) or tomato bushy stunt virus (*B*) resulted in a typical fixation curve with maximal fixation of complement for 50 and 3 μl. of antigen, respectively. The reaction with the mixed antigens (*M*) indicated a composite fixation which approximated to the total of the fixations due to each antigen when

used separately. Thus each antigen/antibody system was effectively responsible for a definite fixation of complement; and neither the formation of specific complexes nor the subsequent fixation of complement by such complexes was modified by the simultaneous reactions of other antigens. The curve of composite fixation (*M*) shows the maximal fixations due to each component of the mixture of antisera almost as well as these are shown by the individual reaction curves (*F* and *B*). Similar data are shown in Fig. 9 for the mixed system of foot-and-mouth disease virus + bovine plasma albumin, reacting with their homologous rabbit antisera.

The consequence of reactions between related antigen/antibody systems is shown in Fig. 10. The antigens in this experiment were the separated 7 and 25 mμ components of the foot-and-mouth disease virus system (type O/VI). These antigens

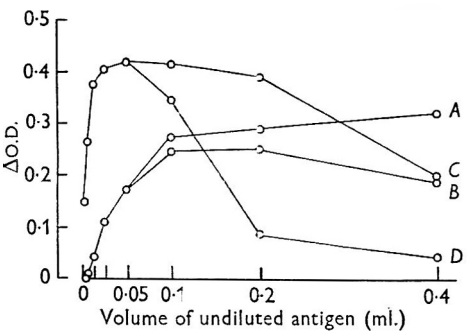

Fig. 7

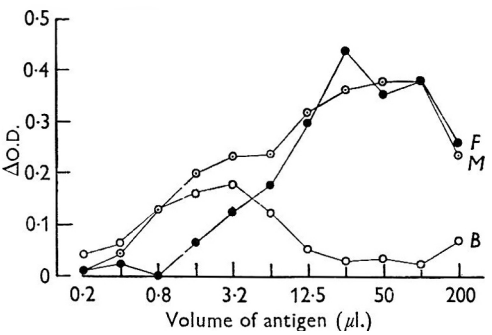

Fig. 8

Fig. 7. Demonstration of availability of antibody-combining sites in regions of nominal antigen excess. Dilutions of fractions of foot-and-mouth disease virus system (type A/GB) in reactions with successive equal volumes of homologous hyperimmune guinea-pig serum. *A:* (7 mμ component + serum/300) + (*C*′ 70 + serum/300); *B:* (7 mμ component + serum/300) + (*C*′ 70 + veronal buffer); *C:* (25 mμ component + serum/150) + (*C*′ 70 + serum/150); *D:* (25 mμ component + serum/150) + (*C*′ 70 + veronal buffer); Volumes: (0·4 ml. + 0·4 ml.) + (1·0 ml. + 0·4 ml.). The reaction mixtures were incubated for 30 min. at 37° before (first bracket) and after the addition of the 70 % haemolysis unit of complement (*C*′ 70).

Fig. 8. Demonstration of independent fixation of complement by two systems reacting simultaneously. Mixed rabbit antiserum used throughout contained anti-bushy stunt serum diluted 1/64,000 and foot-and-mouth disease virus antiserum at 1/32. Pre-incubation of reactants for 30 min. at 37° before addition of complement.

F: Reaction with dilutions of suspensions of 25 mμ infective component of foot-and-mouth disease virus (type O/VI).

B: Reaction with dilutions of suspension of tomato bushy stunt virus, optimum at 3·2 μl. or 0·7 μg.

M: Reaction with mixture of antigens *F* and *B* above adjusted to give same concentrations as reactions with single antigens.

were mixed in the proportions shown by the volume scales in Fig. 10. Homologous guinea-pig antiserum at an optimum dilution of 1/40 replaced the mixture of antisera of the previous experiments. After incubation for 30 min. at 37° the fixation by the system containing the 7 mμ component as the only antigen followed the almost proportional relationship previously discussed. The incorporation of 0·25–1 μl. of the 25 mμ component in mixtures with the 7 mμ component did not disturb this proportionality. The fixation of complement was greatly decreased, however, by

the incorporation of 2 μl. of the 25 mμ component and wholly suppressed by the incorporation of 4 μl. Similar data were obtained when increasing proportions of the 7 mμ component were mixed with the 25 mμ component before reaction with antiserum.

Such data indicate that the 7 and 25 mμ components of the foot-and-mouth disease virus system were competitive antigens which reacted with common antibodies in guinea-pig and rabbit antisera in accordance with their relative concentrations and activities. Such hyperimmune sera apparently contained no significant proportion of antibody which reacted with only the 25 mμ or the 7 mμ component of the virus system. These data extend those of earlier reports (Bradish *et al.* 1960; Bradish *et al.* 1962).

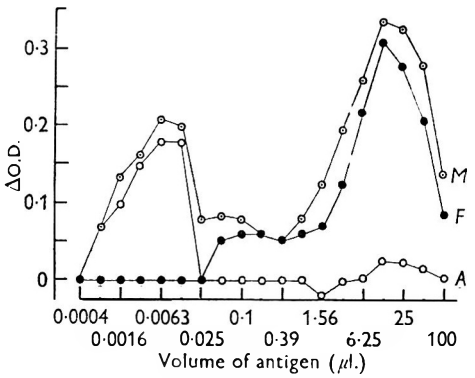

Fig. 9

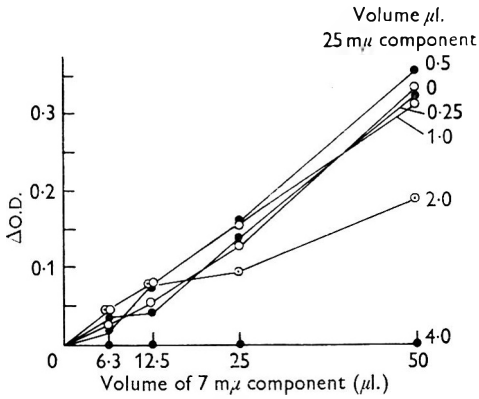

Fig. 10

Fig. 9. Demonstration of independent fixation of complement by two systems reacting simultaneously. Mixed rabbit antiserum used throughout contained anti-bovine plasma albumin serum diluted 1/300 and foot-and-mouth disease antiserum diluted 1/15. Pre-incubation of reactants for 1·5 hr at 37° before addition of complement.

F: Reaction with dilutions of suspension of 25 mμ infective component of foot-and-mouth disease virus (type C/M 11).

A: Reaction with dilutions of solution of bovine plasma albumin, optimum at 0·0063 μl. or 0·18 μg.

M: Reaction with mixture of antigens *F* and *A* above adjusted to give same concentrations as in reactions with single antigens.

Fig. 10. Demonstration of competition for available antibody by 25 mμ and 7 mμ components of foot-and-mouth disease virus system (type O/VI). A constant 0·4 ml. hyperimmune guinea-pig serum at dilution 1/40 reacts for 30 min. at 37° with 0·8 ml. of a series of mixed antigens prepared from fractions of the virus components. The scales show the final content of each antigen in the reaction mixtures. The 70 % haemolysis dose of complement was added for a final 30 min. at 37°.

Proportions of antigen and antibody in relation to extent of fixation

The discussion of antigen/antibody reactions in terms of the combination of a constant concentration of one reactant with a series of concentrations of the other requires extension to the general case in which both reactants are used in a single experiment over a range of concentrations. Figures 11 and 12 show fixation data of this kind for a series of reaction mixtures containing up to 9 μg. tomato bushy stunt virus and up to 1·6 μl. rabbit antisera. Such data are representative of other experiments with bovine plasma albumin or the components of the foot-and-mouth

disease virus system. In Fig. 11 each curve represents a single antibody content and in Fig. 12 a single antigen content. The constant initial slope of the family of curves in Fig. 11 characterizes the fixation due to antigen as limiting reactant in presence of a slight excess of antibody; such data have been discussed in an earlier section. The subsequent inhibition of fixation by excess of antigen is shown clearly by the curves for the lowest contents of antibody. At higher concentrations of antibody, with antigen still in excess, the fixation of complement was limited by the content of antibody, but was not proportional to the concentration. An overall maximum of fixation occurred for a reaction mixture containing 1 μg. virus and about 0·4 μl. rabbit antiserum.

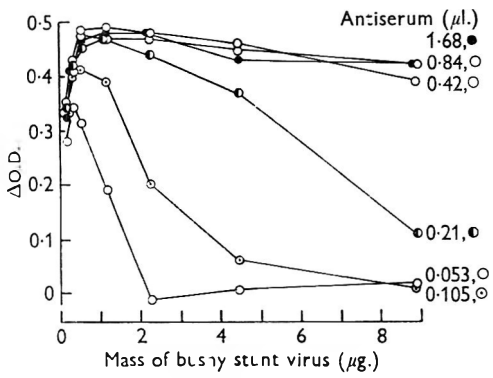

Fig. 11

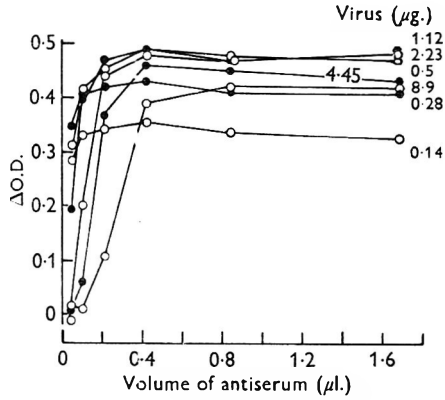

Fig. 12

Figs. 11 and 12. Fixation characteristics for reaction of tomato bushy stunt virus with hyperimmune rabbit serum. Incubation of 0·4 ml. of each reactant for 30 min. at 37° before and after addition of 70 % haemolysis dose of complement in 1 ml. In Fig. 11 the data are shown as curves of constant antiserum content and in Fig. 12 as curves of constant virus content. The scales show the actual content of virus in μg. and of undiluted antiserum in μl.

In Fig. 12 the curves of constant antigen content do not show a common initial slope, indicative of the antiserum activity, since fixation was inhibited by the initial excess of antigen. At the highest values of antigen content the initial fixation was completely depressed and the activity of antiserum was indicated only by the steepest initial slope for the curves of lowest antigen content. Later fixation was limited by the antigen content of the reaction mixtures. The fixation of complement in this reaction series was proportional to the antigen content only below 0·14 μg.; the overall maximum of fixation is shown as in Fig. 11.

Figures 11 and 12 show that a given degree of fixation may be shown by many different values of antigen and antibody concentration. A quantitative titration of the concentration of either reactant required that attention be confined to the initial linear region in which the extent of fixation was limited proportionally by the concentration of one reactant and was not limited or inhibited by the concentration of the other. Similar data for the 25 mμ infective component of the foot-and-mouth disease virus system (type O/VI) are shown in Table 3. The significant inhibition of complement fixation by excess of rabbit antiserum or guinea-pig antiserum has been regularly observed, but the effect seems to be more frequent with rabbit antiserum.

It is apparent from these data (Table 3; Figs. 11, 12) that, although the maximal fixation observed in the presence of a given concentration of antiserum occurred at a definite concentration of antigen, an increased concentration of antiserum did not correspond at the new optimum with a proportionally increased concentration of antigen. If a law of simple proportionality was applicable, then the maximal fixations in the rows and columns of Table 3 would lie on a single diagonal, and there would be no overall maximum of fixation. All of these data indicate that the concentrations of antigen and antibody giving maximal fixation of complement cannot be related by a law of simple proportions. The optimal concentrations of antigen and antibody are, in fact, almost independent since the maximal fixation of complement is given by an almost constant concentration of antigen regardless of the antiserum dilution employed. The concentration of antiserum for maximal fixation is similarly very little influenced by variation of antigen concentration.

The formation of complexes in the presence and absence of complement

In these experiments the 25 mμ infective component of foot-and-mouth disease virus used as antigen was separated from the supernatant fluids of cultures of baby hamster kidney cells. Complexes with homologous guinea-pig antiserum were formed in the presence and absence of the 70 % haemolysis unit of complement. After incubation for 1·5 hr at 37° these reaction mixtures were centrifuged in Spinco L 40 tubes for 1 hr at 30,000 rev./min. Supernatant fluids of 5 ml. volumes were then carefully withdrawn from the 8 ml. volumes loaded, and taken forward for fixation assay together with non-centrifuged controls. Samples containing no complement received the 70 % haemolysis unit after ultracentrifugation and all samples were then incubated for a further 30 min. at 37°. The 'virus-only' controls received antiserum and complement after ultracentrifugation.

The data of Fig. 13 show that, when complexes were formed in the presence of complement, the supernatant fluids (*F*) and non-centrifuged controls (*E*) retained the same high degree of fixation activity. Thus the presence of complexes with bound complement (*E*) did not contribute to the haemolysis by free complement. When complexes were formed in absence of complement, the activity of the supernatant fluids (*D*) was eliminated although the non-centrifuged control (*C*) showed a fixation activity like that of the complexes formed in the presence of complement (*E*, *F*). Thus, in *F* the complexes responsible for fixation were removed together with their bound complement, whereas in *D* the complexes were removed before complement was added. When virus only was centrifuged (*B*) the activities of the supernatant fluids were almost eliminated, whereas those of the non-centrifuged controls (*A*) were almost the same as those of the complexes formed in presence or absence of complement (*E*, *F*, *C*). Hence, as in experiments with bushy stunt virus as antigen, complement was effectively bound by preformed 'soluble' complexes.

The irreversibility of complex formation and complement binding

The irreversibility of complex formation and complement binding was tested by forming antigen/antibody complexes under optimal conditions and adding to these equilibrated mixtures more antigen in excess. Incubation and addition of complement then continued as defined previously. For the foot-and-mouth disease virus and bovine plasma albumin systems quoted in Table 4, the reactions under optimal

Table 3. *Complement-fixation data for infective 25 mμ component of foot-and-mouth disease virus separated from suspension of guinea-pig pad vesicular epithelium (type O/VI) in reaction with homologous rabbit and guinea-pig antisera*

Volume of antigen in reaction mixture (μl.)	Rabbit antiserum (μl.)					Guinea-pig antiserum (μl.)					
	6·3	12·5	2·5	50	100	0·63	1·25	2·5	5·0	10	20
	Depression of optical density Δ O.D.										
1	0·01	0·06	0·09	0·02	−0·02	0·13	0·19	0·20	0·19	0·18	0·16
2	0·04	0·09	0·14	0·01	0·00	0·22	0·33	0·36	0·33	0·33	0·27
4	0·12	0·17	0·17	0·08	0·04	0·31	0·43	0·45	0·45	0·47	0·42
8	0·13	0·21	0·20	0·10	0·09	0·22	0·34	0·38	0·36	0·39	0·36
16	0·18	0·27	0·30	0·09	0·08	0·10	0·28	0·31	0·31	0·33	0·31

The lines in the table show the positions of the α-optimal (horizontal) and β-optimal (near vertical) reactions.

Table 4. *Demonstration of irreversibility of complex formation following addition of antigen in excess*

Antigen (G)	Homologous Antiserum (S)	Reaction conditions	Reaction mixture (first incubation mixture shown in brackets)	Incubation time at 37° after each addition	Complement fixation as depression of optical density (Δ O.D.)
Suspension of 25 mμ component of foot-and-mouth disease (type C/GC) from guinea-pig pads	Guinea-pig at dilution 1/150	Optimal proportions	(400 μl. G/16 + 400 μl. S) + 400 μl. buffer	30 min.	0·32
		Initially optimal	(400 μl. G/16 + 400 μl. S) + 400 μl. G		0·30
		Antigen excess	(400 μl. G/16 + 400 μl. G) + 400 μl. S		0·00
Solution of bovine plasma albumin, 100 μg./ml.	Rabbit at dilution 1/300	Optimal proportions	(400 μl. G/30 + 400 μl. S) + 80 μl. buffer	90 min.	0·36
		Initially optimal	(400 μl. G/30 + 400 μl. S) + 80 μl. G		0·36
		Antigen excess	(400 μl. G/30 + 80 μl. G) + 400 μl. S		0·10

conditions gave maximal fixation of complement. This degree of fixation was not disturbed by further incubation with excess of antigen. However, when the antiserum and the total excess of antigen were incubated together from the start, fixation of complement was decreased. Thus, once formed, the complexes responsible for the later optimal fixation of complement were not modified by continued incubation with antigen in excess. This effective irreversibility of the reaction between antigen and antibody paralleled that already shown as a feature of the neutralization of the infectivity of the foot-and-mouth disease virus. The dissociation of antigen/antibody complexes by modifications of medium introduces additional features not involved in the present considerations of irreversibility with respect to changes of reactant concentrations.

DISCUSSION

The most obvious conditions which define the reaction between antigen and antibody are the concentrations of the reactants; the reactant concentrations control the rate at which antigen/antibody complexes are formed and the nature of the reaction product at equilibrium. However, the rate effect cannot properly be investigated by complement fixation since the initial combination of antigen and antibody is not only more rapid than the binding of complement, but produces many small early complexes which fix little or no complement. Thus complement fixation procedures are restricted to studies of antigen/antibody combination at or near equilibrium, where the rate factors which arise from the distinct primary and secondary reactions are not involved.

In many of the experiments described here the complement system was added to the antigen/antibody mixture only after the completion of the primary reaction. It has been shown, subject to the stability of complement and the completion of the primary and secondary reactions, that the time at which complement is added to the primary reaction mixtures has little effect on the degrees of fixation subsequently observed. It thus became possible to impose experimental modifications upon the fundamental primary reaction without alteration to the standardized secondary reaction involving complement and haemolytic indicator.

The distinction between the complement-binding capacity of antigen/antibody complexes formed in the presence or absence of complement was summarized by Barbaro & Becker (1962). These authors studied the albumin/anti-albumin reaction and suggested that the greater fixation shown by complexes formed in the presence of complement was a unique property of rabbit antisera, since the reverse situation was observed with certain horse and sheep antisera. Although the present studies are not consistent with this conclusion, it may be pointed out that the fixation tests involved are very different. Barbaro & Becker (1962) used the procedure of Mayer, Osler, Bier & Heidelberger (1948) in which the reactants combine in the presence or absence of 50–100 *C′* 50 units and are subsequently chilled and diluted before addition of the haemolytic indicator. Complement-fixation tests in virology use reactants at much lower concentrations; with a total complement load of usually not more than 5 *C′* 50 units there is no dilution before addition of the haemolytic indicator. In the present work only one *C′* 70 unit or about 1·2 *C′* 50 units of complement was used.

Preliminary experiments with the bovine plasma albumin system have shown that the degree of fixation at the optimum is very similar by the present and by the high-complement-load procedures but that the sharpness of the fixation optimum increases markedly as the complement load is decreased to the present low value. Clearly it cannot be assumed that the complement-binding properties of complexes formed by reactants at low concentrations will correspond with those formed at high reactant concentrations.

The relative activities of antigen and antibody are reflected in the present complement-fixation data by sharp maxima of fixation. Such maxima occur for definite concentrations of antigen and antibody, which are thereby defined as optimal. Although limitations of antigen concentration have caused this feature of the reaction to be exa-

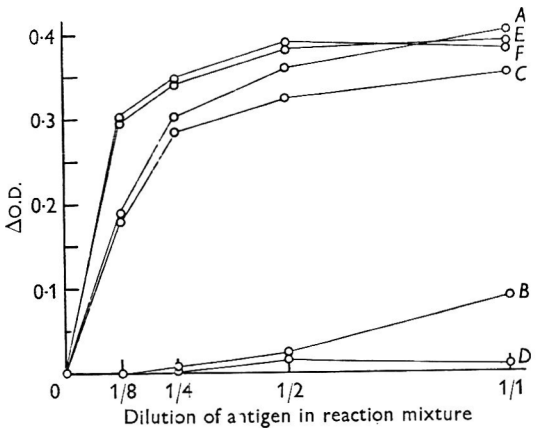

Fig. 13

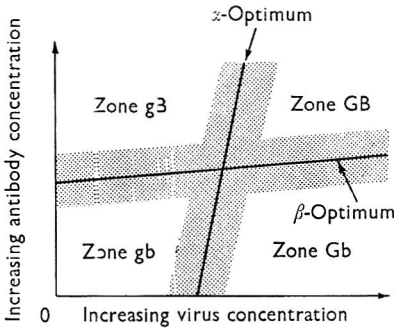

Fig. 14

Fig. 13. Fixation of complement by preformed 'soluble' complexes in reactions of 25 mμ component of foot-and-mouth disease virus (type O/M 11, 10th passage on BHK cells) with an equal volume of hyperimmune guinea-pig serum at dilution 1/300. Sequence in reactions: *A*. [((Virus) + antiserum) + complement]. *B*. [((Virus supernatant) + antiserum) + complement]. *C*. [(Virus + antiserum) + complement]. *D*. [Supernatant fluid of (virus + antiserum) + complement]. *E*. (Virus + antiserum + complement). *F*. Supernatant fluid of (virus + antiserum + complement). The round bracket indicates incubation for 1·5 hr at 37° and the square bracket for 30 min. at 37°. Virus and antiserum were present as 0·4 ml. each and complement as the 70 % haemolysis dose in 1 ml.

Fig. 14. Pattern of reactions shown in complement fixation by virus/antibody complexes. 'g' and 'G' signify antigen concentrations below and above the α-optimum value. 'b' and 'B' signify antibody concentrations below and above the β-optimum value. The α-optimum is that antigen concentration giving maximal reaction against a constant antibody content. The β-optimum is that antibody concentration giving maximal reaction against a constant antigen content.

mined only rarely for animal virus systems (Mayer *et al.* 1957; Fulton, 1958; Bradish & Farley, 1960), it has long been recognized as a feature of the reactions of the small protein and bacterial antigens (Dean, 1912; Opie, 1923; Brown, 1934) and may be regarded as the demonstration by complement fixation of the classical precipitation curve (Heidelberger, 1956). Such parallel fixation and precipitation studies indicate the reactions of common antigen/antibody systems; distinct antibody species for each experimental procedure were not postulated. In appropriate tests the observations of complement-fixation reactions relate only to 'soluble' complexes, and differ

from those in precipitation or flocculation studies by responding to much lower concentrations of reactants and by not requiring the formation of gross insoluble complexes and the interpretation of a variety of non-specific physico-chemical factors. The distribution of antigen/antibody complexes formed in the system at equilibrium is interpreted as being determined by concentrations of reactants and by their valencies (Goldberg, 1952, 1953). This model has received some confirmation in virology through studies on tomato bushy stunt virus and its rabbit antiserum (Bradish & Crawford, 1960). In terms of this model, the region of limited antibody excess or near-equivalence is such that all reactive sites on the antigen are saturated by specific antibody, and the number of antigen/antibody bonds or of complexes formed will be approximately proportional to the concentration of antigen in the system. Since it has been shown that in this restricted zone the fixation of complement is proportional to the concentration of antigen, it thus appears that each virus particle or virus + antibody bond is associated with the fixation of a definite 'unit' of complement. Subject to restrictions related to the quality of the antiserum used, the viruses of foot-and-mouth disease, tomato bushy stunt and poliomyelitis all require about 3×10^9 particles for the maximal fixation of the complement content of 1 μl. normal guinea-pig serum. About 6×10^{11} molecules of bovine plasma albumin are required for this degree of fixation.

The region of antigen excess is characterized by a lower degree of complement fixation for mixtures of higher antigen concentration. Most reactive sites on the antibody are now saturated by specific antigen and the degree of fixation is controlled by the sizes of the complexes formed in relation to the degree of antigen excess. Since increasing antigen excess is associated with the elimination of the larger complexes, it appears that complement is bound effectively only by such complexes and not significantly by greater masses of antigen and antibody distributed as smaller complexes.

The interpretation of complement-fixation data in the region of the optimum presents some difficulties, since the results for different systems do not support the assumption that equivalent concentrations of antigen and antibody are proportional. The optimal fixation of complement occurs at a definite concentration of antigen which varies little with the constant concentration of antibody used in the test. Similarly, in tests with constant antigen content, the optimal fixation occurs at a definite concentration of antibody which is almost independent of the homologous antigen. In the nomenclature of plant virus serology (Matthews, 1957) this is equivalent to the statement that the α-optimum line (value of antigen concentration giving maximal fixation in 'constant-antiserum' tests) and the β-optimum line (values of antibody concentration giving maximal fixation in 'constant-antigen' tests) are almost at right angles and intersect at that unique antigen/antibody mixture which gives the overall maximum of fixation; this situation is summarized in Fig. 14.

The dilution of a virus sample to its α-optimum concentration allows maximal fixation against a range of dilutions of homologous antisera. Similarly, the dilution of an antiserum to its β-optimum concentration allows maximal fixation against a range of dilutions of homologous antigens. Such α- and β-optimum dilutions constitute a sensitive indication of the combining activities of the antigen and the antiserum used, and indicate an alternative procedure for titration of these reactants and

for standardization of the fixation test. The near-constancy of the optimal concentrations of antigen and antibody distinguishes the present complement-fixation data from the more familiar pattern of simple proportions which appears to characterize most gross flocculation data (Matthews, 1957; Belyavin, 1957; Smith, 1958) in which the α- and β-optimum lines pass diagonally across the reaction area from the origin.

It is apparent that the intersection of the α- and β-optimum lines (Fig. 14) divides the area of the antigen/antibody reaction into four zones of distinct fixation character. These zones may be considered as follows:

gB: the zone of antibody excess in which antigen is the limiting reactant.

Gb: the zone of antigen excess in which antibody is the limiting reactant.

gb: the zone in which both antigen and antibody are present at sub-optimal concentration.

GB: the zone in which both antigen and antibody are present at supra-optimal concentration.

Thus, for example, a fixation experiment with variable antigen and constant antibody represents a horizontal section of the reaction area with maximal fixation at the α-optimum. The detailed character of such an experiment depends upon the 'quality' of the antibody and the choice of antibody concentration as low, optimal or high. At constant low antibody content the reaction zones gb–Gb indicate an initial fixation proportional to the antigen content (zone gb) which passes rapidly, after maximal fixation at the α-optimum, into the region of antigen excess inhibition (zone Gb). These reaction zones have been discussed previously in relation to the neutralization of infectivity (Bradish *et al.* 1962). At constant high-antibody content in the zones gB–GB the initial fixation (zone gB) may be inhibited by antibody excess. The later fixation increases non-proportionally with the antigen content and, after maximal fixation at the α-optimum, passes into the supra-optimal zone GB in which, again according to the antiserum used, the degree of fixation may remain near-maximal or decrease only slowly with increasing antigen content. These regions (gB–GB) typify the data previously published from this Institute in which relatively high concentrations of antiserum were used (Bradish *et al.* 1960). The marked inhibition of fixation by antigen excess is a feature of the zone Gb only. The data obtained by Mayer *et al.* (1957) for the poliomyelitis virus/rabbit antiserum system show many of these features.

Similar considerations apply to the vertical sections of the reaction area which represent fixation experiments with constant antigen and a range of antibody concentrations. However, the complexity of the reaction area shown in Fig. 14 indicates that the quantitative assay of antibody by complement fixation requires particular attention (Fulton, 1958; Rice, 1947, 1959*b*). Wallace, Osler & Mayer (1950) have emphasized that in the bovine serum albumin/rabbit antiserum system the degree of complement fixation is greatly influenced by the quality of the antiserum and does not relate simply to the quantity or content of antibody. These difficulties in the present systems are largely due to the limited range of antibody concentration over which fixation is proportional to the antibody content without being inhibited by antigen excess or limited by approach to antibody excess. The observed antibody activity (depression of optical density/μl. undiluted antiserum) is acceptable as an assay conclusion only if independent of the concentration of both antigen

and antibody over a significant range of these variables. Subject to adequate controls, the analysis of the data for this region of confirmed proportional fixation then follows as described for the titration of antigen. The most sensitive titration of antibody is thus available in the region of the α-optimum line (shaded in Fig. 14), between the reaction zones gb and Gb. Similarly, and as previously discussed, the most sensitive titration of viral antigen is obtained in the region of the β-optimum line between the reaction zones gb and gB.

Fulton & Almeida (1962) have discussed a procedure for titration of antiserum in antigen excess which depends upon a linear relationship between the amount of antiserum and the degree of complement fixation in this region (GB and Gb in Fig. 14). Fixation data for the systems studied here do not confirm this relationship as a basis for the titration of antiserum. These authors also discuss the selection of linear or logarithmic scales for the presentation of fixation data. In the present paper both scales have been used as a matter of convenience according to the experiment and the range of variables to be presented in the figure. The generally non-monotonic character of the fixation data is such that neither linear nor logarithmic scales have any special significance in relation to the description of the overall reaction. Over the limited regions of proportional fixation already discussed, the use of linear relationships and scales appears to be well justified.

The failure of the law of simple proportions in the correlation of fixation data blocks any statement about an absolute molecular equivalence of virus and antibody. Thus, the direct enumeration of virus particles by electron microscopy cannot be related through complement fixation to an indirect enumeration of antibody molecules. It is clear that fixation data may not be extended in terms of simple proportions or of constancy of antigen/antibody ratio to other conditions of reactant concentrations.

Notwithstanding the complexity of the fixation of complement by the antigen/antibody complex it has been shown that many features of the reaction may be indicated very sensitively by such methods. Thus, the state of irreversibility of the reaction, the recognition of independent or cross-combining systems, the indirect assay of virus particle concentration, the correlation of fixation with neutralization and adsorption data, the location of the optimum reaction and the confirmation of equivalence, all illustrate the information available beyond that of the routine assay or typing experiment.

Parallel studies on the components of the complex virus system of vesicular stomatitis (major infective component 175×70 mμ) have confirmed that the present methods and general conclusions are not confined to the smaller solvent-insensitive viruses.

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The Effect of Nitrogenous Substances on the Time of Flocculation of *Saccharomyces cerevisiae*

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SUMMARY

The point in the growth cycle at which a strain of brewer's yeast became potentially flocculent could be delayed by supplementing the medium with ammonia, basic amino acids, glutamine, asparagine, γ-aminobutyric acid or urea. Other amino acids were ineffective. β-Alanine and 2-chloro-4-aminobenzoic acid led to an abnormally early appearance of potential flocculence. No development of flocculence occurred in the absence of glucose. It is suggested that the maintenance of non-flocculence is dependent upon the presence in the cell wall of a nitrogenous compound; potential flocculence will develop when this compound is not synthesized at a rate sufficient to maintain its concentration in the wall. The nitrogenous nutrients which delay flocculation would then act by enhancing this rate of synthesis.

Brewer's yeast cells are freely dispersed in the early stage of logarithmic growth, but as this continues they tend to adhere to each other, forming clumps or flocs. The tendency to flocculate varies with different strains and is, to some extent at least, under genetic control (Gilliland, 1951; Thorne, 1951). Cells which have become flocculent may be reversibly separated by a variety of treatments, even washing in saline will suffice. But such redispersed cells differ from the disperse cells of the early logarithmic phase, in that when the former are transferred to a suitable medium containing calcium, they will flocculate at once, whereas the latter will not. Clearly the two types of cells must have structural differences. Those cells which have been reversibly separated by simple changes of environment may be called potentially flocculent, in distinction from those which are dispersed as a consequence of their structure, and which may truly be called non-flocculent.

Three distinct areas of study may be recognized in approaching the phenomenon of flocculation and it is necessary that information obtained in one field should not be confused with that from another: (i), there is the inherited nature of the cell; (ii) there is the nature of the change from non-flocculence to potential flocculence, together with the stimulus which provokes this change; (iii) there is the nature of the interactions of the cells with each other and with the environment, which manifest potential flocculation in the actual formation of flocs.

The present paper is concerned with the second of these fields of study, more especially with the nature of the stimulus which leads to a change to potential flocculence. It will be shown that this change is connected with a specialized part of the nitrogen metabolism of the organism.

METHODS

Yeast. A Strain 7002 of *Saccharomyces cerevisiae* was used throughout. This was a typical class III yeast (Gilliland 1951) and was obtained by Mr J. C. Holdaway from a single cell isolated from a stout-brewing strain.

Media. Glucose yeast-extract salts ammonium medium (GYSA) consisted of 75 g. glucose, 5 g. Difco yeast extract, 2 g. $(NH_4)_2SO_4$, 12·75 ml. 2M-CH_3COOH, 12·25 ml. 2M-CH_3COONa and 100 ml. salts solution (see below) in 1 l. distilled water, adjusted to pH 4·6 with dilute HCl.

Glucose yeast-extract agar consisted of a 2% (w/v) solution of agar in GYSA medium.

Glucose peptone growth-factor (GPF) medium consisted of 150 g. glucose, 10 g. 'Oxoid' mycological peptone, 25·5 ml. 2M-CH_3COOH, 24·5 ml. 2M-CH_3COONa, 200 ml. salts solution, 8 mg. calcium pantothenate, 4 mg. inositol, 2 mg. thiamine and 2 mg. pyridoxine HCl in 1 l. distilled water, adjusted to pH 4·6. This medium was always diluted with an equal volume of water or test solution for use, and 0·4 ml. of a solution of biotin (25 μg./ml. 95% (v/v) ethanol in water) was added to each litre of working medium, after sterilization and just before inoculation.

All media were sterilized by autoclaving for 15 min. at 120°.

The salts solution contained: 50 g. $MgSO_4.7H_2O$, 5 g. NaCl, 10 g. KH_2PO_4, 5 mg. $(NH_4)_2MoO_4$, 10 mg. $MnCl_2$, 5 mg. $FeCl_3$, 5 mg. $ZnSO_4$, 5 mg. H_3BO_3, 5 mg. KI, 5 mg. $CuSO_4$; made up to 1 l. in distilled water.

Inocula. The yeast was maintained on GYSA agar slopes. Inocula were always prepared from slopes which had been incubated for 40–70 hr at 25°. The yeast from one of these was suspended in 10 ml. GYSA medium, and then shaken at 25° for 6 hr. The cells were then collected by gentle centrifugation and resuspended in distilled water. The concentration of the suspension was estimated in an EEL colorimeter. This suspension was used at once to inoculate the cultures, the equivalent of about 9 mg. dry wt. cells being added to each litre of medium.

Cultures. Cultures were grown in 500 ml. conical flasks containing 200 ml. of medium; these were agitated on a rotatory shaker at 25°.

Measurements of cell concentration. Cell concentrations were estimated by centrifuging a sample of the culture, resuspending the yeast in 0·01 N-HCl and measuring the opacity of the suspension in an EEl colorimeter with a Chance filter OB4. The colorimeter was calibrated with dilutions, in 0·01 N-HCl, of a suspension of washed cells harvested during the early phase of logarithmic growth. The dry weight of the cells in this suspension was determined after heating a sample at 95° until an approximately constant weight was obtained. Flocculent cells harvested after prolonged growth gave a very similar curve, provided that the comparisons were made on the basis of dry weights. Comparisons based on cell numbers gave considerable differences.

Measurements of potential flocculence. Potential flocculence was determined with cells suspended in a 0·05 M-sodium acetate buffer (pH 4·6) containing 0·1% $CaCl_2$. The gross rate at which the cells sediment in this buffer is almost exclusively dependent on the size of the flocs. In general, cells obtained by centrifuging a culture were washed, first in water and then in the buffer; they were then resuspended in the buffer at a concentration equivalent to about 4 mg. dry wt./ml. The suspension

was agitated vigorously and then poured quickly into the sample tube of the EEL colorimeter. Readings of the colorimeter were taken at timed intervals and the readings converted to apparent dry wt. cells in the light path of the instrument. A graph of these apparent cell dry-wt. values against time could then be constructed; this was approximately sigmoid in shape, and the steepest negative slope of this graph was taken as a measure of the sedimentation rate (SR). The logarithm of this (log SR) was considered to provide a good measure of the relative flocculence of the cells in the buffer, and hence of the potential flocculence of the cells in the culture. Values of SR obtained, ranged from equiv. 10 μg. dry wt./ml. suspension/min., for a completely non-flocculent culture, to about equiv. 15,000 μg. dry wt./ml./min. for a very flocculent one. The inaccuracies of this procedure are of little significance in comparison with the enormous changes encountered during the development of a culture.

RESULTS

Figure 1 shows the development of flocculence in a culture of the yeast in GPF medium. The potential flocculence increased rapidly in the later half of the logarithmic phase of growth. When considering why the development of flocculence should occur at this time, at least three possible reasons, or stimuli, come to mind. First, is there an internal clock mechanism which leads the yeast through cycles of flocculence and non-flocculence? This possibility is presumably ruled out by the well-known observation that in continuous culture the yeast may remain either flocculent or non-flocculent, depending upon the particular conditions.

Table 1. *The potential flocculence of yeast cells incubated in mixtures of media components*

Components present in (+), or absent from (−), the incubation mixture			Potential flocculence (log SR)	
Glucose (1·25 %)	Yeast extract (0·25 %)	Ammonium succinate (0·06 M)	After 4 hr	After 24 hr
+	−	−	2·37	2·82
+	+	−	2·47	2·92
+	+	+	1·42	2·05
+	−	+	1·42	1·70
−	+	+	1·32	1·20
−	+	−	1·32	1·20
−	−	+	1·20	1·20
−	−	−	1·27	1·20

All the mixtures of media components also contained 10 % (v/v) of salts solution. The cells had a log SR of 1·20 before incubation.

Secondly, does the yeast flocculate as a response to the accumulation in the medium of some substance, e.g. a product of its metabolism? A culture of yeast was prepared in GYSA medium and during early logarithmic phase the yeast was separated by centrifugation. It was divided into three equal portions, one of which was resuspended in one-third of the original culture fluid, another in a similar volume of fresh medium and the third in a similar volume of 5 % glucose solution. As growth continued potential flocculence first appeared in the culture in glucose

alone, later in the culture in the used medium and finally in the culture in the fresh medium. This rules out any simple explanation based on the accumulation of a metabolite in the medium, for on such a basis flocculation should have occurred first with the culture in the used fluid.

Thirdly, does the yeast flocculate as a response to the exhaustion of a factor present in the fresh medium? A similar experiment was performed in which the freshly harvested yeast was added to solutions of glucose, yeast extract and ammonium salts, in various combinations. No flocculence occurred in the absence

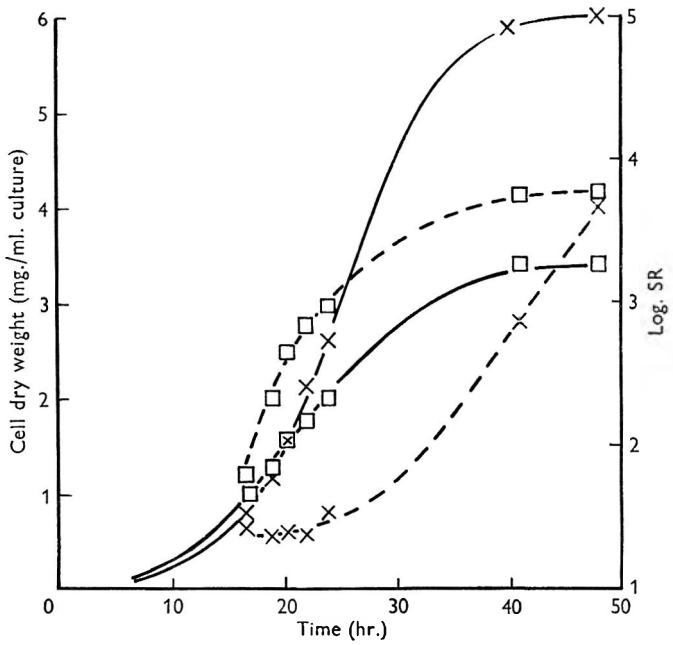

Fig. 1. Growth (solid lines) and potential flocculence (broken lines) of *Saccharomyces cerevisiae* in (*a*) GPF medium, □; (*b*) GPF medium + 0·012 M-ammonium succinate, ×. Log SR = log equivalent μg. dry wt. organism/ml./min.

of glucose. Flocculence developed rapidly in the presence of glucose alone or glucose + yeast extract, but was much delayed when ammonia was also present (Table 1). Experiments were therefore devised to follow this effect more closely and to test a number of nitrogenous compounds in this system.

The effect of nitrogenous compounds on the development of potential flocculence

Media were prepared by adding the double strength (GPF) mixture to equal volumes of solutions of the substances being tested, and growth and the development of potential flocculence were followed in shaken cultures. Controls using the unsupplemented (GPF) medium were included and all cultures were duplicated. Figure 1, curve *b*, shows the effect obtained with 0·012M-ammonium succinate in such a system. There was a marked delay in the onset and development of potential flocculence (expressed as log SR).

To evaluate the efficacy of various substances in such a system, the amount of growth which had occurred by the time the culture reached an arbitrarily selected potential flocculence was noted, and expressed as a percentage of the corresponding

Table 2. *The effects of some α-amino acids and ammonia on the stage of growth at which potential flocculence of yeast cells developed*

Compound tested	Concentration (M)	Growth as a % of that in control at log SR = 2·70
Ammonium succinate	0·012	230
L-Glutamine	0·025	220
L-Glutamic acid	0·025	100
L-Asparagine	0·025	210
L-Asparagine	0·012	200
L-Aspartic acid	0·025	100
L-Lysine	0·025	210
L-Arginine	0·025	170
L-Ornithine	0·025	180
DL-Methionine	0·025	110
L-Cysteine*	0·025	—
DL-Threonine	0·025	110
L-Serine	0·025	110
L-Phenylalanine	0·020	100
DL-Tyrosine	Sat. soln.	110
DL-Tryptophan	0·020	110
DL-Proline	0·020	100
L-Histidine	0·020	100
Glycine	0·025	100
L-α-Alanine	0·025	100
DL-Valine	0·025	100
DL-Leucine	0·025	110

* Cysteine inhibited growth almost completely at this concentration.

Table 3. *The effect of certain nitrogenous substances on the stage of growth at which potential flocculence of yeast cells developed*

All substances were tested at a concentration of 0·025 M, except pantothenate 100 μg./ml. and folic acid 25 μg./ml.

Substance tested	Growth as a % of that in control at log SR = 2·70	Substance tested	Growth as a % of that in control at log SR = 2·70
β-Alanine	60	Orotic acid	100
γ-Aminobutyrate	150	5-Methylorotic acid	100
Glucosamine	100	8-Azaguanine	100
Pantothenate	100	2,6-Diaminopurine sulphate	100
2-Chloro-4-aminobenzoate	60	Oxamic acid	100
p-Aminobenzoate	100	Urea	200
Folic acid	100	Barbiturate	120

amount of growth attained by the control cultures when they had reached the same potential flocculence. A value of log SR = 2·70 was chosen for the comparison, since this value is reached soon after the increase in the potential flocculence of the

culture becomes apparent; moreover, the measurement of potential flocculence is most readily carried out at about this value. The comparison of cultures on a basis of the amount of growth which has taken place by the time that a standard potential flocculence has been reached, rather than by a direct measurement of the time required to attain this flocculence, eliminates difficulties associated with synchronizing the growth of different cultures. It was found in practice that supplementation of GPF medium with nitrogenous substances had little effect on growth itself, although in a few cases a prolongation of the logarithmic phase was noted. Such a prolongation cannot account for the results described here.

Over a period of about 6 months, the replicated controls to these experiments showed a standard deviation of 140 about a mean of equiv. 1500 μg. dry wt./ml. at log SR = 2·70 (i.e. about 10%). Variations from the control outside 70–130% were therefore considered to be highly significant, falling outside three standard deviations ($P < 0{\cdot}01$). Table 2 presents results obtained in this way with ammonium succinate and some amino acids. Significant delays in the development of potential flocculence were found only with ammonia, the basic amino acids and the amides of the dibasic acids. Such a delay occurred with asparagine for example, but not with aspartic acid, even when the amount of total nitrogen supplied was the same.

Table 3 shows similar results with certain other nitrogenous compounds; of these, urea and γ-aminobutyric acid delayed potential flocculation, whereas β-alanine and 2-chloro-4-aminobenzoic acid advanced potential flocculation.

Figure 1 also shows a point which seems generally valid for all the effective compounds: those substances which were effective in postponing potential flocculence did not exert their action by stimulating growth until potential flocculence developed at its normal time, but actually postponed this development.

DISCUSSION

Implicit in this work is the assumption that changes in the potential flocculence of the yeast cells reflect changes in the chemical structure of their cell walls, and that these changes are a result of metabolic processes of the organism which to some extent may be controlled by its nutrition. To investigate such changes, it is essential to measure the flocculence of the yeast cells when they are supported in a carefully controlled physico-chemical environment; only thus is it possible to ensure that measured differences in flocculence truly reflect changes in the chemical structure of the cell.

Kusserow (1897) observed that asparagine inhibited the flocculation of yeast, whereas peptone enhanced it, but it is difficult now to decide whether the effects he described were due to actual changes in the cell or to changes in the suspending medium. Nielsen (1937) observed an increase in sedimentation rates of yeast grown in media containing added amino acids or peptones. Since, however, Nielsen measured the sedimentation of the washed yeast in water, without added calcium, it is difficult to be sure that his results did not reflect the availability of calcium to the cell during growth. The results described in the present paper amplify those of earlier workers. A well characterized group of compounds, namely, ammonia, the basic amino acids and the amides of the dicarboxylic amino acids, will delay the

onset of potential flocculation. This group of substances does not correspond to those compounds which form the most readily assimilable nitrogen sources of yeast (see, for example, Nielsen & Hartelius, 1938; Thorne, 1946). It is interesting to contrast the activity of glutamine and asparagine with the lack of activity of glutamic and aspartic acids. Indeed it appears that the α-amino group is not an effective source of nitrogen for this reaction. The only compounds outside of this narrow group which were found to be effective were urea, which readily yields ammonia, and γ-aminobutyric acid.

Two compounds were found to act in the opposite manner, leading to an earlier onset of flocculence, one of these was β-alanine; it is known that asparagine will antagonize the synthesis of pantothenic acid from β-alanine + pantoic acid, in certain organisms (Atkin, Williams, Schultz & Frey, 1944), and this raises the possibility that β-alanine leads to an early flocculation of yeast by antagonizing the action of asparagine. The other substance which was found to induce early flocculation was 2-chloro-4-aminobenzoic acid, a structural analogue of p-aminobenzoic acid which is an intermediate in the synthesis of pantothenic acid. However, there is no evidence that this connexion with pantothenate is other than fortuitous. Since β-alanine would promote, and 2-chloro-4-aminobenzoic acid antagonize, the synthesis of pantothenate, these two compounds might be expected to have opposed, and not similar, effects on flocculation were their action connected with pantothenate.

These findings can be interpreted by the hypothesis that the cell walls of non-flocculent yeasts contain a nitrogenous substance (XN) whose presence in the walls in a sufficient concentration determines their non-flocculence; so that if the concentration of XN were to decrease then the walls would show increasing potential flocculence. If now the rate of synthesis of XN by the cell were limited, then the concentration of this compound in the wall would diminish following rapid growth. The concentration of XN might also diminish as a response to the depletion in the medium, of XN itself or of materials required for its synthesis. That the change from non-flocculence to potential flocculence involves an active process is clear from the finding that no change took place in the absence of glucose. The group of substances which delay potential flocculation might then include XN itself, together with any intermediates in its synthesis, whose availability in high concentrations might increase the rate at which XN is produced. Certain corollaries should follow if this hypothesis is correct. First, there would be no definite identifiable stimulus, leading to the development of potential flocculence; for this would appear whenever a strain of yeast grew under conditions such that the rate of synthesis of XN was less than that of the other components of the cell wall. Secondly, the differences in the tendency to flocculate shown by different strains of yeast might reflect their different capabilities of synthesizing XN.

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The Nature of the Interactions between Flocculent Cells in the Flocculation of *Saccharomyces cerevisiae*

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SUMMARY

The flocculation of a strain of brewers' yeast was absolutely dependent upon the presence of calcium; a concentration of 200 mM-$CaCl_2$ was sufficient to ensure almost complete flocculation. No other metal could replace calcium; several metals aggregated potentially flocculent cells but also aggregated non-flocculent cells. Sodium ions antagonized the action of the calcium. The effects of pH value and esterification suggested that carboxyl groups were involved. The floes had a 'melting temperature' of 50–60° and were dispersed by urea, suggesting that hydrogen bonds were also present.

Non-flocculent yeast was aggregated when the dielectric constant of the medium was decreased by the addition of organic solvents, but this aggregation was also dependent on the presence of traces of calcium. Conversely, increase of the dielectric constant of the medium, by adding formamide, dispersed flocculent yeast. Certain specific sugars also dispersed flocculent yeast. It is suggested that flocculent yeast cells are linked by salt bridges formed by calcium atoms joined with two carboxyl groups in the surfaces of different cells and that this structure is stabilized by hydrogen bonds formed between complementary patterns of carbohydrate hydrogens and hydroxyls in the cell surfaces.

INTRODUCTION

The phenomenon of yeast flocculation may conveniently be separated into fields of study comprising: the inherent tendency of a yeast to flocculate, the nature of the change from a non-flocculent to a potentially flocculent cell, and the interactions of potentially flocculent cells to form flocs (Mill, 1964). Several theories have been advanced to explain the formation of flocs (St Johnston, 1949; Hartong, 1951; Trolle, 1950; Masschelein & Devreux, 1957), but none of them gives a completely satisfactory explanation of the phenomenon. The present paper describes experiments designed to elucidate the physico-chemical nature of the forces which bind flocculent cells together. Both ionic and hydrogen bonds seem to be involved. It is suggested that the flocculent yeast cells interact to form a calcium chelate complex.

METHODS

The strain of brewers' yeast, the media and the method of measuring flocculence have been described (Mill, 1964).

In many cases, where the flocculation had to be gauged in other than aqueous media, simple visual observations were used, the degree of flocculence being graded

from 0 (completely dispersed) through ±, +, + +, + + +, + + + + to + + + + + (very highly flocculent).

Inorganic salts. When possible, AnalaR grade salts were used, but because of the extreme sensitivity of the test system to calcium, it was occasionally found necessary to purify these further. Recourse was made to normal inorganic chemical techniques such as recrystallization from water, but organic solvents were used with some salts of transition elements. Ferrous sulphate was obtained free from ferric sulphate by adding 70 % (v/v) ethanol to an aqueous solution of the commercial material. The ferrous salt precipitated and was thoroughly washed with ethanol and used at once.

Water. In experiments with solutions containing low calcium concentrations, it was found necessary to use double-distilled water which was passed through a mixed bed of cationic and anionic exchange resins immediately before use.

Glassware. For experiments involving low calcium concentrations, the glassware was cleaned in *aqua regia* overnight, rinsed well in de-ionized water and used immediately.

Calcium chloride + sodium acetate buffer. This was prepared by adding 0·1 % (w/v) of calcium chloride to 0·05 M-sodium acetate + acetic acid buffer (pH 4·6).

Yeast. Saccharomyces cerevisiae strain 7002 was used throughout and was grown in shaken cultures in glucose yeast extract salts ammonium medium (GYSA; Mill, 1964). Incubation was continued for 16 hr to give non-flocculent yeast and for 40 hr to give flocculent yeast.

Dispersed, potentially flocculent yeast was obtained by washing flocculent yeast three times in 1 % NaCl solution and then three times in de-ionized water.

RESULTS

Effect of washing flocculent yeast. It was very difficult to deflocculate yeast by washing with water as described by Jansen & Mendlik (1951); a diminution but not a complete loss of flocculence was observed, unless the treatment was repeated many times. However deflocculation was readily achieved by washing the yeast with 1 % NaCl solution, and the yeast then remained dispersed when it was thoroughly washed with de-ionized water. It reflocculated immediately when a trace of calcium chloride was added to the suspension.

The concentration of calcium required to induce flocculence. Samples of dispersed potentially flocculent yeast were suspended in de-ionized water and various amounts of calcium chloride added. Figure 1*a* shows that the yeast expressed almost its maximum flocculence at a calcium concentration of about 200 μM.

The effect of sodium and potassium ions. The experiment described above was repeated in the presence of M-NaCl (Fig. 1*b*) or M-KCl (Fig. 1*c*). The NaCl antagonized the action of the $CaCl_2$ so that higher concentrations of the latter were needed to attain any given flocculence; KCl had little effect. This result is in accordance with the dispersal of flocculent yeast on washing with NaCl.

The effect of some other metallic ions. In testing the action of metallic salts on potentially flocculent yeast, it is necessary to bear in mind that aggregation of cells as a response to the addition of an ion does not necessarily imply that that ion can replace the Ca^{2+} ion in the flocculation phenomenon; the other ion may aggregate the cells by some different mechanism. The criterion was therefore established that

an ion was only to be considered as acting in the flocculation system when it aggregated potentially flocculent, but not non-flocculent, cells. Yeast samples were suspended in water at a concentration equivalent to about 4 mg. dry wt. yeast/ml., and small volumes of solutions of metallic salts were added to give final concentrations of 2 mM. Table 1 shows the degree of flocculence obtained (measured as log SR; see Mill, 1964). Only Ca^{2+} flocculated dispersed potentially flocculent yeast cells, but not non-flocculent cells; whereas Fe^{3+}, Sn^{2+}, Ag^{+}, Y^{2+}, UO_2^{2+}, Ce^{2+}, and Ti^{2+} aggregated both types of cells. These aggregates differed in appearance from those obtained with Ca^{2+}; in particular Ag^{+} caused the cells to darken in colour and it was possible to see a deposit of black material around the cells when they were observed under the microscope. Feeble reactions with potentially flocculent cells were at first encountered with several salts, but disappeared when rigorous precautions were taken to eliminate traces of calcium.

Table 1. *Flocculence of yeast cells in the presence of metal ions*

Washed, potentially flocculent and non-flocculent cells were suspended in 2 mM solutions of the salts shown and the flocculence was measured. The flocculence is expressed as log SR (log μg. equivalent dry wt. yeast/ml./min.).

Salt	Potentially flocculent cells (Log SR)	Non-floceulent cells (Log SR)	Salt	Potentially flocculent cells (Log SR)	Non-floceulent cells (Log SR)
$CaCl_2$	4·1	1·0	$MnCl_2$	1·7	1·0
$FeCl_3$	3·4	3·5	$FeSO_4$	1·7	1·0
YCl_3	4·2	4·2	$Co(NO_3)_2$	1·7	1·0
$SnCl_2$	4·0	4·0	$NiCl_2$	1·5	1·0
$TiCl_3$	3·7	4·1	$CuSO_4$	1·5	1·0
$CeCl_3$	3·9	4·1	$ZnSO_4$	1·7	1·0
$UO_2(C_2H_3O_2)_2$	2·0	1·9	$SrCl_2$	1·4	1·0
$AgNO_3$	3·4	3·3	$CdSO_4$	1·7	1·0
$BeSO_4$	1·7	1·0	$BaCl_2$	1·7	1·0
$MgSO_4$	1·7	1·0	$HgCl_2$	1·7	1·0
$Al_2(C_4H_4O_6)_3$	1·7	1·0	$PdCl_2$	1·7	1·0
$CrCl_3$	1·7	1·0			

The effect of pH value. A range of 0·05M-acetate or glycine buffers was prepared and $CaCl_2$ was added to them to give a final concentration of 5 mM. Samples of potentially flocculent yeast were suspended in these mixtures at a final concentration equivalent to about 4 mg. dry wt./ml. and the flocculence of the suspensions was measured (Fig. 2). The flocculence was low at pH 2 but rose with increasing pH and reached a maximum between pH 4·5 and 5·5.

Esterification. When potentially flocculent yeast was shaken overnight in 0·05M-acetate buffer (pH 4·0) containing 5% 1,2-epoxypropane, it became completely non-flocculent. When ethylene glycol was substituted for the epoxypropane, little change in potential flocculence occurred.

The effect of temperature. A suspension of flocculent yeast in calcium chloride + sodium acetate buffer was placed in a thin-walled tube dipping into a large bath of water. Both the water and the yeast were thoroughly stirred whilst the water was gently heated. The flocculence of the yeast was graded by eye at intervals and at the same moment the temperature of the suspension was measured with a thermo-

meter which dipped into it. Table 2 shows that there was at first a slight decrease in flocculence as the temperature was increased and that a rapid change occurred between 50° and 60°. At 60° the yeast was virtually completely dispersed. As the temperature was lowered again, the flocculenee reappeared.

The effect of urea. Samples of flocculent yeast were suspended in portions of the calcium chloride+sodium acetate buffer to which different amounts of urea had been added. The yeast was completely dispersed at a urea concentration of 40 %, but when the urea was removed by washing the yeast with buffer, the flocs reappeared.

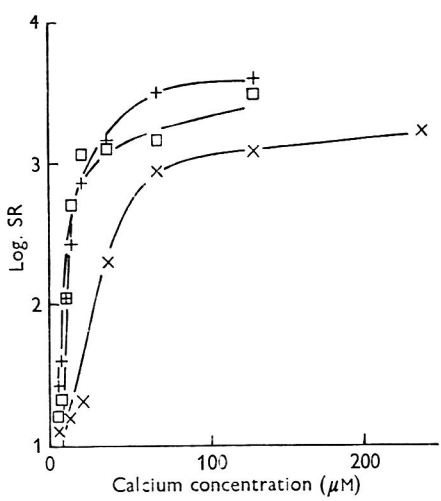

Fig. 1

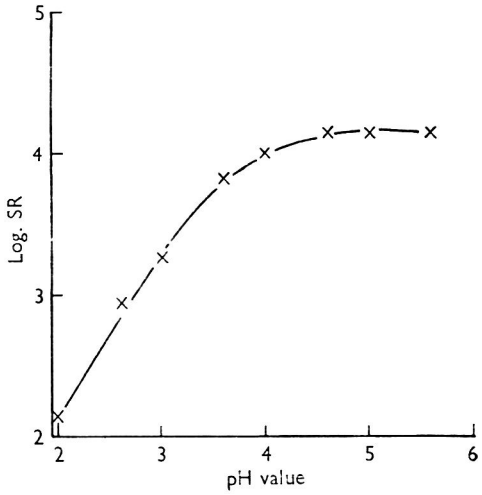

Fig. 2

Fig. 1. Dependence of flocculence on calcium concentration with potentially flocculent cells of *Saccharomyces cerevisiae* suspended in (*a*) water, + − + ; (*b*) M-NaCl, × − × ; (*c*) M-KCl, □−□ . , Flocculence is given as log SR = log μg. equivalent dry wt. of cells/ml./min.

Fig. 2. The flocculence of potentially flocculent cells of *Saccharomyces cerevisiae* suspended in media at various pH values. Flocculenee is given as log SR = log μg. equivalent dry wt. of cells/ml./min.

Table 2. *The effect of temperature on flocculence of yeast cells*

Flocculent cells suspended in sodium acetate+calcium chloride buffer were gently heated and their flocculence was graded by eye at different temperatures.

Temperature (°)	Flocculence	Temperature (°)	Flocculence
23	+ + + + +	51	+ +
45	+ + + + +	54	+
47	+ + + +	58	±
50	+ + +	62	−

The effect of organic solvents. There was no effect when organic solvents were added to flocculent yeast or to non-flocculent yeast which had been washed in saline. However, when organic solvents were added to suspensions of non-flocculent yeast in the presence of traces of calcium chloride, flocculation occurred. On removing the solvent the yeast dispersed once more.

Methanol, ethanol, propanol, acetone and dioxane were each effective when used

Table 3. *Aggregation of non-flocculent yeast by organic solvents*

Organic solvents were added to suspension of non-flocculent yeast in 2 mM-$CaCl_2$ and the flocculence of the suspension was graded by eye.

Solvent concentration (%, v/v)	Apparent dielectric constant	Flocculence
	Methanol	
29	74	−
38	71	+
41	70	+ +
48	68	+ + + +
52	66	+ + + + +
	Ethanol	
23	76	−
29	75	+
33	73	+ +
41	70	+ + + +
44	69	+ + + + +
	*iso*Propanol	
23	74	−
29	73	+
33	71	+ +
41	67	+ + + +
44	64	+ + + + +
	Acetone	
23	75	−
29	74	+ +
31	71	+ + + +
38	69	+ + + + +
	Dioxane	
9	76	−
17	73	+ +
23	68	+ + + +
29	66	+ + + + +

Table 4. *Dispersal of flocculent yeast by sugars*

Sugar	Sugar concentration (%)					
	0	5	10	15	20	30
	Flocculence (graded by eye)					
Glucose	+ + + + +	+ + +	+	+	−	−
Mannose	+ + + + +	+	+	+	−	−
Galactose	+ + + + +	+ + + +	+ + +	+ + +	+ + +	+ +
Fructose	+ + + + +	+ + + + +	+ + + +	+ + + +	+ + +	+ +
Maltose	+ + + + +	+ +	+	+	+	+
Sucrose	+ + + + +	+	+	−	−	−
Lactose	+ + + + +	+ + + +	+ + +	+ + +	+ + +	+
Mannitol	+ + + + +	+ + + + +	+ + + +	.	.	.
Glucosamine	+ + + + +	+ + + +	+ + + +	+ + +	+ +	+

. = not tested.

in concentrations such that the mixture had a dielectric constant approaching 68 (Table 3). (This value is an apparent one obtained from published tables; it takes no account of the contribution of the electrolyte, but this will be approximately the same in each case.)

The effect of sugars. The finding by Eddy (1955*a*) that certain specific sugars could disperse flocculent yeast was confirmed. The sugars were tested by dissolving them, at various concentrations, in calcium chloride + sodium acetate buffer, and suspending washed potentially flocculent yeast in these mixtures at a final concentration equivalent to about 4 mg. dry wt./ml. The flocculence was graded by eye (Table 4).

The effect of formamide. The ability of formamide to disperse flocculent yeast was tested in a similar manner. Samples of flocculent yeast suspended in mM-$CaCl_2$ were dispersed by 4% formamide. This effect was however dependent upon the electrolytes present; in a solution 0·1 M with respect to NaCl or KCl and mM with respect to $CaCl_2$, 20% formamide did not disperse flocculent yeast.

DISCUSSION

The work described here is concerned with elucidating the nature and location o the forces which bind together the flocculent yeast cells; it does not consider the changes which convert a non-flocculent yeast to a flocculent one. A single strain of yeast was used throughout to minimize variations due to differences in the structure of the yeast. Similarly, the control for the flocculent yeast was sought in the non-flocculent stage of the same strain, rather than in a different non-flocculating strain.

That calcium is an essential requirement for flocculation was one of the earliest known features of the phenomenon. It has been claimed that a number of different metallic ions can replace calcium (Jansen & Mendlik, 1953; Lindquist, 1953; Eddy, 1955*b*), but it was not possible to confirm this. Provided that the reagents employed were free of calcium, only a few other metals aggregated the yeast cells used, and every one of these also aggregated non-flocculent yeast cells. Thus there seems to be no evidence that these other ions act in the same manner as calcium or combine with the same groups in the cell surface. Silver ions apparently acted by depositing metallic silver on the cell surfaces, but it is noteworthy that all the other ions which were effective were polyvalent. The case of iron is particularly striking in that Fe^{3+} caused an intense aggregation but Fe^{2+} ions were inactive. Since, of all the ions tested, only calcium appeared to act in the normal flocculation mechanism, it must be supposed that strict steric relationships exist between the yeast cells and the ions in the flocculent state.

That sodium antagonizes the action of calcium suggests that the latter normally combines with the cell wall at some definite site from which it can be competitively displaced. Sodium might be expected to act in such a system since its crystal ionic radius (0·98 Å) is very near that of calcium (0·99 Å).

Two roles can be envisaged for calcium: (i) it might sterically block a group in the cell surface whose presence maintained the non-flocculent state; (ii) it might form a 'salt bridge' between two yeast cells, i.e. its two valencies might enable it to form a bond between a combining site on each of two different cells (Harris, 1959). Of these two roles, the former should be equally well performed by univalent sodium

whereas the latter role could not. Since sodium reacts with the combining site and in doing so deflocculates the yeast, the evidence favours the salt bridge hypothesis. This does not exclude the possibility that the action of calcium is in part due to the blocking of charged groups on the cell surface.

Groups which might exist in the cell surface, and be capable of combining with calcium at pH 4·6, comprise carboxyl, phosphate and sulphate. The effect of pH value on flocculation indicates that the phenomenon is dependent upon the ionization of a group of pK about 4, suggesting that carboxyl groups are involved. This is supported by the effect of esterification: under the conditions used, epoxypropane preferentially combines with carboxyl groups to form propylene glycol esters (Fraenkel-Conrat, 1944). The objection might be raised that the action of the epoxypropane was due to its solvent and toxic properties, but ethylene glycol, which should have similar properties, did not destroy potential flocculence and of course did not form esters under these conditions. There is thus some evidence that carboxyl groups are involved in flocculation, but there is as yet no direct evidence that these groups are so involved as combining sites for calcium ion.

A salt bond between calcium and a carboxyl group would normally be largely ionic in character and dissociated at pH 4·6, so that some further type of bonding must be sought to account for flocculence. The effect of temperature on the flocs is in fact an indication that weak secondary, almost certainly hydrogen, bonds are present. The flocs show a 'melting temperature' of 50–60°. This accords well with values found for the helices of collagen (Verzar, 1963) and nucleic acids (Doty, 1962); these helices are maintained by hydrogen-bonding. The lack of sharpness in the 'melting' may simply result from the method used, but it is also in accord with the picture, here developed, of a mutual stabilization of salt and hydrogen bonds. The deflocculation of the yeast by urea also supports the view that hydrogen bonds are involved. Since the cells regain their potential flocculence immediately the urea is removed, it is not to be supposed that this acts by disrupting structures in the cell surface, but rather merely by interfering with the interactions between cells.

At first sight the action of organic solvents in promoting aggregation of non-flocculent yeast cells might be dismissed as unconnected with the true flocculation phenomenon were it not for the requirement for calcium. This dependence on the presence of calcium strongly suggests that the aggregation of the yeast cells is in fact a manifestation of the flocculation phenomenon. The organic solvents most probably exert their action by decreasing the dielectric constant of the solution, for the aggregation occurs at about the same value of dielectric constant with each solvent. A decrease in the value of the constant will have two effects: it will increase the strength of hydrogen bonds and it will diminish the ionization of salt bonds, i.e. it will increase their strength also. Both types of bonding envisaged here will thus be intensified. Any groups in the yeast cell surface, which are charged by ionization, will tend to become reassociated and hence discharged when the dielectric constant is decreased; but the aggregation of the cells may not be exclusively ascribed to this, in view of the calcium requirement. The reverse process is seen in the dispersal of flocculent cells when the dielectric constant of the medium is increased by adding formamide.

A clue to an origin of the postulated hydrogen bonds was provided by the observation that certain specific sugars would dissociate floes at relatively low concentra-

tions (Eddy, 1955*a*). In agreement with this author, it was found that of the four naturally occurring hexoses, only glucose and mannose showed a high dispersing activity. Sucrose and maltose were also effective, but lactose much less active. Modifications of the structures of the hexoses, as in mannitol and glucosamine, resulted in a loss in activity. Glucose and mannose are the dominant sugar residues of the yeast cell wall (Northcote & Horne, 1952), which suggests that the hydrogen-bonding involves complementary patterns of carbohydrate hydroxyls and hydrogens in the wall surface.

The general picture which emerges from these considerations is of a calcium atom forming a bridge between receptor sites on two different yeast cells; and these receptors may possibly be carboxyl groups. The bonds involved are at first essentially ionic, but once established the cells will transitorily approach closely to one another and hydrogen bonding will be set up between complementary carbohydrate structures in the walls of the two cells. The resulting complex will thus assume a chelate character, with the calcium-complexing groups held in a definite spatial relationship to each other.

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A Temperate Phage Specific for Female Strains of *Escherichia coli* K 12

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SUMMARY

A temperate bacteriophage named '*tau*' which forms plaques on F− strains but not on F+ strains of *Escherichia coli* K 12 has been isolated. This phage adsorbs equally well to F+ and F− strains, which indicates that some later step of the growth cycle is inhibited by the presence of the sex-factor *F*. The frequency of lysogenization of the phage is also affected by the *F* factor. The genetic material of phage *tau* may be DNA. The phage is 40–50 mμ in diameter.

INTRODUCTION

Genetic studies with *Escherichia coli* have shown that some transmissible elements such as the temperate phage *lambda* (Lederberg & Lederberg, 1953), the sex-factor *F* (Lederberg, Cavalli & Lederberg, 1952; Hayes, 1953) and colicinogenic factors (Fredericq, 1948, 1953) show certain common features in their behaviour. These are named 'episomes' (Jacob & Wollman, 1958). It is well known that lysogenic bacteria are not susceptible to infection by phage particles which are homologous with the phage lysogenized. This phenomemon is ascribed to the presence of a prophage on the bacterial chromosome which is homologous to the superinfecting temperate phage. Various temperate phages, such as *lambda* or P1, interfere with the growth of the T series of phages when they are present as prophages in their hosts (Benzer, 1955; Lederberg, 1957). If analogous phenomena could be found between different episomic elements, such as the *F* factor and temperate phages, the interaction between them might open the way to further understanding of their nature. For this reason, we have searched for temperate phages which have some relationship to the sex-factor *F* of *E. coli*.

Recently, Zinder (1961) succeeded in isolating a phage which grows in female (F−), but not in male (F+ or Hfr) strains of *Salmonella typhimurium*. The present paper describes a new temperate phage named '*tau*' which forms plaques on F− strains but not on F+ or F′ (F prime) strains (Hirota & Sneath, 1961; Jacob, Schaeffer & Wollman, 1960). Preliminary studies were reported previously (Hakura & Hirota, 1961).

METHODS

Nutrient broth used for these experiments consisted of 10 g. Kyokuto meat extract, 10 g. polypeptone and 2 g. NaCl. in 1 l. distilled water; adjusted to pH 7·2. Nutrient agar plates were prepared from the same medium +1 % agar. In some

experiments, tris + glucose medium was used. This contained: tris 10^{-2}M; Na_2HPO_4, 5×10^{-4}M; $CaCl_2$, 10^{-3}M; $MgSO_4 7H_2O$, 10^{-3}M; $FeCl_3$, 10^{-5}M; Casamino acid, 2 g; NaCl, 5 g.; NH_4Cl, 1 g.; glucose 3 g.; in 1 l. distilled water; adjusted to pH 7·2.

The characteristics of *Escherichia coli* K 12 and its various mutants used in these experiments are summarized in Tables 1 and 2. Strains of *E. coli* independently isolated from clinical specimens were obtained from the Research Institute for Microbial Diseases, Osaka University, and tested for lysogenicity. Single colonies grown on Bacto S-S agar medium were spotted on a nutrient agar plate which was to serve as master plate in testing lysogenicity of bacteria isolated. After incubation for 16 hr at 37°, the master plate was replica-plated on soft agar containing strain 58–161 F+ or strain Y-70 F−. The replica plate was exposed to ultraviolet light (15 W. Toshiba germicidal lamp) for 40 sec. at a distance of 45 cm. and then incubated at 37° for 14 hr. Colonies on the master plate corresponding to inhibition zones were picked out and their properties examined.

RESULTS

Among eighty-four lysogenic strains isolated, it was found that two strains released bacteriophages which formed plaques only on F− strains but not on F+ strains of *Escherichia coli* K 12. Both phages appeared to be closely related, if not identical, by the criteria of plaque morphology, latent period, burst size, and immunity to superinfection. Therefore, only one of these phages was used for the succeeding experiments; it was named '*tau*'.

Table 1. *The relationship between growth of phage tau and the presence of the sex-factor F of Escherichia coli*

	Genotype*					
Strain	Nutritional requirement	Sugar fermentation	Lp	S	Sex	Response to phage tau
W4574	P⁻	Ara_2^- Xyl_2^- Mal_1^- Mtl^- Gal_2^- Lac_{85}^-	+	*r*	F−	+
JE-23†	P⁻	Ara_2^- Xyl_2^- Mal_1^- Mtl^- Gal_2^- Lac_{85}^-	+	*r*	F+	−
W3110	Prototroph	Fermenter	−	*s*	F−	+
JE-24†	Prototroph	Fermenter	−	*s*	F+	−
58-161	M⁻	Fermenter	+	*s*	F+	−
JE-25‡	M⁻	Fermenter	+	*s*	F−	+

* The abbreviations of genetic markers used here are as follows: P, proline; M, methionine; Lp, prophage lambda; *F*, sex factor; Ara, arabinose; Xyl, xylose; Mal, maltose; Mtl, mannitol; Gal, galactose; Lac, lactose; S, Streptomycin, *r* or *s* indicate resistance or sensitivity.

† JE-23 and JE-24 were isolated from W4573 and W3110 after infection with *F* factor.

‡ JE-25 was isolated from 58–161 by eliminating *F* factors (Hirota & Iijima 1957).

An experiment was done to determine the growth of the phage on different strains of *Escherichia coli* K 12. As seen from Table 1, plaques of the phage were formed on all the F− strains used, but not on any of the F+ strains tested. These results indicate that formation of plaques of the phage is prevented by the F factor, and bacterial characteristics such as sugar fermentation, drug resistance, nutritional requirements, or the presence or absence of prophage *lambda* are not associated with the capacity of the phage *tau* to form plaques.

To test this hypothesis two experiments were carried out. In one experiment, an F− strain which could support growth of the phage was converted into an F+ strain by infection with the *F* factor from an F+ strain. It was found that as a result of its conversion from F− to F+, the strain lost its ability to support growth of phage *tau*. In the other experiment, F− strains were isolated from an F+ strain after growth in the presence of acridine (Hirota & Iijima, 1957). These F− strains, in contrast to the original F− strain, supported growth of the phage. These results further show that for growth of the phage in the host cells the sex-factor *F* must be absent.

Table 2. *Comparison on the efficiency of adsorption of phage tau to Escherichia coli* K *12 F+ and F−*

Exponentially growing broth cultures of Y-70 and JE-26 were centrifuged and resuspended in the same volumes of broth. They were infected with the phage at a multiplicity of about 10^{-2}, and incubated at 37° for 30 min. and then were centrifuged at 5000 r.p.m. for 10 min. Phage particles remaining in the supernatant were assayed by plating on F−.

Strain	No. of cells per ml.	Phage particles added	Multiplicity of infection	Adsorbed phage
Y-70*	$2{\cdot}3 \times 10^8$	$1{\cdot}8 \times 10^7$	$7{\cdot}4 \times 10^{-2}$	$1{\cdot}2 \times 10^7$
JE-26†	$3{\cdot}2 \times 10^8$	$1{\cdot}8 \times 10^7$	$5{\cdot}6 \times 10^{-2}$	$1{\cdot}2 \times 10^7$

* Y-70: TLB_1^- Lac^-. F−
† JE-26: Y-70 F+

Table 3. *The effect of the F factor on lysogenization of phage tau*

Exponentially growing broth cultures of F−, F′ and F+ strains were mixed with a phage lysate which had been prepared by u.v.-irradiation of JE-103 for induction of phage *tau*. The mixtures were incubated at 37° for 30 min. and then washed twice with distilled water to remove unadsorbed free phage. They were then resuspended in the original volume of broth.

Appropriate dilutions of these infected cells were spread on individual nutrient agar plates, and incubated at 37° overnight. Colonies formed on these plates were replica-plated to nutrient agar plates which had been overlaid with 0·55 % (w/v) soft agar containing one drop of an overnight broth culture of w3110.

The replica plates were exposed to u.v.-light (15 W. of Toshiba germicidal lamp) for 20 sec. at a distance of 45 cm, and then incubated at 37° for 14 hr.

Where colonies were lysogenized by the phage, zones of inhibition of growth appeared around the replica-plated colonies.

	F−*	F+†	F′‡
Multiplicity of infection	3·3	3·6	3·4
No. of colonies lysogenized	41	1	3
No. of colonies tested	128	79	100

* F− used: w3110, Lp^s Prototroph F−.
† F+ used: JE-24, w3110 carrying F factor.
‡ F′ used: JE-340, w3110 carrying an F prime, F_{ilv}.

The inability of the phage to grow on F+ strains might have been due to its failure to be adsorbed, F− but not F+ bacteria having a specific receptor for the phage, as occurs with *Escherichia coli* B/2 and phage T2. The data presented in Table 2 indicate that the phage adsorbs to F+ strains as efficiently as to F− strains. Therefore, it was concluded that the growth cycle of the phage is inhibited at some later stage by the presence of the sex-factor *F*.

The F+ strain as well as the F− strain may become lysogenic with the phage. Ultraviolet (u.v.) irradiation or treatment with mitomycin C induces growth of the phage in lysogenic cells. The frequency of lysogenization by the phage of F− and F+ strains were compared. As shown in Table 3, the phage lysogenized a smaller proportion of F+ and F′ cells than F− cells under these experimental conditions. This result indicates that the *F* factor inhibits not only plaque formation but also lysogenization. A one-step growth curve of the phage after u.v.- induction of an F− strain of *Escherichia coli* lysogenized by phage *tau* was made. The progeny was released after a latent period of about 120 min. and 200 infective particles/bacterium were obtained. The sedimentation constant of purified phage was measured in a Spinco analytical ultra-centrifuge, Model E. There was a single schlieren peak and the sedimentation constant was about 200*S*. Electron micrographs of this phage shows an approximately spherical particle of 40–50 mμ diameter.

DISCUSSION

We can assume that the genetic material of phage *tau* is injected into F+ cells, since the phage adsorbs equally well to F+ as F− strains of *Escherichia coli*, and can lysogenize F+ as well as F− strains, although with different efficiencies. It is well known that lysogenic bacteria are immune to superinfection by homologous phage. To explain the immunity of the lysogenic bacteria, it is assumed that there exists a cytoplasmic repressor in lysogenic bacteria, which inhibits multiplication of homologous temperate phages (Jacob & Campbell, 1959). The relationship between the *F* factor and the phage may be somewhat analogous to the phenomenon seen in the lysogenic bacteria. By analogy, we may assume that the *F* factor directs the synthesis of a cytoplasmic repressor which inhibits multiplication of phage *tau*.

Loeb & Zinder (1961) reported that the bacteriophage specific for F+ strains of *Escherichia coli* contain RNA. Preliminary experiments indicate that phage *tau* contains DNA, but it is not yet possible to give exact values for the amount of DNA/phage particle, because of difficulties in obtaining pure preparations without loss of plaque-forming capacity (Otsuji, 1961).

Recently two other bacteriophages which are capable of growth on F− but not on F+ strains have been isolated (Iijima & Eguchi, personal communication). One of these shows the same immunity as phage *tau*, but the other one is able to grow in bacteria which were lysogenized with phage *tau*. Such lysogenic bacteria carrying different kinds of prophage, the growth of which is affected by the *F* factor, may be used as tools for genetic analysis of the *F* factor; experiments on this are in progress.

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Inactivation of Vaccinia Virus by Ascorbic Acid

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SUMMARY

Ascorbic acid undergoing auto-oxidation inactivated vaccinia virus. Copper ion was shown to have a catalytic effect on the inactivation. Neither unoxidized ascorbic acid nor its oxidation product, dehydroascorbic acid, were inhibitory. When ascorbic acid was oxidized at high pH in the absence of copper ion no inactivation took place. Similarly, enzymic oxidation of ascorbic acid in the absence of copper was without effect on the virus. Catalase prevented inactivation but not the oxidation of ascorbic acid. Glutathione prevented both inactivation and the oxidation of ascorbic acid. Inhibition experiments with ascorbic acid under anaerobic conditions were inconclusive. The mechanism of ascorbic acid inactivation is discussed in the light of these data and that of other authors with different viruses.

INTRODUCTION

During experiments on the stability of vaccinia virus in the presence of various reducing agents, it was found that ascorbic acid was strongly inhibitory. Kligler & Bernkopf (1937) noted this phenomenon, and Jungeblut (1935) reported that ascorbic acid inactivated poliovirus. Similar results have been reported for herpes virus (Holden & Resnick, 1936; Holden & Molloy, 1937), bacteriophages (Lominski, 1936) and tobacco mosaic virus (Lojkin, 1936). Lojkin made a systematic study of the inhibitory effects of ascorbic acid on tobacco mosaic virus infectivity; our results with vaccinia virus are in many respects like hers.

METHODS

Virus. The Lister Institute vaccine strain of vaccinia virus was used throughout the experiments. It was prepared from sheep dermal pulp in 0·004 M-McIlvaine buffer (pH 7·2). The virus was partially purified by extraction with 'Arcton 113' (trifluorotrichloroethane, I.C.I.) followed by one cycle of high-speed centrifugation. It was stored in small volumes at $-70°$. Such preparations produced cytopathic changes in monkey kidney tissue cultures in dilutions of 10^{-6} to 10^{-9}.

Virus titrations. Suitable dilutions of samples were inoculated in 0·1 ml. volumes into roller tube cultures of 2nd cycle monkey kidney cells; four tubes were used per dilution. Cytopathic changes were evident in 48 hr with high concentrations of virus, but the tubes were examined daily until the seventh day after inoculation, when no further changes were recorded. Fifty % end-points (TCD50) were calculated by the method of Reed & Muench (1938).

Buffers were prepared from McIlvaine's 0·2 M-disodium phosphate + 0·1 M-citric

acid solutions, which were mixed and diluted to give the desired pH value and molarity. Unless otherwise stated, all experiments were done in buffer at pH 6·9.

Enzymes. Catalase was a commercial beef-liver preparation (Light and Co.). It was used in different experiments in final concentrations of 10 and 4·0 mg./ml.

Ascorbic acid oxidase (ascorbate oxidase) was prepared from fresh cabbage by freezing whole cabbage-heart at −70° and then expressing the juice in a press. The juice was dialysed overnight against distilled water, and an equal volume of cold acetone was added to the dialysis residue. The resulting precipitate was dissolved in distilled water of one-tenth of the original volume of juice. This preparation was stored at −70°; at pH 6·8 and 37°, 0·2 ml. of this solution in a total volume of 2·0 ml. completely oxidized 180 μg. ascorbic acid in 2 hr.

Copper solution was 0·1 M-$CuSO_4$ in distilled water. It was added to ascorbic acid + virus mixtures to a final copper concentration of 5 μg./ml.

Ascorbic acid (British Drug Houses Ltd.) was prepared in solutions of various strengths in pH 6·8 buffer immediately before use. The concentration of ascorbic acid was estimated by titration against standard B.D.H. dichlorophenolindophenol.

Cystein hydrochloride, glutathione and thioglycollic (mercaptoacetic) acid were dissolved in distilled water and diluted in buffer of the desired pH value.

RESULTS

In preliminary experiments samples of virus containing 5×10^3 TCD50/ml. were exposed to cysteine, glutathione, thioglycollic acid or ascorbic acid at 100 μg./ml. for 18 hr at 37° and pH 7·4. Whereas the three thiol-containing reducing substances were without effect, ascorbic acid decreased the virus titre to < 10 TCD50/ml. This inactivation was not abolished by dialysis of the virus + ascorbic acid mixture against water. Tissue culture cells, pretreated for 2 days at 37° with ascorbic acid 200 μg/ml. had no altered susceptibility to virus. The inactivating effect of ascorbic acid on vaccinia virus was also shown by titration of samples on the chorioallantoic membranes of chick embryos.

Effect of copper. The autoxidation of ascorbic acid is a well-known phenomenon and is catalysed by copper ions (Barron, Demilio & Klemperer, 1936). Preliminary experiments showed that Cu^{2+} 5 μg./ml. was itself without effect on the virus, but it increased both the rate and degree of inactivation by ascorbic acid in ascorbic acid + vaccinia virus mixtures. The Cu^{2+} also increased the rate of oxidation of ascorbic acid in the mixture (Fig. 1).

Effect of concentration of ascorbic acid. Vaccinia virus has an optimum stability at pH 6·8–7·0 at 37°; accordingly, quantitative experiments with ascorbic acid were performed in undiluted McIlvaine buffer at pH 6·9. Vaccinia virus with an initial titre of $10^{8.5}$ TCD50/ml. was diluted 1/100 and mixed with different quantities of ascorbic acid. The mixtures were incubated at 37° and samples examined at various times for infectious virus. A control sample of virus alone was titrated after incubation for the maximum time. All the mixtures contained 5 μg. Cu^{2+}/ml. The concentration of ascorbic acid and the time of exposure influenced the inactivation of vaccinia virus; quantities as small as 50 μg. ascorbic acid/ml. fully inactivated $10^{6.5}$ TCD50 in 2 hr at 37° and amounts as little as 10 μg. caused partial inactivation after 24 hr (Table 1).

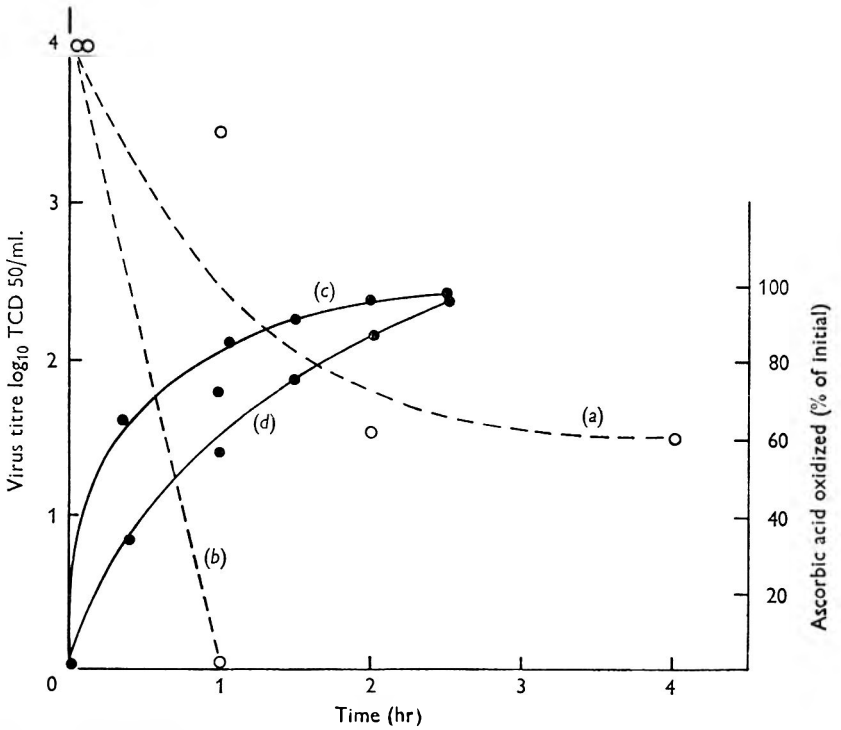

Fig. 1. Vaccinia virus 10,000 TCD 50 that was exposed to ascorbic acid 100 μg./ml. at 37° and pH 7·4. Curve (*a*) open circles virus titre without Cu^{2+}. Curve (*b*) open circles virus titre + Cu^{2+} 5 μg./ml. Curve (c) closed circles % ascorbic acid oxidized in the presence of Cu^{2+} 5 μg./ml. Curve (*d*) closed circles % ascorbic acid oxidized in the absence of Cu^{2+}.

Effect of dehydroascorbic acid. An inhibitory concentration of ascorbic acid (100 μg./ml.) was oxidized with an equivalent quantity of potassium permanganate. Although potassium permanganate inactivates vaccinia virus, the amount required to oxidize 100 μg. ascorbic acid was calculated to be less than the inhibitory concentration. Only partial inactivation occurred when the sample of oxidized ascorbic

Table 1. *Effect of ascorbic acid on the infectivity of vaccinia virus suspensions*

Ascorbic acid (mg./ml.)	Time of exposure (hr)						Amount of ascorbic acid oxidized at time of inactivation of virus (μg.)
	0	0·25	0·5	1·0	2·0	24·0	
	Relative degree of virus inactivation						
1·0	−	+	+	+	+	+	80–190
0·5	−	−	±	+	+	+	140
0·25	−	−	+	+	+	+	70
0·125	−	−	+	+	+	+	65
0·0625	−	−	−	±	+	+	50
0·05	−	−	−	−	−	±	50
0·01	−	−	−	−	−	±	10

+ = complete inactivation; ± = partial inactivation; − = > 10 TCD 50 active virus still present.

acid was held with virus overnight at 37°. Ascorbic acid (125 μg./ml.) completely oxidized with ascorbic acid oxidase did not inactivate vaccinia virus after 2 hr at 37°. Controls of unoxidized ascorbic acid in this concentration inactivated completely. The infectivity of untreated virus under these conditions was unaltered.

Effect of ascorbic acid oxidase (ascorbate oxidase). The rapid oxidation of ascorbic acid can be catalysed without added Cu^{2+} by the enzyme hexoxidase. Virus and ascorbate oxidase preparation in pH 6·9 buffer were mixed to give a virus titre of 10^6 TCD50/ml. and a concentration of enzyme preparation of 20 % (v/v). To one portion of this mixture ascorbic acid was added to 125 μg./ml. The second portion was adjusted to the same volume by adding pH 6·9 buffer. Controls consisting of virus alone and virus + ascorbic acid + Cu^{2+} were set up at the same time. All mixtures were incubated at 37° for 2 hr. There was no inactivation of vaccinia virus when the ascorbic acid used had been completely oxidized by ascorbate oxidase in the absence of added Cu^{2+}. The enzyme alone was without effect on the virus, which was completely inactivated in the presence of the same concentration of ascorbic acid when oxidation was catalysed by Cu^{2+}. These results confirmed that the oxidation product of ascorbic acid (dehydroascorbic acid) did not inactivate and indicate that the copper catalysed oxidation of ascorbic acid (probably in the presence of oxygen) was necessary for inactivation, and that the enzyme-catalysed oxidation proceeds by different mechanism. These findings are like those of Lojkin (1936) with tobacco mosaic virus.

Effect of catalase. The addition of catalase to ascorbic acid – tobacco mosaic virus mixtures abolishes the inactivating effect of ascorbic acid (Lojkin, 1936). Similarly, the addition of beef-liver catalase 4 mg./ml. completely prevented the inactivation of vaccinia virus in a buffered mixture containing $10^{6·5}$ TCD50 virus/ml. + ascorbic acid 125 μg./ml. + Cu^{2+} 5 μg./ml. held at 37° for 2 hr. Inactivation occurred when the catalase was omitted from a similar mixture or when the enzyme was inactivated by boiling before addition. The enzyme alone was without effect on the virus, and did not prevent the oxidation of ascorbic acid. The ascorbic acid in the mixture was about 90 % oxidized.

Effect of high pH values. Barron *et al.* (1936) showed that the autoxidation of ascorbic acid was increased in alkaline media in the absence of catalytic Cu^{2+}. Vaccinia virus and ascorbic acid were mixed without added Cu^{2+} in pH 10·0 buffer to final concentrations of 10^6 TCD50 and 125 μg./ml. respectively. The mixture, and a virus control at the same pH value, were incubated for 2 hr at 37°. No inactivation of virus took place, although more than 40 % of the ascorbic acid was oxidized. Exposure to pH 10·0 for 2 hr at 37° had no effect on the virus alone.

Effect of glutathione. The inhibition of vaccinia virus by reduced glutathione reported by Kligler & Bernkopf (1937) was not confirmed. Not only were glutathione concentrations up to 1·0 mg./ml. without effect on the virus, but appeared to prevent the oxidation of ascorbic acid in vaccinia virus + ascorbic acid mixtures and consequently the inactivation of virus. Ascorbic acid was added to mixtures of virus and freshly prepared reduced glutathione. The final mixture, containing virus $10^{6·5}$ TCD50/ml., glutathione 125 μg./ml., ascorbic acid 125 μg./ml., and Cu^{2+} 5 μg./ml. was held for 2 hr at 37°; suitable controls were included. Glutathione decreased the inactivation by ascorbic acid at 125 μg./ml., and abolished it at 1 mg./ml. Glutathione prevented the autoxidation of ascorbic acid, but did not reactivate virus when

added to virus + ascorbic acid mixtures in which inactivation of the virus had already occurred. The substitution of cysteine in the same concentration for glutathione had no effect on the inactivation by ascorbic acid. These experiments also indicate that ascorbic acid itself is not inhibitory when its autoxidation is prevented.

Effect of an atmosphere of nitrogen and anaerobiosis on ascorbic acid inactivation. Several attempts were made to prevent the autoxidation of ascorbic acid in vaccinia virus + ascorbic acid mixtures by gassing the system with oxygen-free nitrogen. In a typical experiment the buffer to be used as diluent was boiled to drive off dissolved air. Oxygen-free nitrogen (British Oxygen Company Limited) was bubbled through a mixture containing 10^6 TCD50/ml. virus and 5 μg. Cu^{2+}/ml. for 30 min. before a freshly prepared solution of ascorbic acid was added to 125 μg./ml., the nitrogen bubbling was continued during the 2 hr incubation at 37°. The virus was completely inactivated, although only 15–20 % of the ascorbic acid was oxidized. A Thunberg tube was prepared containing similar concentrations of virus and Cu^{2+}. Ascorbic acid solution was placed in the side arm and the tap opened. This, together with a virus control, was placed in an anaerobic jar. After the usual procedure for producing anaerobic conditions within the jar, the ascorbic acid was tipped into the virus. Again, after incubation at 37° the virus was inactivated, although only about 11 % of the ascorbic acid was oxidized. The control virus retained its original infectivity. It appears that under these conditions even a small degree of autoxidation of ascorbic acid was sufficient to inactivate the vaccinia virus.

DISCUSSION

The observations reported here for the inactivation of vaccinia virus by ascorbic acid are similar to those of Lojkin with tobacco mosaic virus. The inactivation of other animal viruses by ascorbic acid has been investigated from different viewpoints. In at least one case the inactivation can be ascribed to a direct pH effect; in other cases no cognizance was taken of the ready autoxidation of ascorbic acid, the catalysis of this oxidation by minute amounts of cupric ion, or the effect of pH on the autoxidation process. Milas (1932), in a review of autoxidation, stated that autoxidations induce the oxidation of other substances that are relatively unaffected by free oxygen. The inactivation of tobacco mosaic virus by ascorbic acid has been explained not as a reduction, but an oxidation effected by an intermediate unstable peroxide formed during the autoxidation of ascorbic acid catalysed by Cu^{+}. Although the identity of the peroxide was not established, the addition of catalase to ascorbic acid + tobacco mosaic virus mixtures abolished the inactivation (Lojkin, 1936). Catalase likewise prevented the inhibition of vaccinia virus by ascorbic acid, presumably by destroying peroxides, although it did not prevent the autoxidation of ascorbic acid. Peloux, Loire, Cier & Colobert (1962) inactivated poliovirus with hydrogen peroxide and ascorbic acid and suggested that free hydroxyl radicals induced by iron and copper ions were responsible. Berneis (1963) showed that ascorbic acid degraded deoxyribonucleic acid, a degradation that could be abolished by catalase or peroxidase; he too concluded that free hydroxyl radicals rather than intermediate peroxides were involved. Free radicals drastically alter the properties of both synthetic and natural polymers. The inactivation of vaccinia virus might similarly be explained on this basis. Whether viral protein or nucleic acid is attacked remains to be established.

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Enzymic and Genetic Control of Polyphosphate Accumulation in *Aerobacter aerogenes*

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SUMMARY

Addition of orthophosphate to *Aerobacter aerogenes* strain A3(0) organisms previously subjected to phosphate starvation induced accumulation of inorganic polyphosphate within the organisms. With resumption of growth the polyphosphate was degraded and served as a source of nucleic acid phosphorus. During phosphate starvation the specific activity of polyphosphate kinase and inorganic polyphosphatase increased five- to tenfold while the amount of alkaline phosphatase increased 50 times. The results suggest that synthesis of polyphosphate kinase and alkaline phosphatase was subject to repression by exogenous orthophosphate. Two mutant strains blocked in polyphosphate accumulation were found to carry defects in the synthesis of these enzymes. Mutants of class Pn-1 contained normal amounts of all three enzymes, but repression of their synthesis was not annulled by phosphate starvation. Mutants of class Pn-2 contained no polyphosphate kinase. It is suggested that synthesis of polyphosphate kinase is controlled by two genes, a structural gene and a regulator gene; the latter gene also appears to control the synthesis of alkaline phosphatase and perhaps polyphosphatase. The patterns of polyphosphate accumulation under various nutritional conditions are discussed in relation to the amounts and activities of the enzymes of polyphosphate synthesis and degradation.

INTRODUCTION

Jeener & Brachet (1944) discovered that addition of orthophosphate to phosphate-starved yeast resulted in rapid uptake of phosphate and accumulation of a basophilic substance within the organisms. Originally thought to be ribonucleic acid (RNA), the basophilic material was later identified as inorganic polyphosphate (Wiame, 1947; Schmidt, 1951). Its accumulation corresponded to the appearance of structures known as 'volutin granules'; both disappeared together when growth of the organisms was resumed. This basic pattern has since been described in various fungi and bacteria (Kuhl, 1960; Harold, 1962; Liss & Langen, 1962; Smith, Wilkinson & Duguid, 1954; Zaitseva, Belozerskii & Novozhilova, 1960; Kaltwasser, 1962). The term 'polyphosphate overplus' is proposed to designate the accumulation of polyphosphate upon addition of orthophosphate to phosphate-starved organisms, in order to distinguish this from polyphosphate accumulation under other conditions of nutrient imbalance (Smith *et al.* 1954). The term corresponds to 'Polyphosphat-Überkompensation' as used by Liss & Langen (1962).

The physiological basis of the polyphosphate overplus has remained obscure despite repeated investigation. Liss & Langen (1962) found an increased rate of

polyphosphate synthesis in yeast previously subjected to phosphate starvation and attributed this to an imbalance of phosphate uptake and utilization. In the present paper evidence will be offered to relate the polyphosphate overplus to the control of the enzymes of polyphosphate metabolism in wild-type *Aerobacter aerogenes* and in mutants of it which carry genetic defects in polyphosphate synthesis. Preliminary accounts of these results have been published (Harold, 1963*a*, *b*).

METHODS

Organisms. *Aerobacter aerogenes* strain A3 (0) was obtained from Professor J. F. Wilkinson. The isolation of auxotrophic mutants requiring uracil (Sl_u) and methionine ($0_{met.}$) was described by Harold (1963*c*). Mutants defective in polyphosphate accumulation were isolated by a ^{32}P-suicide technique (Harold & Harold, 1963). These mutants, were of two types: (i) Pn-1 mutants which did not show the overplus effect but accumulated polyphosphate upon sulphur starvation; (ii) Pn-2 mutants which did not accumulate polyphosphate under any conditions. Most of the present experiments were conducted with the strains 1025D(Pn-1) and 1023C (Pn-2) previously used.

Growth experiments. The bacteria were grown in $T_{subscript}$ media as described earlier (Harold, 1963*c*; Harold & Harold, 1963). This is a mineral medium containing sulphate as sole source of sulphur, glucose as carbon + energy source and buffered with tris (pH 7·6, 10 g./l.). The subscript indicates the phosphorus (P) content as μg. P/ml. All cultures were grown on a rotary shaker at 37°. The population density was normally about 10^9 cells/ml.; multiplication was followed turbidimetrically at 600 mμ. Overnight cultures were grown in T_{230} medium; the organisms were then harvested, washed, and resuspended in fresh pre-warmed T medium containing orthophosphate and growth factors as described for the individual experiments. Samples were withdrawn periodically and the organisms centrifuged down. Samples intended for enzyme assay were washed with chilled tris buffer. Phosphorus analyses were performed on unwashed organisms. The fractionation and estimation of phosphorus compounds was described earlier (Harold, 1963*c*). The bulk of the polyphosphate was acid-insoluble polyphosphate; acid-soluble phosphate, when present, was measured separately and the two fractions added to give the amount of total inorganic polyphosphate.

Enzyme assay. Bacterial cells from 30 ml. culture were resuspended in 1 ml. tris buffer (0·1 M, pH 7·0) in a plastic tube. Glass beads (Superbrite, Minnesota Mining and Manufacturing Co., Type 115; 0·50 g.) were added, and the tube exposed for 20 min. to sonic treatment in a Raytheon 10 Kc oscillator cooled with ice water. The tubes were then centrifuged at 4° for 10 min. at 20,000 *g*; the supernatant fluid was used for the estimation of polyphosphate kinase as described below, and for protein estimation by the biuret method (Layne, 1957).

Assay of polyphosphatase activity in these extracts gave variable results. As a rule, high activity was found only in extracts of bacterial cells which had been allowed to accumulate polyphosphate following phosphate starvation. This apparent production of polyphosphatase in response to polyphosphate accumulation was ultimately found to be an artifact: the enzyme is readily lost, apparently by adsorption to the glass beads, unless inorganic polyphosphate is present. Consequently,

for the assay of polyphosphatase the bacterial cells were sonically treated in tris buffer containing polyphosphate (synthetic, chain length about 170, 100 μg./ml. P).

Polyphosphate kinase. This enzyme was assayed in crude extracts by a modification of the procedure described by Muhammed (1961). It had an obligatory requirement for Mg^{2+} and an optimum at about pH 7. Adenosine diphosphate (ADP) was inhibitory at concentrations above 2×10^{-3} M, but polyphosphate itself had little effect at 15×10^{-3} M. Each assay tube received 2 μmole acetyl phosphate-^{32}P, 2 μmole $MgCl_2$, 1·5–5 units acetokinase (Rose, 1955), 0·2 μmole ADP, 25 μmole glycylglycine or tris buffer (pH 7·0), and 0·05–0·10 ml. enzyme extract in a final volume of 0·33 ml. The tubes were incubated for 10 min. at 37°. At that time, 0·25 ml. N-perchloric acid was added and then 0·50 ml. bovine serum albumin solution (5 mg./ml.) to precipitate the polyphosphate formed. The precipitate was washed twice with 0·5 N-perchloric acid, dissolved in 0·5 N-NaOH and plated for counting. The course of the reaction was linear with time for 20 min. and proportional to enzyme concentration over the range of 0·02 to 4 units/tube. The unit is defined as that amount of enzyme which produces 0·01 μmoles polyphosphate in 10 min. The identity of the product was established by its behaviour in the standard fractionation scheme and by its complete lability to acid (N-HCl, 15 min., 100°).

Polyphosphatase. The polyphosphatase activity of crude extracts had a broad pH optimum between 7 and 9. A requirement for Mg^{2+} was observed only in the presence of EDTA, but high concentrations of KCl (about 0·3 M) were necessary for maximal activity. No dialysable cofactors were detected. The assay of polyphosphatase was modified from that described by Muhammed, Rodgers & Hughes (1959). Each assay tube received 100 μmole KCl, 0·25 μmole $MgCl_2$, 1·6 μmole polyphosphate-^{32}P, 10 μmole tris buffer (pH 9) and 0·05 ml. enzyme extract in a final volume of 0·30 ml. (the amount of polyphosphate introduced with the extract was neglected). The tubes were incubated at 37° for 30 min., then excess polyphosphate and protein were precipitated by adding 1·0 ml. 0·5 N-perchloric acid and 0·1 ml. bovine serum albumin (10 mg./ml.). The tubes were centrifuged and the supernatant fluids decanted. After addition of ammonium molybdate the ^{32}P-orthophosphate was extracted with isobutanol (Weil-Malherbe & Green, 1951), plated and counted. The hydrolysis of polyphosphate was linear with time for 60 min. and proportional to the enzyme concentration over a wide range. A unit of polyphosphatase is defined as that amount of enzyme which liberates 0·01 μmole ^{32}P-orthophosphate in 10 min.

Alkaline and acid phosphatase. These enzymes were in general assayed with intact *Aerobacter aerogenes* organisms as described by Torriani (1960). For consistency, the unit is defined as that amount of enzyme which liberates 0·01 μmole orthophosphate from *o*-nitrophenyl phosphate in 10 min.

Chemicals. Acetyl phosphate-^{32}P was synthesized as described by Kornberg (1957), polyphosphate-^{32}P by the method of Muhammed *et al.* (1959). Other reagents were purchased from Sigma Chemical Co. and the California Corporation for Biochemical Research.

RESULTS

The polyphosphate overplus in wild-type and mutant strains of Aerobacter aerogenes A 3(O). *Aerobacter aerogenes* organisms in the logarithmic or the stationary phase of growth contained only traces of polyphosphate. When orthophosphate was added to organisms which had been incubated in phosphate-deficient medium

for 3–4 hr dramatic accumulation of polyphosphate occurred, together with the appearance of microscopically visible volutin granules (Smith *et al.* 1954). Induction of this polyphosphate overplus was dependent upon protein synthesis during the starvation phase: no polyphosphate accumulation was observed when orthophosphate was added to strain A3(0) organisms subjected to phosphate starvation in absence of glucose or of sulphur, or to organisms of strain 0_{met-} incubated in medium lacking orthophosphate and methionine. Protein synthesis was not, however, required for polyphosphate accumulation itself since chloramphenicol (10 μg./ml.), sulphur deficiency or methionine deprivation had no effect once starvation was complete.

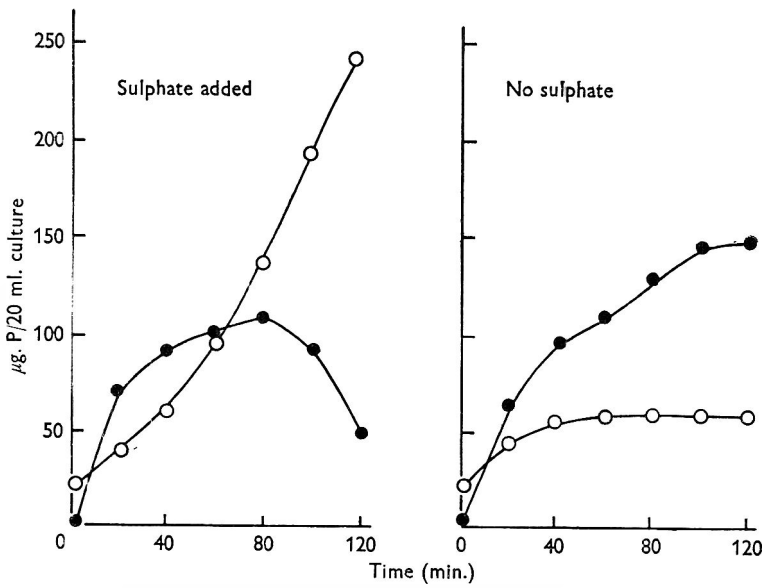

Fig. 1. The polyphosphate overplus in *Aerobacter aerogenes* strain A3(0). The organisms were subjected to phosphorus starvation for 4 hr, collected, washed and resuspended in T_0 medium with and without sulphate. Orthophosphate was added to both flasks at 0 min. ●—●, Polyphosphate; ○—○, total nucleic acids.

A typical experiment illustrating the accumulation of polyphosphate and its subsequent fate is shown in Fig. 1. *Aerobacter aerogenes* A3(0) organisms were subjected to phosphorus starvation in T_0 medium for 4 hr. The bacteria were then harvested, washed, and resuspended in T_{100} medium, with and without sulphate. Both cultures rapidly accumulated polyphosphate and smaller amounts of nucleic acids. In the flask supplemented with sulphate, growth of the bacteria resumed and was accompanied by extensive nucleic acid synthesis and degradation of polyphosphate. In the sulphur-deficient culture growth was prevented, and a high polyphosphate concentration was maintained. The degradation of polyphosphate upon resumption of growth was shown to be a consequence of nucleic acid synthesis. In the presence of chloramphenicol protein synthesis and growth were inhibited, but nucleic acids accumulated within the bacteria and polyphosphate was concurrently degraded. In a typical experiment, organisms of the uracil-requiring mutant Sl_{u-} were subjected to phosphate starvation; the bacteria were then

harvested and allowed to accumulate polyphosphate in radioactive T_{100} medium without uracil (to prevent nucleic acid synthesis). After 1 hr the ^{32}P-orthophosphate was diluted with excess unlabelled orthophosphate, and uracil + chloramphenicol (10 μg./ml.) were added. Nucleic acid accumulated within the bacteria while the ^{32}P-polyphosphate was degraded and the ^{32}P transferred to the nucleic acid fraction. These results are analogous to those described previously for the degradation of polyphosphate accumulated during nutrient deprivation (Harold, 1963*c*).

Mutants defective in polyphosphate accumulation showed quite a different pattern of phosphorus metabolism. *Aerobacter aerogenes* A3(0), Pn-1 and Pn-2 organisms were subjected to phosphorus starvation for 4 hr. The bacteria were then transferred to sulphur-deficient medium and ^{32}P-orthophosphate (100 μg./ml.) was added. As illustrated in Fig. 2, some nucleic acid was synthesized by all three strains but only strain A3(0) accumulated polyphosphate. Mutant and wild-type bacteria incorporated about equal amounts of ^{32}P into the orthophosphate and acid-soluble organic phosphate fractions.

Differential synthesis of enzymes of phosphate metabolism in Aerobacter aerogenes during phosphate starvation. From the results presented in the preceding section it is clear that the overplus phenomenon is a consequence of events which occur during the starvation phase and require protein synthesis. Clues to the nature of these events came from studies on the amount of polyphosphate kinase in wild-type and mutant *Aerobacter aerogenes* under various growth conditions. When bacteria of strain A3(0) were incubated in phosphate-deficient medium, the optical density and protein content of the culture increased by about 50 %, and concurrently there was a marked increase in the specific activity of polyphosphate kinase. This increase in enzyme content was abolished by chloramphenicol and, in a methionine auxotroph, was dependent upon the presence of methionine (Fig. 3). The differential increase in the specific activity of polyphosphate kinase was induced specifically by phosphorus starvation: sulphur starvation and amino acid deprivation in auxotrophic mutants had no such effect. Upon addition of orthophosphate to phosphate-starved bacteria the differential rate of enzyme synthesis decreased until the value characteristic of growing bacteria (2–5 units/mg. protein) was attained. These results suggest that the synthesis of polyphosphate kinase is subject to repression by extracellular orthophosphate and thus proceeds at a higher differential rate during phosphate starvation.

The two polyphosphateless mutants exhibited clear defects in the synthesis of polyphosphate kinase. Organisms of strains A3(0), Pn-1 and Pn-2 were grown on T_{230} medium and then transferred to T_0 medium. As shown in Fig. 4, de-repression of polyphosphate kinase occurred only with strain A3(0). Mutant strains of class Pn-1 contained normal amounts of enzyme (2-4 units/mg. protein) in growing organisms, but there was no increase in specific activity during phosphorus starvation. Mutants of class Pn-2 contained no detectable polyphosphate kinase under any nutritional conditions. No evidence was obtained for destruction of polyphosphate kinase in these extracts, nor for the presence of an inhibitor.

The effects of nutritional conditions on the amount of polyphosphatase paralleled those described for polyphosphate kinase. Bacteria from overnight cultures contained very little polyphosphatase (2–4 units enzyme/mg. protein) and incubation of such organisms in medium deficient in sulphur produced no increase in its amount.

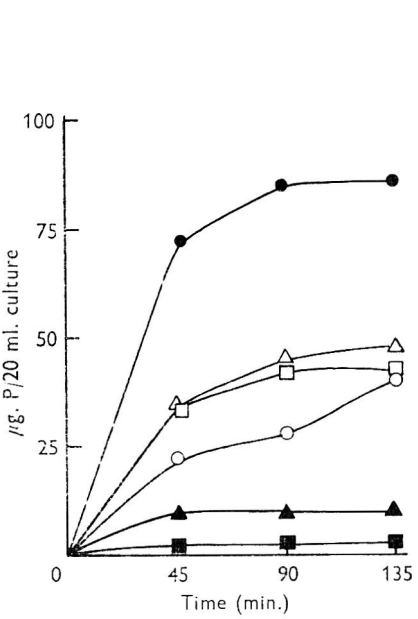

Fig. 2

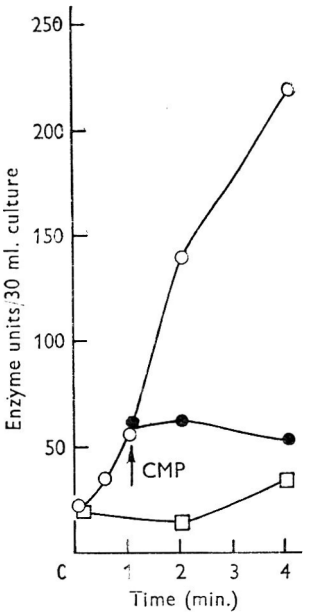

Fig. 3

Fig. 2. Accumulation of polyphosphate and nucleic acids in *Aerobacter aerogenes* strains A3 (0), Pn-1 and Pn-2. The bacteria were subjected to phosphorus starvation for 4 hr, collected, washed and resuspended in T_0 medium without sulphate. ^{32}P-orthophosphate was added to all flasks at 0 min. Closed symbols, polyphosphate; open symbols, nucleic acids. ●, ○, Strain A3(0); ▲, △, mutant Pn-1; ■, □, mutant Pn-2.

Fig. 3. Synthesis of polyphosphate kinase in *Aerobacter aerogenes* strain O_{met} during phosphate starvation. Washed organisms were suspended in T_0 medium at 0 hr, and the culture distributed among three flasks. Flask no. 1 (○—○) was supplemented with methionine (30 μg./ml.); no. 2 (□—□) received no methionine; no. 3 (●—●) received methionine but chloramphenicol (CMP; 10 μg./ml.) was added after 1 hr.

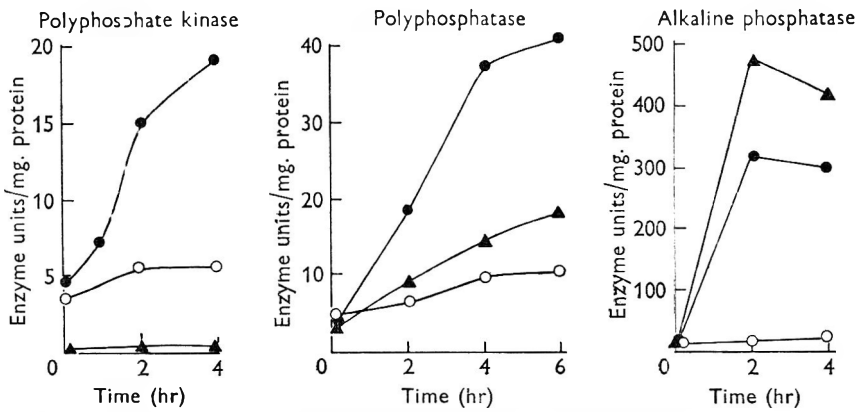

Fig. 4. Effect of phosphate starvation on the amounts of polyphosphate kinase, polyphosphatase and alkaline phosphatase in *Aerobacter aerogenes* strains A3(0), Pn-1 and Pn-2. Bacteria from an overnight culture were collected, washed and resuspended in T_0 medium at 0 hr. ●—●, strain A3(0); ○—○, mutant Pn-1; ▲—▲, mutant Pn-2.

Bacteria harvested during the logarithmic phase of growth contained 6–10 units enzyme/mg. protein. During incubation in phosphate-deficient medium, a substantial increase in the specific activity of polyphosphatase occurred in strain A 3 (0) organisms and, to a lesser degree, in Pn-2 organisms; the specific activity of polyphosphatase in strains of class Pn-1 was not affected (Fig. 4). The production of polyphosphatase required protein synthesis and thus presumably reflected differential synthesis of the enzyme, but repression upon subsequent addition of orthophosphate was not unequivocally demonstrated.

Differential synthesis of alkaline phosphatase during phosphate starvation in *Escherichia coli* was demonstrated by Torriani (1960) and by Horiuchi, Horiuchi & Mizuno (1959), and shown to be due to repression of the synthesis of this enzyme by exogenous orthophosphate. It therefore seemed of interest to examine whether the synthesis of alkaline phosphatase and of the enzymes of polyphosphate metabolism might be under joint genetic control. As shown in Fig. 4, phosphate starvation induced extensive synthesis of alkaline phosphatase in organisms of *Aerobacter aerogenes* strains A 3 (0) and Pn-2, but not in organisms of strain Pn-1. The amount of acid phosphatase was unaffected.

DISCUSSION

Accumulation of inorganic polyphosphate in micro-organisms is generally associated with nutritional conditions unfavourable to growth. Two quite distinct procedures for the induction of polyphosphate accumulation in *Aerobacter aerogenes* were described by Smith *et al.* (1954). On the one hand, polyphosphate accumulates when growth of the organisms ceases because of lack of certain essential nutrients, e.g. in sulphur starvation. Deposition of polyphosphate under these conditions has been shown to be due primarily to the cessation of nucleic acid synthesis while assimilation of phosphate from the medium continues (Harold, 1963*c*). On the other hand, polyphosphate accumulates upon addition of orthophosphate to a phosphate-starved culture. This phenomenon, for which the term 'polyphosphate overplus' is proposed, is the subject of the present paper. The patterns of polyphosphate accumulation under these two nutritional conditions are quite different. Polyphosphate accumulation due to nutrient deprivation is relatively slow, and is prevented by concurrent nucleic acid synthesis as well as by other environmental factors which accelerate polyphosphate degradation (Harold & Sylvan, 1963). The polyphosphate overplus is more rapid and is quite independent of nucleic acid synthesis (Fig. 1) and of changes in the composition of the medium. The physiological basis of these differences appears to reside in the amount of polyphosphate kinase. Wild-type *A. aerogenes* organisms normally contain a low concentration of polyphosphate kinase, and the rate of polyphosphate synthesis *in vivo* during nutrient deprivation agrees well with that calculated from the amount of enzyme present. During phosphate starvation the specific activity of polyphosphate kinase increases up to tenfold, and a corresponding increase in the rate of polyphosphate accumulation is observed when orthophosphate is made available to the starved organisms. It thus appears that the rate of polyphosphate synthesis is directly proportional to the amount of polyphosphate kinase within the organisms. Results with mutants blocked in polyphosphate accumulation (Harold & Harold, 1963) support this conclusion. *Aerobacter aerogenes* mutants of class Pn-2 lack polyphosphate

kinase, and do not accumulate polyphosphate under any conditions. Hence polyphosphate kinase catalyses the main, if not the only, reaction responsible for polyphosphate synthesis. Mutants of *A. aerogenes* class Pn-1 contain normal amounts of polyphosphate kinase and are therefore capable of slow polyphosphate accumulation when subjected to nutrient deprivation. However, repression of the synthesis of this enzyme is not annulled by phosphate starvation and hence no polyphosphate overplus occurs upon addition of orthophosphate.

The intracellular concentration of polyphosphate must be a function of the rates of synthesis and of degradation. From the present results and from those reported earlier (Harold, 1963*c*), it seems clear that polyphosphate degradation is coupled to nucleic acid synthesis. Except under special nutritional conditions (Harold & Sylvan, 1963), little polyphosphate degradation occurs until growth and nucleic acid synthesis resume. The molecular basis underlying the control of polyphosphate breakdown is unknown. Studies with cell-free extracts have provided evidence for four possible routes for the mobilization of inorganic polyphosphate: transfer of phosphate to ADP by reversal of polyphosphate kinase (Kornberg, 1957); transfer of phosphate to adenosine monophosphate (Winder & Denneny, 1957); direct phosphorylation of hexoses by polyphosphate (Dirheimer & Ebel, 1962; Szymona, Szymona & Kulesza, 1962); hydrolytic degradation to orthophosphate by inorganic polyphosphatase. The limited information available from work with intact organisms (Hughes & Muhammed, 1962; Harold, 1962) seems to favour hydrolysis as the chief route of polyphosphate degradation. Should this prove to be the case in *Aerobacter aerogenes*, an explanation will be required for the accumulation of polyphosphate in organisms subjected to phosphate starvation despite their elevated content of polyphosphatase (Fig. 4).

It remains to consider the genetic control of polyphosphate metabolism. During phosphate starvation differential synthesis of alkaline phosphatase, polyphosphate kinase and polyphosphatase occurs in wild-type *Aerobacter aerogenes*; the synthesis of the first two enzymes is repressed when orthophosphate is restored. Two mutations were found to affect this metabolic region. Mutants of class Pn-1 contain all three enzymes but repression of their synthesis is not annulled by phosphate starvation. Mutants of class Pn-2 lack polyphosphate kinase, but synthesize alkaline phosphatase and a small amount of polyphosphatase in phosphate-deficient medium. It is thus reasonable to postulate two genes, a regulator gene which controls the synthesis of all three enzymes (Pn-1) and a structural gene for polyphosphate kinase (Pn-2). The diminished formation of polyphosphatase in Pn-2 shows that this simple picture is no more than a first approximation. Nevertheless, the finding that these three enzymes form a unit of genetic regulation suggests that they constitute also a physiological unit, functionally concerned with phosphate storage (Harold, 1963*b*).

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The Metabolism of ^{14}C-Glycine and ^{14}C-Bicarbonate by Washed Suspensions of the Rumen Ciliate *Entodinium caudatum*

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SUMMARY

Washed suspensions of *Entodinium caudatum* incubated anaerobically in the presence of penicillin and neomycin incorporated ^{14}C-glycine in the cell 'pool' as *N*-acetylglycine and into the cell protein as glycine without conversion to any other amino acid. At low external concentrations the glycine was present in the protozoa themselves and was not free in the gastric sac or present solely in the intracellular or extracellular bacteria. The incorporation reached a constant value after incubation for 24 hr; after this time the uptake of glycine was balanced by an equal loss of *N*-acetylglycine from the organisms. These suspensions also incorporated ^{14}C from $NaH^{14}CO_3$ into carbon atoms 3 and 4 of protozoal glucose which was probably present as starch in the intact protozoa.

INTRODUCTION

Although *Entodinium caudatum* can be grown *in vitro* in the presence of bacteria on a substrate of rice starch grains, dried grass, autoclaved rumen fluid and chloramphenicol (Coleman, 1960*a*), it has not so far proved possible to grow these protozoa in the absence of bacteria (Coleman, 1962) and very little is known about the biosynthetic capabilities of this or any other Entodiniomorphid protozoon. Abou Akkada & Howard (1962) found that although chloramphenicol-treated *E. caudatum* taken directly from the rumen rapidly hydrolysed casein, there was little simultaneous increase in cell nitrogen. However, DL-alanine, DL-leucine and DL-valine were incorporated by *Ophryoscolex caudatus* (Williams, Davis, Doetsch & Gutierrez, 1961) and *Epidinium ecaudatum* (Gutierrez & Davis, 1962) although with neither organism was the cellular distribution of the incorporated amino acid investigated. The present investigation was undertaken in an attempt to determine if uniformly labelled ^{14}C-glycine, ^{14}C-glucose, ^{14}C-maltose, ^{14}C-starch and ^{14}C-bicarbonate were incorporated by washed *E. caudatum* grown under controlled conditions *in vitro* and whether any such uptake was into the intracellular bacteria or protozoal material.

METHODS

Source of protozoa. The protozoa were grown and 'inoculum cultures' prepared and treated as described by Coleman (1962) except that 'inoculum cultures' were treated each day with 15 mg. rice starch and about 10 mg. dried grass. Where the

metabolism of ^{14}C-starch or ^{14}C-glucose was studied the amount of rice starch was reduced to 5 mg. on the day before the experiment.

Preparation of protozoa for inoculation. The protozoa were taken from the 'inoculum cultures' after removal of the surface scum and most of the supernatant liquid, and allowed to stand in 9 in. × 1 in. tubes until any grass present had sunk to the bottom. The supernatant fluid containing the protozoa was transferred to centrifuge tubes, the residual grass washed with salt solution B (Coleman, 1960*b*) and the washings added to the supernatant fluid. The protozoa were spun down and washed five times in salt solution B, which contained 0·03 % (w/v) L-cysteine, on an angle-head centrifuge for 45 sec. from starting. The maximum speed was equivalent to 300 ***g***. The organisms were finally inoculated at a population density of 100,000–250,000/ml. incubation medium.

Incubation conditions for incorporation experiments. Except where otherwise stated, the basal medium consisted of 1·0–2·0 ml. (chosen so that the final volume was 4·0 ml.) salt solution B (Coleman, 1960*b*) autoclaved (115° for 20 min.) with 0·8 ml. water, 1·5 ml. 2 % soluble starch (dissolved in salt solution B) and glycine (when added at the beginning of the experiment) in a 5 in. × $\frac{1}{2}$ in. cotton-plugged test tube. Immediately after removal from the autoclave and cooling, the following additions were made aseptically: 0·1 ml. 1 % L-cysteine hydrochloride (neutralized and Seitz filtered), 0·1 ml. 5 % $NaHCO_3$ (Seitz filtered), 0·2 ml. penicillin G (25,000 units/ml.), 0·2 ml. 1 % neomycin sulphate solution, 0·2 ml. autoclaved rumen fluid residue (AR) and any other additions. After inoculation, the tubes were gassed for 10 sec. with 95 % (v/v) N_2 + 5 % (v/v) CO_2, sealed with a sterile rubber bung and incubated at 39°. Where larger quantities of protozoa were required 5–10 times these quantities were used. For experiments on the metabolism of carbohydrates the soluble starch was omitted and for experiments on the incorporation of ^{14}C from $NaH^{14}CO_3$ the $NaHCO_3$ was omitted and the tubes gassed with 100 % N_2. ^{14}C-glucose and $NaH^{14}CO_3$ were always added immediately before the tube was sealed. At the end of experiments where the uptake of a ^{14}C-compound was investigated the protozoa were centrifuged and washed twice at 300 ***g*** for 1 min. in salt solution B containing about 0·005 % of the ^{12}C form of the ^{14}C-compound added initially.

Bacterial viable counts. These were carried out on media A and C as described by Coleman (1962). The number of viable bacteria inside each protozoon was determined after breakage of washed protozoa in a Potter (Potter & Elvehjem, 1936) homogenizer. The number of protozoa was estimated by the method of Coleman (1958). Only those protozoa which showed no signs of disintegration were counted.

Sterile rumen fluid fractions. All rumen fluid was taken from Clun Forest wethers fed on hay and oats. Protozoa-containing autoclaved rumen fluid (PARF) was prepared from fresh rumen contents by straining through two layers of muslin and autoclaving under 95 % (v/v) N_2 + 5 % (v/v) CO_2 in sealed McCartney bottles at 115° for 20 min. Autoclaved rumen fluid (ARF) was prepared similarly to PARF except that the strained material was centrifuged at 500***g*** for 3 min. before autoclaving. Autoclaved residue (AR) was the pellet from the above centrifugation resuspended in 15 % of the original volume and autoclaved at 115° for 20 min.

Estimation of ^{14}C. ^{14}C in whole protozoa was estimated by washing the organisms with water on to an aluminium disc of area 4·7 cm.^{2} carrying a disc of lens tissue.

The sample was spread by one drop of cetyltrimethyl-ammonium bromide solution (5 mg./ml.) and fixed by one drop of polyvinyl alcohol (2 mg./ml.). The disc was dried *in vacuo* and the ^{14}C estimated by using a thin mica end-window GM tube and conventional scaler equipment. Over 1000 counts at a rate greater than five times background were recorded for all fractions. Most determinations were carried out with less than 0·5 mg. of material per cm.^{2} of disc. Where more material was present, the results were corrected to infinite thinness by using corrections determined from adding known weights of glycine, mineral salts or rumen fluid fractions to tracer quantities of ^{14}C-glycine. The amount of glycine incorporated by protozoa was calculated from the known specific activity (usually 1·0–4·0 μC./mg.) of glycine added initially. To determine the relative ^{14}C contents of spots on a chromatogram a thin mica end-window GM tube was placed directly on the spot, the position of which had been determined by radioautography. $^{14}CO_2$ was estimated by the method of Coleman (1956).

Fractionation of organisms. The protozoa after harvesting, washing as described above and resuspension in a small volume of salt solution, were first broken by treatment in an all-glass Potter homogenizer at room temperature until 98–100 % of the protozoa were broken (usually about 90 sec.) and then homogenate made up to a known volume and centrifuged at 7000*g* for 20 min. The supernatant liquid from this centrifugation is hereafter referred to as the 'broken-cell supernatant fluid' and the pellet after washing once in water as the 'broken-cell pellet'. This latter fraction contained all the viable bacteria in the homogenate.

These two fractions were further fractionated by a method based on that of Schneider (1945) and Roberts *et al.* (1955). The fraction was treated with 5 % trichloroacetic acid (TCA) and allowed to stand at 4° for 30 min. The precipitate was centrifuged down and washed once with 5 % TCA. The supernatant fluid and the washing formed the 'cold TCA soluble fraction'. The precipitate was then extracted with 5 % TCA at 100° for 30 min. The residue was centrifuged down and washed once with 5 % TCA. The supernatant fluid and washings formed the 'hot TCA soluble or nucleic acid fraction'. The TCA was removed from both fractions by washing three times with ether before plating out for the estimation of ^{14}C. The residue after further washing, once with acidified ethanol and once with ether, formed the protein fraction.

The complete acid hydrolysis of any fraction was done by heating to 105° in 6 N-HCl for 16 hr in a sealed tube. At the end of this period the tube was cooled and opened and the acid removed on a boiling water bath in a current of air.

Paper chromatography. The following solvents were used: A, *sec*-butanol + formic acid + water (70 + 10 + 20, by vol.); B, phenol + ammonia (sp.gr. 0·880) + water (80 g. + 0·3 ml. + 20 ml.; Roberts *et al.* 1955); C, *n*-propanol + ethyl acetate + water (24 + 13 + 7, by vol.); D, *n*-butanol saturated with 1 % (w/v) aqueous ethylamine (Elsden & Lewis, 1953); E, *n*-butanol + ethanol + water (10 + 1 + 2 by vol.); F, *n*-butanol + acetone + water + diethylamine (10 + 10 + 5 + 2, by vol.; Hardy, Holland & Nayler, 1955); G, isopropanol + HCl solution (sp.gr. = 1·18) + water (162 + 42 + 46, by vol.); H, *n*-butanol + cyclohexane + propylene glycol + water + NH_4OH (sp.gr. = 0·880) + morpholine (30 + 30 + 10 + 3·5 + 0·7 + 0·07, by vol.; Guillaume & Osteux, 1955); J, benzene + isopropanol + propylene glycol + water + NH_4OH (sp.gr. = 0·880) (30 + 20 + 10 + 3 + 0·6, by vol.; Guillaume & Osteux, 1955).

Protein hydrolysates were chromatographed in two dimensions in solvents A and B (Roberts *et al.* 1955).

Purification of glycine-X. The method finally adopted was as follows. The supernatant fluids obtained after incubation of protozoa for 3 days in the standard medium containing 0·05 M ^{12}C-glycine or tracer [u-^{14}C]glycine were combined and shaken with Zeo Karb 225 (H^+) and the resin discarded. The supernatant fluid was neutralized with 8 % $Ba(OH)_2$ and the precipitate centrifuged down. Glycine-X was then absorbed on to a column of Dowex 2 (OH^-) which was then washed successively with water, N-acetic acid, water. Glycine-X was then eluted with N-HCl and the eluate evaporated to dryness *in vacuo*. The material was further purified by chromatography in solvent A and its position determined by radioautography. The spot was eluted and this material used for analytical tests.

Estimation of soluble glycine in protozoa. Washed protozoa were broken in a Potter homogenizer and the homogenate centrifuged at 7000*g* for 20 min. Free glycine was then estimated in the supernatant fluid, after the removal of protein with tungstic acid, by the method of Alexander, Landwehr & Seligman (1945). Combined and free glycine were estimated together after hydrolysis of the protein-free supernatant in 6 N-HCl for 16 hr at 105° in a sealed tube.

Chemicals. ^{14}C-compounds were supplied by the Radiochemical Centre, Amersham, Buckinghamshire. All the compounds were uniformly labelled except for [1-^{14}C] sodium acetate. The specific activity of the compounds as supplied was in μC. per mg.: glucose 450, glycine 41, maltose 16, sodium acetate 260, sodium carbonate 250, starch 23. The specific activity of these compounds was usually lowered to a more convenient level by the addition of carrier ^{12}C-compound before use. *N*-Acetylglycine was supplied by L. Light and Co. Ltd.

RESULTS

Metabolism of glycine

Washed suspensions of *Entodinium caudatum* prepared from growing cultures and incubated anaerobically in the presence of soluble starch, penicillin and neomycin incorporated ^{14}C-glycine. Initially each protozoon contained 10–100 viable bacteria in its gastric sac and there were 10–20 viable bacteria/protozoon free in the medium as determined by growth on media A and C (Coleman, 1962). The number of viable bacteria in these cultures measured immediately after inoculation was only 10 % of that found previously (Coleman, 1962). This finding is probably associated with the addition of less rice starch each day to the protozoal cultures and the use of lower centrifuge speeds when washing the protozoa. When the basal medium was supplemented by 5 % autoclaved residue (AR) 80–100 % of the protozoa survived for over 3 days compared with 40 % in its absence, during which time the bacterial viable count on the complete medium decreased to 10 % of the initial. Figure 1 shows that ^{14}C-glycine added to the culture at any time during the first 24 hr was incorporated into the protozoal fraction although the initial rate and the final level of incorporation dropped the later the glycine was added.

Effect of salt concentration and rumen fluid fractions. The concentration of salts in the medium had a marked effect on the incorporation of glycine measured after incubation for 7 hr although there was no effect on the number of apparently

healthy protozoa over this time and only a small effect over 48 hr. The uptake of ^{14}C-glycine was stimulated by the addition of 10 % of all the rumen fluid fractions tested of which 10 % AR was the most effective (Fig. 2). However, the magnitude of this stimulation with AR varied from 380 % in salt solution B to 55 % in salt solution B diluted by 20 %. AR was therefore added to all media for incorporation experiments. Control experiments carried out in the absence of protozoa showed that there was no adsorption of ^{14}C on to the particles of AR. The supernatant obtained after centrifugation of AR had only 10–20 % of the incorporation-promoting activity of the original material.

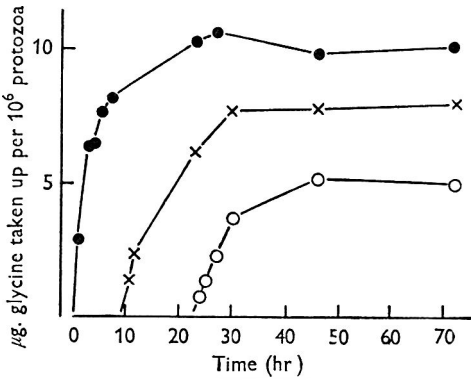

Fig. 1

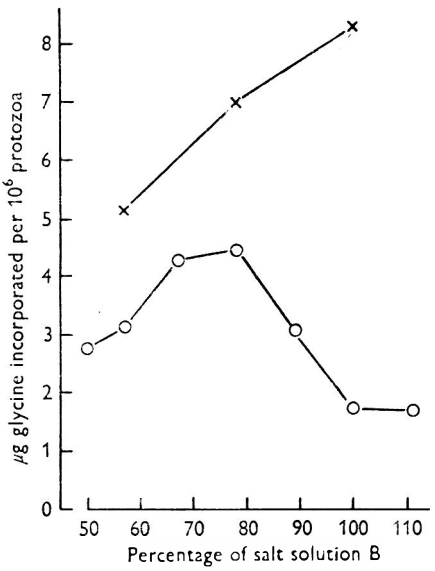

Fig. 2

Fig. 1. Incorporation of glycine into *Entodinium caudatum* incubated in presence of penicillin and neomycin. ●——●, 28 μg ^{14}C-glycine/ml. added initially; ×——×, 28 μg ^{14}C-glycine/ml. added after 9½ hr; ○——○, 28 μg. ^{14}C-glycine/ml. added after 23 hr. Results calculated from uptake of ^{14}C from ^{14}C-glycine of specific activity 130 counts/min./μg.

Fig. 2. Effect of salt concentration on incorporation of ^{14}C-glycine during the first 7 hr of incubation. The concentration of cysteine, $NaHCO_3$, penicillin and neomycin were maintained constant throughout and only the concentration of the constituents of salt solution B varied. ○——○, no addition; ×——×, +10 % autoclaved residue (AR).

Effect of glycine concentration. To determine whether the glycine incorporated by the protozoa was free in the gastric sac, the protozoa were incubated with various concentrations of [u-^{14}C]glycine and the uptake measured. At the lowest concentrations of glycine (up to 20 μg./ml. in the presence of 2×10^5 protozoa/ml.) over 7 % of the total ^{14}C was taken up in 4 hr by organisms, the packed cell pad volume of which was less than 1 % of the total. This shows that glycine was concentrated inside the protozoa. At low external glycine concentrations most of the non-protein glycine was in a combined form, but as the external concentration was increased there was a marked increase in the amount of free glycine present (Fig. 3). It is possible that some of this glycine was not intracellular but was present in the

gastric sac, as the method used does not separate glycine in the cell 'pool' from that in the gastric sac.

Effect of antibiotics and breakage of the protozoa. Although the above results show that glycine was incorporated into the micro-organisms in the suspensions, this uptake might have been into the extracellular or intracellular bacteria rather than into the protozoal protoplasm. In an attempt to eliminate this possibility the uptake of ^{14}C-glycine by intact protozoa was compared with that obtained with protozoa which had been broken under conditions that did not break the bacteria, i.e. in a Potter homogenizer. As a further test for the possible uptake of ^{14}C-glycine into bacteria, all the experiments were done in duplicate; one tube was centrifuged under

Table 1. *Incorporation of ^{14}C-glycine by broken and intact Entodinium caudatum in the presence and absence of antibiotics*

Protozoa were incubated at a concentration of $1{\cdot}4 \times 10^5$ protozoa/ml. in salt medium containing 0·75 % soluble starch, 0·025 % L-cysteine, 0·12 % $NaHCO_3$, 25 μg. ^{14}C-glycine (130 counts/min./μg.)/ml. in the presence or absence of 0·05 % neomycin sulphate and 1250 units penicillin G/ml. for 5½ hr under 95 % N_2 + 5 % CO_2. To determine ^{14}C in intact protozoa the culture was centrifuged for 45 sec. from starting the centrifuge, at the end of which time the speed was equivalent to 300 *g*. To determine ^{14}C in protozoa + bacteria free in the medium the culture was centrifuged at 7000 *g* for 20 min. and the pellet washed twice in salt medium in each case. All results are expressed per ml. culture.

Protozoa	Antibiotics	Viable bacteria Medium A	Viable bacteria Medium C	^{14}C in protozoal fraction (counts/min.)	^{14}C in protozoa + bacteria (counts/min.)
Initial					
Intact	–	10^5	5×10^5	.	.
Broken	–	10^5	30×10^5	.	.
Final					
Intact	+	10^5	$< 10^5$	196	194
Broken	+	10^5	5×10^5	10	12
Intact	–	10^8	150×10^6	222	226
Broken	–	10^7	300×10^6	144	624

* The viable counts were carried out on media A and C respectively (Coleman, 1962).

conditions such that only protozoa were sedimented (45 sec. at up to 300*g*) and the other such that protozoa + free bacteria were sedimented (20 min. at 7000*g*). Table 1 shows that in the presence of antibiotics breakage of the protozoa decreased the incorporation into the protozoal fraction to 5 % and that the same result was obtained under both sets of sedimentation conditions, indicating that uptake by intracellular and extracellular bacteria was negligible. In the absence of antibiotics there was an increase (range 10–100 % depending on the experiment) in the incorporation by intact protozoa. However, the experiments made in the absence of antibiotics are difficult to interpret due to the rapid bacterial growth under these conditions.

Intracellular products of glycine metabolism. As a further test as to whether the uptake of ^{14}C-glycine was into the bacteria, protozoal suspensions which had incorporated glycine were broken in a Potter homogenizer. The homogenate was then centrifuged to produce a supernatant fraction free of bacteria and a pellet fraction which contained all the bacteria. Table 2 shows that after 1 hr incubation in the

presence of ^{14}C-glycine over 80 % of the ^{14}C incorporated into intact protozoa was present in the broken-cell supernatant fluid and that 88 % of that was in the cold 5 % TCA soluble or small molecular weight fraction. The low incorporation of ^{14}C into the pellet shows that less than 20 % of the total incorporation could have been into the bacteria. Incubation in the absence of antibiotics resulted in a tenfold increase in the ^{14}C in the pellet fraction and this was probably due to increased incorporation by the intracellular bacteria which were present in this fraction; the incorporation into the supernatant fraction was unchanged.

Table 2. *Distribution of ^{14}C in Entodinium caudatum during the incorporation of ^{14}C-glycine*

Protozoa were incubated at a concentration of $3{\cdot}5 \times 10^5$ protozoa/ml. in 8 ml. salt medium containing 0·75 % soluble starch, 0·025 % L-cysteine, 0·12 % $NaHCO_3$, 0·05 % neomycin sulphate, 1250 units penicillin G/ml. and 28 μg. ^{14}C-glycine (440 counts/min./μg.)/ml. under 95 % N_2 + 5 % CO_2. One 8 ml. tube was harvested for each time point. All results are expressed in counts/min.

Time (hr)	Broken-cell supernatant fluid			Broken-cell pellet		
	Cold TCA soluble	Hot TCA soluble	Protein (counts/min.)	Cold TCA soluble	Hot TCA soluble	Protein
1	1710	64	140	95	123	180
5½	2170	168	580	230	232	380
23	1120	160	1440	110	76	700

In antibiotic-treated cultures all the protein ^{14}C was present as glycine. This was determined by chromatography in solvents A, B, C, D and E and by the fingerprint method of Roberts *et al.* (1955). Solvent F separated glycine and serine better than the other solvents and no trace of ^{14}C was found at the R_F of the serine marker. The cold TCA-soluble material from the supernatant fraction prepared from protozoa incubated in the presence or absence of antibiotics contained only one ^{14}C-component at low external glycine concentrations and this had different chromatographic properties from glycine (see below).

Extracellular products of glycine metabolism. Figure 1 shows that although glycine uptake apparently proceeded for only 24 hr when glycine was added initially, glycine first added after 24 hr was still incorporated. This suggested that the amount of ^{14}C found in the protozoa at any time may have represented a balance between the uptake of glycine and the liberation of glycine or a glycine metabolite by the organisms. To test this theory the following experiments were carried out.

Protozoa were incubated under otherwise identical standard conditions in tubes containing: (*a*) 3 μmole ^{12}C-glycine + 0·2 μC. ^{14}C-glycine; (*b*) 3 μmole ^{12}C-glycine; (*c*) no glycine. When the rate of ^{14}C incorporation in tube (*a*) began to decrease, i.e. after 10 hr, 0·2 μC. ^{14}C-glycine was added to tube (*b*) and 3 μmole ^{12}C-glycine and 0·2 μC. ^{14}C-glycine to tube (*c*). The rate and total uptake of ^{14}C was the same in tubes (*b*) and (*c*). Similar results were obtained when the additions were made after 24 hr. This shows that even when there was an equilibrium amount of ^{12}C-glycine in the protozoa, uptake of glycine, presumably balanced by an equivalent loss of 'glycine', still occurred.

In an attempt to demonstrate this loss of glycine an excess of ^{12}C-glycine was

added to protozoa that contained an amount of ^{14}C-glycine in equilibrium with ^{14}C-glycine in the medium. Under these conditions decrease in the specific activity of the medium glycine to about 10 % of the initial value decreased the ^{14}C in the protozoa by 50 % after 7 hr. In the absence of any added ^{12}C-glycine there was no loss of ^{14}C. This shows that when the rate of uptake of ^{14}C was decreased by lowering the specific activity of the medium glycine, then the protozoa lost ^{14}C presumably due to the liberation of high specific activity 'glycine'. If the plateau in the glycine incorporation were due to the inability of the protozoa to take up further glycine, then this loss would not have occurred. The addition of equivalent amounts of L-alanine, L-aspartic acid or sarcosine produced no fall in the protozoal ^{14}C.

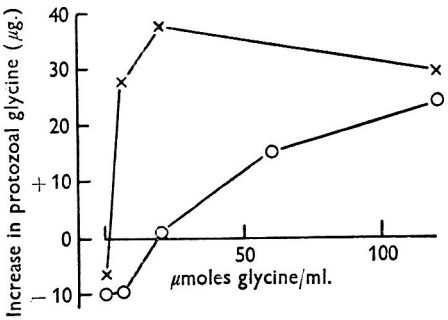

Fig. 3

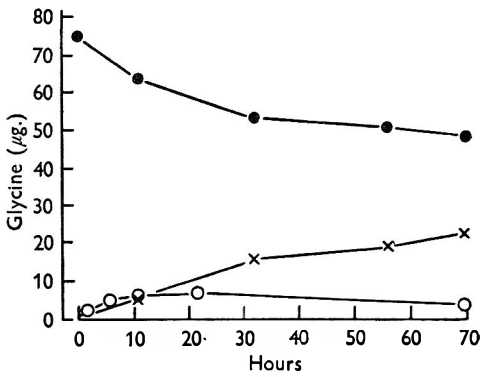

Fig. 4

Fig. 3. Effect of glycine concentration on the amount of free (○——○) and combined (×——×) glycine present in *Entodinium caudatum* after removal of protein. The results are expressed per $9{\cdot}26 \times 10^5$ protozoa which were incubated in 5 ml. medium for 6 hr. The initial glycine amounts were: free 10·2 μg.; combined 15·5 μg.

Fig. 4. Metabolism of glycine by *Entodinium caudatum*. ○——○, glycine incorporated into protozoa; ●——●, free glycine in the supernatant fluid; ×——×, glycine-metabolite in supernatant fluid. Results calculated from uptake of ^{14}C from ^{14}C-glycine (440 counts/min./μg.) by $1{\cdot}34 \times 10^6$ protozoa suspended in 10 ml. medium.

It was not possible to demonstrate a glycine-induced loss of ^{14}C from washed equilibrium-state protozoa because after washing once in salts 0·02 % L-cysteine medium and resuspension in the standard medium, the protozoa lost 50 % of their ^{14}C in 7 hr in the absence of external glycine. The rate of ^{14}C release under these conditions was the same at 38°, 33°, 27° and 20°. That this was not the result of damage to the protozoa was shown by the finding that washed equilibrium-state protozoa inoculated into medium containing glycine of the same specific gravity as that present in the first incubation medium lost no ^{14}C.

Chromatographic separation of the materials present in the incubation medium from equilibrium-state protozoa revealed two ^{14}C spots only. One was glycine and the other ran with the same R_F as (*a*) ^{14}C-material found in the cold TCA-soluble fraction from the broken-cell supernatant fluid, and (*b*) the material liberated from equilibrium-state protozoa by ^{12}C-glycine or washing. The amount of this material increased throughout the incubation and could account for 30 % of the ^{14}C-glycine added initially (Fig. 4). This unknown material was also produced when the sodium

propionate in the medium was replaced by an equimolar amount of sodium acetate or sodium chloride.

The composition of the glycine-metabolite. Preliminary experiments carried out with incubation media taken after 3 days showed that this material was not absorbed by Zeo Karb 225 (H^+) which removed free glycine, but was absorbed by Dowex 2 (OH^-) from which it was eluted by N-HCl but not by N-acetic acid; this shows that the metabolite was a comparatively strong acid. By using a method based on these properties considerable purification was achieved. The ^{14}C-material in the final eluate was chromatographed in solvent A where it ran as a discrete spot with R_F value 0·9. This spot, which was detected by radioautography or by spraying with bromocresol green, was eluted, found to be a single spot when chromatographed in solvents A, B, C, D, E, J, and analysed.

After complete acid hydrolysis, the material was chromatographed in two dimensions in solvents A and B. The only ^{14}C- and ninhydrin-positive spot was glycine. When 0·5 % collidine was incorporated in the ninhydrin spray the spot had the characteristic glycine colour. The absence of serine was shown by chromatography in solvent F. However, the same quantity of the unhydrolysed material, after chromatography in solvent A, was ninhydrin negative, indicating the probable absence of a free amino group, contained no phosphate as shown by spraying with molybdate + perchloric acid (Bandurski & Axelrod, 1951) and no carbohydrate as shown by spraying with aniline + oxalate (Horrocks & Manning, 1949).

These properties suggested that the glycine metabolite might be a compound such as an *N*-acylglycine, possibly *N*-acetylglycine. The unknown material was chromatographed in solvents A, B, C, D, F, H and J and shown to run with the same R_F as marker *N*-acetyl glycine, which was detected by spraying with bromocresol green, in all solvents. The unknown had different chromatographic properties from *N*-formylglycine in solvents A and J. To obtain satisfactory results it was essential to adjust the unknown material and the *N*-acetylglycine to the same pH value before developing the chromatograms. The glycine metabolite prepared from tracer quantities of ^{14}C-glycine only also corresponded with *N*-acetylglycine by the fingerprint technique of Roberts *et al.* (1955) after chromatography in solvents A and B.

The presence of an acetyl group in the glycine metabolite was shown in the following way. The protozoa were incubated for 3 days under standard conditions in the presence of 0·02 M-^{12}C-glycine and 1·44 μg (0·4 μC.) 1-^{14}C-sodium acetate/ml. The metabolite in the medium was purified and finally chromatographed in solvent J (opposite *N*-acetylglycine). The ^{14}C spot was eluted, shown to have the same chromatographic properties as *N*-acetylglycine in solvents A, B, H and J, hydrolysed in N-NaOH for 2 hr and finally chromatographed, without removal of the alkali, in solvent H which separates the lower fatty acids. The ^{14}C material which was acid volatile chromatographed with the same R_F as marker sodium acetate.

Incorporation of ^{14}C from ^{14}C-bicarbonate

^{14}C (added to the medium as $NaH^{14}CO_3$) was incorporated linearly by *Entodinium caudatum* for 10 hr (Fig. 5) before the uptake reached a plateau. The addition of further $NaH^{14}CO_3$ at 10 or 24 hr in order approximately to double the original specific activity (of the CO_2) produced a further incorporation of ^{14}C. Diminution in the specific activity of the CO_2 to approximately 20 % of that of the original $^{14}CO_2$

by the addition of $NaH^{12}CO_3$ at 10 hr produced a small decrease in the ^{14}C in the protozoa, but the effect was smaller than in the case of glycine. As there was a uniform increase in the amount of non-volatile ^{14}C-material in the medium over 24 hr these results suggested that ^{14}C was incorporated by the protozoa and then liberated again into the medium.

The ^{14}C uptake was dependent on the presence of intact protozoa and breakage of the protozoa reduced it by over 90 %. The results obtained in the presence and absence of antibiotics with protozoa harvested at the end of the incubation by high- or low-speed centrifugation were similar to those obtained with glycine.

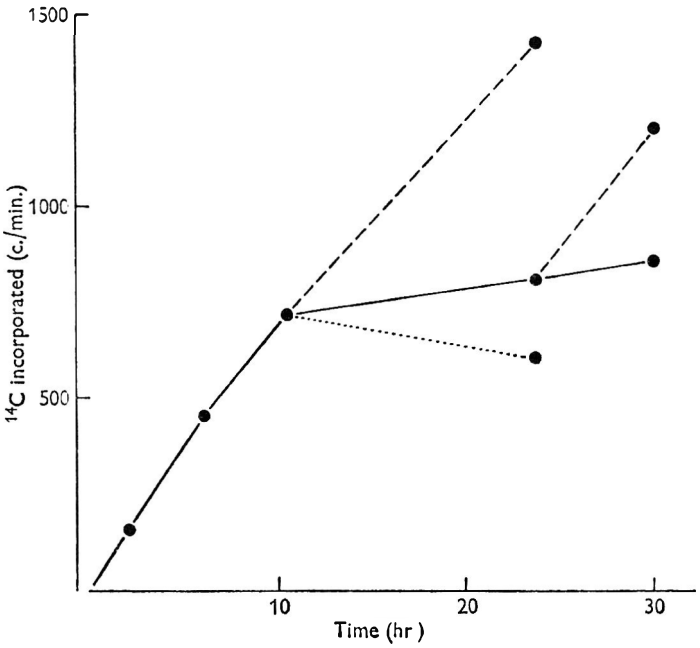

Fig. 5. Incorporation of ^{14}C into *Entodinium caudatum* incubated ●——● in presence of $NaH^{14}CO_3$ (100 μg. and 4000 counts/min./ml.). ● – – – ●, Additional $NaH^{14}CO_3$ (0·2 μg. and 4000 counts/min./ml.) added; ●·····●, 500 μg./ml. $NaH^{12}CO_3$ added.

Table 3 shows that over 60 % of the ^{14}C incorporated from $NaH^{14}CO_3$ was found in the hot TCA-soluble fraction of the broken-cell pellet. After removal of the TCA with ether the material was hydrolysed in N-HCl for 1 hr at 100° and chromatographed in solvent G. The ^{14}C material, which ran as a single spot, as detected by radioautography, did not correspond with any nucleic acid components as detected by ultraviolet photography. The spot was eluted and identified in the following way. When ^{12}C-glucose was added to the ^{14}C material and an osazone prepared the ^{14}C was found in the precipitated osazone, indicating that the ^{14}C material was glucose or fructose. This material also gave the characteristic hexose reaction when treated with aniline oxalate reagent (Horrocks & Manning, 1949). Chromatography against markers in solvents A, B, C, E, G and H indicated that the unknown material was glucose which was probably present in the intact protozoon as starch. As confirmation of its identity the unknown material was chromatographed in solvent E before and after treatment with glucose oxidase. As a result of enzyme action the original

spot disappeared and was replaced by another which ran with the same R_F as that produced from pure ^{14}C-glucose under the same conditions.

To determine which of the glucose carbon atoms were labelled with ^{14}C the glucose was fermented under nitrogen by baker's yeast (Barnet & Wick, 1950) in a Conway unit and the $^{14}CO_2$ collected on an aluminium disc containing $Ba(OH)_2$ (Coleman, 1956). At the end of the reaction the baryta was dried *in vacuo* and the ^{14}C estimated. Under these conditions 33 % of the ^{14}C in uniformly labelled glucose and 95 % of the ^{14}C in glucose isolated from protozoa incubated with $^{14}CO_2$ for $\frac{1}{2}$, 4 or

Table 3. *Distribution of ^{14}C in Entodinium caudatum after incorporation of ^{14}C-bicarbonate*

Protozoa were incubated at a concentration of $2{\cdot}0 \times 10^5$ protozoa/ml. in salt medium containing 0·6 % soluble starch, 0·025 % L-cysteine, 0·05 % neomycin sulphate, 1250 units penicillin G/ml. and 500 μg. $NaH^{14}CO_3$ (270,000 counts/min.) under N_2 for 6 hr.

	Cold TCA soluble (counts/min.)	Hot TCA soluble (counts/min.)	Protein (counts/min.)
Broken-cell supernatant fluid	890	105	270
Broken-cell pellet	430	4005	510
Non-volatile ^{14}C in incubation medium	5300		

11 hr was liberated as CO_2. As CO_2 is produced only from glucose carbon atoms 3 and 4 by this method, the result shows that *Entodinium caudatum* was similar to higher animals (Abraham & Hassid, 1957) in that ^{14}C from $^{14}CO_2$ was incorporated only into these carbon atoms of glucose. The protein from the supernatant fluid and pellet contained ^{14}C-aspartic acid, ^{14}C-glutamic acid, ^{14}C-alanine and ^{14}C-leucine, isoleucine or phenylalanine which were not separated by the chromatographic solvents used. Insignificant amounts of ^{14}C were found in lipid extracted by the method of Roberts *et al.* (1955).

Incorporation of ^{14}C from uniformly labelled ^{14}C-starch, ^{14}C-maltose and ^{14}C-glucose. Soluble ^{14}C-starch was rapidly taken up by *Entodinium caudatum* and the maximum incorporation was reached inside 15 min. Thereafter the ^{14}C was slowly lost from the protozoa until only 10 % remained after 24 hr. At this time over 85 % of this residual ^{14}C was in the hot TCA-soluble fraction and none was present in the protein amino acids. Any starch present in the protozoa would be extracted by hot TCA and appear in that fraction. The total ^{14}C in the culture was constant over 24 hr, at which time the principal ^{14}C materials in the medium were found to be maltose and glucose. It seems probable therefore that the soluble starch was absorbed by the protozoa and then slowly hydrolysed to maltose and glucose without appreciable conversion into cellular material. Incorporation of ^{14}C from [u-^{14}C]glucose and [u-^{14}C]maltose was small; less than 0·2 % of the carbon was incorporated at sugar concentrations of 1 μg.–10 μg./ml. Glucose disappearance at these concentrations was also measured by the method of Huggett & Nixon (1957) but none was detected.

DISCUSSION

Although Williams *et al.* (1961) and Gutierrez & Davis (1962) showed that *Ophryoscolex caudatus* and *Epidinium ecaudatam* washed free from most of their extracellular bacteria but probably still containing bacteria in their gastric sacs, incorporated three amino acids, these authors gave no evidence that the uptake was directly into protozoal material. Although materials in the gastric sac can obviously

be digested by a protozoon, they must be regarded as extracellular. The present studies show that at low external glycine concentrations at least, much more glycine (free and combined) was found inside the protozoa than could be accounted for by free diffusion of external medium into the gastric sac. Since after incubation for 1 hr over 70 % of the glycine had been metabolized to *N*-acetylglycine and no free glycine could be detected in the broken-cell supernatant fluid, this suggests that, if the glycine entered the organism via the gastric sac, it was rapidly metabolized from it. Although this evidence and the finding that breakage of the protozoa almost completely abolished incorporation indicate that the reactions probably represent protozoal metabolism, it is impossible to be certain that the incorporation was not mediated by the bacteria in the gastric sac. It has been assumed in these studies that the material in the broken-cell supernatant fluid was of protozoal origin, but it is uncertain to what extent protozoal protein and nucleic acid were carried down with bacteria, starch and other cell debris in the pellet fraction.

The liberation of *N*-acetylglycine into the medium is not considered to be the result of lysis of the protozoa because: (*a*) as much *N*-acetylglycine was released every 7–10 hr as was found in the protozoa at any one time (Fig. 4); (*b*) the number of protozoa remained constant over the period of the experiment, although this may have been the result of the death of some protozoa balanced by the division of an equal number of other protozoa; (*c*) after washing equilibrium-state protozoa there was only a decrease in protozoal ^{14}C when the protozoa were inoculated into fresh medium containing no glycine or glycine of a lower specific activity to that used in the first incubation. The observation that *N*-acetylglycine was liberated from protozoa after washing and that the rate of release was independent of temperature suggests that the process was passive diffusion rather than active secretion. In view of the known action of penicillin on bacterial cell-wall synthesis (Park, 1958) it is of interest that *N*-acetylglycine was also produced in the absence of penicillin. Although glycine is often converted into serine in mammals (Bach, 1952), some bacteria (e.g. Roberts *et al.* 1955) and in Tetrahymena (Dewey & Kidder, 1955), there is no evidence for such a reaction in *Entodinium caudatum*.

Although the protozoa were capable of incorporating glycine into their cellular protein no evidence was obtained for the synthesis of protein amino acids from carbohydrate. The protozoa were therefore either unable to synthesize amino acids under any conditions or only when multiplying. As shown previously (Coleman, 1962) high concentrations of some carbohydrates prolonged the life of starved, almost bacteria-free protozoa. The results quoted here with protozoa containing bacteria show that if this effect was concerned with the metabolism of the carbohydrates then the proportion of the carbohydrate that was metabolized by or incorporated into the protozoa was very small. In contrast there was rapid incorporation of ^{14}C from ^{14}C-bicarbonate into carbon atoms 3 and 4 of protozoal glucose, probably in the form of starch in the living organism, suggesting that the enzymes of the Embden–Meyerhof pathway are present in these protozoa.

It is not claimed that the conclusions reached here with non-multiplying protozoa in the presence of antibiotics can be used as good evidence for the source of the amino acids required for the growth of these protozoa. However, the results do show that certain biosynthetic reactions can probably be carried out by these entodinia without assistance from their associated bacteria.

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Further Studies of Ultraviolet-sensitive Mutants of *Escherichia coli* Strain B

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SUMMARY

A group of twelve ultraviolet-sensitive mutants has been isolated after u.v. irradiation of *Escherichia coli* strain B. The use of crystal violet during irradiation and subsequent growth appears to increase the frequency of such mutants among colonies formed by surviving cells. This effect may be due to post-irradiation selection. Eleven of the mutants were more crystal violet resistant than their parent.

The mutants were compared by determining their u.v. survival curves, the extent of elongation after u.v. irradiation, their ability to repair u.v. induced damage to bacteriophage T1, and their resistance to furacin. They comprised nine different phenotypes. In all but one case, the mutants differed from the parent in more than one property.

INTRODUCTION

The genetic basis of ultraviolet (u.v.) sensitivity in the bacterium *Escherichia coli* has been established in a number of recent investigations (Adler & Copeland, 1962; Rörsch, Edelman, van der Kamp & Cohen, 1962; Howard-Flanders, Boyce, Simson & Theriot, 1962; Rörsch, Edelman & Cohen, 1963). These studies have usually consisted in the genetic transfer of properties previously found in some u.v. sensitive or u.v. resistant mutants and the subsequent demonstration that the recipient organism has acquired the expected change in u.v. sensitivity. The properties employed have been the ability to repair u.v. induced damage in bacteriophage and the formation of snake-like filaments after u.v. irradiation. The former property is thought to be dependent upon the organism's ability to repair u.v. induced damage to DNA; the latter is considered to be indicative of u.v. induced damage to cell division.

It is apparent from these studies that u.v. radiation produces more than one effect. Hence it might be expected that mutants whose increased u.v. sensitivity is due to increased susceptibility to one effect should be like the parent strain with respect to susceptibility to other effects. The present report concerns an investigation of this question in the case of u.v. sensitive mutants derived from *E. coli* strain B.

METHODS

The bacterial strains employed were *E. coli* n, a u.v. resistant (B/r) mutant, and twelve u.v. sensitive (B_S) mutants. Two of the u.v. sensitive mutants, $B_{S\text{-}1}$ and $B_{S\text{-}2}$, had been isolated previously and some of their properties described (Hill & Simson,

1961). The ten additional mutants were isolated as follows. Cultures of B were grown to the logarithmic stage by aeration in nutrient broth. Samples containing 10^6–10^7 organisms were spread on nutrient broth agar. In some isolation experiments the nutrient broth agar was supplemented with crystal violet. The significance of this will be discussed later.

After spreading, the organisms were u.v. irradiated with doses allowing 10^{-4}–10^{-5} survival and incubated at 37°. Colonies formed by surviving cells were subcultured in nutrient broth and loopfuls were spread over small areas on nutrient broth agar. These areas were u.v. irradiated with a dose (160 ergs/mm.^{2}) allowing 1% survival of B cells. After 3 hr incubation, the spread areas were examined microscopically. Areas containing very short cells or mixtures of short cells and slightly elongated cells were tentatively diagnosed as containing B_s mutants, in comparison with areas showing extremely long filaments ('snakes'), which is typical of B at the given dose, or with areas showing a moderate amount of cellular elongation and microcolony formation, typical of B/r at the same dose (Payne, Hartman, Mudd & Phillips, 1956; Hill & Simson, 1961).

The tentative diagnosis of B_s mutants was confirmed or refuted by macroscopic re-examination of the spread areas, after re-incubation overnight. Areas containing B or B/r showed dense confluent growth while areas containing u.v. sensitive mutants showed considerably less growth. The most sensitive showed either no growth or only a few isolated colonies. Occasionally, areas which had been tentatively diagnosed as containing B_s mutants showed a faint film of growth. Subsequent determination of the survival curves showed that these were slow-growing B/r types. In two cases, the tentative diagnosis of B on the basis of the formation of snakes was refuted by the scanty growth overnight and the survival curves for these showed that they were B_s mutants.

The procedure used to determine the u.v. survival curves for the bacteria was the same as the preceding except that measured samples were spread on nutrient broth agar and irradiated with a range of doses. Bacteriophage T1 was irradiated while suspended in 2% ammonium acetate. Its survival, using the different bacterial strains as hosts, was determined by the agar layer method (Adams, 1950). The apparatus used for irradiation has been described previously (Hill & Rossi, 1952). The present dose rate of the u.v. lamp at 56 cm. is 8·1 ergs/mm.^{2}/sec. as measured by a calibrated Jagger dosimeter (Jagger, 1961).

The extent of elongation of *Escherichia coli* cells after u.v. irradiation is dependent upon the strain and the dose (Payne *et al.* 1956). In order to compare more precisely the ability of the different B_s mutants to elongate, the procedure described above for the tentative diagnosis of type was modified. After the survival curves were obtained, each mutant was irradiated with a dose giving about 1% survival. The extent of elongation by 3 hr after irradiation was then classified as 1+ to 4+ by comparison with the length (4+) of the snakes formed by B at 1% survival.

Sensitivity to crystal violet was determined by spreading 0·1 ml. samples of appropriate saline dilutions of broth-grown cultures on nutrient broth agar containing varying concentrations of the dye, followed by incubating at 37°. Exposure to crystal violet during growth slows the development of colonies. By counting the numbers of colonies appearing by a maximum of 4 days of incubation, survival

curves for colony formation as a function of dye concentration were obtained. The times required for the appearance of colonies were also noted.

Sensitivity to furacin was determined by the gradient plate technique (Szybalski, 1952).

RESULTS

Effect of crystal violet on the isolation of B_s mutants

The numbers of B_s, B/r and parental B colonies found in different isolation experiments are shown in Table 1. The presence of B/r colonies could be due to selection of pre-existing mutants as well as to the induction of new mutations of this type. Selection undoubtedly occurs since B/r mutants are more resistant both to u.v. radiation and to crystal violet than B (Bryson *et al.* 1951–52). Unfortunately, the evidence available on the u.v. induction of B/r mutations in B organisms is scanty and equivocal (Witkin, 1947). In two reported experiments, the frequencies of B/r mutants before and after u.v. irradiation of B were determined. In one case,

Table 1. *Frequency of radiological types among survivors of u.v. irradiation of Escherichia coli* B

Expt.	u.v. dose (sec.)	Concentration of crystal violet (μg./ml.)	No. of colonies examined	No. of colonies of B/r	No. of colonies of B_s	No. of colonies of B
1	80	1	11	1	1	9
2*a*	80	0	100	7	1	92
b	0	0	100	0	0	100
3*a*	60	2	27	23	3	1
b	40	2	54	47	2	5
c	0	2	217	107	0	110
d	80	1	60	13	2	45
e	60	1	21	5	1	15
4	60	0	132	3	2	127

the post-irradiation frequency was about ten times the frequency expected on the basis of u.v. selection, as calculated from the survival curves. However, in the second, the post-irradiation frequency was very nearly equal to the expected frequency. In the absence of definite information to the contrary, it was assumed that B/r mutations may not be u.v. inducible in B and therefore that the presence of B/r mutants among surviving colonies was due only to selection. These colonies were regarded as unavoidable 'contaminants'.

The significance of this is that in calculating the frequency of B_s colonies, B/r colonies should not be included in the total scored if they are merely contaminants. However, the possibility that some of the B_s colonies might have resulted from u.v. induced mutation in the B/r 'contaminants' also has to be considered. The unlikelihood of this can be demonstrated. For example, in experiments 3*a* and 3*b* (Table 1), 27 and 54 total colonies respectively were scored. In each case, these numbers were the totals appearing after the irradiation of 10^7 organisms. The spontaneous frequency of B/r in populations of B is about 10^{-5} (Witkin, 1947). Thus the

total number of irradiated organisms must have included about 100 B/r mutants. Since only three and two B_s colonies were found in experiments 3*a* and 3*b* respectively, the u.v. induced mutation rate for the change from B/r to B_s would have to be extremely high. However, in separate experiments where B/r populations were irradiated to 1 % survival in the presence of 1 μg. crystal violet per ml. of nutrient broth agar, no B_s mutants were found among 412 colonies formed by surviving cells. A similar result has been reported for the u.v. irradiation of *E. coli* strain K 12, a strain resembling B/r in u.v. resistance. No u.v. sensitive mutants were found among 500 colonies (Howard-Flanders & Theriot, 1962). Thus although u.v. sensitive mutants may be u.v. induced in B/r, the rate of induction cannot be high enough to account for the B_s mutants found after the u.v. irradiation of B populations. The B_s mutants must therefore have resulted from mutation in B only.

In view of the above considerations, the frequency of B_s mutants was calculated as the ratio of B_s colonies to the total of B and B_s colonies. As shown in Table 2, the frequency is increased markedly when increasing concentrations of crystal violet are present during irradiation and subsequent growth.

Table 2. *Effect of crystal violet concentration on frequency of B_s mutants*

Concentration of crystal violet (μg./ml.)	B_s/B_s+B* (%)	Mean
0	1·0	
0	1·6	1·3
1	10·0	
1	4·3	
1	6·7	7·0
2	75	
2	30	53

* The reason for excluding B/r mutants from the denominator of this fraction is explained in the text.

Properties of B_s mutants

The u.v. survival data for small and large doses to the B_s mutants are shown in Fig. 1. Although there was a wide distribution in the positions of the curves and variation in shape, all of the mutants were more u.v. sensitive than the parent B strain (Fig. 1*b*).

The curves for the survival of colony formation when the strains were grown on agar containing varying concentrations of crystal violet were of three types. The strains were therefore compared by assigning each to a type, as illustrated in Fig. 2. Type A includes strains whose survival shows a sharp decrease at crystal violet concentrations of 1–2 μg./ml. Type B strains show a sharp decrease at 2–4 μg./ml. The survival curves for strains of type C show a gradual decrease for concentrations up to at least 8 μg./ml. Type C is heterogeneous; some strains showed an accelerated decrease in survival at 8–16 μg./ml. while others continued to decrease at the same rate even at concentrations as high as 32 μg./ml. In addition, colonies of the latter appeared within 24 hr even at 32 μg./ml. Thus, type C strains could be distinguished as C_1, C_2 and C_3, in order of increasing resistance.

Of the twelve mutant strains, nine were classified as type C, two as type B and only one as type A, the most crystal violet-sensitive group. The only other type A strain was the parent B (Table 3). Thus eleven of the mutants were more crystal violet-resistant than their parent. The only strain (B_{s-11}) which was like the parent,

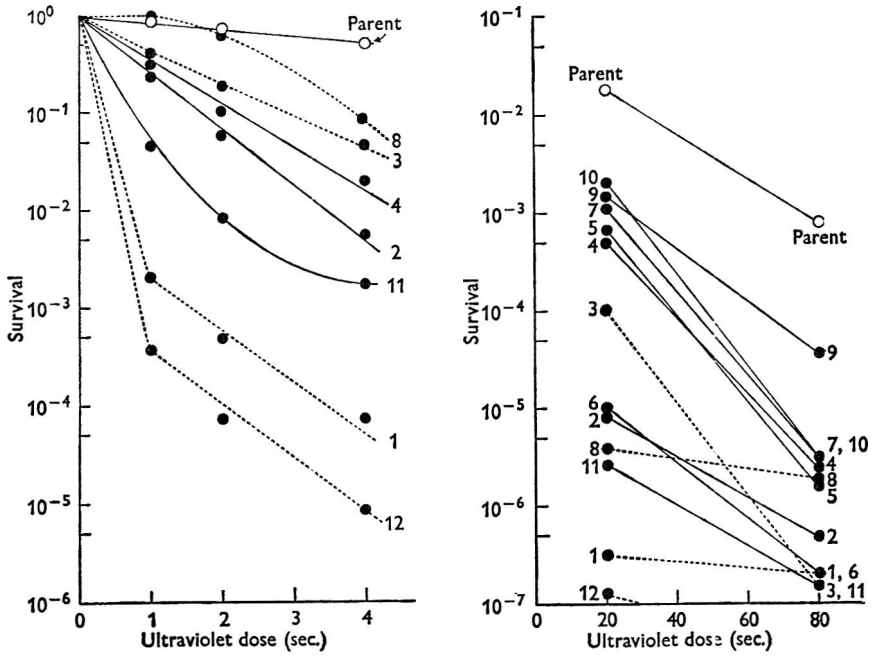

Fig. 1 (*a*) Fig. 1 (*b*)

Fig. 1. Survival of u.v. sensitive mutants of *Escherichia coli* B after small and large doses of u.v. radiation. Dotted curves are for those mutants which are unable to repair u.v. induced damage in bacteriophage T1 (*hcr*⁻).

Table 3. *Properties of* B_s *mutants*

Strain	Isolated in expt.*	Elongation after u.v. irradiation	Sensitivity to crystal violet (type)	Inhibitory concentration of furacin (μg./ml.)	Ability to repair T1 (*hcr*⁺ *or hcr*⁻)
B	—	4+	A	0·25	+
B/r	1	2+	C3	3·3	+
B_{s-1}	1	1+	C2	0·23	−
B_{s-2}	2	1+	C1	0·53	+
B_{s-3}	3*a*	4+	B	0·25	−
B_{s-4}	3*a*	2+	C2	0·75	+
B_{s-5}	3*a*	2+	C2	0·67	+
B_{s-6}	3*b*	2+	C2	0·53	+
B_{s-7}	3*b*	2+	C2	0·59	+
B_{s-8}	3*d*	2+	C3	0·87	−
B_{s-9}	3*d*	2+	C3	0·59	+
B_{s-10}	3*e*	2+	C2	0·73	+
B_{s-11}	4	1+	A	0·20	+
B_{s-12}	4	4+	B	0·20	−

* See Table 1.

had been isolated without the use of crystal violet. However, this is probably without significance since, although B_{S-2} and B_{S-12} were also isolated in this way, they were classified as types C and B, respectively.

Two different survival curves were obtained when the mutants were used as hosts for u.v. irradiated bacteriophage T1. The change in slope shown in the upper curve of Fig. 3 has been interpreted as indicating that the host organism can repair

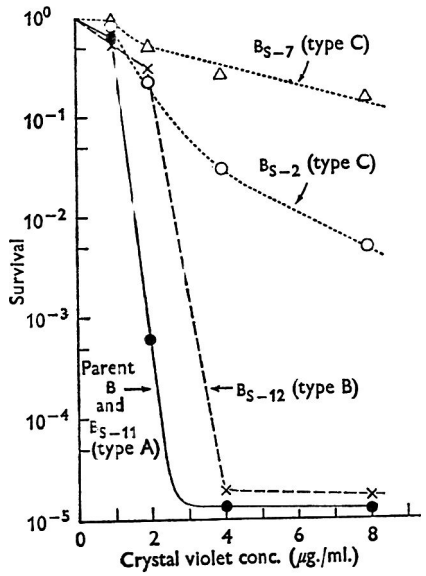

Fig. 2

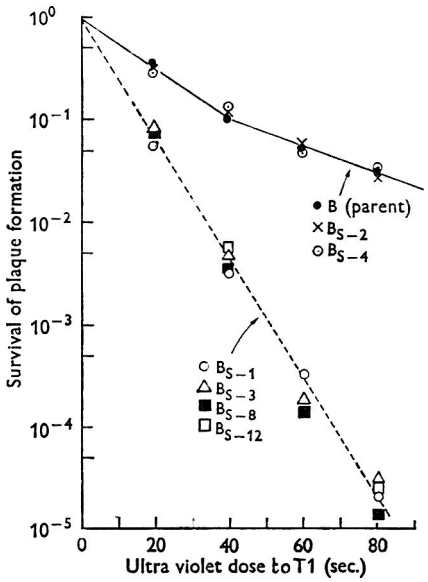

Fig. 3

Fig. 2. Types of survival curves shown by B_s mutants when grown in the presence of crystal violet.

Fig. 3. Survival of u.v. irradiated bacteriophage T1 when measured by using different B_s mutants as host organisms.

a fraction of u.v. induced damage in T1 (Garen & Zinder, 1955; Harm, 1963). Strains giving this curve have been designated as *hcr*$^+$ (host cell reparability is positive). The lower curve of Fig. 3 is characteristic of *hcr*$^-$ strains. Eight of the mutants gave the same *hcr*$^+$ curve and four gave the same *hcr*$^-$ curve.

In regard to sensitivity to furacin, four of the mutants were as sensitive as the parent and eight were more resistant (Table 3). None of the latter were as resistant as the B/r strain used for comparison. Similarly, none of the mutants of type C with respect to crystal violet resistance were as resistant as this B/r. Therefore other B/r strains in our collection were examined. One B/r, a tryptophan-deficient auxotroph used in other studies, showed a crystal violet survival curve identical to that shown by B_{S-2}. Two other B/r mutants were inhibited by about 0·75 μg. furacin/ml., as were two of the B_S mutants.

Comparison of the twelve B_S mutants (Table 3, Figs. 1 and 2) showed possibly nine different phenotypes. Four of these were represented by the four *hcr*$^-$ strains. Although B_{S-3} and B_{S-12} were identical in snake formation and antibiotic resistance,

their u.v. survival curves were distinctly different. B_{s-1} and B_{s-8} differed from B_{s-3} and B_{s-12} in snake formation and crystal violet resistance and differed from each other in crystal violet resistance, in furacin resistance and in their u.v. survival curves. The eight *hcr*$^+$ strains gave an additional five phenotypes. B_{s-11} differed from all the other *hcr*$^+$ strains in that it had the same sensitivity to the antibiotics as the B parent. B_{s-9} was like the *hcr*$^-$ B_{s-8} in having the greatest resistance to crystal violet and in addition, had the greatest resistance to high doses of u.v. radiation of all the B_s strains. Of the other six *hcr*$^+$ strains, B_{s-2} could be differentiated by its crystal violet and u.v. curve and B_{s-6} by the fact that, although it was like B_{s-4}, B_{s-5}, B_{s-7} and B_{s-10} in degree of elongation and antibiotic resistance, its u.v. curve was very close to that for B_{s-2}. Only B_{s-4}, B_{s-5}, B_{s-7} and B_{s-10} may have been identical.

DISCUSSION

The finding that B_s mutants tend to be more crystal violet resistant than B suggests that the mechanism by which crystal violet increases their frequency among colonies formed by u.v. irradiated B organisms is probably selective. This would not be a selection of pre-existing mutants. Rather, those parental organisms which survive irradiation but mutate to the B_s form would have a selective advantage during subsequent growth in the presence of the dye over those survivors which do not mutate. Since the use of crystal violet alone does not select B_s mutants to a detectable extent (Table 1), it seems likely that they originated by u.v. induced mutation rather than by spontaneous mutation during post-irradiation growth on agar. In this respect, mutation to increased u.v. sensitivity may differ from mutation to increased u.v. resistance since, as mentioned previously, the latter may not be u.v. inducible.

The wide distribution which was observed for the positions of the u.v. survival curves is independent of whether the B_s mutants are *hcr*$^+$ or *hcr*$^-$. A similar spread has been reported for *hcr*$^-$ u.v. sensitive mutants of *Escherichia coli* K 12 (Howard-Flanders & Theriot, 1962). It has been suggested that the *hcr* property is determined by the ability of the organism to produce an enzyme for the repair of u.v. induced damage to DNA (Sauerbier, 1961). The fact that *hcr*$^-$ mutants are not equally u.v. sensitive may mean that the amount of this enzyme is more critical for the survival of the u.v. irradiated bacterium. In addition, it seems clear that u.v. sensitivity is determined by a number of factors rather than by only one (Howard-Flanders & Theriot, 1962). A second factor which has been proposed is injury to cell division, manifested by the formation of snake-like filaments after irradiation. According to cytological studies, 'snake' formation is due to continuous nuclear replication and cytoplasmic increase without cell division (Payne *et al.* 1956). A third factor is suggested by the present finding that the *hcr*$^-$ mutants B_{s-3} and B_{s-12} formed 'snakes' after u.v. irradiation like the parent B strain. Apparently these mutants can replicate their DNA in spite of inability to repair it. It has been suggested that DNA replication without repair results in mutation (Witkin, 1961). Therefore the increased u.v. sensitivity of B_{s-3} and B_{s-12} might be due to a combination of the *hcr*$^-$ property and the production of new mutations which subsequently reduce the probability of recovery from the u.v. induced damage to cell division even more than it is reduced in the case of the *hcr*$^+$ parent B.

Injury to cell division (manifested by snake-formation) is produced by exposure to other agents besides radiation. These include crystal violet and furacin. It has been suggested that snake formation and sensitivity to these antibiotics are correlated properties (Payne *et al.* 1956; Rörsch *et al.* 1962). However, the present results show that this is not always the case. Although $B_{s\text{-}3}$ and $B_{s\text{-}12}$ are snake formers after irradiation like their parent, they show an increased resistance to crystal violet but not to furacin. A possible explanation would involve a combination of (*a*) differences in the efficiency with which different agents injure the mechanism of cell division, and (*b*) quantitative differences in whatever metabolic property is responsible for eventual recovery from injury to cell division. The results with $B_{s\text{-}3}$ and $B_{s\text{-}12}$ suggest that the order of decreasing efficiency may be u.v. radiation, furacin, crystal violet. This is supported by the observation that unlike *Escherichia coli* strain B, in *E. coli* strain s, selection for increased furacin resistance is not always equivalent to selection for increased u.v. resistance (Szybalski & Nelson, 1954; Woody-Karrer & Greenberg, 1963). Furthermore when strain s mutates to increased u.v. resistance, there may or may not be a reduction in snake formation (Curry & Greenberg, 1962). Thus an increased ability to recover from injury to cell division may not be the only cause of increased resistance to u.v. radiation. It would be interesting to know whether strain s is *hcr*$^-$. If it is, then increased u.v. resistance could be due to mutation to *hcr*$^+$. If it is already *hcr*$^+$, the increased u.v. resistance of mutants which still form snakes after u.v. irradiation would suggest that there is a fourth mechanism of u.v. induced lethality in addition to those already mentioned. In any case, it is clear from the results with strain s and with the u.v. sensitive mutants of strain B that neither snake formation nor sensitivity to crystal violet and furacin are infallible indicators of u.v. sensitivity.

The diversity of B_s phenotypes reported here is not surprising since mutants with increased radio-resistance are also heterogeneous (Witkin, 1947; Woody-Karrer & Greenberg, 1963; Greenberg & Woody-Karrer, 1963). An interesting feature of the present results is that with the exception of $B_{s\text{-}11}$, all of the u.v. sensitive mutants differed from their B parent in more than one property. If failure to form snakes, crystal violet resistance and furacin resistance are considered as manifestations of a single property affecting cell division even though the magnitude of the change may vary so that all three aspects may not appear simultaneously, at least two properties changed in eleven of the twelve mutants. One change, either increased resistance to crystal violet alone or to both crystal violet and furacin is presumably related to a *decreased* u.v. sensitivity while the other, loss of ability to propagate u.v. irradiated T1 or some unknown change, presumably *increases* u.v. sensitivity.

The occurrence of multiple phenotypic changes as a result either of spontaneous mutation or of exposure to u.v. radiation is a fairly common phenomenon. In some cases, such as mutants of bacteriophage showing both a sensitivity to the presence of a suppressor gene in the host organism and an altered plaque morphology, there has been mutation at different loci (Campbell, 1961). The involvement of different genes is frequently the result of a deletion as for example in bacterial mutants exhibiting both resistance to T1 and a requirement for tryptophan (Yanofsky & Lennox, 1959). On the other hand, mutation at a single site can also cause multiple phenotypic changes. In some cases, the underlying common factor is known. Examples of this include bacterial mutation causing joint resistance to

bacteriophages T3, T7 and the unrelated T4 (the result of impaired production of the lipocarbohydrate portion of a bacterial surface site for adsorption) and bacteriophage mutation causing increased sensitivity to a suppressor gene in the host, to temperature and to pH extremes (the result of impaired production of an enzyme (Goebel & Jesaitis, 1952; Campbell, 1961)). It is also known that although allelic mutants show the same primary property governed by a gene, they may differ in other respects (Demerec, 1956). Since all of the *hcr*$^+$ mutants except $B_{s\text{-}11}$ show inhibitory concentrations of furacin within a narrow range and 'type C' crystal violet survival curves, the differences between them may be allelic. $B_{s\text{-}3}$ and $B_{s\text{-}12}$ may also be allelic mutants since they are both *hcr*$^-$, both snake-formers and show the same response to crystal violet and furacin. In any case, decision as to which of the various possibilities of genetic change is responsible for each of the B_s phenotypes or groups of phenotypes must await the results of genetic analysis.

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Numerical Classification of Salmonella Serotypes

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SUMMARY

Numerical methods were applied to an analysis of the relationships among Salmonella serotypes listed in the Kauffmann–White schema. Although the result suggested a possible new basis for schematic arrangement of these serotypes, a complete and satisfactory classification could not be derived entirely from the computer results. Examination of this outcome suggests some cautions to be observed in the design and interpretation of experiments in numerical taxonomy.

INTRODUCTION

In most numerical studies of bacterial classification, the 'operational taxonomic units' (or OTU's) have been actual cultures. Although numerical analysis might equally well be applied to descriptions of taxa or to pooled data taken from the literature (Sneath, 1962), this approach has been used infrequently (e.g. Lysenko & Sneath, 1959). This paper reports an attempt to develop a numerical classification of the genus *Salmonella*, using Kauffmann's (1961) descriptions of Salmonella serotypes as the OTU's.

METHODS

Descriptions of each of the 700 Salmonella serotypes listed in the Kauffmann–White schema (Kauffmann, 1961) were recorded in form suitable for computer analysis, using a method previously described (Lockhart & Hartman, 1963: Lockhart, 1963). Entries for which Kauffmann lists two biochemical types (e.g. *Salmonella arechavaleta* 4, 5, 12 and *S. arechavaleta* 4, 12) were treated as two separate serotypes. For each listed property of a given serotype, a code symbol (A, B, C or D) was assigned for each alternative state in which the property might occur. A total of 155 properties was used, scored as follows: *Somatic antigens* 1 *through* 56 (56 *features*); A = present, B = absent, D = incomplete or variant (listed in parentheses by Kauffmann, 1961). The Vi antigen was not scored. *Flagellar antigens—phase variation* (1 *feature*); A = antigens listed in only one phase, B = antigens listed for two phases, C = no H antigens listed. *Individual flagellar antigens a through z* (26 *features*), z_1 *through* z_{47} (47 *features*) *and* 1 *through* 11 (11 *features*); A = antigen listed in first phase, B = antigen listed in second phase, C = antigen not listed in either phase, D = antigen sometimes present in either phase (listed in parentheses by Kauffmann, 1961). *Biochemical characteristics* (14 *features*); A = +, B = + + or + with a superscript, C = − or *x*, D = *d* (symbols as defined by Kauffmann, 1961). A number of the antigens listed here (e.g. somatic antigens 26, 29, 31, 32, 33, 36, 37 and 49) are not actually found in the 1961 schema. Such anti-

gens, forty-five in all, were scored as 'absent' for all OTU's, reducing the total of effective features to 110. This did not affect the outcome; the question of whether or not one should score a similarity when both of two OTU's *lack* a particular antigen (or when both give a negative response to a particular biochemical test) does not arise, since the computer program used was one which enumerates differences only.

During computations, two serotypes were considered 'different' with respect to a given property whenever different symbols were recorded for each in that property. For somatic antigens, then, a difference was recorded for any antigen possessed by one serotype but not by the other. Two serotypes score a difference with respect to any flagellar antigen unless it is possessed by both in the same phase. No attempt was made, in scoring the biochemical features, to discriminate among varying degrees of 'lateness' in positive reactions ($+^{1-3}$ *vs.* $+^{2-4}$, etc.), although these were distinguished from strong positives (+) and from negatives (−). Kauffmann's symbol (++) is not really equivalent to (+) with a superscript, but he never uses both in describing the same reaction. His symbol (*x*), meaning 'late, irregularly positive, often negative', seems nearly equivalent to an outright negative, and it is so scored. The symbol (*d*) apparently indicates that individual strains of the serotype in question may give almost any response from strong positive to outright negative, and is scored so that it records a difference from any serotype showing constancy with regard to the property in question.

Groupings were obtained according to the monothetic criterion of Lockhart & Hartman (1963), using the Cyclone computer (Iowa State University). In this method, the operator designates a starting OTU, and the machine then locates the second individual which (of all those under study) is most like the first. All the features in which these two are not identical are then eliminated from consideration, and the computer locates the third OTU which is most like the original pair with respect to the remaining features. This process is repeated many times, with the computer output designating the new OTU added at each step and the total number of features eliminated from consideration thus far (this quantity is designated the 'cumulative difference', d_c). All members of the groups obtained in this way are thus identical with respect to the common core of remaining properties, presumably producing a classification in harmony with the hierarchical character of most currently accepted taxonomic schemes. Since previous work (Lockhart & Hartman, 1963) had indicated that little useful information is obtained after a group under formation has coalesced with a taxonomically adjacent group, the computer program has an instruction which makes it possible to stop the calculation after the group has reached a predetermined size. All the present experiments were terminated after 100 serotypes had been grouped around the original pair. The program also has an optional instruction permitting certain properties to be eliminated from consideration during formation of a group, and this was done in some instances specified later.

RESULTS

A preliminary examination of the Kauffmann (1961) data shows that the Kauffmann–White schema is essentially a monothetic classification based on somatic antigens. Within the major groups thus designated, subdivisions are based on the flagellar antigens found in the first phase. Antigenic composition in the second

phase and a number of biochemical properties are listed for each type, but neither shows any obvious correlations with the somatic or first-phase antigens. Certain rather characteristic combinations of antigens may be found in the second phase, however. Frequently there is no second phase, but when one exists antigen 1 seems to occur most frequently—nearly always paired with antigens 2, 5, 6 or 7 (sometimes a combination like 1, 5, 7 may be seen). If antigen 1 is not present, the most likely alternatives are the combinations e, n, x or e, n, z_{15} (and, occasionally, such combinations as d, e, n, z_{15} or e, n, x, z_{15}). Less often, the combination l, w or the single antigen z_6 may be found. Quite rarely, a few other antigens are reported for the second phase. Although most of these antigens are sometimes found also in the first phase, the characteristic combinations (and their mutual exclusiveness) in second phase are rather striking.

Table 1. *Composition of a monothetic group formed around Salmonella cairo, considering all features*

Cumulative difference	No. of individuals (serotypes) added	New Kauffmann groups represented	New antigens represented		
			Somatic	Phase 1	Phase 2
0	1	B	1,4,12,27	d	1,2
4	2	.	.	.	5,7
8	2	.	.	b	.
11	1	.	.	y	.
13	1	.	.	a	.
14	2	.	.	r	.
16	1	.	.	.	6
17	3	.	.	e	.
19	1	.	.	i	.
20	7	.	5	.	.
24	3	.	.	z	.
25	5	.	.	z_{10}	.
29	8	.	.	.	z_6
33	1	.	.	.	absent
34	2	.	.	z_{29}	.
35	1	.	.	z_{38}	.
37	2	A	2	.	.
38	21	D_1	9	.	.
39	1	.	.	.	z_{39}
40	2	.	.	.	z_{35}
41	1	.	.	k	.
42	9	D_2	46	.	.
44	3	C_3	8	.	.
45	18	C_2	6	.	.
46	4	C_1	7	.	.

When the 14 biochemical features were subjected to a conventional card-sorting routine, it was found that many of the serotypes were alike in all or most of their biochemical characters, but there was no discernible pattern that could be correlated with antigenic structure. More than a third of the 700 serotypes, including all the species listed in *Bergey's Manual* (1957), were unique; their patterns of physiological characters resembled none of the other types.

The first attempt at formation of groups using the entire body of data was made with *Salmonella cairo* (1, 4, 12, 27; d; 1, 2) specified as the original OTU. *Salmonella*

cairo appears to be a 'typical' member of Kauffmann's Group B, and it had been found in our preliminary study to be biochemically similar to a number of other serotypes. The results of this computation are presented in Table 1, which shows the number of serotypes added at each level of cumulative difference (d_c) and the Kauffmann groups to which they belong. In an earlier trial of this computer program

Table 2. *Composition of a monothetic group formed around Salmonella cairo, with biochemical features omitted from consideration*

Cumulative difference	No. of individuals (serotypes) added*	New Kauffmann groups represented	New antigens represented		
			Somatic	Phase 1	Phase 2
0	1	B	1,4,12,27	d	1,2
2	1	.	.	.	5
3	1	.	.	.	7
5	2	.	.	b	.
7	9	.	5	a	.
11	1	.	.	.	6
12	1	.	.	.	absent
13	4	.	.	c	.
14	2	.	.	.	z_6
15	2	.	.	i	.
16	3	.	.	r	.
17	3	.	.	y	.
18	4	.	.	z	.
19	4	.	.	z_{10}	.
20	2	.	.	z_{29}	.
21	1	.	.	z_{38}	.
23	2	A	2	.	.
24	21	D_1	9	.	.
25	1	.	.	.	z_{39}
26	2	.	.	.	z_{35}
27	1	.	.	k	.
29	9	D_2	46	.	.
30	3	C_3	8	.	.
31	18	C_2	6	.	.
32	4	C_1	7	.	.

* The Table is condensed in those instances when an increase in d_c occurred as a result of admission of a serotype that lacked an antigen possessed by all previous members of the group, rather than one having a 'new' antigen. The specific serotypes added at various stages of the computation were as follows: $d_c = 0$, *S. cairo*; $d_c = 2$, *S. eppendorf*; $d_c = 3$, *S. schwarzegrund*; $d_c = 5$, *S. limete*; $d_c = 6$, *S. uppsala*; $d_c = 7$, *S.* '*hessarek*' 4, 12, 27; $d_c = 8$, *S. hessarek* 4, 12, *S.* '*arechavaleta*' 4, 12; $d_c = 9$, *S. kisangani*, *S. fulica*, *S. arechavaleta* 4, 5, 12, *S. paratyphi*-B, *S. stanley*; $d_c = 10$, *S. java*; $d_c = 11$, *Salmonella* 4, 12: b: 1, 6; $d_c = 12$, *S. schleissheim*; $d_c = 13$, *S. legon*, *S. abortus-ovis*, *S. altendorf*, *S. womba*; $d_c = 14$, *S. bury*, *S. ayinde*; $d_c = 15$, *S. typhimurium*, *S. agama*; $d_c = 16$, *S. heidelberg*, *S. bradford*, *S. remo*; $d_c = 17$, *S. coein*, *S. teddington*, *S. kamoru*; $d_c = 18$, *S. shubra*, *S. kiambu*, *S. indiana*, *S. entebbe*; $d_c = 19$, *S. haifa*, *S. ituri*, *S. tudu*, *S. fortune*; $d_c = 20$, *S. brancaster* 1, 4, 12, *S.* '*brancaster*' 1, 4, 12, 27; $d_c = 21$, *S. Wilhelmsburg*; $d_c = 23$, *S. paratyphi*-A, *S. paratyphi*-A var. *durazzo*; $d_c = 24$, *S. sendai*, *S. miami*, *S. os*, *S. saarbruecken*, *S. onarimon*, *S. frintop*, *S. blankenese*, *S. goeteborg*, *S. ipeko*, *S. ridge*, *S. typhi*, *S. ndolo*, *S. zega*, *S. seremban*, *S. jamaica*, *S. lawndale*, *S. stellenbosch*, *S. angola*, *S. portland*, *S. canastel*, *S. gallinarum-pullorum*.

(Lockhart & Hartman, 1963), a substantial increase in cumulative difference (d_c) was noted when a new subgroup fused with the group under formation. The peculiar nature of the present data prevented this; addition of a new subgroup might occur

with only a slight increase in d_c. The existence of the subgroup could be detected however, by the fact that an appreciable number of new individuals would join the main group at the same time. In Table 1, for example, 21 types were added as a group at $d_c = 38$, when somatic antigen 9 was admitted. For simplicity, no lists of individual serotype designations are given in the tables; instead, the new antigens possessed by serotypes added at each successive level of d_c are indicated. These last three columns of the tables thus show which features have been eliminated during formation of the group. This information was not included in the computer output, but could easily be deduced by comparing the results with Kauffmann's (1961) tables. As we will discuss later, members of the group may or may not have any of the 'eliminated' features, but cannot possess a feature which has not yet been listed.

Table 3. *Composition of a monothetic group formed around Salmonella saarbruecken, considering all features*

Cumulative difference	No. of individuals (serotypes) added	New Kauffmann groups represented	New antigens represented		
			Somatic	Phase 1	Phase 2
0	1	D_1	1,9,12	a	1,7
6	2	B	4,5	.	.
12	1	.	.	c	.
13	1	.	.	z	.
15	2	.	.	.	5
16	6	.	.	.	2
19	1	.	.	b	.
20	3	.	.	.	6
21	4	.	27	.	.
22	4	.	.	d	.
23	2	.	.	i	.
24	4	.	.	r	.
25	2	.	.	y	.
26	8	.	.	z_{10}	.
32	15	.	.	.	z_6
36	3	.	.	.	absent
37	2	A	2	.	.
38	3	.	.	z_{29}	.
39	1	.	.	z_{38}	.
40	1	.	.	.	z_{39}
41	1	.	.	z_{35}	.
42	1	.	.	k	.
44	9	D_2	46	.	.
45	3	C_3	8	.	.
46	18	C_2	6	.	.
47	4	C_1	7	.	.

The fate of biochemical features is not indicated in the tables, for reasons that become obvious on examination of Table 2, which shows the result when the computation was repeated with the 14 biochemical features eliminated. The groups formed in Tables 1 and 2 are identical. The order in which individual serotypes were added to the groups was slightly different in the early stages of the two computations, but the eventual composition of the groups was the same. The process of group formation may appear more clear after examination of the footnote to Table 2, wherein is indicated the order in which specific serotypes were added to the group.

It will be noted that the d_c level at which the second computation ended (32) is just 14 less than that at which the first had ended (46). The only essential difference is in the elimination of the 14 biochemical features; that is, *any* combination of biochemical characteristics is acceptable for admission to the group.

Table 4. *Composition of a monothetic group formed around Salmonella dar-es-salaam, considering all features*

Cumulative difference	No. of individuals (serotypes) added	New Kauffmann groups represented	New antigens represented		
			Somatic	Phase 1	Phase 2
0	1	D_1	1,9,12	l,w	e,n,x
3	1	.	.	b	.
7	1	B	4	.	.
10	1	.	.	z_{10}	.
12	1	.	27	d	.
13	1	.	.	y	.
15	1	.	.	.	z_{15}
16	3	.	.	a	.
19	3	.	.	c	.
21	3	.	5	.	.
22	1	.	.	k	.
23	4	.	.	v	.
28	2	.	.	z	.
30	1	.	.	z_{13}	.
31	1	.	.	z_{28}	.
33	3	.	.	.	1,w
34	1	.	.	.	absent
35	9	.	.	.	z_6
36	4	.	.	e	.
37	1	.	.	i	.
38	1	.	.	r	.
39	2	.	.	z_{29}	.
40	1	.	.	z_{38}	.
41	1	.	.	.	z_{39}
42	3	.	.	.	z_{35}
44	8	D_2	46	.	.
46	2	A	2	.	.
47	3	.	.	absent	.
49	12	F	11	.	.
50	11	I	16	.	.
51	6	J	17	.	.
52	4	L	21	.	.
53	4	M	28	.	.

It was concluded that the primary basis for group formation had been possession of the 1, 2; 1, 5; 1, 6 or 1, 7 antigenic grouping in the second phase, with a secondary subgrouping based on somatic antigens. Although the machine eventually began to accept such single second-phase antigens as z_6, z_{39}, and z_{35}, it rigorously excluded the second phase complexes, e, n, x or e, n, z_{15}. The antigenic composition in first phase, and the biochemical characteristics, appear to have no influence on the composition of the group. *Salmonella cairo* gathered about it neither the rest of Group B nor a biochemical group, but one based on second-phase antigens.

When the calculation was repeated using *Salmonella typhimurium* (1, 4, 5, 12;

i; 1, 2) as the starting OTU, the resulting group was again the same as that shown in Tables 1 and 2. Thus the group formed around one of the classical species also is based on second-phase antigenic composition rather than on the Kauffmann groupings. Essentially similar results were obtained in several further experiments. By way of illustration, two of these are shown in Tables 3 and 4. The starting types for these groupings were *S. saarbruecken* (1, 9, 12; a; 1, 7) and *S. dar-es-salaam* (1, 9, 12; 1, w; e, n, x), both members of Kauffmann's Group D_1. In both cases the initial groups formed are mixtures of Kauffmann Groups D_1 and B, and are based primarily on the characteristic second-phase antigenic complexes 1, 2–5–6 or 7 and e, n, x or z_{15}, respectively. In these and other computations, a few single second-phase antigens were eventually included in the group under formation (as was the lack of a second phase), but the two principal kinds of groups (1, 2, etc., *vs.* e, n, x) were never mixed. In no case was there any evidence that biochemical features had an influence on group formation, or that more distinctive groups were formed around species listed in *Bergey's Manual* (1957) than around serotypes chosen at random as a starting point.

DISCUSSION

Unfortunately, the physiological characters listed by Kauffmann (1961) for each serotype were of no help in achieving a numerical classification. Relationships based entirely on the biochemical features did not include all the serotypes, and could not be correlated either with the Kauffmann–White serological groups or with any of the groupings obtained by computer analysis. The fact that exclusion of the biochemical features did not effectively alter the result of computations (cf. Tables 1 and 2) indicates that not even a selected few of the physiological features were involved in group formation. This conclusion applies primarily to attempts to rearrange the Kauffmann data, however, and not necessarily to all efforts to achieve a classification of salmonellas. The physiological data given by Kauffmann for each 'type' appear not to be based on the characters of a single type strain, but to represent a summary of the reactions shown by an indeterminate number of isolates having the requisite antigenic composition. The result of this, for any 'type' of which a number of individual isolates had been studied, is that a great many of the biochemical results are recorded as 'variable'. This seems to be especially true of the frequently encountered species, so that attempts to form groups around these serotypes, as they are characterized by Kauffmann, become almost hopeless. A better result might be obtained if data were available for the reactions obtained either from a great many individual isolates or from single, type strains (using 'type' in its nomenclatural sense). Since our study was restricted to Kauffmann's (1961) data, we could hope—at best—only to produce a classification of his classification.

In our results, the primary criterion for group formation seems to have been antigenic composition in second phase, with somatic antigens furnishing a basis for further subdivisions. Members of a Kauffmann–White somatic group were rejected by the computer if they did not fit the established second-phase pattern of the numerical grouping. In Tables 1 and 2, for example, all members of Group B which showed the e, n, x or e, n, z_{15} pattern were excluded, while members of Kauffmann's Groups A, D_1, D_2, etc. were admitted if they possessed the prevailing 1, 2–5–6 or 7 combination of antigens in second phase. This trend is even more strikingly demon-

strated in Tables 3 and 4, where the numerical groups (though consistent for second phase antigens) are composed from the outset of a mixture of Kauffmann (somatic) groups. The illustrations shown in the Tables were selected because the 'starting' serotypes were those with complex somatic antigens. Other computations, in which the original serotype was one listed by Kauffmann as possessing only a single somatic antigen, produced groups of even more mixed somatic composition—though still consistent for second-phase antigens.

First-phase antigens seem to be so diverse that they contribute little or nothing to the pattern of a group; the Tables indicate that first-phase components occur more or less at random among the groups obtained. However, serotypes with a complex first phase, such as d, e, h or g, m, s, t, were consistently excluded.

Members of these numerical 'groups' did not necessarily possess common properties, but all members of any group were commonly lacking in certain properties (antigens). As a group was formed, it was defined in terms of a list of the properties a member might possess without being excluded from the group. The groupings, therefore, are not really monothetic (Sneath, 1962). They are partially polythetic or polythetic, but restricted; that is, a given individual may be considered a member of a group if the properties it possesses are restricted to within the range of those characteristic of the group. But any given member does not necessarily have all the properties that are included in this definition of the group.

Whatever the actual nature of groups, even if they are altogether polythetic, it may be necessary to effect simplifications of this sort for purposes of diagnosis. Practical definition of groups may require application of some such monothetic criterion as accepting the possession or lack of a very few correlated properties as sufficient evidence for assigning an individual to a group or excluding it therefrom. Perhaps any really useful classification is necessarily somewhat arbitrary. The definition of taxa, and particularly the diagnosis of unknown isolates as members thereof, might otherwise prove operationally impossible—or at least so cumbersome as to be hardly worth the bother.

In this light, a computer in some cases is less useful for a complete analysis of groupings than for obtaining hints as to possible criteria for group formation (as we have done in this case). With some sets of data, the multidimensional nature of relationships within and between polythetic groups is such that even lengthy computation produces numerical results that confuse rather than illuminate. One can propose a revision of the Salmonella scheme, based partly on second-phase antigens (Holt & Lockhart, unpublished), but the scheme will be 'better' than Kauffmann's only if it is more practical—not simply because it is derived by numerical methods. In any case, such an arrangement is no more than suggested by these results. The computer certainly did not produce this or any other classification; it merely indicated, in a vague and incomplete manner, some trends in the data. The machine, in fact, was severely handicapped both by the nature of the data and by the inadequacy of the directions we provided. The result is instructive, for it provides some insight into the limitations as well as the advantages of numerical approaches to classification.

We have seen that an OTU may be omitted from a group simply because it has an 'excluded' property—i.e. one that has not yet been included in the definition of the group. This seems to have occurred a number of times in our experiments, when

possession of an obscure antigen (usually in the first phase) caused rejection of individual serotypes that might otherwise have been included. An investigator may simply ignore such anomalies, but the computer, of course, cannot. The particular properties which become 'accepted' as characteristic of a group depend not only on the properties of the OTU designated as a starting point, but also on the order in which similar individuals happen to be listed in the data. Thus, if two serotypes have the same number of differences, though in different specific properties, from the common group pattern, the computer will 'accept' whichever one is encountered first, and eliminate from further consideration any of its properties which are not also possessed by the rest of the group. The properties thus eliminated are now, in effect, admitted to the definition of the group, and the computer has reversed the investigator's intended criterion for group formation! This is illustrated in Tables 2 and 3, where although the predominant grouping in both cases is around the 1, 2–5–6 or 7 complex of second-phase antigens, the secondary subdivision in one case is composed of Kauffmann Group B (somatic antigens 1, 4, 5, 12, 27) individuals only, and in the other case is a mixture of Group B and Group D_1 (somatic antigens 1, 9, 12) serotypes. In the latter case the Group D_1 antigens were accepted because the starting OTU possessed them, but the search for similar individuals then began in Group B (serotypes were stored in the computer 'memory' in the order in which they are listed by Kauffmann), and somatic antigens 4 and 5 were accepted at once. Similarly, all the results reflected a tendency for acceptance of somatic antigens listed near the start of Kauffmann's data (i.e. groups A, B, C, D) rather than equally appropriate representatives of groups listed later and thus less likely to be encountered during searches of the stored data. These 'position effects' are a serious defect in the computer program, for they lead sometimes to a quite trivial property of a single individual becoming one of the criteria for group formation.

Further, the program necessarily shows a bias against complex antigens. A 'group' will eventually include in its definition a variety of antigens that can be added individually with small increments in d_c, but will exclude antigen complexes whose admission would involve a larger increase in d_c. A group formed about a starting OTU possessing antigens 1 and 5 in its second phase, for example, will accept, singly, antigens 2, 6 and 7, and eventually other single antigens such as z_6, but it will reject a complex like e, n, x.

Although these particular difficulties are characteristic of the 'monothetic' method we have used, other techniques for group-formation have deficiencies of their own (Lockhart & Hartman, 1963). Numerical taxonomy has enormous potential, but depending on the nature and complexity of the data, and the adequacy of the programs used, computer analysis may or may not provide complete and satisfactory solutions for specific problems in classification. The machine will not automatically transform confusion into order; on the contrary, it will perpetuate faithfully any errors or misconceptions the investigator has unwittingly included in his program. One must therefore examine carefully all the assumptions, intended or otherwise, that are implied in the way the data were scored or in the directions given the machine for calculation of similarities and formation of groups. The computer will follow all such instructions quite literally, and will exercise neither discrimination nor good judgement in any instance—however obvious—unless these are explicitly specified beforehand by the investigator.

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Mechanism of Chloramphenicol and Tetracycline Resistance in *Escherichia coli*

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SUMMARY

The protein-synthesizing activity of cell-free preparations of *Escherichia coli* was estimated by adding radioactive amino acids according to the system of Matthei & Nirenberg (1961). Cell-free systems were prepared from antibiotic-sensitive strains of *E. coli* and from resistant variants, and the sensitivity of amino acid incorporation to inhibition by chloramphenicol and tetracycline determined. The same sensitivity was shown for the sensitive as for the resistant strains.

INTRODUCTION

Several explanations have been proposed to account for bacterial resistance to chloramphenicol, for example: increased production of chloramphenicol reductase (Merkel & Steers, 1953; Miyamura, 1961), changes in permeability (Kuschner, 1955), and alteration of the process of protein synthesis (Ramsey, 1958). However, for none of these explanations is the experimental evidence conclusive. In a preliminary account (Okamoto & Mizuno, 1962), we reported that a cell-free system for amino acid incorporation into protein prepared from a chloramphenicol-resistant strain of *Escherichia coli* B was almost as sensitive to chloramphenicol inhibition as was that from the wild type of *E. coli* B. The present paper gives a more detailed description of this work, together with further experimental work. The extended programme included examination of resistance to tetracycline exhibited by this chloramphenicol-resistant strain as well as by *in vitro*-developed tetracycline-resistant strains, and also by another type of chloramphenicol and tetracycline resistance, i.e. the multiple drug resistance transferable by 'R factor' (review by Watanabe, 1963). Results similar to those previously reported were obtained in all these cases.

METHODS

Strains used. The following five strains were examined: (1) *Escherichia coli* B, wild strain; (2) a chloramphenicol-resistant strain (B CMR) developed *in vitro* from *E. coli* B as described by Okamoto & Mizuno (1962); (3) *E. coli* K12 cs-2 (Skaar & Garen, 1956); (4) a multiple drug-resistant strain (K MR) obtained by transfer of resistance into *E. coli* K12 cs-2 from a resistant strain of Shigella isolated from a natural source, kindly supplied by Mr H. Ikeda of our laboratory; (5) tetracycline-resistant strains isolated from *E. coli* K12 cs101 (Hfr), (Skaar & Garen, 1956) by successive passages through liquid media containing increasing concentrations of tetracycline (strains K TCR). This procedure was performed chiefly by Mrs N. Okamoto.

Twelve independent cultures were used, but only two of them (nos. 3 and 5) attained resistance to tetracycline 100 μg./ml. without losing their Hfr character. Several independent cultures from each of the two were further developed to higher degrees of resistance, and five strains were obtained which could grow in the presence of more than 300 μg. tetracycline/ml. They were designated as TCR 5A, 5B, 5–350, 5–900, TCR 3C. Since precise genetic analysis was not performed, it is not known whether these strains were genetically different or not.

Table 1. *Sensitivity of the strains of Escherichia coli to various antibiotics*

The figures indicate the concentrations (μg./ml.) of antibiotics which produce 50% inhibition of growth as determined by the method described by Treffers (1956).

	Antibiotic					
Escherichia coli strain	Chloramphenicol	Tetracycline	Penicillin	Erythromycin	Streptomycin	Polymyxin B
	Concentration (μg./ml.) for 50% growth inhibition					
B	0·3	1·3	7	10	1	1·2
B CMR	300	22	100	170	1·3	0·3
K12	0·6	3·6	15	22	0·75	1·8
K MR	90	25	16	25	10	0·4
K TCR 5–900	15	100	130	100	0·25	0·25

The sensitivity of the strains to various antibiotics is shown in Table 1 as the concentration required to inhibit the bacterial growth by 50%. As shown, strain B CMR was about a thousand times as resistant to chloramphenicol as the wild strain and exhibited cross-resistance against tetracycline, penicillin and erythromycin, but was sensitive to streptomycin, as reported earlier by Szybalsky & Bryson (1952); strain K MR was resistant to chloramphenicol, tetracycline and streptomycin, but not to erythromycin and penicillin. Strain K TCR shows similar patterns of cross-resistance to those of strain B CMR. All resistant strains were somewhat more sensitive to polymyxin B than were their wild types.

Culture medium and cultural conditions. The culture medium consisted of the following ingredients: 0·1 M-solution of $MgSO_4$, 10 ml.; 0·01 M-solution of KH_2PO_4, 3·2 ml.; 20% (w/v) solution of glucose, 5 ml.; NaCl, 3 g.; Polypepton (Daigo Eiyo Co.), 10 g.; in a total volume of 1000 ml.; adjusted to pH 7·2. An overnight shaken culture (incubated at 37°) was transferred to about 50 vol. of fresh medium and the culture incubated with aeration. In the case of strains B CMR and K MR, chloramphenicol was added to the medium at 30 μg./ml. The TCR strains were pre-cultured in the presence of tetracycline 100 μg./ml. and then grown in a large-scale culture (5 l.) in medium containing tetracycline 20 μg./ml.

Preparation of cell-free system. When the culture reached early logarithmic phase, it was chilled rapidly with ice; the organisms were harvested (centrifugation) and washed twice with 0·05 M-tris buffer (pH 7·6) containing 8×10^{-3}M-$MgCl_2$. In later experiments this buffer, which was also used for the preparatory procedure and dialysis, was modified according to Matthei & Nirenberg (1961) to include 0·06 M-KCl and 0·006 M-β-mercaptoethanol. The washed organisms were suspended in about 2 vol.

of the buffer and disrupted by sonic treatment (Okamoto & Mizuno, 1962) or, in later experiments, by passage through a French pressure cell. The latter procedure gave more active preparations. The disrupted-cell suspension was centrifuged twice at 30,000 *g* for 30 min. to remove whole organisms and large debris, and the final supernatant fluid was kept as '3s fraction'. The 3s fraction was further fractionated by centrifugation at 100,000 *g* for 60 min. to give supernatant fraction (10s) and pellet fraction (ribosome fraction); the ribosome fraction was washed once by resuspending and sedimenting at 100,000 *g*, and the 10s fraction was dialysed overnight against tris buffer (pH 7·6) containing 8×10^{-3} M-$MgCl_2$ at 4°. In later experiments the 3s fraction was directly dialysed against buffer containing β-mercaptoethanol and kept frozen before use.

Assay of incorporation of ^{14}C-amino acids into protein by the cell-free system. The ribosome fraction (equiv. about 2 mg. protein) together with the 10s fraction (equiv. about 1 mg. protein) were incubated with ^{14}C-amino acids and necessary cofactors as described previously. In later experiments dialysed 3s fraction was chiefly employed and the reaction mixture was modified according to Matthei & Nirenberg (1961), as follows: 3s fraction, equiv. 2–3 mg. protein; tris buffer (0·05 M, pH 7·6); $MgCl_2$, 8×10^{-3} M; KCl, 6×10^{-2} M; adenosine triphosphate (ATP) Na salt, 1 μmole/ml.; creatine phosphate, Na salt, 5 μmoles/ml.; creatine kinase, equiv. 0·1 mg. protein/ml.; guanosine triphosphate (GTP), 0·03 μmole/ml.; β-mercaptoethanol 0·006 M; ^{14}C-amino acid mixture (hydrolysed algal protein, specific activity 6·88 mc./mM, supplied by the Institute of Applied Microbiology, University of Tokyo), 2×10^5 c.p.m./tube (counted with the windowless gas-flow counter); antibiotic to be tested at the required concentration; the total volume was 0·3 or 0·5 ml. After incubation at 37° for 45 min., the reaction was stopped by adding about 5 ml. of 5 % trichloroacetic acid (TCA). The radioactivity incorporated into the hot-acid insoluble fraction was counted, and the degree of inhibition by the antibiotic assayed as described (Okamoto & Mizuno, 1962). Counting was performed with a windowless gas-flow counter or with a thin-window gas-flow counter equipped with an automatic sample changer; the counting efficiency of the latter was about one-fourth that of the former. All the data are presented as values to be obtained with the windowless gas-flow counter.

RESULTS

Inhibition of the amino acid incorporation with chloramphenicol in the cell-free system of Escherichia coli

The inhibition of amino acid incorporation in the cell-free preparations of *Escherichia coli* B and strain B CMR was examined. The results are shown graphically in Fig. 1. Almost identical inhibition curves were obtained with the wild-type and the chloramphenicol-resistant strains. The same kind of experiment with the cell-free systems of strains K and K MR gave similar inhibition patterns, as shown in Fig. 2. In both cases the amino acid incorporation system of the resistant strain was as sensitive, within the experimental errors, to inhibition by chloramphenicol as that of the sensitive strain.

It should be noted that the amino acid incorporation in these systems was inhibited about 50 % by 2·5 μg. chloramphenicol/ml. and about 80 % with 40 μg./ml.;

the inhibition was not complete even in the presence of 200 μg./ml., where the inhibition was about 80–90 %.

Inhibition with tetracycline

Amino acid incorporation by the cell-free system of the wild strain, *Escherichia coli* K12, was inhibited also by tetracycline; the inhibition was about 40–50 % at 40 μg./ml. and about 80 % at 160 μg./ml. Thus the concentration of tetracycline required to inhibit the cell-free system was considerably higher than that required for growth inhibition. However, when growth inhibition with tetracycline was examined in the medium supplemented with $MgCl_2$ (0·008 M) and KCl (0·06 M), the concentration of tetracycline which caused 50 % inhibition of growth was about

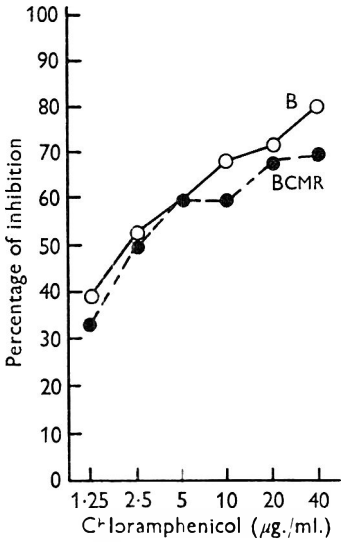

Fig. 1

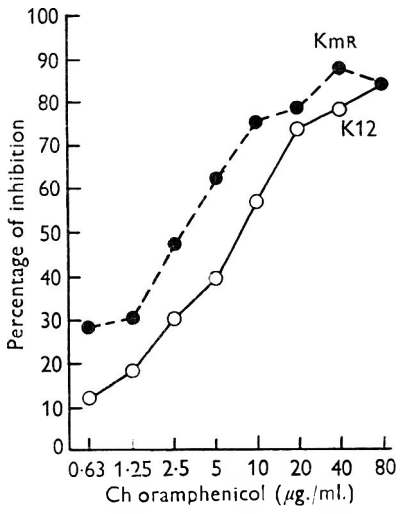

Fig. 2

Fig. 1. Effect of chloramphenicol on amino acid incorporation by cell-free systems of *Escherichia coli* B and strain B CMR. The reaction mixture contained about equiv. 1–3 mg. protein of ribosomal fraction and about 1 mg. protein of 10s fraction. Incubation was at 37° for 20-min.; other conditions as described in the text. Each point represents the average value of two or three independent experiments. The value for complete system without antibiotic ranged from 120 to 600 counts/min./mg. protein, depending on the experiment. The radioactivity is corrected for the value of the 20–0 time control of 20–80 counts/min./mg. protein, and not corrected for self-absorption.

Fig. 2. Effect of chloramphenicol on amino acid incorporation by cell-free systems of *Escherichia coli* strains K and K MR. Conditions are similar to Fig. 1. Average data of from two to four experiments are shown.

20 μg./ml. for *E. coli* K12, about 100 μg./ml. for K MR, and about 300 μg./ml. for K TCR strains. Under these circumstances, therefore, the tetracycline concentration which caused 50 % inhibition of growth of *E. coli* K12 was not very different from that which caused 50 % inhibition of the cell-free system.

As shown in Table 1, strain B CMR exhibited considerable cross-resistance to tetracycline, and strain K MR was also resistant to it. The sensitivity to tetracycline of cell-free amino acid incorporation by these resistant strains was identical with that of their wild strains (Figs. 3, 4).

Since it was reported by Yokota & Akiba (1962) that a tetracycline-resistant strain of *Escherichia coli* selected *in vitro* by using tetracycline gave tetracycline-resistant ribosomes, we examined the sensitivity to tetracycline of cell-free preparations from several *in vitro*-developed tetracycline-resistant strains of *E. coli* K12. Representative results are shown in Table 2. The cell-free systems of the TCR strains investigated were, again, as sensitive to the effect of tetracycline as that of *E. coli* K wild strain, with some slight differences, especially in the case of TCR 3C. Two other TCR strains tested gave similar results.

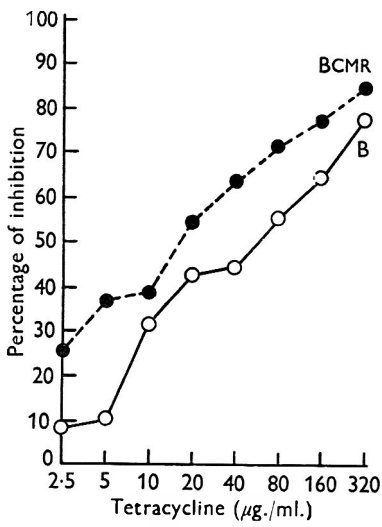

Fig. 3

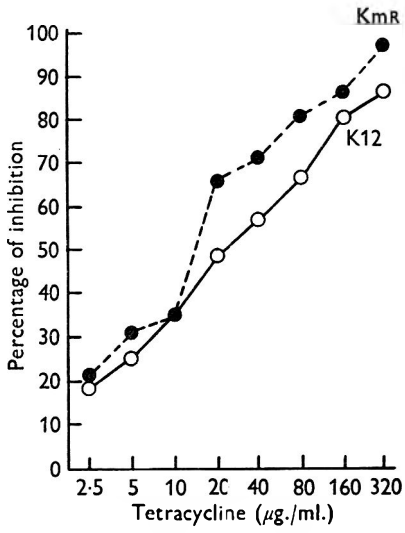

Fig. 4

Fig. 3. Effect of tetracycline on amino acid incorporation by cell-free systems of *Escherichia coli* B and strain B CMR. Conditions are similar to Fig. 1. Average data of from two to four experiments are shown.

Fig. 4. Effect of tetracycline on amino acid incorporation by cell-free systems of *Escherichia coli* strains K and K MR. Conditions are similar to Fig. 1. Average data of from two to four experiments are shown.

Inhibition of amino acid incorporation by other compounds

As mentioned above, strain B CMR was also cross-resistant to penicillin, erythromycin, and strain K MR to streptomycin. Since it has been observed that erythromycin (Brock & Brock, 1959) and streptomycin (Eaton & Caffrey, 1961; Erdös & Ullmann, 1959; Hahn *et al.* 1962) have inhibitory effects on protein synthesis, the effects of these compounds on the cell-free system of *Escherichia coli* B and strain K12 were examined.

Streptomycin (100 μg./ml.) exerted diverse effects. With amino acid incorporation by cell-free preparations of relatively low activity, streptomycin exhibited no inhibitory effect and occasionally even an acceleration was observed, especially at higher concentrations (500 μg./ml. or more). However, amino acid incorporation by preparations with higher activity obtained by the modified method used in later experiments was inhibited by streptomycin to a variable extent. These complicated effects produced by streptomycin are now under investigation in view of the findings

on the effect of streptomycin on the synthesis of polyphenylalanine directed by polyuridylic acid in a cell-free system (Speyer, Lengyel & Basilio, 1962; Flaks, Cox & White, 1962).

Erythromycin inhibited the amino acid incorporation up to 30% at concentrations of 100–1000 μg./ml. Penicillin (40 μg./ml.) had no effect on amino acid incorporation, suggesting that this process is not related to the synthesis of cell-wall substance, which is sensitive to inhibition by penicillin (Park, 1958; Mandelstam & Rogers, 1959).

Table 2. *Inhibition by tetracycline of amino acid incorporation by cell-free preparations of various in vitro-developed tetracycline-resistant strains of Escherichia coli* K12

	Strain						
	TCR 5A		TCR 5–900		TCR 3C		K12 wild
	Degree of resistance (tetracycline μg /ml.)*						
	> 100		About 120		About 50		3·6
Tetracycline added (μg./ml.)	counts/min. incorporated†	Inhibition %	counts/min. incorporated	Inhibition %	counts/min. incorporated	Inhibition %	Average value of % of inhibition‡
0	1588	0	1176	0	2024	0	0
10	1216	22	952	19	1732	14	32 (16–45)
20	1144	28	842	28	1523	25	38 (19–47)
40	1004	37	648	45	1400	31	51 (45–67)
80	704	56	536	54	1284	36	62 (52–80)
160	412	74	264	78	883	56	77 (74–84)
320	244	85	116	90	443	78	81 (68–90)

Each tube contained equiv. 2–3 mg. protein of dialysed 3s fraction in a total volume of 0·3–0·5 ml. Incubation: 37° for 45 min. Other conditions are as described in the text. Each experiment made in duplicate.

* The figures indicate the concentration (μg./ml.) of tetracycline required for 50% growth inhibition; tests were made directly on the organisms used for preparation of the cell-free system.

† Total counts measured on samples containing equiv. 1·5–2 mg. protein; the value for the 0 time control, about 100 counts/min., was subtracted. Not corrected for self-absorption.

‡ Average value of four to six experiments. The range of variation is shown in parentheses.

DISCUSSION

It is well established that chloramphenicol specifically inhibits protein synthesis in bacteria (Gale & Folkes, 1953; Wisseman, Smadel, Hahn & Hopps, 1954). The amino acid incorporation system of *Escherichia coli* used in the present work can be considered to approximate to the process of protein synthesis in many respects, and is sensitive to inhibition by chloramphenicol at a concentration slightly higher than that required for growth inhibition. Thus the cell-free amino acid incorporation system may well be regarded as the site of action of chloramphenicol on bacteria. The data presented above indicate that the resistant strains investigated lost their chloramphenicol resistance when they were disrupted to a cell-free preparation. Thus it can be concluded that the primary cause of chloramphenicol resistance in these strains is not alteration(s) in the process of protein synthesis, but is some change(s) in the process by which the compound reaches its site of action, i.e. some change(s) in permeability.

Since tetracycline acts as a chelating agent, and consequently may exert action on several cellular activities (see review by Weinberg, 1957), its mode of action is not so well defined as is that of chloramphenicol. However, evidence has been given that, at the growth inhibitory concentration, tetracycline inhibits bacterial protein synthesis preferentially (Gale & Folkes, 1953) as chloramphenicol does. Therefore, it seems likely that the inhibitory effect of tetracycline on the amino acid incorporation system reflects the inhibitory effects of tetracycline on bacterial growth. Although the concentration of tetracycline required for inhibition of the cell-free system is considerably higher than that required for growth inhibition, this may partly be explained by the presence of a rather high concentration of Mg^{2+} (see Results).

If it be assumed that the inhibition by tetracycline of cell-free amino acid incorporation represents its antibacterial effect, tetracycline resistance in strains B CMR, K MR and K TCR should also be due to changes in permeability as in chloramphenicol resistance. A certain degree of cross-resistance between chloramphenicol, tetracycline and other compounds in *in vitro*-developed chloramphenicol- and tetracycline-resistant strains of *Escherichia coli* has been observed (Szybalsky & Bryson, 1952; Cavalli, 1952); changes in permeability for these compounds would be a plausible explanation for such cross-resistance.

Independently of our preliminary work (Okamoto & Mizuno, 1962), Akiba & Yokota (1962) made similar experiments and reached the same conclusion as we did except for the case of the *in vitro*-developed tetracycline-resistant strain. They reported that the cell-free system of the latter strain was resistant to tetracycline and that this resistance resided in the ribosomal fraction (Yokota & Akiba, 1962). As described above, all the tetracycline-resistant strains that we isolated, through multiple steps from *Escherichia coli* K12 strain CS101, gave tetracycline-sensitive cell-free systems. These findings do not, of course, exclude the possibility that tetracycline-resistant mutants equipped with tetracycline-resistant protein-synthesis machinery may occur. We can only say that, in the most easily obtainable tetracycline-resistant strains of *E. coli* K12, their cell-free systems are not resistant to tetracycline. Although only one *in vitro*-developed chloramphenicol-resistant strain was investigated, this strain was so resistant (about one thousand times as resistant as the wild strain) that it may be assumed that many of its loci responsible for chloramphenicol resistance have mutated, so that the above considerations can also be applied to chloramphenicol resistance.

The contradiction of our conclusion with that of Ramsey (1958) may be explained by the staphylococcus he used possessing a different mechanism of chloramphenicol resistance from that of *Escherichia coli*, or by the possibility that the disrupted cell system used by Ramsay still retained some sort of permeability barrier.

Our results do not exclude the possible role of a chloramphenicol-inactivating enzyme (Merkel & Steers, 1953; Miyamura, 1961) in the chloramphenicol resistance of *Escherichia coli*, since they only showed that cell-free protein synthesis of resistant organisms was not resistant, and that resistance was probably due to changes other than at the immediate site of protein synthesis. However, the enzymic degradation of chloramphenicol is not sufficiently rapid to decrease the concentration of chloramphenicol in the medium, and our experiments indicate that such an enzyme is not operating in the cell-free system. Hence, if a chloramphenicol-

inactivating enzyme plays some role in chloramphenicol resistance in *E. coli*, its mode of action might be to decrease intracellular concentration of chloramphenicol by acting on the compound at its entrance into the cell, and this might be one type of change in the permeability barrier. Spheroplasts of chloramphenicol-resistant *E. coli* remain resistant to chloramphenicol (Hirokawa, Abe & Mizuno, 1959). The most probable alteration of permeability, if any, is therefore within the spheroplast.

Resistant strains of *Escherichia coli* derived *in vitro* and by transferred resistance are somewhat more sensitive than their wild types to polymyxin B, a surface active antibiotic which combines with some surface structure of Gram-negative bacilli. However, attempts to reverse the resistance by combined action of chloramphenicol and polymyxin B were unsuccessful.

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Studies on the Nutrition and Growth Requirements of *Mycoplasma gallisepticum*

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SUMMARY

A complex tissue culture medium supplemented with swine serum and peptone supported optimal growth of *Mycoplasma gallisepticum* strain 293. Media lacking any of these components supported little or no growth. However, when 5′-monophosphate nucleotides replaced the peptone growth was supported. The minimal nucleotides necessary to support good growth were adenylic, cytidylic, guanylic and thymidylic acids. In some cases the addition of the four ribonucleotides and the four deoxyribonucleotides in place of peptone improved growth; the four ribonucleotides alone supported poor growth. Thymidylic acid seemed essential for growth, and uridylic acid appeared to be inhibitory.

The mixture of ribonucleosides and deoxyribonucleosides, but not of purines and pyrimidines, when substituted for the peptone, also supported good growth. The concentrations of nucleosides and nucleotides had a significant effect on growth. Although the nutritional factors of swine serum were not defined, the effects of different sera on growth were investigated. Rabbit, horse, turkey and swine serum supported optimum growth; human serum supported less, whereas PPLO serum fraction (Difco) or bovine serum supported poor growth. Dog serum did not support growth.

INTRODUCTION

The nutritional requirements of some *Mycoplasma* species have been reported by Edward (1947, 1953, 1954), Smith & Morton (1951), Smith, Lecce & Lynn (1954), Smith (1955), Chalquest & Fabricant (1960), Rodwell (1960), Razin & Knight (1960*a*, *b*), Razin (1962), and Razin & Cohen (1963). It is apparent from these studies that the pathogenic as well as the saprophytic species have complex nutritional requirements. The main purpose of the present work was to evolve a defined medium which would support the growth of the avian pathogen *Mycoplasma gallicepticum* (Edward & Kanarek, 1960) strain 293 and to determine its nutritional requirements. A partially defined medium described by Razin & Knight (1960*a*), or modifications of it, did not support growth of the test organism. However, excellent growth was supported by a medium containing tissue culture (TC) medium 199 (Morgan, Morton & Parker, 1950), swine serum and Bacto-peptone. This medium was selected as a basis for further work to replace the undefined components with defined ones. Media without the serum did not support

growth. However, growth of the test organism did occur when the 5′-monophosphate ribonucleotides and deoxyribonucleotides, or the ribonucleosides and deoxyribonucleosides replaced the peptone component.

METHODS

Organism. Mycoplasma gallisepticum strain 293 was selected as the test organism. This strain was originally isolated from a field case of chronic respiratory disease in chickens by Dr J. R. E. Taylor in 1957; its morphological and physiological characteristics were described by Fabricant *et al.* (1959). Since isolation the organism has been maintained in continuous subculture on the same laboratory medium.

Media. Adler's overlay medium (Adler, Yamamoto & Bankowski, 1954) was used for maintaining stock cultures and for growing the organism for inocula in the nutritional experiments.

Difco PPLO broth without crystal violet, enrichments or inhibitors was used as diluent in preparation of serial dilutions for colony counts. Difco PPLO agar enriched with 10 % (v/v) pooled swine serum, 1000 units penicillin/ml. and 0·05 % (w/v) thallium acetate was used for colony counts. The pooled swine serum was obtained from a local slaughtering plant and sterilized by passage through a Seitz EK filter. All batches of serum were checked for ability to support growth of several Mycophasma species and then stored at −25° until needed.

The medium used for the nutritional experiments was prepared from Difco TC medium 199 (based on the tissue culture medium of Morgan *et al.* 1950) liquid ten-fold concentrate. This medium was diluted to double concentration with sterile glass-distilled water, buffered with 0·3 % (w/v) K_2HPO_4, rather than with sodium bicarbonate. After the addition of 10 % (v/v) pooled swine serum, and various supplements, the appropriate volume of distilled water was added to adjust the TC medium 199 to single concentration; when necessary, this was readjusted to pH 7·6–7·8 with 10 % (w/v) KOH. Hereafter the TC medium 199 + swine serum but without other supplements will be referred to as the basal medium. The control medium used in all nutritional experiments consisted of basal medium to which had been added 1 % (w/v) Bacto-peptone.

Except for Adler's overlay medium all media, including all reagents and supplements, were prepared with glass-distilled water. Pyrex glassware was used in all experiments. Before use it was cleaned with chromic + sulphuric acid mixture and thoroughly rinsed with distilled water.

All nucleic acid derivatives and coenzymes were sterilized by passage through sintered glass filters. Undegraded DNA preparations were sterilized with a few drops of chloroform and then decanted from the chloroform. All other inorganic reagents and media used for colony counts were sterilized by autoclaving at 120° for 15 min.

Conditions of growth. Five ml. of basal medium with various supplements were dispensed in 13 × 100 mm. tubes with stainless steel closures. These were inoculated with 0·05 ml. of a 48 hr stock culture. The cultures were incubated statically in air at 37°. Colony counts showed that the inoculum contained about 10^6 organisms/ml. medium.

Assessment of growth. The estimation of growth by turbidimetric methods or acid titration was unreliable under our experimental conditions; the basal medium

was too turbid and there was no consistent correlation between acid production and colony counts. Therefore, growth was measured by the colony count technique described by Fabricant, VanDemark & Fabricant (1962). All plates for colony counts were incubated in candle jars with excess moisture at 37°.

Chemicals. Undegraded DNA, the purines and pyrimidines adenine, cytosine, guanine, uracil and thymine, and the 5′-monophosphate deoxyribonucleotides, deoxyadenylic acid disodium salt.$3H_2O$ (dAMP), deoxycytidylic acid.$2H_2O$ (dCMP), deoxyguanulate.NH_4 (dGMP), and thymidylic acid sodium salt (TMP) were products of the California Corporation for Biochemical Research (Los Angeles, California). The 5′-monophosphate ribonucleotides muscle adenylic acid (AMP), guanylic acid sodium salt (GMP), cytidylic acid sodium salt (CMP), and uridylic acid disodium salt (UMP) were products of the Pabst Laboratories (Milwaukee, Wisconsin) or the Sigma Chemical Company (St Louis, Missouri). The 5′-triphosphate nucleotides of adenosine, disodium salt (ATP), cytidine, sodium salt (CTP), guanosine, sodium salt (GTP), and uridine, sodium salt (UTP) were also products of the Pabst Laboratories. The ribonucleosides adenosine, cytidine, guanosine, uridine, and the deoxyribonucleosides, deoxyadenosine, deoxycytidine.HCl, deoxyguanosine, and thymidine were products of the Nutritional Biochemical Corporation (Cleveland, Ohio).

RESULTS

The partially defined medium described by Razin & Knight (1960*a*) for a strain of *Mycoplasma laidlawii* did not permit survival of *Mycoplasma gallisepticum* strain 293. Attempts to improve this medium by addition of PPLO serum fraction (Difco), 10 % (v/v) swine serum, increased amounts of glucose, or biotin and folic acid also failed and further work with it was abandoned.

Initial attempts to grow the test organism in TC medium 199 with or without 10 % (v/v) swine serum also failed. Since this medium changed from pH 7·6 to 8·9 within 3 hr of static incubation at 37°, the medium was subsequently buffered with 0·3 % (w/v) K_2HPO_4 rather than with sodium bicarbonate as recommended. Inocula of the test organism survived better in this medium, without serum and buffered with K_2HPO_4, than in media buffered with sodium bicarbonate or tris buffer (pH 7–9; Sigma).

However, minimal growth of strain 293 occurred when the TC medium 199 was supplemented with 10 % (v/v) swine serum (basal medium). Addition of 0·1–0·4 % (w/v) of glucose to this basal medium did not improve growth.

Other studies indicated that the basal medium lacked some essential nutritional factor, or factors, which could be supplied by Difco PPLO broth (without crystal violet). The factor, or factors, could be supplied equally by 1 % (w/v) Bacto-peptone, one of the PPLO broth ingredients (see Table 1), and to a lesser degree by beef heart for infusion, the other PPLO broth ingredient.

The basal medium supplemented with 1 % (w/v) Bacto-peptone was used as the control medium in all later experiments. Optimal growth of strain 293 was obtained only in the control medium. No growth occurred in media when either TC medium 199 or serum were omitted.

Optimal growth of the test organism also occurred in media where the concentration of peptone (in the control medium) was altered from 0·4 to 4 % (w/v).

Concentration of 6, 0·2 and 0·1 % peptone yielded less growth, while concentrations above 6 % were inhibitory.

Essentially the same amount of growth occurred in media where rabbit, horse, or turkey serum was substituted for one lot of swine serum in the control medium (see Table 2). Human serum supported moderate growth, Difco PPLO serum fraction or bovine serum supported poor growth, while dog serum did not support growth. Other experiments indicated that some batches of swine serum were superior to horse serum in supporting growth of strain 293 in the control medium. Neither tryptic digests of casein nor peptone prepared according to the method of Roberts & Snell (1946), when substituted for the peptone of the control medium, allowed growth of strain 293.

Table 1. *The growth of Mycoplasma gallisepticum strain 293 in TC medium 199 + swine serum (basal medium) supplemented with Difco PPLO broth, Bacto-peptone, or beef heart for infusion (Difco)*

	Time (days)			
	1	2	3	4
Media*	Counts†/ml. medium ($\times 10^6$)			
PPLO broth + swine serum‡	81	139	124	380
Basal medium	7	5	5	3§
Basal medium + PPLO broth	3	10	1255	240
Basal medium + 1 % (w/v) peptone	180	1555	0	0
Basal medium + 5 % (w/v) beef heart for infusion	65	196	90	44

* Since the inoculum did not survive in any of the above mixtures of medium components without serum, these counts are not given in the Table.

† The inoculum contained 4×10^6 viable particles/ml. medium. All counts have been rounded off to the nearest 10^6 figure.

‡ Swine serum was added in 10 % (v/v) to all media.

§ Marked decrease in all counts after initial growth was due to acid production.

Table 2. *The growth of Mycoplasma gallisepticum strain 293 in TC medium 199 supplemented with peptone and various pooled sera or PPLO serum fraction (Difco)*

	Time (days)			
	1	2	3	4
Media* supplement	Counts†/ml. medium ($\times 10^6$)			
Rabbit serum	295	855	3‡	0
Horse serum	795	500	< 1	0
Swine serum	705	530	< 1	0
Turkey serum	605	< 1	0	0
Human serum	105	230	< 1	< 1
Bovine serum	22	6	10	6
PPLO serum fraction	9	6	8	7
Dog serum	< 1	< 1	< 1	< 1

* TC medium 199 was buffered with 0·3 % (w/v) K_2HPO_4 supplemented with 1 % (w/v) peptone and 10 % (v/v) of the various sera or 1 % (v/v) of Difco PPLO serum fraction.

† The inoculum contained 7×10^6 viable particles/ml. medium; all counts have been rounded off to the nearest 10^6 figure.

‡ Marked decrease in counts after initial growth was due to acid production.

Further work involved attempts to replace the serum and peptone of the control medium with chemically defined factors. However, none of the factors tested were capable of replacing the serum and only data concerning replacement of the peptone will be included here.

Media in which the peptone of the control medium was replaced by the 5′-monophosphate ribonucleotides or ribonucleosides, where these were added in the same concentration as the free purine and pyrimidine bases already present in TC medium 199, supported slight growth of the test organism. The addition of coenzyme A, diphosphopyridine nucleotide (DPN), flavin adenine dinucleotide (FAD) and α-lipoic acid (added in 0·25, 0·7, 0·1, 0·001 mg./100 ml. medium, respectively) to these media did not stimulate growth. Nor did the addition of reduced glutathione and L-cysteine.HCl.H_2O (100 mg. each/100 ml. medium) improve the growth in these media.

Table 3. *The influence of ribonucleotides AMP, CMP, GMP, deoxyribonucleotide TMP, and DNA on the growth of Mycoplasma gallisepticum strain 293 in the basal medium*

Media* (Basal medium† supplemented with:)	Time (days) Counts‡/ml. medium ($\times 10^6$)				
	1	2	3	4	5
1 % (w/v) peptone (control medium)	320	600	34	24‖	—
No supplements added	71	3	2	4	—
AMP, CMP, GMP, TMP§	4	8	92	48	—
DNA§	1	30	53	140	29
AMP, CMP, GMP, TMP, DNA	71	8	75	350	42

* Media combinations without serum have been omitted since they did not support growth.

† Basal medium contained TC medium 199 buffered with 0·3 % (w/v) K_2HPO_4 and 10 % (v/v) swine serum.

‡ The inoculum contained 13×10^6 viable particles/ml. medium. All counts have been rounded off to the nearest 10^6 figure.

§ Nucleotides and undegraded DNA each added in 1 mg./100 ml. medium.

‖ Marked decrease in all counts after initial growth was due to acid production.

However, as shown in Table 3, growth was stimulated when the 5′-monophosphate ribonucleotides AMP, CMP, GMP and the same isomer of deoxyribonucleotide TMP were added in 1 mg./100 ml. basal medium (nucleotides replacing the peptone of the control medium). As further shown in Table 3, growth was markedly stimulated when DNA (1 mg./100 ml. medium) was added to the basal medium supplemented with these four nucleotides.

In view of the growth of strain 293 in basal medium + DNA only, and the stimulation of growth by DNA in the medium containing the nucleotides, AMP, CMP, GMP, and TMP, the four 5′-monophosphate deoxyribonucleotides were substituted for the DNA to determine their comparative effects on growth. Table 4 shows that the basal medium supplemented with the four ribonucleotides alone supported the least growth. The preparation of DNA or the nucleotides AMP, CMP, GMP, UMP and TMP supplementing the basal medium supported the same amount of growth. Better growth was supported in the medium containing AMP, CMP, GMP, and TMP, while slightly less growth occurred in the medium containing both the

ribonucleotides and the deoxyribonucleotides. The medium containing the deoxyribonucleotides alone supported growth intermediate between the last two media named.

As also shown in Table 4, the addition of the 5′-triphosphate nucleotide ATP to the medium containing the eight monophosphate nucleotides, further stimulated growth of strain 293. The addition of the four triphosphate nucleotides ATP, CTP, GTP and UTP to this medium stimulated only slightly more growth than ATP alone. In this experiment the triphosphate nucleotides were prepared and stored frozen for 1 week before use. In other experiments freshly prepared ATP did not show the same growth stimulation.

Table 4. *The influence of 5′-monophospho-ribonucleotides, deoxyribonucleotides, DNA and triphosphonucleotides on the growth of Mycoplasma gallisepticum strain 293 in the basal medium*

Media (basal medium† supplemented with:)	Time (days) 1	2	3	4
	Counts*/ml. medium ($\times 10^6$)			
I. 1 % (w/v) peptone (control medium)	190	640	480	54¶
II. AMP, CMP, GMP, UMP‡§	6	18	18	2
III. AMP, CMP, GMP, UMP, TMP	10	26	47	< 1
IV. AMP, CMP, GMP, TMP	28	56	77	< 1
V. AMP, CMP, GMP, TMP, DNA	8	101	8	< 1
VI. DNA‡	8	22	48	39
VII. dAMP, dCMP, dGMP, TMP‡‖	18	56	60	< 1
VIII. AMP, CMP, GMP, UMP+DNA	12	69	115	< 1
IX. AMP, CMP, GMP, UMP, dAMP, dCMP, dGMP, TMP	9	67	50	< 1
X. Medium IX with ATP‡	23	190	4	< 1
XI. Medium IX with ATP, CTP, GTP, UTP	12	215	10	< 1

* Inoculum contained 13×10^6 organisms per ml. of medium. All counts have been rounded off to the nearest 10^6 figure.

† Basal medium contained TC medium 199 buffered with 0·3 % (w/v) K_2HPO_4 and 10 % (v/v) swine serum.

‡ All ribo- and deoxyribonucleotides and DNA were added in 1 mg. each/100 ml. of medium. The triphosphonucleotides were added in 0·1 mg. each/100 ml. of medium.

§ The 5′-monophosphoribonucleotides.

‖ The 5′-monophosphodeoxyribonucleotides.

¶ Marked drop in all counts after initial growth was due to acid production.

Further effects on the growth of strain 293 in the basal medium supplemented with various mixtures or concentrations of nucleotides are illustrated in Table 5. The results indicate that the concentration of the eight monophospate nucleotides significantly influenced the amount of growth. Growth was essentially doubled when the concentration of these nucleotides was halved, whereas doubling their concentration supported approximately half as much growth. In this experiment the medium containing the eight monophosphate nucleotides supported slightly more growth than that containing only AMP, CMP, GMP and TMP. The omission of UMP from the medium containing the seven other nucleotides yielded more growth than the medium including UMP. In this same experiment, a week-old preparation of ATP had a stimulatory effect on growth in the medium containing the eight monophosphate nucleotides, whereas adenosine diphosphate (ADP) seemed to have an inhibitory effect. When ATP and ADP were added together, the resulting growth was essentially the same as that in the medium where ATP was added alone.

Table 6 compares the effects of growth of strain 293 in the basal medium supplemented with the eight monophosphate nucleotides, the corresponding eight nucleosides, or the free purine and pyrimidine bases. The concentrations of the nucleosides

Table 5. *The influence of various combinations and concentrations of 5′-monophosphonucleotides and the adenine diphospho- and triphosphonucleotides (ADP, ATP) on growth of Mycoplasma gallisepticum strain 293 in the basal medium*

Media (basal medium* supplemented with:)	Time (days) 1	2	3
	Counts†/ml. medium ($\times 10^6$)		
I. 1 % (w/v) peptone (control medium)	435	745	80
II. AMP, CMP, GMP, UMP, dAMP, dCMP, dGMP, TMP‡	15	66	7
III. Medium II without UMP	38	94	3
IV. AMP, CMP, GMP, TMP	13	51	43
V. Medium II with nucleotides at 1/2 concentration	32	114	2
VI. Medium II with nucleotides at 2 × concentration	28	37	1
VII. Medium II plus ATP§	22	102	9
VIII. Medium II plus ADP§	23	18	23‖
IX. Medium II plus ADP and ATP	33	99	29

* Basal medium contained TC medium 199 buffered with 0·3 % (w/v) K_2HPO_4 and 10 % (v/v) swine serum.

† The inoculum contained 12×10^6 viable particles/ml. of medium. All counts have been rounded off to the nearest 10^6 figure.

‡ The first four listed are the 5′-ribonucleotides, the others are the 5′-deoxyribonucleotides; each was added in 1 mg./100 ml. of medium. (Monophosphate nucleotides).

§ ADP and ATP were each added in 0·1 mg./100 ml. of medium.

‖ Slight increase in growth at 3 days was followed in four days by a drop to less than 1×10^6. Marked decrease in all counts after initial growth was due to acid production.

and the purine and pyrimidine bases added were calculated to approximate the concentrations of the nucleotides, each of which was added at 1 mg./100 ml. medium. Growth was slightly greater in the medium containing nucleosides than that containing nucleotides. Although growth was increased in both media when the concentration of nucleosides or nucleotides was halved, considerably more growth resulted in the medium with the lower concentration of nucleosides. However, it was noted in the course of colony counts, that subcultures to plating agar from media containing nucleosides resulted in a large number of atypical rough colonies. Atypical colonies were rarely, if ever, noted in subcultures during the growth phase of strain 293 from media containing nucleotides. The substitution of purines and pyrimidines for the peptone of the control medium resulted in minimal growth. It was further noted that all colonial growth on subcultures to plating agar was extremely rough and atypical.

DISCUSSION

Since the tissue culture medium (TC medium 199) of Morgan *et al.* (1950) contains a large complement of amino acids, vitamins, purines, pyrimidines, inorganic salts and cholesterol, it was selected as the base medium from which to evolve a defined medium capable of supporting growth of *Mycoplasma gallisepticum* strain 293.

Table 6. *The relative influence of the 5′-monophospho-, ribo- and deoxyribonucleotides, the corresponding nucleosides, or the free purine and pyrimidine bases on growth of Mycoplasma gallisepticum strain 293 in the basal medium*

Media (basal medium* supplemented with:)	Time (days) 1	2	3	4
	Counts†/ml. medium ($\times 10^6$)			
I. 1 % (w/v) peptone (control medium)	630	705	120§	—
II. AMP, CMP, GMP, UMP, dAMP, dCMP, dGMP, TMP‡	59	81	12	< 1
III. Medium II with nucleotides at 1/2 concentration	53	105	62	< 1
IV. Nucleosides‡	54	100	6	< 1
V. Medium IV with nucleosides at 1/2 concentration	86	167	8	< 1
VI. Purines and pyrimidines‡	5	12	12	< 1
VII. Medium VI with purines and pyrimidines at 1/2 concentration	8	9	12	2

* Basal medium contained TC medium 199 buffered with 0·3 % (w/v) K_2HPO_4 and 10 % (v/v) swine serum.

† The inoculum contained 10×10^6 organisms/ml. of medium. All counts have been rounded off to the nearest 10^6 figure.

‡ The first four listed are the ribonucleotides, the others are the deoxyribonucleotides; each was added in 1 mg./100 ml. of medium. Calculated equivalent concentrations of the corresponding nucleosides (adenosine, cytidine, guanosine, deoxyadenosine, deoxycytidine, deoxyguanosine, and thymidine) and purines and pyrimidines (adenine, cytosine, guanine, uracil and thymine) were added to media IV and VI respectively.

§ Marked drop in all counts after initial growth was due to acid production.

This medium, however, was found to lack essential nutrients which could be supplied by adding serum + Bacto-peptone. Optimal growth of strain 293 occurred only in media containing the TC medium 199 + serum + peptone, omission of any of these three components resulted in little or no growth. It is likely that all the components of TC medium 199 are not essential for growth of strain 293. The present work and the report of Razin (1962) suggest that the purines and pyrimidines might be eliminated. It is also possible that the essential ingredients of this medium are not at optimal concentrations. However, it was felt that until defined reagents were found capable of adequately replacing the serum and peptone parts of the control medium, it would be unprofitable to determine the effects of the individual components of TC medium 199 on growth of strain 293.

The reports of Edward (1954), Chalquest & Fabricant (1960) and Razin & Knight (1960*b*) suggested that nucleic acid precursors or other known protein derivatives might replace either or both undefined components (serum, peptone) of the control medium. Professor R. W. Holley suggested (personal communication) that the preformed ribonucleosides or the 5′-monophosphate ribonucleotides of adenine, cytosine, guanine and uracil, rather than purines and pyrimidines might be essential for growth of organisms with a limited content of enzymes. Therefore these ribonucleosides or ribonucleotides were substituted for either or both serum and peptone of the control medium. Initial studies indicated that the ribonucleotides, rather than the ribonucleosides, would partially replace the peptone, but not the serum, in supporting growth of strain 293. However, better growth resulted when

peptone was replaced by the ribonucleotides AMP, CMP, GMP and the deoxyribonucleotide (5′-monophosphate isomer) thymidylic acid (TMP). It was found that the best growth occurred when each of these nucleotides was added in the same concentration. These findings parallel those of Razin (1962) in which he reported that mixtures of the 5′-monophosphate ribonucleotides were active in growth promotion of *Mycoplasma laidlawii* strain A.

Comparison of growth in media where the 5′-monophosphate nucleotides of RNA, or DNA or mixtures of these were substituted for the peptone (each nucleotide added to 1 mg./100 ml. medium) showed that the four RNA nucleotides, AMP, CMP, GMP and UMP, gave the poorest growth. The four DNA nucleotides, dAMP, dCMP, dGMP and TMP, or the RNA and DNA nucleotides together gave about four times as much growth as the RNA nucleotides alone. However better growth was given by the nucleotides AMP, CMP, GMP and TMP than by the four DNA nucleotides only. The experimental evidence indicates that in these media TMP may be essential for growth while UMP may be somewhat inhibitory.

In one experiment when undegraded DNA replaced the peptone in the control medium there was more growth than in the media containing nucleotides. However, in a later experiment various mixtures of nucleotides (supplementing the basal medium) were superior to undegraded DNA. Further work may resolve this discrepancy. The effect of ATP on growth of strain 293 in basal medium supplemented with nucleotides also needs clarification. Week-old preparations of ATP had a stimulatory effect on growth, while fresh preparations did not. The difference did not seem to be due to the possible breakdown of ATP to ADP, since ADP seemed to have an inhibitory effect on growth in one trial.

Comparison of growth of *Mycoplasma gallisepticum* strain 293 in the basal medium supplemented by the RNA and DNA nucleosides, 5′-monophosphate nucleotides or purines and pyrimidines, parallels in part the results reported by Razin (1962) for *M. laidlawii* strain A. He found that purines and pyrimidines would not stimulate growth of *M. laidlawii* strain A in his partially defined medium. The minimal growth of strain 293 in our basal medium supplemented with purines and pyrimidines was probably due to the basal medium alone. Furthermore, the markedly rough and atypical colonial growth from these media subcultured on plating agar, suggests that purines and pyrimidines may disrupt typical colonial development of strain 293. Growth of *M. gallisepticum* strain 293 was better in media containing nucleosides than in those containing nucleotides; similar results were reported by Razin (1962) for *M. laidlawii*. However, we noted that a large proportion of colonies plated from media with nucleosides was rough and atypical. Atypical colonies were not noted during the growth phase of strain 293 in media containing nucleotides. Since the only difference between the nucleosides and nucleotides was the phosphate linkage of the latter it is possible that the preformed nucleotides may be better utilized by strain 293 for subsequent typical colonial growth.

Further work is needed to establish the optimum concentrations and mixtures of nucleosides or nucleotides for growth of strain 293 in the basal medium. The concentration of 0·5 mg. of each nucleoside or nucleotide/100 ml. medium supported more growth than double this concentration. Further increase in concentration of nucleotides (and possibly nucleosides) was inhibitory.

We found that the minimal nucleotides which supported good growth of *Mycoplasma gallisepticum* strain 293 were adenylic, cytidylic, guanylic and thymidylic acids (5′-monophosphates). Future work may establish that the minimal nucleoside requirements of strains 293 may be adenosine, cytidine, guanosine and thymidine. This would parallel the minimal nucleoside requirements for growth of *M. laidlawii* strain A as reported by Razin (1962) in his partially defined medium.

We wish to thank Professor R. W. Holley (U.S. Plant, Soil and Nutrition Laboratory at Cornell University, Ithaca, New York) for his assistance and guidance in the work with nucleosides and nucleotides. We also thank Professors S. A. Zahler, R. E. MacDonald, H. B. Naylor and E. A. Delwiche of the Laboratory of Bacteriology at Cornell for helpful advice and the loan of some chemicals and equipment. This work was supported in part by Grant No. AI-04318-02 from the U.S. National Institutes of Health.

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Repression by Methionine of Cystathionase Formation in *Escherichia coli*

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SUMMARY

Cystathionase catalyses the formation of homocysteine from cystathionine; its formation in cultures of *Escherichia coli* is repressed by the presence of methionine in the growth medium, and to a similar extent to that shown with homocysteine methylase (the enzyme complex which catalyses the conversion homocysteine → methionine). Cystathionase, again like homocysteine methylase, is formed rapidly without concomitant growth when repressed organisms are transferred to a medium free from methionine; such enzyme formation is prevented by chloramphenicol, suggesting that *de novo* synthesis of protein is required. The co-repression by methionine of the two enzyme systems fortifies the evidence that cystathionase is a component of the normal pathway of methionine synthesis by *E. coli* and consequently that its substrate, cystathionine, is a normal intermediate. Cystathionase preparations also formed pyruvate from cysteine; this activity paralleled that of cystathionase itself when the methionine status of the medium was changed, and it is concluded that the same protein is responsible for both activities.

INTRODUCTION

In previous work in this laboratory Wijesundera & Woods (1962) showed that L-cystathionine was converted to homocysteine, pyruvate and ammonia by a cystathionase enzyme present in several strains of *Escherichia coli*. Since homocysteine is undoubtedly the immediate precursor of methionine in this organism (see review by Guest & Woods, 1962), these observations supported the view, originating from the properties of methionine auxotrophs of *E. coli* and other micro-organisms, that cystathionine is an earlier intermediate, though there remained some evidence to the contrary (see Discussion). The final reaction by which homocysteine is converted to methionine is a complicated one requiring folic acid derivatives and, in certain circumstances, also cobalamin as cofactors (Guest & Woods, 1962). However, the formation of the enzyme complex (homocysteine methylase), and indeed individual components of it, is repressed by the presence of methionine in the culture medium; the enzymes are, however, rapidly resynthesized when methionine is removed (Rowbury & Woods, 1961; Foster, Rowbury & Woods, 1963). The main objective of the present work was to examine the effect of growth with methionine on the cystathionase content of *E. coli* with conditions under which it could be strictly compared with the known effect on the homocysteine methylase complex.

METHODS

Organisms. The several auxotrophic strains of *Escherichia coli* used were maintained on slopes of tryptic digest of meat agar; stocks were subcultured monthly and stored at about 4° after incubation for 18 hr at 37°. Their growth requirements were: strain PA 15 (originally obtained from Dr Barbara Wright) serine or glycine; strain 121/176 (Davis & Mingioli, 1950) methionine or cobalamin; strain 26/18 (obtained from Dr B. D. Davis) homocysteine or methionine.

Organisms for the preparation of washed suspensions or cell-free extracts were grown on the glucose+lactate medium (*GL*) of Guest, Helleiner, Cross & Woods (1960) supplemented with glycine (10 mM), DL-methionine (0·3 mM) and DL-homocysteine (mM) for strains PA 15, 121/176 and 26/18, respectively; media were autoclaved for 7 min. at 115°. Growth was in conical flasks (1 l., containing 200–500 ml. medium) which were shaken in air for 16–18 hr at 37° with a circular motion of 220 rotations/min. and radius 2 cm. The size of the inoculum was so adjusted that growth reached the equivalent of 0·4–0·7 mg. dry wt. organism/ml. The cultures were centrifuged at 2200*g* for 15 min. and the organisms washed once with the culture volume of water. Inocula were derived from cultures (6 ml.) in the same medium seeded from an 18 hr culture on tryptic meat agar and incubated in ⊥-shaped tubes (150 × 150 × 16 mm.) which were shaken for 8 hr at 37° at 36 oscillations/min. of excursion 10 cm.

Cell-free extracts. Washed organisms were resuspended in water (equiv. 20–30 mg. dry wt./ml.) and subjected to ultrasonic vibration and further treatment as described by Guest *et al.* (1960); in some experiments the dialysed extract was also treated with Dowex-1 resin by the method of these authors. The protein content of the final preparation was determined spectrophotometrically according to Layne (1957).

Extract of heated *Escherichia coli* (strain PA 15) was prepared as by Guest *et al.* (1960); it was normally used at 0·1 ml./ml. of reaction mixture.

Cell-free extracts of certain other organisms used in one group of experiments only (Table 3) were made available by other workers in this laboratory.

Study of enzyme formation. Organisms in which enzyme formation had been repressed were obtained by growth in the presence of DL-methionine (10 mM) in medium *GL* supplemented with any other growth factor required by the strain under test. After washing in the culture volume of water, the harvested organisms were suspended (equiv. 3 mg. dry wt./ml.) in the same medium, but without methionine. The suspensions (300–500 ml. in 1 l. conical flasks) were shaken at 37° as described for the original growth of cultures. Samples were taken at intervals (usually 1 hr) for assessment of the extent of growth and for the preparation of cell-free extracts in which enzyme was determined.

Assay of cystathionase, serine hydratase (deaminase) and cysteine desulphhydrase (deaminase). Cell-free extract (equivalent to 1–5 mg. protein) was incubated at 37° for 30 min. in 2 ml. volumes of solution *B* which contained (in 133 mM phosphate buffer, pH 7·4): $MgSO_4$, 0·25 mM; pyridoxal phosphate, 0·25 mM; L-cystathionine, 10 mM (or DL-allocystathionine, 20 mM). The cystathionine was replaced, when required, by D- or L-serine (10 mM) or by L-cysteine (10 mM). The reaction was stopped (and proteins precipitated) by addition of trichloroacetic acid (0·5 ml.;

25 %, w/v). The supernatant fluid after centrifugation was used for the estimation of pyruvate by the method of Friedemann & Haugen (1943), as modified by Wijesundera & Woods (1962).

Methionine synthesis by cell-free extracts. This assay was based on the experiments of Guest *et al.* (1960) and was done by the procedure described by Rowbury & Woods (1961). When required DL-homocysteine (5 mM) was replaced as substrate by L-cystathionine (5 mM) or DL-allocystathionine (10 mM).

Detection and assay of homocysteine. The microbiological method described by Wijesundera & Woods (1962) with *Escherichia coli* strain 26/18 (which responds to homocysteine and methionine) was used, as was the chromatographic procedure used by those authors.

Chemicals. The L-cystathionine used was the batch described by Wijesundera & Woods (1962) isolated from the mycelium of a mutant of *Neurospora crassa*. DL-Allocystathionine was obtained from the California Corporation for Biochemical Research (Los Angeles, U.S.A.). Other special chemicals were as described by Guest *et al.* (1960).

RESULTS

Cystathionase activity of various strains of Escherichia coli

Earlier experiments in this laboratory on the cystathionase of *Escherichia coli* (Wijesundera & Woods, 1962) were made with a pyridoxin auxotroph (B166) and a wild strain (F); furthermore the cell-free extracts then used were prepared by shaking with minute glass balls. It was first necessary to establish that the strain (PA15) chosen for the present work contained the enzyme, and that it survived ultrasonic treatment of the organisms. Cell-free extracts of strain PA15 prepared in this way produced both a keto acid and homocysteine from L-cystathionine; the

Table 1. *The effect of pyridoxal phosphate and magnesium ions on cystathionase activity*

Ultrasonic extract (*Escherichia coli* PA15) was dialysed for 18 hr and one sample treated with Dowex-1 resin. Cystathionase activity was tested in solution *B* with the stated omissions. Incubation was for 30 min.

Omission	Cystathionase activity (μmole pyruvate/mg. protein/hr) with extract: Dialysed only	Dialysed and treated with Dowex-1
None	0·66	0·71
$MgSO_4$	0·70	0·63
Pyridoxal phosphate	0·27	0·08

keto acid was identified as pyruvic acid by the absorption spectrum of its 2,4-dinitrophenyl hydrazone, and the homocysteine both by paper chromatography and by the growth response of *E. coli* strain 26/18. Formation of pyruvate was used thereafter for the routine assay of cystathionase activity in ultrasonically prepared extracts. Pyruvate increased linearly with time up 60 min. and was maximal at a cystathionine concentration of 8 mM; the conditions chosen for normal tests were 10 mM-cystathionine and an incubation period of 30 min.

Cystathionase activity of dialysed extracts was increased about twofold by pyridoxal phosphate; a considerable increase in resolution of the enzyme was obtained by treatment with Dowex-1 resin (Table 1). Contrary to the results of Wijesundera & Woods (1962) the omission of Mg^{2+} had little effect with either the crude or dialysed enzyme preparations (Table 1).

Table 2. *Effect of homocysteine and methionine on the activity of cystathionase*

Cystathionase activity of an extract of *Escherichia coli* PA 15 was assayed in solution *B* with the stated additions. Incubation was for 30 min.

Addition	Concn. (mM)	Cystathionase activity (μmole pyruvate/mg. protein/hr)	Inhibition (%)
None	—	1·94	—
DL-Methionine	1·0	1·94	0
	10·0	1·96	0
DL-Homocysteine	0·1	1·84	5
	1·0	1·28	35
	10·0	0·16	92

Table 3. *Cystathionase activity of various strains of Escherichia coli and of other organisms*

The strains of *Escherichia coli* and *Salmonella typhimurium* were grown on basal medium *GL* and harvested at a similar stage of growth.

Organism	Cystathionase activity (μmole pyruvate/mg. protein/hr)
E. coli PA 15	1·05
3/62	1·05
121/176	0·93
113/3	0·76
26/18	0·00
Rhodopseudomonas spheroides	0·57
Acetobacter rancens	0·84
Micrococcus denitrificans	0·77
Salmonella typhimurium	0·68
Pseudomonas AM1	0·03
Penicillium Q176	0·03

The breakdown of cystathionine was not affected by the presence of equimolar concentration of DL-methionine, but was inhibited (90 %) by a similar concentration of DL-homocysteine and perceptibly by even one-hundredth of this (Table 2). Wijesundera & Woods (1962) also reported marked inhibition by homocysteine (and also by cysteine and glutathione). It was concluded that the cystathionase enzyme present in *Escherichia coli* strain PA 15 was essentially similar to that studied previously in other strains.

Distribution. The enzyme was absent from ultrasonic extracts of *Escherichia coli* strain 26/18 (Table 3), thus confirming the results obtained by Wijesundera & Woods (1962) who used a different method of disintegration. This strain 26/18 grows with either homocysteine or methionine, but not with cystathionine; a metabolic lesion in the conversion cystathionine → homocysteine was therefore expected. The enzyme was present in three other strains of *E. coli*, and in four other organisms, in activity

comparable to that of *E. coli* PA 15; with two organisms however (neither of which required methionine for growth) only slight activity was found with the type of preparation tested.

Conversion of cystathionine to methionine. In later work it was desired to compare the effect of growth of the organism in the presence of methionine on the conversion of: (*a*) cystathionine to homocysteine, (*b*) homocysteine to methionine, (*c*) cystathionine to methionine. It was first necessary to establish that (*c*) occurred by the successive action of the enzymes which catalysed (*a*) and (*b*); a direct reductive fission of cystathionine to methionine and glycine was a theoretical possibility, although Wijesundera (1954) found no evidence for this with intact organisms.

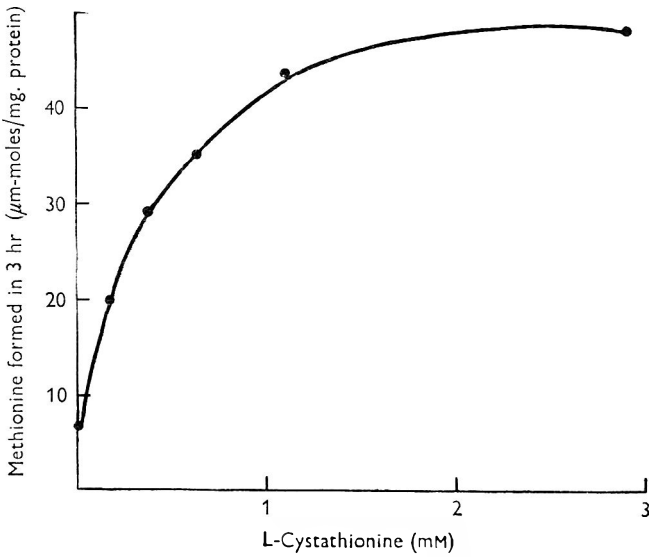

Fig. 1. The effect of the concentration of cystathionine on its conversion to methionine by cell-free extracts of *Escherichia coli* PA 15.

Table 4. *Conversion of cystathionine to methionine*

Cell-free extracts of the stated strains of *Escherichia coli* were used. The concentration of DL-allocystathionine was 10 mM; other substrates, 5 mM. Incubation was for 3 hr.

Strain of *E. coli*	Substrate	L-Methionine formed (μm-mole/mg. protein in 3 hr)
PA 15	L-Cystathionine	31
	DL-Allocystathionine	32
	DL-Homocysteine	70
26/18	L-Cystathionine	1
121/176	L-Cystathionine	0
121/176 + 26/18	L-Cystathionine	12

Methionine was formed by ultrasonic extracts from cystathionine (and allocystathionine) at about half the rate at which it was formed from homocysteine (Table 4). Synthesis increased linearly with time up to about 4 hr, and with increasing cystathionine concentration up to about 1·5 mM (Fig. 1); the K_m was

about 0·4 mM. At the lowest concentration of cystathionine tested (0·2 mM) there was about 30 % conversion to methionine.

Serine was required for significant synthesis of methionine from cystathionine (Table 5), as it is also for synthesis from homocysteine (Guest *et al.* 1960). Furthermore, a source of the folic acid cofactor system (either extract of heated organisms or tetrahydropteroylglutamate + cobalamin) was essential (Table 5). Tetrahydropteroylglutamate (in the absence of cobalamin) strongly inhibited the action of the extract of heated *Escherichia coli*. These properties are the same as those described for the conversion of homocysteine to methionine by ultrasonic extracts of the same strain (Guest *et al.* 1960) and would not be expected if cystathionine were cleaved directly to methionine and glycine.

Table 5. *Cofactor requirements for the conversion of cystathionine to methionine*

Methionine synthesis by extracts of *Escherichia coli* PA 15 was tested with L-cystathionine (5 mM) replacing homocysteine and with the other modifications to the reaction mixture indicated. Present, +; absent, −; EHC, extract of heated *E. coli* (see Methods section); H_4PtG, tetrahydropteroylglutamate.

L-Serine	EHC	H_4PtG	Cobalamin	L-Methionine formed (μm-mole/mg. protein in 3 hr)
+	+	−	−	41
−	+	−	−	4
+	−	−	−	0
+	−	+	+	46
+	+	+	−	7
+	−	−	+	0
+	−	+	−	0

It has been shown (Table 3) that extracts of *Escherichia coli* 26/18 lack cystathionase whilst it was known that extracts of strain 121/176 lack homocysteine methylase activity (i.e. ability to convert homocysteine to methionine) unless cobalamin is present (Guest *et al.* 1960; Foster, Tejerina & Woods, 1961). Neither extract alone converted cystathionine to methionine, but a mixture of the two was effective (Table 4), thus confirming the view that methionine formation from cystathionine requires the successive action of cystathionase and homocysteine methylase, and that in this particular case each organism provides the enzyme or enzymes lacking in the other.

Repression of the formation of cystathionase

Although it had already been established that growth in the presence of methionine represses the formation of homocysteine methylase by *Escherichia coli* PA 15 (Rowbury & Woods. 1961), further experiments with this enzyme system were included in the present series with cystathionase so that a strict quantitative comparison might be made. Ultrasonic extracts derived from organisms grown with DL-methionine (10 mM) had only about 20 % of the cystathionase activity of preparations from organisms grown without methionine. Loss of activity increased with increasing concentration of methionine in the growth medium; the sharpest decrease occurred between 0·1 and 1·0 mM, a range over which the effect on homocysteine methylase production was also greatest (Table 6). The degree of repression of enzyme formation at all methionine concentrations was however slightly greater with homocysteine methylase than with cystathionase (Table 6). The overall synthesis of

methionine from cystathionine by ultrasonic extracts of organisms grown with methionine was also greatly decreased compared with the controls, and to an extent similar to that of the decrease in homocysteine methylase activity (Table 7).

Following the terminology of Wijesundera & Woods (1960) organisms and derived enzyme preparations obtained after growth in the presence and absence of methionine will be referred to as 'inactive' and 'active', respectively, the former term now indicating a severely restricted content of both cystathionase and homocysteine methylase.

Table 6. *Effect of methionine on the formation of cystathionase*

The figures in parentheses indicate the percentage decrease in activity due to the presence of methionine during growth. Enzyme assays were on extracts of *Escherichia coli* PA 15 grown with the stated concentration of methionine.

DL-Methionine in growth medium (mM)	Homocysteine methylase (μm-mole methionine/mg. protein/hr)	Cystathionase (μmole pyruvate/ mg. protein/hr)
0	60	1·8
0·001	58 (4)	1·6 (10)
0·01	50 (16)	1·5 (17)
0·1	39 (35)	1·4 (22)
1	11 (81)	0·6 (67)
10	8 (86)	0·4 (78)

Table 7. *Effect of growth with methionine on the conversion of cystathionine to methionine*

The figures in parentheses indicate the percentage decrease in methionine formed due to the presence of methionine in the growth medium. Extracts of *Escherichia coli* PA 15 harvested after growth with the stated concentration of methionine were used.

DL-Methionine in growth medium (mM)	L-Methionine formed (μm-mole/mg. protein in 3 hr) from	
	Homocysteine	Cystathionine
0	83	52
0·001	75 (10)	42 (19)
0·01	71 (15)	36 (31)
0·1	67 (19)	30 (42)
1	21 (75)	15 (71)
10	18 (78)	10 (81)

When 'active' organisms were transferred to a medium containing methionine and incubated, the cystathionase activity of ultrasonic extracts decreased exactly in proportion to the extent of growth. It appears that, as in the case of homocysteine methylase (Rowbury & Woods, 1961), there is no destruction of cystathionase and that it is simply 'diluted out' by new organisms containing only limited amounts of the enzyme.

Effect of homocysteine. Methionine is the ultimate product of the metabolic pathway in which cystathionase presumably functions and it represses the formation of this enzyme. Homocysteine is the immediate product and has been shown above to inhibit the activity of the enzyme; it has however only a small effect on the formation of the enzyme. The presence of DL-homocysteine (10 mM) during growth resulted only in a 20 % decrease in cystathionase activity, an effect which given was by DL-methionine at one-hundreth of the concentration (Table 6).

Relief from repression. When 'inactive' organisms were transferred to a methionine-free medium their cystathionase content (as judged by the activity of ultrasonic extracts) increased rapidly for 3 hr (Fig. 2); the major part of this increase occurred during the first 2 hr, a period during which there was no significant growth. The rate of increase was similar to that of homocysteine methylase, which was assayed in the same cell-free preparations. As expected, the organism also

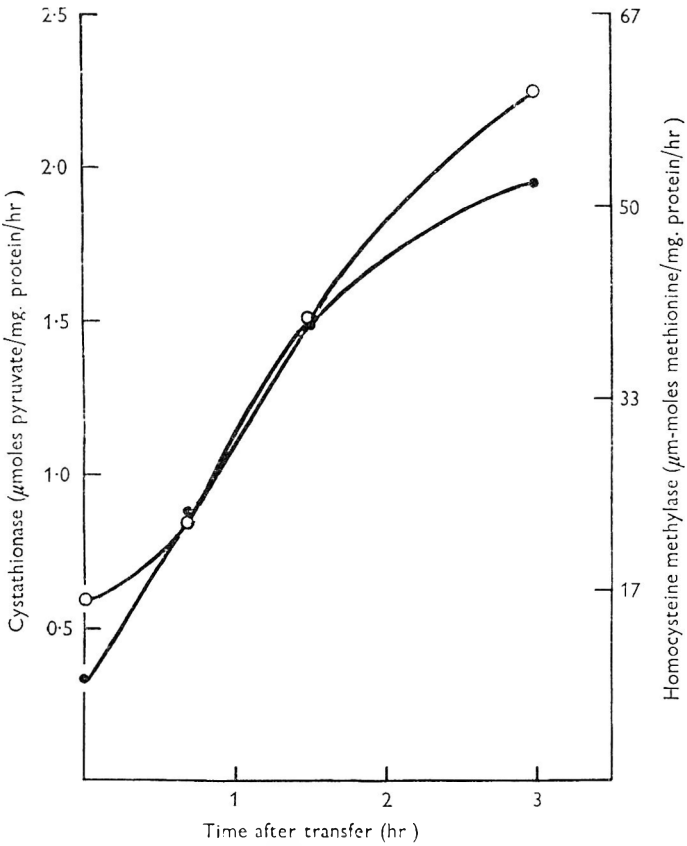

Fig. 2. Appearance of cystathionase (○) and homocysteine methylase (●) activity in *Escherichia coli* PA 15 following transfer from a methionine-rich to a methionine-free medium. Enzyme activity determined in cell-free extracts of organisms harvested at the stated time.

regained the ability to bring about the over-all conversion (in the presence of serine) of cystathionine to methionine (Fig. 3); the relative rate of synthesis of methionine from cystathionine and homocysteine remained constant during the recovery period.

The increase in cystathionase (and homocysteine methylase) activity on transfer of 'inactive' organisms to methionine-free medium did not occur when chloramphenicol was added (Fig. 4). Since the main increase takes place in advance of growth this effect cannot be a secondary one due to inhibition of growth and provides evidence that relief from repression requires synthesis of new protein; further evidence for this in the case of homocysteine methylase was given by Rowbury & Woods (1961).

Specificity of cystathionase

Allocystathionine. This stereoisomer of the substrate was tested (DL-form) arbitrarily at twice the optimal concentration of L-cystathionine and was degraded at about the same rate (Table 8). The individual isomers of DL-allocystathionine were

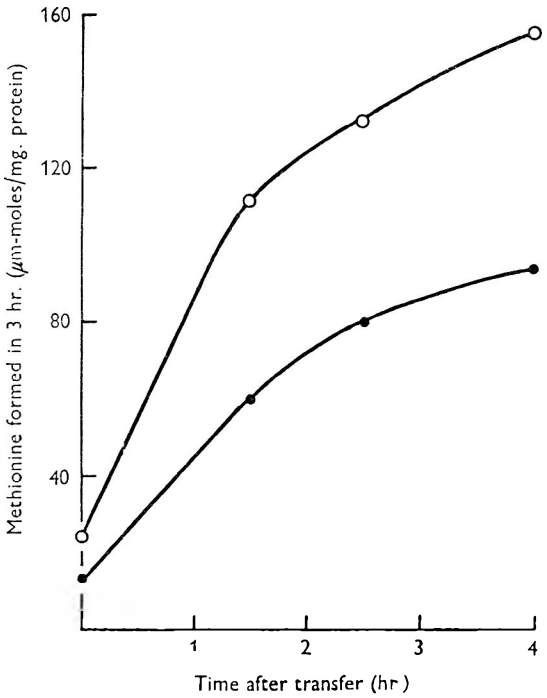

Fig. 3. Relative activity of *Escherichia coli* PA 15 in converting homocysteine (○) and cystathionine (●) to methionine following transfer from a methionine-rich to a methionine-free medium. Activity determined in cell-free extracts of organisms harvested at the stated time.

Table 8. *Pyruvate production from certain amino acids by extracts of Escherichia coli*

All substrates except DL-allocystathionine (20 mM) were present at a final concentration of 10 mM.

Strain of *E. coli*	Substrate	Pyruvate formed (μmole/mg. protein/hr)
PA 15	L-Cystathionine	1·3
	DL-Allocystathionine	1·2
	D-Serine	0·8
	L-Serine	0·1
	DL-Serine	0·8
	L-Cysteine	0·6
	DL-Homoeysteine	0·0
26/18	L-Cystathionine	0·0
	L-Cysteine	0·0
	L-Serine	0·1
	D-Serine	0·3

not available for testing, but, unless the enzyme preparations contained a homocysteine racemase, it is certain that D-allocystathionine (which contains a L-homocysteine residue) is the isomer attacked since L-methionine is formed by the further action of homocysteine methylase (Table 4). It cannot, however, be con-

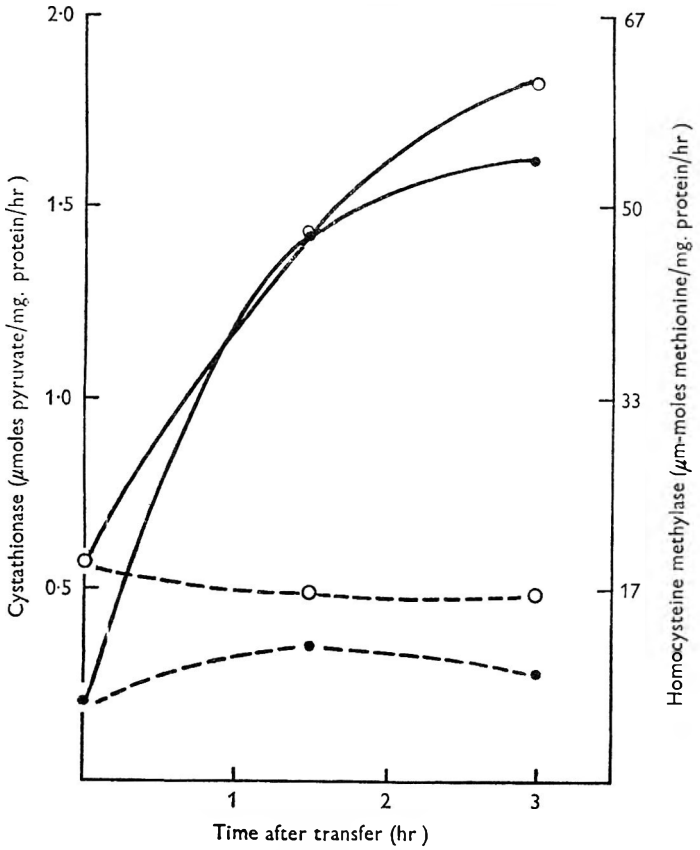

Fig. 4. Formation of cystathionase (○) and homocysteine methylase (●) in the presence (– – –) and absence (——) of chloramphenicol (0·125 mM). Activity determined in cell-free extracts prepared at intervals after transferring the organisms from methionine-rich to methionine-free medium.

Table 9. *Effect of methionine on the formation of* D-*serine dehydratase (deaminase) and* L-*cysteine desulphhydrase (deaminase)*

Extracts of *Escherichia coli* PA 15 harvested after growth with the stated concentration of methionine were used for the enzyme assay.

DL-Methionine in growth medium (mM)	Pyruvate formed (μmole/mg. protein/hr) from	
	D-Serine	L-Cysteine
0	0·64	0·31
0·001	0·73	0·28
0·01	0·72	0·24
0·1	0·68	0·18
1	0·59	0·05
10	0·59	0·03

cluded that L-allocystathionine (containing a D-homocysteine residue) is not attacked since any D-methionine formed would not have been detected by the microbiological assay used. DL-Allocystathionine, which, unlike cystathionine, was available commercially, was used in some later experiments.

Cysteine. Crude cell-free extracts formed pyruvate from L-cysteine at about a half the rate from L-cystathionine (Table 8). No attempt was made by purification procedures to determine whether cysteine deaminase activity was due to a distinct enzyme or was a function of the cystathionase enzyme itself. Instead, techniques suggested by the preceding work were used.

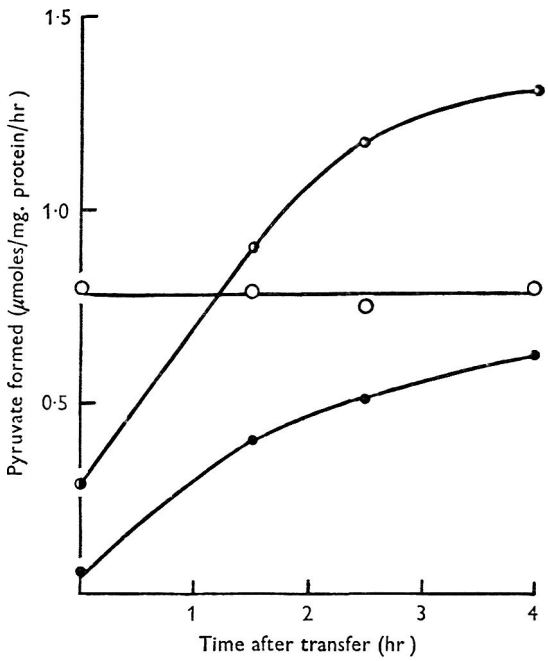

Fig. 5. Changes in the activity of the L-cysteine desulphhydrase (●), D-serine dehydratase (○) and cystathionase (◑) of *Escherichia coli* PA 15 following transfer from methionine-rich to methionine-free medium. Activity, assessed in each case in terms of pyruvate formed, was determined in cell-free extracts of organisms harvested after the time stated.

Ability to metabolize L-cysteine was decreased to 10 % by growth of the organism in the presence of methionine; as with cystathionase the sharpest fall of activity occurred in the range 0·1–1·0 mM-methionine (Table 9). Furthermore, reappearance of cystathionase on transfer of the organisms to methionine-free medium was accompanied by reappearance of cysteine desulphhydrase activity (Fig. 5). *Escherichia coli* strain 26/18, which lacks cystathionase, also lacks cysteine desulphhydrase (Table 8) it is unlikely that a single mutation would affect the production of two proteins. Finally the degradation of cysteine by cell-free preparations of strain PA 15 was inhibited 90 % by DL-homocysteine (10 mM) as was also the case with cystathionine as substrate (Table 2). It is concluded provisionally that cystathionine and cysteine are attacked by the same enzyme.

Serine. Crude cell-free extracts of *Escherichia coli* PA 15 formed pyruvate from

D-serine (though not from L-serine) at two-thirds the rate from cystathionine (Table 8). In this case, however, it is clear from investigations analogous to those used with cysteine that a different enzyme catalyses the reaction. Activity was not significantly decreased by growth in the presence of methionine (Table 9), nor did it increase in parallel with increase of cystathionase (Fig. 5). The strain of *E. coli* (26/18) deficient in cystathionase contained an active D-serine deaminase (Table 8).

DISCUSSION

The growth response of methionine auxotrophs of *Escherichia coli* to possible precursors suggests strongly that cysteine is converted to methionine, as in Neurospora, through the successive intermediate formation of cystathionine and homocysteine (Lampen, Roepke & Jones, 1947; Gots & Koh, 1950; Horowitz, 1947). Cystathionine is, however, contra-indicated as an intermediate in *E. coli* by the work of Bolton, Cowie & Sands (1952), who showed that the addition of unlabelled cystathionine to cultures growing with [^{35}S] sulphate as sole source of sulphur did not, as would be expected, decrease the incorporation of ^{35}S into methionine. Wijesundera & Woods (1962) established the presence in the organism of an enzyme which attacked cystathionine (cystathionase) and whose specific products were homocysteine, pyruvate and ammonia; the case for cystathionine as an intermediate was thus strengthened since active enzymic mechanisms (homocysteine methylase) for the further conversion of homocysteine to methionine had been described (see review by Guest & Woods, 1962).

Furthermore, in the present work cell-free extracts of the test strain of *Escherichia coli* catalysed the over-all formation of methionine from cystathionine under conditions, discussed in Results, which make it clear that homocysteine is an intermediate. With intact organisms of the same strain, Wijesundera (1954) found only slight conversion of cystathionine to methionine (10 % of the rate with homocysteine); it is possible that the intact organism has limited permeability to exogenous cystathionine, a possibility which might also explain the results (quoted above) of Bolton *et al.* (1952).

The phenomenon of enzyme repression (in the terminology of Vogel, 1957) provides an interesting and novel approach to the determination of the status of a possible intermediate in a biosynthetic pathway. In several cases the ultimate product is known to repress the formation of enzymes which catalyse a number of steps in the reaction sequence, though not always to the same extent. Examples of this are the biosynthesis of arginine and pyrimidine by *Escherichia coli* and of histidine by *Salmonella typhimurium* (Gorini & Maas, 1958; Yates & Pardee, 1957; Ames & Garry, 1959). One of the earliest described cases of enzyme repression was that of homocysteine methylase, the enzyme complex catalysing the last step of methionine synthesis by *E. coli*; the initial observations of Wijesundera & Woods (1953) and Cohn, Cohen & Monod (1953) were amplified by Wijesundera & Woods (1960) while Rowbury & Woods (1961) showed that relief from repression entailed synthesis of new protein. The present results establish that formation of cystathionase, as well as of homocysteine methylase, is repressed by methionine. It seems reasonable to take this as *a priori* evidence that cystathionase (and therefore cystathionine) is a functional entity in methionine biosynthesis, especially since cystathionase

formation is not markedly repressed by its own immediate product (homocysteine). It is difficult to envisage any explanation other than a control mechanism for this specific effect of methionine. The slight repression caused by homocysteine (at high concentrations) is probably due to methionine formed from it by the growing organism. It may be noted that homocysteine does exhibit the second type of feedback control shown by products, that is, inhibition of the activity of the enzyme.

The extent of repression of cystathionase (and the rate of new enzyme formation on relief from repression) was quantitatively similar to that with homocysteine methylase. Recent studies in this laboratory, reported briefly by Rowbury (1962), show that enzymes which catalyse the synthesis of cystathionine are also repressed, and to a similar degree, by methionine. It is possible therefore that control of methionine synthesis is another example of the 'co-ordinate repression' shown to occur in the histidine pathway by Ames & Garry (1959).

Application of the principles of enzymic repression also permits a novel approach to obtaining evidence, without purifying an enzyme preparation, as to whether two distinct chemical events are or are not manifestations of the activity of the same enzyme. In the present case cysteine desulphydrase (deaminase) activity disappeared and reappeared in parallel with that of cystathionase; this and other evidence led to the conclusion that both activities are a function of the same protein. On the other hand, D-serine dehydratase (deaminase) activity was not affected when cystathionine was repressed or resynthesized; this supports the conclusion of Wijesundera & Woods (1962), based on purification and selective inactivation, that the enzymes which attack D-serine and cystathionine are distinct entities.

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Correlation of the *in vivo* Action of Streptomycin on Survival and on Protein Synthesis by *Mycobacterium fortuitum*

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SUMMARY

When a sensitive population of *Mycobacterium fortuitum* is exposed to streptomycin, two effects of the drug on viability can be experimentally differentiated: (1) a sublethal injury from which the cells recover once streptomycin is removed, and (2) a lethal injury from which no recovery occurs. Protein synthesis is markedly inhibited by streptomycin before sublethal or lethal effects on viability are noted. The effect on protein synthesis of exposure to streptomycin cannot be reversed by washing the cells by centrifugation, but protein synthesis recovers its original rate on further incubation after streptomycin is removed. It is postulated that streptomycin causes both sublethal and lethal injury to cells by irreversibly blocking protein synthesis, possibly by inactivating ribosomes.

INTRODUCTION

It has been suggested (Hurwitz & Rosano, 1962) that exposure of sensitive cells to streptomycin may result in two categories of reactions: (1) primary reactions preceding or accompanying the loss of viability of the cell, and (2) secondary reactions following the death of the cell and possibly having no relation to the lethal effect of the drug. Many of the reactions which have been shown to occur after exposure of sensitive cells to streptomycin may fall into the second category. A comparison of the kinetics of the proposed lethal reactions with the kinetics of the effects of the drug on growth and on survival should help resolve these two types of reaction.

Evidence has been presented supporting the hypothesis that streptomycin exerts its lethal action on sensitive cells by blocking a late stage of protein biosynthesis. Erdös & Ullmann (1959) reported that streptomycin blocked the reactions concerned with the transfer of amino acids from aminoacyl ribonucleic acid (RNA) to ribosomes. Spotts & Stanier (1961) later proposed that streptomycin might interfere with the attachment of messenger RNA to ribosomes in sensitive cells. Flaks, Cox & White (1962) showed that the antibiotic inhibited the polyuridylic acid enhancement of synthesis of polyphenylalanine by ribosomal preparations from sensitive *Escherichia coli* cells.

To prove that the site of the lethal action of streptomycin occurs at a late stage of protein synthesis, one must show that the block of protein synthesis occurs before

or at the same time as loss of viability, that the block is complete enough to account for loss of viability or other possible intermediate effects, and that the block is irreversible and irreparable when the cells lose their viability.

Some attempts at evaluating the kinetics of the effects of streptomycin on protein synthesis and on survival of viable cells have been made (Hurwitz, Rosano & Landau, 1962). The effects of the antibiotic on both parameters were found to be complex. The decline in viable count resulting from exposure to streptomycin was preceded by a period during which the viable count remained unchanged. The duration of this period of apparent bacteriostasis was inversely related to the concentration of streptomycin. The effect of streptomycin on protein synthesis likewise appeared to occur in two stages. The first observed effect was a reduction of the initial rate of synthesis which appeared to coincide with the duration of apparent bacteriostasis. The second stage, which seemed to coincide with the beginning of the decline in viability, was observed as a further decrease in the rate of protein synthesis leading to its eventual cessation.

In another study (Hurwitz, Landau & Doppel, 1962), it was observed that a brief exposure of *Escherichia coli* to 1 μg. streptomycin/ml. caused a delay in onset of growth of survivors plated after the drug was removed. This reaction to a prior exposure indicated the possibility of investigating an early type of lesion not previously described, i.e. a residual effect found after streptomycin was removed.

The slower growing *Mycobacterium fortuitum* was used in the following experiments because it enabled greater resolution of the sequence of events preceding and occurring during the loss of viability. More accurate comparisons of the kinetics of biological events, following exposure to streptomycin, with the kinetics of the proposed biochemical mechanism of the lethal action of streptomycin could therefore be made.

METHODS

The culture of *Mycobacterium fortuitum* was obtained through the courtesy of Dr L. Wayne. It grows in Difco–Dubos broth base predominantly as singly dispersed cells with about 10 % of the units appearing as clumps. Vigorous shaking of the suspension on a Vortex Jr. mixer reduces the incidence of clumps to about 5 %. Generation time in the Dubos broth base is slightly less than 3 hr.

When plated on Dubos broth base containing $1\frac{1}{2}$ % agar, colonies first appear as round, white, dense, discernible dots after 40–48 hr of incubation at 37°. Colony counts were repeated every 24 hr until new colonies failed to appear. Appearance of a colony was marked on the bottom of the plate with a coloured felt-tip marker using a different colour for each day's count.

Each experiment was performed with cells growing exponentially in flasks fitted with a Coleman tube sidearm so that nephelometric changes in cell density could also be measured with a Coleman Model 7 Nephelometer.

Special care was taken both with the broth base and with the agar to avoid presence of dust particles and lint which interfered with nephelometric measurement and with recognition of newly emerged colonies on plates.

The bacteria were brought into log phase for each experiment by inoculating flasks containing 20 ml. of the broth with about 3×10^5 organisms (estimate based on nephelometric readings of a growing suspension) 16 hr before the start of the

experiment. At the beginning of the experiment, the suspension contained about 10^7 bacteria/ml. The cellular suspensions were aerated by rotation on a New Brunswick water bath at 37°.

Protein synthesis was measured as incorporation of ^{14}C-leucine into the hot-acid insoluble fraction of the bacteria. Since labelled leucine was incorporated from a non-defined medium, the true specific activity of the incorporated leucine is not known. Comparative results were therefore always obtained within one experiment.

For viable counts the bacteria were diluted in sterile Dubos broth base before plating. The volume after each dilution was kept at 1·0 ml. and the suspension was shaken vigorously (2 min. on a Vortex Jr. Mixer) before each sampling to ensure maximum dispersion of cells.

RESULTS

Effects of streptomycin on growth and survival of Mycobacterium fortuitum

Mycobacterium fortuitum, when plated on Dubos broth base agar, first appeared as colonies after 40–48 hr incubation at 37°. Less than 1 % of the colonies took longer than 48 hr to appear. As seen from Fig. 1, a considerable number of colonies failed to appear in 48 hr when exposed to streptomycin, but did appear from the 3rd to the 6th day after plating. Furthermore, as the time of exposure to any lethal concentration of streptomycin increased, the percentage of colonies showing delayed appearance also increased.

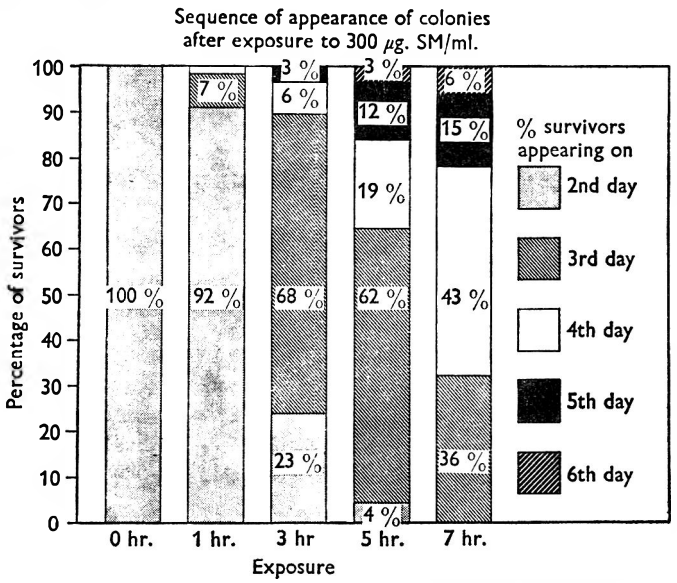

Fig. 1. Delayed appearance of macrocolonies of surviving cells of *Mycobacterium fortuitum* after exposure to 300 μg. streptomycin/ml.

It should be noted that in this illustration the percentage of colonies appearing on any given day is a measure of the percentage of total survivors and not the percentage of the number of bacteria actually plated. Those bacteria which failed to produce macrocolonies, i.e. those rendered non-viable by exposure to streptomycin, represent another parameter which will be described below.

Once the colony had become visible, its subsequent increase in size was not affected by the length of time it took for the colony to appear. From this it is inferred that the delay in appearance of the colony resulted from a delay in onset of growth and not from a slower growth rate.

The delay in onset of growth was regarded as a form of injury to the cell which could be operationally differentiated from total loss of ability to produce macrocolonies. Those colonies which appeared on the 2nd day are defined as 'uninjured', those appearing after the 2nd day as 'injured', and those which fail to appear as 'non-viable'.

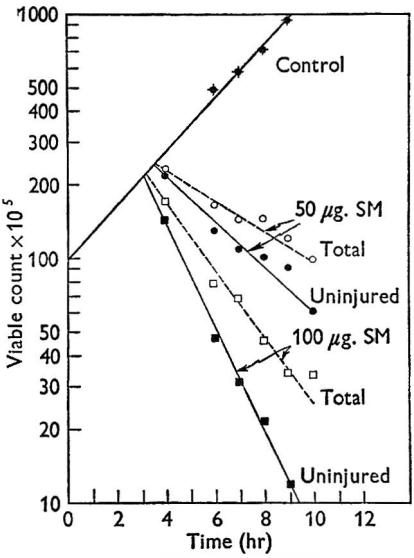

Fig. 2. Kinetics of the decline in total viable count and in number of 'uninjured' cells resulting from exposure of exponentially growing *Mycobacterium fortuitum* to streptomycin. 'Uninjured' organisms are operationally defined as those producing macrocolonies within 48 hr after being plated.

It should be pointed out, however, that a delay in onset of growth equivalent to the time for several generations might not be detectable by the procedure used, since colonies were counted as early as they could be detected. Under these conditions, it was not possible to precisely regulate the size of the colony at the time of counting, and variations in size of one or more diameters might not be noted. It was therefore possible, indeed likely, that many colonies scored as 'uninjured' may have sustained sufficient injury to delay onset of growth by several hours. One cannot, therefore, rule out the possibility that any exposure to streptomycin may cause some delay in onset of growth and that the measurements herein reported represent only the more easily distinguishable results of relatively severe effects of the drug.

Figure 2 illustrates the effect of exposure to streptomycin on 'injury' and on loss of viability at two concentrations of streptomycin. The dotted lines represent the total number of survivors, while the unbroken lines represent the survival of 'uninjured' cells. At both streptomycin concentrations, the total number of survivors and the number of 'uninjured' cells decreased after a preliminary period

of increase at the same rate as the control. As time of exposure to streptomycin increased, the extent of injury, as measured by the decrease in the total number of survivors or by the decrease in the number of 'uninjured' cells, also increased. As concentration of streptomycin increased, the decrease in number of survivors began earlier and the extent of injury became greater.

The results show that two effects of streptomycin on sensitive cells could be experimentally differentiated: (1) a sublethal injury from which the cells recover once streptomycin has been removed, and (2) a lethal injury from which no recovery occurred. Studies were therefore undertaken to determine how the kinetics of the block of protein synthesis were related to the kinetics of the sublethal and lethal effects of streptomycin.

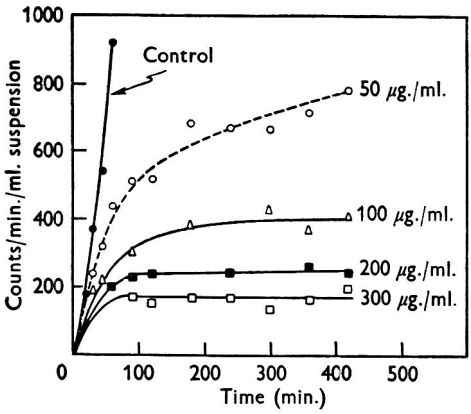

Fig. 3

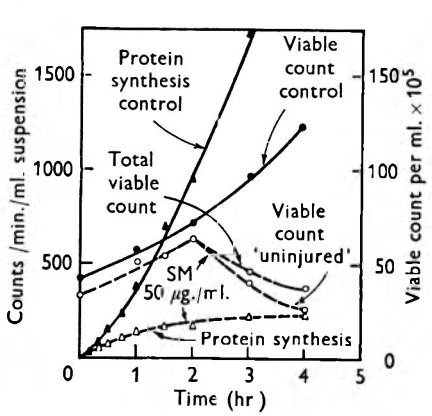

Fig. 4

Fig. 3. Kinetics of the decline in rate of protein synthesis resulting from exposure of exponentially growing *Mycobacterium fortuitum* to streptomycin. Protein synthesis was measured as the incorporation of ^{14}C-leucine into the protein fraction of the bacteria. The numbers at each curve represent the concentration of streptomycin in μg./ml. The viable count at zero time was 1×10^7/ml. The medium, Dubos broth base, was supplemented at zero time with 0·167 μg. ^{14}C-leucine/ml., specific activity 0·20 μC/μg.

Fig. 4. Comparative kinetics of the effect of streptomycin on protein synthesis and on viable count of *Mycobacterium fortuitum*. Streptomycin concentration, 50 μg./ml. Protein synthesis was measured as incorporation of ^{14}C-leucine into the protein fraction. 'Uninjured' organisms are operationally defined as those producing macrocolonies within 48 hr after being plated. The medium was supplemented at zero time with 0·167 μg. ^{14}C-leucine/ml., specific activity 0·20 μC/μg.

Effects of streptomycin on protein synthesis by Mycobacterium fortuitum. Figure 3 shows the effects of various concentrations of streptomycin on protein synthesis by growing suspensions of *Mycobacterium fortuitum*. Protein synthesis was markedly inhibited by as little as 50 μg./ml. of the antibiotic.

In Fig. 4 an experiment is illustrated in which the effects of 50 μg. streptomycin/ml. on the formation of macrocolonies and on protein synthesis were determined on the same cellular suspension. Although protein synthesis was strongly inhibited after 1 hr of exposure, the viable count at the end of 2 hr had increased at the same rate as the control. A decrease in viable count and the appearance of 'injured' cells were first detected after 3 hr of exposure to streptomycin.

Macrocolonies obviously cannot be formed from single cells in the absence of protein synthesis. Since protein synthesis was extensively blocked by 50 μg. streptomycin/ml. 1–2 hr before injury to the cell was detectable by plate counts, some method of recovery of protein-synthesizing ability must be available to the cells after streptomycin is removed. Experiments were therefore performed to determine how much residual protein-synthesizing ability remained after removal of the drug by washing, and whether the early block of protein synthesis could be reversed.

In these experiments, exponentially growing organisms were exposed to 50 μg. streptomycin/ml. for varying periods of time. Streptomycin was removed by centrifugation of the suspension at 14,000 rev./min. for 5 min. and the organisms were resuspended in growth medium containing ^{14}C-leucine for further incubation.

The washing procedure itself resulted in from 20 to 50 % loss of viable organisms. 100 % of the organisms could be recovered if the centrifugation was prolonged for 15 min., a time period which would handicap the experiment. Initial experiments using organisms not exposed to streptomycin showed that the decrease in rate of incorporation of ^{14}C-leucine into the protein fraction corresponded to the loss of viable organisms resulting from incomplete centrifugation. Similar results were obtained when recovery of viable organisms and protein-synthesizing ability were determined with suspensions which had been minimally exposed to streptomycin (zero time sample). It was therefore concluded that the washing procedure itself had no effect on protein synthesis, *per se*, and that streptomycin was effectively removed under these conditions.

In the first of the following experiments, the residual protein-synthesizing ability was determined after exposure of the organisms to streptomycin for 1 hr (see Fig. 5). The rate of incorporation of leucine was linear for at least 1 hr after streptomycin was removed and indicated that about 80 % of the protein-synthesizing ability had been lost after 1 hr of exposure to the drug, although the viable count was still increasing at the same rate as the control. Furthermore, it appeared that this loss of protein-synthesizing ability was not immediately reversible on removal of streptomycin.

In later experiments (Fig. 5), exposure to streptomycin was increased to 6 hr. The progressive loss in residual protein-synthesizing ability corresponded fairly well with the decrease in rate of incorporation of leucine, observed in Fig. 3, after addition of 50 μg. streptomycin/ml. Even after 6 hr of exposure to this concentration of streptomycin some protein-synthesizing ability remained intact.

In the last experiment of this series (Fig. 6), an attempt was made to determine whether the cells could recover their original rate of protein synthesis on prolonged incubation after removal of streptomycin. After exposure to 50 μg. streptomycin/ml. for 20, 40 and 60 min., the cells were washed free of streptomycin and resuspended in the growth medium supplemented with ^{14}C-leucine. Incubation, in the absence of streptomycin, was continued for 5 hr and samples were removed for the determination of the rates of incorporation of label from leucine into the protein fraction. As the duration of the exposure to the drug increased, the initial rate of protein synthesis decreased and it took longer for recovery of protein synthesis to occur.

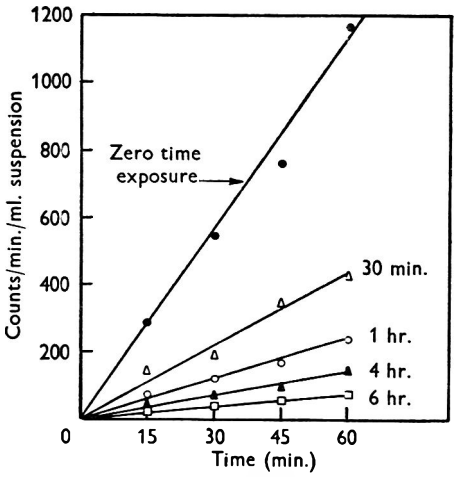

Fig. 5

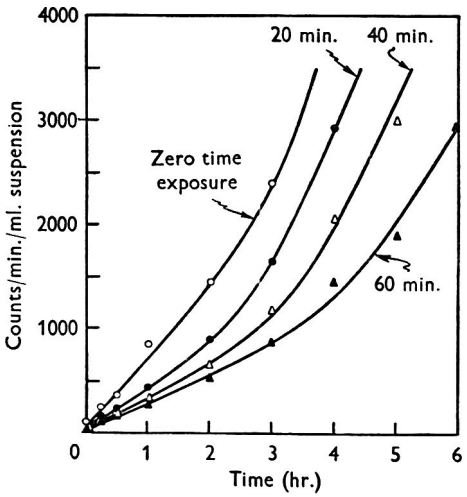

Fig. 6

Fig. 5. Effect of duration of exposure of *Mycobacterium fortuitum* to 50 μg. streptomycin/ml. on the residual rate of incorporation of ^{14}C-leucine into the protein fraction. After the indicated exposure, the organisms were washed by centrifugation to remove streptomycin and resuspended in growth medium. The viable count at zero time was adjusted to 1×10^7 viable organisms/ml., and the medium was supplemented with 0·83 μg. ^{14}C-leucine/ml., specific activity 0·14 μC/μg.

Fig. 6. Kinetics of recovery of protein-synthesizing ability of *Mycobacterium fortuitum* after incubation following removal of streptomycin. The conditions of the experiment were the same as described in Fig. 5 except that the subsequent incubation after removal of streptomycin was extended to 6 hr.

DISCUSSION

The effects of streptomycin on growth and survival of *Mycobacterium fortuitum* have been shown to correspond to the effects observed with *Escherichia coli* (Hurwitz, Rosano & Landau, 1962) but can be measured with greater resolution. In *E. coli*, on exposure to streptomycin, an apparent bacteriostasis was followed by a decline in viability. Under different experimental conditions, a delay in onset of growth was observed (Hurwitz, Landau & Doppel, 1962) which may correspond to the bacteriostasis noted above. All of the above parameters were functions of streptomycin concentration and time of exposure. With *M. fortuitum*, exposure to streptomycin caused a sublethal effect, which could be measured as a delay in onset of growth, and a lethal effect observed as a decline in viability. The extent of both these effects also varied with concentration of streptomycin and with time of exposure.

Streptomycin inhibited protein synthesis in both organisms and the general kinetics of inhibition were similar. With both organisms, there was a decrease in the rate of protein synthesis from the time of addition of the drug, followed by a further decline in rate and eventual stoppage. With *Escherichia coli*, only the secondary decline in rate seemed to correspond with loss of viability (Hurwitz, Rosano & Landau, 1962). With *Mycobacterium fortuitum*, as much as 80 % inhibition of protein synthesis had no apparent effect on the viable count.

As pointed out earlier, if the lethal action of streptomycin is caused by an inhibition of a late stage of protein synthesis, it must be shown that (*a*) the block occurs before or at the same time as the decline in viability, (*b*) that the block is sufficient to account for loss of viability, and (*c*) that the block is irreversible and irreparable. In addition, as a result of the finding of the sublethal injury described in this report, it must also be shown that the block is early and extensive enough to cause the demonstrated delay in onset of growth, and that it is reparable under conditions where the effect of the drug remains sublethal.

The inhibition of protein synthesis begins very early. In fact, an extensive block in protein synthesis was observed considerably before any effect on the rate of cell division became apparent. This finding raised the question of how cells can divide and eventually produce macrocolonies after their protein-synthesizing capacity has been largely inhibited. Either the inhibition of protein synthesis by streptomycin can be reversed or the lesion can be repaired.

Since an immediate reversal of the block resulting from the removal of streptomycin is ruled out by the experiment reported in Fig. 5, recovery of protein-synthesizing ability by repair or replacement seems more likely. It should be pointed out, however, that the experiments do not exclude the possibility that recovery of protein synthesis might result from a slow removal of streptomycin from some inhibitory binding site after plating.

As seen in Fig. 5, 80 % of the protein-synthesizing ability of the cells is lost after 1 hr exposure to 50 μg. streptomycin/ml., yet the rate of cell division is unaffected for at least an additional hour. In addition, no significant delay in onset of growth was observed even after 2 hr of exposure.

The 80 % loss of protein-synthesizing ability after 1 hr of exposure can be looked upon in two ways. Either 80 % of the cells have lost their ability to synthesize protein, while 20 % of the cells have not; or within each cell, on the average, 80 % of its protein-synthesizing ability is destroyed while 20 % remains intact. Recovery of ability to synthesize protein must occur if the cells are to resume their normal growth rate. It seems unlikely that cells which have completely and irreversibly lost their ability to synthesize protein after 1 hr of exposure to streptomycin would be capable of producing macrocolonies after 2 hr of exposure. For this to occur, cells containing no active protein-synthesizing apparatus would have to be able to rapidly synthesize a new apparatus to replace that rendered irreversibly inoperable. If synthesis of a new unblocked apparatus itself involves protein synthesis, as would be the case if ribosome degradation had occurred, it would be difficult to understand recovery of the totally inhibited cells.

On the other hand, if after 1 hr of exposure to streptomycin, 80 % of the protein-synthesizing ability of each cell is destroyed while 20 % remains intact, rapid recovery of protein synthesis and subsequent growth of each cell would be possible. Conforming to the data in Figs. 5 and 6, the progressive increase in delay of onset of growth might result from the progressive decrease in residual protein-synthesizing ability per cell and the consequent increasing time required for recovery of optimal rate of protein synthesis. Death of a cell or loss of viability would presumably result when insufficient residual protein-synthesizing ability remained to initiate synthesis of a new protein-synthesizing apparatus.

Since the observed effects of streptomycin on protein synthesis seem to adequately

explain both the delay in onset of growth and the loss of viability, it is concluded that some reaction in a late stage of protein synthesis is the site of bactericidal action of the drug. The occurrence of a block in a stage preceding peptide bond formation (Erdös & Ullmann, 1959) raises the possibility that exposure to streptomycin may result in an irreversible degradation or alteration of ribosomes in such a way as to prevent their participation in polypeptide formation.

Flaks & Witting (1963) have recently proposed that streptomycin kills by blocking formation of ribosomes. Our data would indicate that it is more likely that streptomycin promotes degradation or inactivation or ribosomes since the killing occurs too rapidly to result from dilution of the ribosomal content by cell division. When *Escherichia coli*, growing exponentially in nutrient broth, is exposed to 1 μg. streptomycin/ml., more than 90 % of the cells are rendered non-viable within 30 min. (Hurwitz, Rosano & Landau, 1962). With *Mycobacterium fortuitum*, under presently described conditions, exposure to 300 μg. streptomycin/ml. causes an 80 % loss of viability within 3 hr, the time required for one cell division.

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