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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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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.

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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 preferences 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.

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Characteristics and Identification of Oxidative Pseudomonads Isolated from Diseased Fish

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(*Received* 14 *August* 1964)

SUMMARY

Several characteristics of 20 strains of oxidative pseudomonads, all but one of which were isolated from freshwater fish, were determined. All strains oxidized glucose and gluconate, produced NH_3 from 1 % peptone, grew at 6, 12, 20 and 30° and produced cytochrome oxidase. Lipase was produced by 17 of the strains, while 15 produced gelatinase, and 16 produced fluorescent pigment. These and other characteristics used to identify fish-spoilage pseudomonads are also useful for identification of fish-pathogenic pseudomonads. Species determinations from the present data were not attempted. However, from previously published data on speciation of *Pseudomonas*, most of the 20 strains appeared to be closely related to or identical with *P. fluorescens*.

INTRODUCTION

Pseudomonads, which usually oxidize carbohydrates and produce fluorescent pigment, have been reported as causing diseases in warm-water and cold-water fish (Wolf, 1937; Rucker & Whipple, 1951; Rucker, Earp & Ordal, 1954; Schäperclaus, 1954; Seaman, 1951). The identification and separation of these organisms from the closely related aeromonads is desirable for diagnosis of the diseases and proper control measures. Current methods used for identification of these pseudomonads lack the precision needed for separation of closely related species or strains isolated from diseased fish. Recent studies on the taxonomy of the genus *Pseudomonas* have shown the inadequacies of the present classification (Rhodes, 1959; Colwell & Liston, 1961). Often speciation within the genus has been a matter of personal opinion, or has been based on particular tests considered important by the investigator. This situation is by no means limited to pseudomonads, since one has only to look to the closely related aeromonads to find the same disagreement on speciation (Ewing, Hugh & Johnson, 1961; Eddy, 1960, 1962). Sneath (1957*a*, *b*) stated that many schemes of taxonomy are catalogues rather than true classifications, and he discussed a computer method for comparing organisms for overall similarity. This method has been applied to several groups of bacteria including those in the genus *Pseudomonas* (Rhodes, 1961; Colwell & Liston, 1961; Lysenko, 1961). In our opinion the computer analysis method while having limitations is a sound and objective approach, which promises improvement in bacterial classification. The present work was undertaken for two reasons. First, it was desirable to determine the degree of homogeneity of characteristics of the oxidative pseudo-

monads isolated from diseased fish, and secondly it was hoped that, by the use of some of their characteristics, better methods of identification of fish-pathogenic pseudomonads could be established.

METHODS

Selection of cultures. The cultures used in this study (Table 1) were Gram-negative, polarly flagellate rods. With the exception of the two ATCC strains, all cultures came from this laboratory's collection, and all except no. 80 were isolated from fish, most of which were diseased. Culture no. 80 was included because it was isolated from a cold-blooded vertebrate. Characteristics of many of the cultures had been previously studied but not reported. To obtain the characteristics of the strains near the time of isolation, lyophilized cultures were used except in the case of the two ATCC strains and the most recent isolates P-1, P-2, P-3. To ensure purity, all test strains were streaked twice before use.

Table 1. *Strains of pseudomonads used*

Designation	Original source or source of isolation
P. fluorescens nos. 11250, 12633	American Type Culture Collection
1, 2, 3	Dr Troutman, Ohio
7	Quitman, Texas
11	Brook trout (*Salvelinus fontinalis*), Berlin, N.H.
21	*Pseudomonas* no. 28, Dr R. R. Rucker, State of Washington
22*b*	Pisgah Forest Hatchery, North Carolina
37	Adult brook trout (*S. fontinalis*)
49	Brook trout fingerling (*S. fontinalis*), Leetown, West Va.
50, 51	*Pseudomonas* Dr R. R. Rucker, State of Washington
66	Dr Prevost, Canada
80	Purple salamander (*Ambystoma* sp.), Leetown, West Va.
81	Black dace (*Rhinichthys atratulus*), Leetown, West Va.
86, 87, 88	Spirit Lake, Iowa
P-1	Fathead minnow (*Pimephales promelas*), Tennessee
P-2, P-3	Brook trout brood stock (*S. fontinalis*), Spring Run Trout Hatchery, Petersburg, West Virginia

Characters examined. Unless otherwise stated, all the following tests were repeated, all media steam sterilized, and all cultures incubated at 20–22° for 1 week.

1. Cell form and Gram reaction of 18–24 hr nutrient agar cultures.

2. Flagellation; Novel's (1939) staining method was used.

3. Hydrogen sulphide in Motility Sulfide Medium (Difco; Difco Laboratories, Detroit, Michigan, U.S.A.).

4. Nitrate reduction in nutrient broth containing 0·1 % (w/v) KNO_3; zinc powder (ZoBell, 1932) was used to detect false negatives. Tested after 1, 2, 7 days.

5. MR-VP in MR-VP (Difco) Broth. VP test performed after 48 hr and 1 week.

6. Gelatinase by Smith's modification of Frazier's technique in the *Manual of Microbiological Methods* (1957).

7. Lecithinase in egg yolk agar; method and medium as in the *Manual of Microbiological Methods* (1957).

8. Levan production on nutrient agar containing 4 % (w/v) sucrose (Klinge, 1960). A thick slimy growth was considered positive for levan production.

9. Ammonia production as indicated by increased alkalinity of 1 % (w/v) peptone broth containing pH indicator.

10. Indole production in 1 % (w/v) tryptone broth by the method of Kovacs, as in the *Manual of Microbiological Methods* (1957).

11. Hydrolysis of starch in nutrient agar containing 0·2 % (w/v) soluble starch. Medium flooded with Lugol's iodine solution after incubation.

12. Oxidation of glucose in O/F Basal Medium (Difco) containing 1 % (w/v) glucose. This commercial medium is almost identical with the medium of Hugh & Leifson (1953).

13. Production of fluorescent pigment was tested on Pseudomonas F Agar (Difco) slopes. This commercial medium is designed to enhance fluorescin production by Pseudomonas. Fluorescence was tested by exposing inoculated slopes to ultraviolet radiation after 24–48 hr incubation. Lyophilized cultures and the same cultures which had been kept for 10 years in stock culture were tested.

14. *Growth at different temperatures.* All cultures were tested for ability to grow at 0, 6, 12, 20, 30, 37 and 42°. The preparation of inocula was a modification of Klinge's (1960) method in that, for each culture tested, a 1/100 dilution, in 0·85 % (w/v) sterile saline, was made from a 24 hr turbid broth culture. After shaking, 0·1 ml. of the dilution was inoculated into each tube of nutrient broth, which contained 5 ml. broth. Inoculated and control tubes were placed in covered pans of water at the indicated temperatures. Glycerol was added to the pan water at 0° to keep the water from freezing. Temperatures were checked at least twice a day during the 2-week incubation period and did not fluctuate more than 0·5–1·0°. It took 30–45 min. from the time the first tubes of a series were inoculated until all tubes of that series had reached proper temperature. Results were recorded daily for the first week and three times during the second week. Growth was measured by visually comparing turbidity of inoculated tubes with uninoculated controls. Tubes were not removed from the incubator for more than 5 min. to record results. Ability to grow at 6, 12, 20 and 30° was determined once, while ability to grow at 0, 37 and 42° was determined twice.

15. Lipase was demonstrated by: (*a*) clearing of 1 % (v/v) tributyrin in nutrient agar; (*b*) change in colour of pH indicator in Rhodes (1959) medium substituting peanut oil for olive oil.

16. The presence of cytochrome oxidase was examined by the method of Ewing & Johnson (1960).

17. The oxidation of gluconate was determined in the medium of Haynes (1951) with the exception that 2 % (w/v) peptone was substituted for tryptone. The basal medium was autoclaved, and 0·5 ml. of a 40·0 % (w/v) Seitz-sterilized potassium gluconate solution was added to a final concentration of 4 % (w/v). The inoculated medium was shaken twice by hand during incubation. Presence of reducing compounds was tested with Benedict's qualitative reagent.

RESULTS

Of the 20 pseudomonads isolated from fish and one salamander, 15 of the strains showed positive reactions for 12 of the 22 applied tests (Fig. 1). Reactions of the strains to the other tests listed in Fig. 1 ranged from 11 to none positive. Detailed

results of all individual strain reactions are given in Table 2. Flagellar stains indicated most strains were lophotrichous but a few appeared monotrichous. Also the oxidative carbohydrate metabolism of the test strains was confirmed in O/F Basal Medium (Difco) containing 1 % glucose. Although the two ATCC strains were similar in their reactions to the test strains, they did differ in lipase production and gluconate oxidation. Also, neither produced gelatinase, which, according to *Bergey's Manual* (1957), is typical for *Pseudomonas fluorescens*. It was also found that, when Pseudomonas F Agar (Difco) was used, fluorescence was not lost after repeated subcultivation.

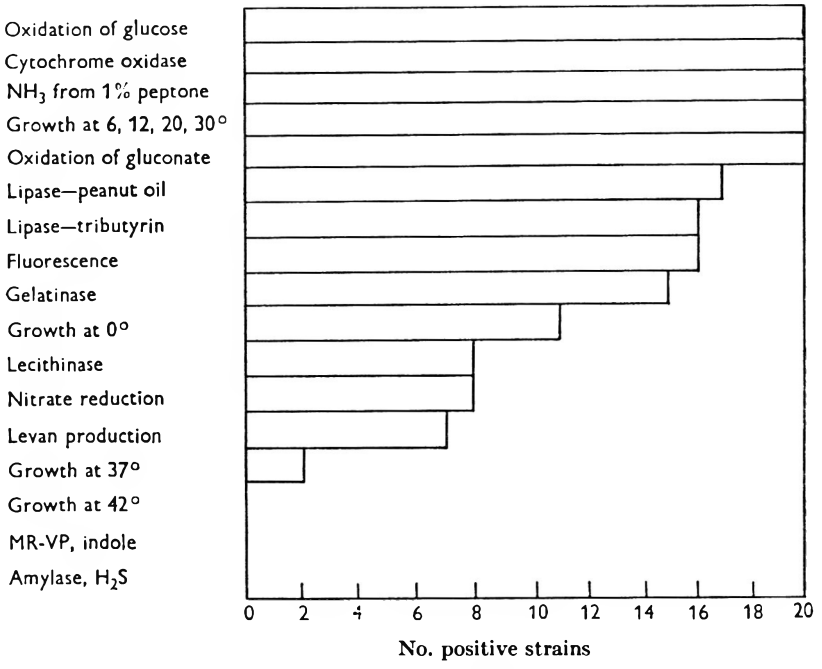

Fig. 1. Number of Pseudomonas strains from fish and one salamander having positive reactions with the applied tests.

DISCUSSION

The pseudomonads isolated from diseased fish used in the present study represent a fairly homogeneous group, especially in those characteristics normally used for identification, namely polar flagellation, fluorescence, carbohydrate metabolism and cytochrome oxidase reaction. These and other criteria were used by Shewan, Hobbs & Hodgkiss (1960*a*, *b*) to identify and group the very important pseudomonad spoilage organisms of marine fish. The scheme of Bullock (1961) used to identify fish pathogens also incorporates some of these characteristics for Pseudomonas identification. However, in this scheme a peptone basal medium + carbohydrate was used to determine carbohydrate metabolism and the more definite O/F Basal Medium (Difco) + carbohydrates is now preferred. A further comparison of fish-pathogenic and fish-spoilage pseudomonads would place the pathogens studied into groups I and II of Shewan *et al.* (1960*a*) and, since 16 of the 20 strains

Table 2. *Characteristics of pseudomonad test strains*

All strains oxidized glucose, were cytochrome oxidase positive and produced NH_3 from 1 % peptone. All strains were MR-VP, amylase, H_2S, and indole negative.

Character determined	Culture number (see Table 1)																
	1, 2 3, P-2	7	11	21	22b	37	40	50, 51	66	80	81	86	87	88	P-1	P-3	ATCC 11250	ATCC 12633
Gluconate oxidation	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+
Lipase																		
Peanut oil	+	+	+	−	+	+	−	+	+	+	+	+	−	+	+	+	+	−
Tributyrin	+	+	−	−	+	+	−	+	+	+	+	+	−	+	+	+	+	−
Growth at: 0°	+	−	+	+	+	+	−	−	+	+	+	−	−	−	−	−	−	−
6°, 12°, 20°, 30°	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
37°	−	−	−	−	−	−	−	−	−	−	−	+	−	−	+	−	−	−
42°	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Fluorescence	+	+	−	+	+	−	+	−	+	+	+	+	+	+	+	+	+	+
Gelatinase	+	+	−	−	+	−	−	+	+	+	+	+	+	+	+	−	−	−
Lecithinase	+	+	−	−	−	−	−	−	−	+	+	+	−	−	−	−	−	−
Levan	+	−	−	−	−	−	−	−	−	−	+	−	+	+	−	−	−	−
Nitrate reduction	+	−	−	−	−	−	+	−	+	−	−	−	−	−	+	+	−	+

produced fluorescent pigment, mainly in group I. Organisms producing an alkaline or no reaction in the medium of Hugh & Leifson (1953) and corresponding to groups III and IV (Shewan *et al.* 1960*a*) are rarely encountered as pathogens of fish and are of no practical importance in this field.

While speciation was not an aim of this work it may be possible on the basis of growth at various temperatures more closely to define our strains. The ability to grow at 42° is considered an important feature in the separation of *Pseudomonas aeruginosa* from other *Pseudomonas* species (Haynes, 1951; Klinge, 1960; Haynes & Rhodes, 1962). Haynes & Rhodes (1962) recommended three successive transfers for determining growth at 42°. This was not used in the present work, but since after two attempts no strain was found to grow at 42° and only two strains grew at 37°, it is unlikely that *P. aeruginosa* plays an important role in diseases of freshwater fish. We are mainly concerned with psychrophilic pseudomonads, which are identical with or closely resemble *P. fluorescens*.

The authors wish to thank Dr K. E. Wolf of this laboratory for his critical review of the manuscript and many helpful suggestions.

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The Influence of Previously X-irradiated Aqueous Solutions on the Infectivity of the Viruses of Foot-and-Mouth Disease and Vesicular Stomatitis

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(*Received* 16 *April* 1964)

SUMMARY

The inactivation of foot-and-mouth disease virus and vesicular stomatitis virus by long-lived inactivating agents in X-irradiated buffer solutions was investigated and compared with the findings of Alper (1952, 1955, 1956) relating to the inactivation of bacteriophage by similar agents. A comparison is made between the effects of these inactivating agents and of hydrogen peroxide to which there is a resemblance. Possible reaction kinetics are discussed in an attempt to explain the form of the survival curves resulting from such inactivation reactions. The radiation sensitization of the viruses to these modes of inactivation is also described and discussed.

INTRODUCTION

Inactivation of bacteriophage by the long-lived products in X-irradiated aqueous solutions was reported by Alper (1952, 1955, 1956). She showed that the inactivation of dilute suspensions of bacteriophage in pre-irradiated buffer solutions could not be attributed entirely to the action of formed hydrogen peroxide and that phage, irradiated but still infective, was more susceptible to subsequent inactivation by hydrogen peroxide or by pre-irradiated buffer solution. During investigation of the indirect action of X-irradiation on certain animal viruses a similar behaviour was observed and the results so obtained can be partly explained in terms of reactions at the surface of the virus particle.

METHODS

Viruses used. Foot-and-mouth disease virus of two immunological types was used: type C strain 997 and type O strain M. 11 (Davie, 1962). Vesicular stomatitis virus of the Indiana type, strain Indiana c, was also used for comparative studies in many experiments.

The foot-and-mouth disease virus was obtained either from the lesions produced following the intradermal inoculation of the plantar pads of guinea pigs or by the propagation of virus on cell monolayers grown from pig kidney (Sellers, 1955) or from a cell line derived from baby hamster kidney (Mowat & Chapman, 1962; Capstick, Telling, Chapman & Stewart, 1962). In general the 25 mμ infective component of these virus preparations was separated and concentrated by ultracentrifugation (Bradish, Brooksby, Dillon & Norambuena, 1952) for use in these experiments. This treatment also decreased the concentration of non-viral protein

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in the samples. The virus suspensions required for experiment were made up in 0·04 M-phosphate buffer (pH 7·6), or 0·1 M-ammonium bicarbonate adjusted to pH 7·6 by acetic acid.

Vesicular stomatitis virus was grown on the chorioallantoic membranes of 7-day developing chick embryos. All data in the text refer to experiments with pooled allantoic and amniotic fluids collected 24 hr after inoculation from batches of about thirty eggs. Because of the sensitivity of vesicular stomatitis virus to light (Skinner & Bradish, 1954) exposure to light was minimized when handling this virus. The experimental procedure was tested in this respect by using suitable virus controls which were exposed to the same illumination as the virus samples in the main experiment. No significant loss of infectivity was observed in these control samples.

Titration of infectivity. The infectivities of suspensions of foot-and-mouth disease virus were estimated by intraperitoneal inoculation of series of tenfold dilutions into groups of five to ten unweaned mice (Skinner, 1951) or by plaque counts of tenfold dilutions on pig kidney cell monolayers (PK monolayers, Sellers, 1955) or on baby hamster kidney cell monolayers (BHK monolayers, Capstick *et al.* 1962). A standard inoculum volume of 0·3 ml. was used in the plaque count technique.

For titration of suspensions of the virus of vesicular stomatitis in mice the intracerebral route of inoculation was used, otherwise the methods of titration were the same as for suspensions of foot-and-mouth disease virus. The results to be described showed no significant dependence on the method of infectivity titration.

In these studies of inactivation the initial infectivity (V_0) and the virus infectivity V surviving a certain inactivation process were the quantities measured. For infectivities measured in mice, V is the reciprocal of the 50% end-point dilution calculated according to Reed & Muench (1938). For infectivities measured by plaque assay, V is the number of plaque-forming units counted per 0·3 ml. inoculum. The fraction of infectivity surviving, V/V_0, is used to express the degree of inactivation.

Estimation of hydrogen peroxide. Hydrogen peroxide (British Drug Houses Ltd. Laboratory reagent grade) was used throughout this work. The starch iodide test was used for the detection and determination of hydrogen peroxide at concentrations 10^{-3} M or less (Savage, 1951). A solution of starch + potassium iodide was added to the samples containing hydrogen peroxide and the colour intensity of the blue starch + iodine complex so formed was compared with that of a colour standard produced by adding starch + potassium iodide to known quantities of hydrogen peroxide. This standard was based upon previous titration of hydrogen peroxide against standard ceric sulphate solution. The extinction coefficients were measured on the Coleman Junior Spectrophotometer at 550 mμ.

X-irradiation. All aqueous solutions, including those containing virus, were X-irradiated in closed metal containers lined with borosilicate glass. During irradiation the samples were maintained at temperatures between 0° and 5°. A Raymax 150 industrial X-ray unit (manufactured by Associated Electrical Industries, London) was used as the source of X-radiation. All irradiations were made with an accelerating voltage of 150 kVp applied to the X-ray tube and with 0·5 mm. duraluminium beam filtration in addition to that provided by the X-ray tube window. This resulted in a beam having a first half-value-layer thickness of 0·5 mm. Cu.

Irradiation dose rates were 1200 or 3000 r./min. according to the specimen container in use. The standard of dose was the ferrous sulphate dosimeter modified by the addition of sodium chloride (Weiss, Allen & Schwarz, 1955).

Because of the proximity of the specimen containers to the X-ray tube anode there was considerable variation of dose rate with depth within the specimen. Thus under the worst conditions within a specimen depth of 1 cm. at 5 cm. mean distance from the focal spot, the difference in dose rate between the top and bottom of the sample on account of the inverse square law was 40% of the value at the mean position. Consequently the doses and dose rates quoted in the text refer to mean values derived from measurements of the total dose delivered to a volume of ferrous sulphate irradiated under geometrical conditions identical with those used in the irradiation of virus or other samples.

RESULTS

Suspensions of foot-and-mouth disease virus and vesicular stomatitis virus were readily inactivated by pre-irradiated buffer solutions provided that the concentration of virus and of non-viral protein or other protective agent was sufficiently low. The same is true of the inactivation of these viruses by hydrogen peroxide. The following features of these reactions were observed.

Inactivation in pre-irradiated buffer

Pre-irradiated samples of 0·04M-phosphate buffer, 0·005M-phosphate buffered saline and 0·1M-ammonium bicarbonate all at pH 7·6 were capable of inactivating virus to about the same extent. Also, when triple-distilled water was irradiated and then added to 0·4M-phosphate buffer solution so as to produce 0·04M final concentration at pH 7·6, this buffer inactivated virus in a similar manner. Over the reaction times involved in these experiments, normally up to 3 hr, the virus suspensions showed no significant decrease of infectivity when mixed with non-irradiated buffer solutions; typical survival curves are shown in Fig. 1. A total of twenty-eight such curves was obtained for various buffer solutions, virus types and irradiation doses, and all were characterized by a slope which decreased continuously with increasing reaction time.

The overall inactivation of a virus suspension for a fixed time in a sample of pre-irradiated buffer solution was found to depend upon the concentration of virus + residual non-viral protein present in the reaction mixture. This is shown in Fig. 2 in which the extent of inactivation of virus samples of different concentration by identical volumes of irradiated phosphate buffer is related to the concentration of virus used. A similar dependence upon virus concentration was shown when the inactivating agent was hydrogen peroxide.

Protection effects

Protein or other protective agents such as glutathione or thiourea, in the absence of any toxic effects, decreased the rate of inactivation of virus infectivity in pre-irradiated buffer solution. When present in sufficiently high concentrations, these agents annulled any observable inactivation. This effect is shown in Table 1 which relates to the inactivation of vesicular stomatitis virus by pre-irradiated phosphate

buffered saline. The infectivity remaining after reaction for 2 hr at 4° in the presence of different concentrations of peptone increased with the concentration of peptone. At peptone concentrations in excess of 1 g./l. no significant inactivation was detected. Thus a convenient method of studying reaction rates is to take samples from the reaction mixture at appropriate times and to stop the reaction in these

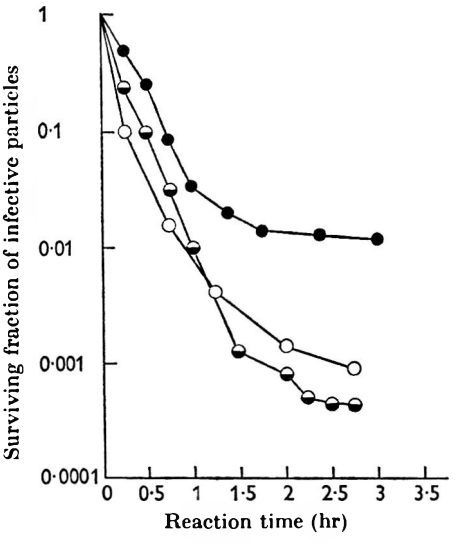

Fig. 1

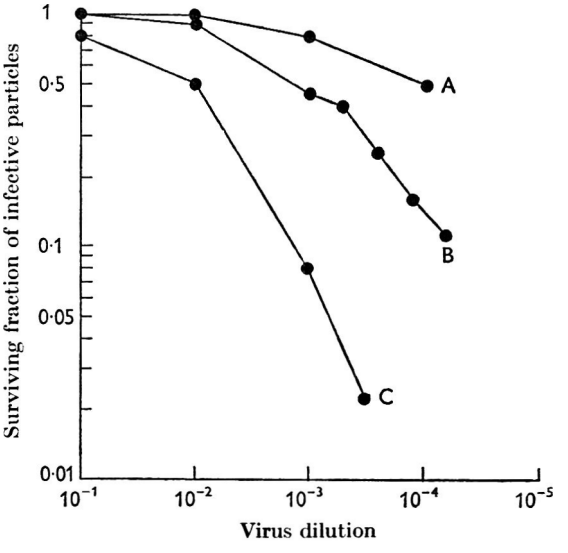

Fig. 2

Fig. 1. Typical survival curves for the inactivation of foot-and-mouth disease (FMD) and vesicular stomatitis (VS) viruses by X-irradiated buffer solutions. Irradiation dose = 30,000 r. ●, Inactivation of FMD virus by irradiated 0·1 M-ammonium bicarbonate buffer. ○, Inactivation of FMD virus by irradiated 0·005 M-phosphate buffered saline. ◒, Inactivation of VS virus by irradiated 0·005 M-phosphate buffered saline. All titrations of infectivity on pig kidney cell monolayers.

Fig. 2. Inactivation of various concentrations of foot-and-mouth disease virus in hydrogen peroxide and pre-irradiated buffer solutions. Constant reaction time of 3 hr at 4°. Infectivities titrated on pig kidney cell monolayers. A, Inactivation in $10^{-3\cdot5}$ M-hydrogen peroxide; B, inactivation in 0·04 M-phosphate buffer irradiated for 30 min. at 3000 r./min.; C, inactivation in $10^{-3\cdot0}$ M-hydrogen peroxide.

Table 1. *Depression of infectivity of vesicular stomatitis virus in egg fluids titrated on pig kidney cell monolayers following reaction for 3 hr with pre-irradiated phosphate buffered saline in the presence of various concentrations of peptone at pH 7·6*

Concentration of peptone in buffer	Surviving fraction of infectivity
g./l.	V/V_0
100	0·95
30	1·10
10	1·00
3	1·05
1	0·95
0·1	0·8
0·01	0·55
Nil	0·10

samples by the addition of an equal volume of, say, a peptone solution at a concentration of 10 g./l. (pH 7·6). The whole set of samples may then be titrated for infectivity at the termination of the reaction.

Rates of inactivation

Apart from the dependence upon concentration of reactants the rate of inactivation of the viruses in pre-irradiated buffer solutions was found to increase with the temperature of the reaction mixture and to decrease with the quantity of air or

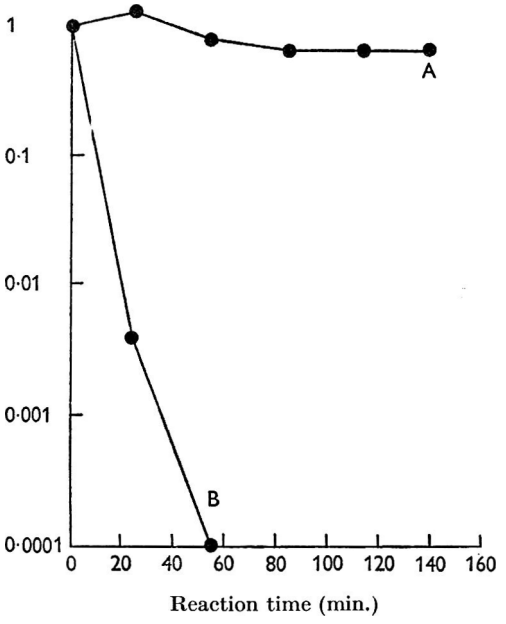

Fig. 3

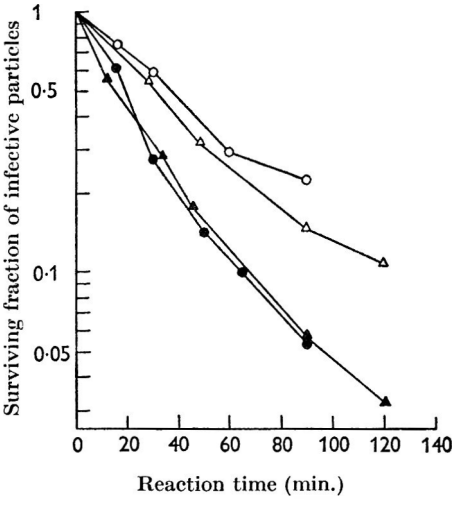

Fig. 4

Fig. 3. Inactivation of vesicular stomatitis virus by pre-irradiated phosphate buffer solutions. A, buffer X-irradiated in frozen state at −60° for 30 min. at 3000 r./min.; B, buffer solution irradiated in liquid state for 30 min. at 3000 r./min.

Fig. 4. Inactivation of vesicular stomatitis (VS) virus in irradiated phosphate buffered saline (○) and in a solution of hydrogen peroxide (△) compared with the inactivation of the pre-irradiated material by the same two inactivating agents, points ● and ▲. The concentration of hydrogen peroxide was arbitrarily selected to give approximately the same rate of inactivation as the irradiated buffer solution. Irradiation doses to buffer solution and virus were each 3×10^4 r. △, Inactivation of VS virus by 10^{-3}M-hydrogen peroxide; ○, inactivation of VS virus by irradiated phosphate buffered saline; ▲, inactivation of irradiated VS virus by 10^{-3}M-hydrogen peroxide; ●, inactivation of irradiated VS virus by irradiated phosphate buffered saline.

oxygen dissolved in the buffer solution at the time of irradiation. All comparisons between rates of reaction subsequently given are for reactions at 4° with air-saturated buffer solutions. No inactivation was observed when buffer solution irradiated in the frozen state was thawed and used with virus at 4°. This is shown in Fig. 3; the upper curve shows no significant loss of infectivity for the reaction between vesicular stomatitis virus and phosphate buffer pre-irradiated at about −60°; the lower curve shows the same virus treated with buffer solution pre-irradiated at a temperature between 0° and 5°.

Virus sensitization effect

The X-irradiation of virus suspensions rendered them more susceptible to subsequent inactivation by pre-irradiated buffer solution and to the action of hydrogen peroxide. Also pre-treatment of virus suspensions with hydrogen peroxide rendered them more sensitive to inactivation by pre-irradiated buffer. These effects have been termed virus sensitization (Alper, 1952). The effect of increasing the susceptibility of virus to the inactivating agents of pre-irradiated buffer and to hydrogen peroxide was shown in a set of experiments of which the results are shown in Figs. 4–6.

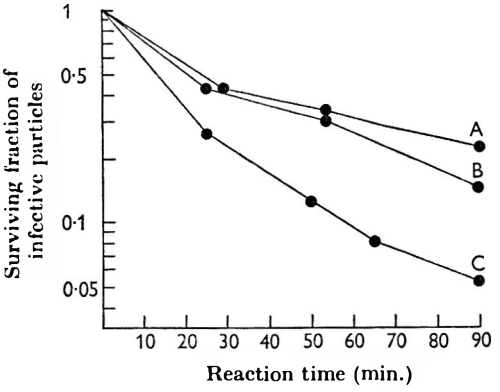

Fig. 5

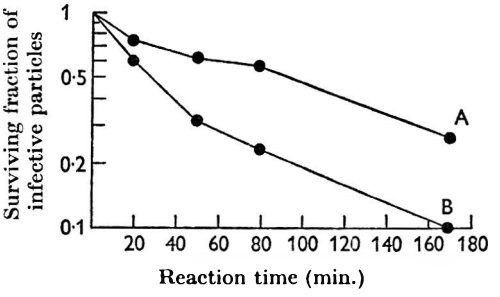

Fig. 6

Fig. 5. Inactivation of irradiated vesicular stomatitis (VS) virus in pre-irradiated phosphate buffered saline. Infectivities titrated on baby hamster kidney cell monolayers. A, inactivation of non-irradiated VS virus; B, inactivation of VS virus pre-irradiated for 30 min. at 3000 r./min.; C, inactivation of VS virus pre-irradiated for 90 min. at 3000 r./min.

Fig. 6. Inactivation of stomatitis (VS) virus in pre-irradiated phosphate buffered saline before and after treatment with hydrogen peroxide. Infectivities titrated on baby hamster kidney monolayers. A, VS virus inactivated by pre-irradiated phosphate buffered saline; B, VS virus treated with hydrogen peroxide and subsequently inactivated by pre-irradiated phosphate buffered saline.

A dose of 3×10^4 r. of X-radiation was delivered to a 1/10 dilution of pooled egg fluids infected with vesicular stomatitis virus. This resulted in a 60% loss of infectivity, probably due primarily to the action of short-lived free radicals in solution (Lea, 1946). The irradiated virus was then immediately further diluted 1/300 into: (*a*) phosphate buffer which had received an irradiation dose of 3×10^4 r. at the same time as the virus itself; (*b*) into hydrogen peroxide diluted to 10^{-3}M in phosphate buffer solution. Similar reaction mixtures were prepared from non-irradiated virus. Fig. 4 shows the resulting inactivation of virus in the four reaction mixtures. It is apparent that 10^{-3}M-hydrogen peroxide inactivated the virus at a very similar rate to that of the pre-irradiated buffer and that in each case the pre-irradiation of the virus suspensions resulted in an increased rate of inactivation. The concentration of hydrogen peroxide in the four reaction mixtures was determined at the start and end of the reaction time by the starch iodide test. The concentration of hydrogen peroxide, as detected by this test, was more than one hundred times greater in the samples containing 10^{-3}M-hydrogen peroxide than in those containing pre-irradi-

ated buffer solution. Also the concentration of hydrogen peroxide did not decrease significantly during the reaction, but in the case of pre-irradiated buffer the concentration of formed hydrogen peroxide was near the limit of detection. Thus this observation could not be made fully quantitative. This experiment was repeated with only irradiated phosphate buffer as the inactivating agent, but two identical samples of virus were pre-irradiated for different times at the same dose rate. The subsequent rates of inactivation of the surviving virus were compared with that of the non-irradiated virus in the control samples. The rate of inactivation increased with the extent of pre-irradiation (Fig. 5).

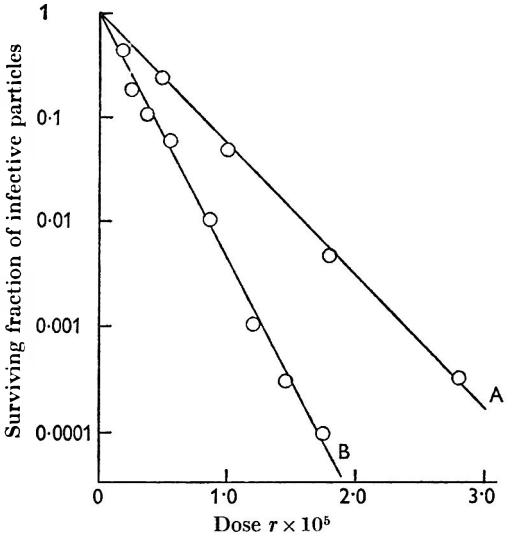

Fig. 7

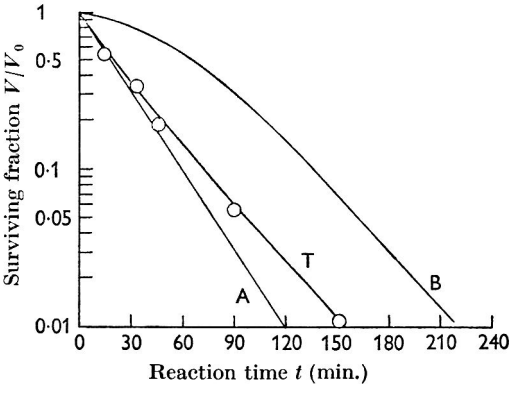

Fig. 8

Fig. 7. Inactivation by the indirect effect. A, inactivation of foot-and-mouth disease (FMD) virus (strain c/997) by X-irradiation in aqueous solution of low non-viral protein concentration; infectivities titrated in unweaned mice. B, inactivation of vesicular stomatitis (VS) virus (Indiana) by X-irradiation in aqueous solution of low non-viral protein concentration; infectivities titrated on baby hamster kidney cell monolayers.

Fig. 8. Theoretical survival curve T derived by combining curve A for a single-step mechanism with curve B for a three-step mechanism, weighted in the ratio 9:1. The experimental points are for the inactivation of vesicular stomatitis (VS) virus by irradiated phosphate buffered saline. Curve A has the equation $V/V_0 = e^{-kt}$ and curve B has the equation $V/V_0 = e^{-kt}(1+kt+\frac{1}{2}k^2t^2)$ where k has the numerical value 0·033 when time t is in minutes.

To examine whether virus sensitization could be produced by pre-treatment of virus with hydrogen peroxide, a suspension of vesicular stomatitis virus was diluted 1/10 in 10^{-3} M-hydrogen peroxide. After 3 hr at 4° this virus suspension showed a 20 % loss of infectivity, indicating that sufficient hydrogen peroxide had been used to produce a significant effect. However, when further diluted 1/300 in irradiated phosphate buffer solution, this treated virus was inactivated much more rapidly than normal virus at the same overall dilution. The results of an experiment of this type are shown in Fig. 6.

It may be noted that the high dose rates used in these irradiations were necessary

in order that the time of irradiation should be short compared with the time required for observation of the inactivation by the long-lived agents. In this way the two distinct modes of inactivation, the instantaneous indirect effect and the post-irradiation effect, were separated. In the discussion of the effects of pre-irradiation on virus samples it is justifiable to assume that the depression of infectivity caused by the irradiation was almost wholly due to the indirect effect of short-lived free radicals in solution.

As a supplement to the data on post-irradiation effects, typical exponential survival curves for the inactivation of the infectivity of suspensions of foot-and-mouth disease virus and vesicular stomatitis virus by free radicals in aqueous solutions are presented in Fig. 7. These curves were obtained by irradiating aqueous suspensions of the viruses at high dose rates. Samples of virus taken for titration of infectivity during the irradiation were immediately diluted into a solution of peptone (50 g./l.) in order to prevent any subsequent inactivation by the post-irradiation effects. Exponential survival curves result from this mode of inactivation.

DISCUSSION

From the data presented here three main points emerge. First, in connexion with the effect of hydrogen peroxide solutions on virus infectivity, it is clear that there are some similarities between hydrogen peroxide and the inactivating agents present in X-irradiated buffer solutions. One agent responsible for inactivation in the latter case may be formed hydrogen peroxide. There is, however, a considerable quantitative difference between the amount of hydrogen peroxide formed in an X-irradiated buffer solution and that which must be added to a non-irradiated buffer solution in order to achieve the same rate of inactivation. This difference has been attributed to the presence of stabilizers in commercial preparations of hydrogen peroxide (Alper, 1954).

The second observation, which is of more immediate interest in virology, is that X-irradiation in aqueous solution can modify the viruses of foot-and-mouth disease and vesicular stomatitis in such a way that, although still infective, they become more sensitive to the subsequent action of hydrogen peroxide or irradiated buffer solution. This implies a modification of the intact virus particle of such a nature that its ability to infect the animal cell is not impaired. As this modification is probably brought about by free radicals in solution and as some free radicals can also themselves produce inactivation, it is reasonable to suppose that the virus sensitization effect is a result of those reactions between the virus and the free radicals which do not result in direct inactivation. It is thought that there are many such reactions for every one which causes an inactivation (Allen, 1961).

The third observation relates to the shape of the inactivation curves which, as shown in Fig. 1, are non-linear and in all cases show a gradual decrease in rate of inactivation as the reaction proceeds. When inactivation of virus is caused by the indirect effect, exponential survival curves are obtained for both foot-and-mouth disease virus and vesicular stomatitis virus (Fig. 7). The reaction kinetics which describe this form of inactivation make the following assumptions (Allen, 1961): (1) That a constant dose rate is maintained and hence a constant free radical concentration exists during the inactivation. (2) That the virus particle is capable of

reacting with free radicals to produce either loss of infectivity or other modifications which may not be detected. (3) That inactivation results from reactions of the single-event type occurring at random among the other reactions. (4) That a virus after inactivation continues to react as readily with the free radicals as it did before the inactivation. If the post-irradiation inactivation process were due to a similar single-event interaction produced by a constant concentration of inactivating agents capable of combining with infective and non-infective virus alike, then exponential survival curves would be expected. The observation that such curves are not obtained implies that one or more of the conditions set out above is not fulfilled.

The shape of the survival curves is generally consistent with what might be expected if the concentration of inactivating agents became less, either as a result of loss due to recombination or decay, or as a result of combination with virus and other protein within the reaction mixture. It was shown, however, that inactivation curves of the same characteristic shape were obtained when 10^{-3}M-hydrogen peroxide was used as the inactivating agent (Fig. 4) and also that in this instance the concentration of hydrogen peroxide remained unchanged during the course of the reaction. Nevertheless, it is possible that, when the inactivation is due to agents in X-irradiated buffer solution, the inactivating agent does, in fact, become less concentrated during the reaction; and in the experiment with hydrogen peroxide mentioned above, some other agent, undetected by the starch iodide test, might account for the inactivation. It might be possible for this associated agent to become less concentrated during the reaction, thus producing a survival curve of the observed shape, without any apparent decrease in the concentration of hydrogen peroxide itself. This might also partly account for the relatively high concentration of hydrogen peroxide needed to simulate the effect of irradiated buffer solutions.

An alternative explanation of the shape of the survival curves is that the inactivation is in part a multi-step process. Fig. 8 shows how a virus sample of which 90% is inactivated by a single-step reaction and 10% by a cumulative three-step reaction can result in an overall survival curve of the observed shape. There are many parameters in an analysis of this type; for example, the reaction constant k may not be the same for the one-step and the three-step reactions, and higher-order reactions may be involved. Consequently a considerable variety of theoretical shapes is possible when the different processes are combined. On a model of this form it is necessary to assume that the proportion of virus particles which are inactivated by, say, the three-step process cannot be inactivated by the single-step mechanism, otherwise the latter would dominate. In other words there are different degrees of sensitivity to inactivation by irradiated buffer within the virus sample. The one-step reaction may be merely the last stage of the multi-step reactions. Another possibility is that the inactivating agents in irradiated buffer do not combine equally readily with infective and non-infective virus, thus modifying the reaction kinetics as the ratio of infective to non-infective particles changes.

Any acceptable model of the types outlined above must attempt to explain the virus sensitization effect which is observed in parallel with the inactivation process. Although it is difficult to correlate the virus sensitization effect with a single-step inactivation mechanism, the effect can be broadly accounted for in terms of the multi-step inactivation mechanism. If the mean number of steps required to

produce inactivation in a given sample is decreased then there will be an overall increase in the rate of inactivation compared with that of the original virus sample. There may also be some change in the shape of the inactivation curve, but consideration of this is outside the accuracy and scope of this work.

If, as a result of sensitization irradiation, a virus suspension had lost 60% of its initial infectivity by the indirect effect, the residual infective virus would have combined with free radicals in the types of reaction not producing direct inactivation. These reactions might have modified the virus sample in such a way that the relative population of infective particles requiring only single-step inactivation in pre-irradiated buffer was increased. This would have the net effect of increasing the sensitivity of the virus sample to inactivation by pre-irradiated buffer. It would therefore be expected that the degree of sensitization would depend upon the pre-irradiation dose delivered to the virus; this was shown to be so in Fig. 6. There is an obvious limit to the amount of pre-irradiation which may be employed without destroying the majority of the virus infectivity.

It is interesting to speculate on the possibilities that the virus sensitization effect may be demonstrated by means other than these observations on the rate of inactivation in hydrogen peroxide or pre-irradiated buffer solutions. It is conceivable that the specific reactions between virus and its antiserum may be altered by irradiation treatment of the virus. This treatment may also modify the sensitivity to thermal and other degradation processes which influence the complex surface of the virus particle.

I wish to thank Miss H. Wigman, Miss R. Jowett and Mr M. Ryal for their valuable assistance; Miss H. E. N. Ferrier for supplying much of the virus used, and Dr C. J. Bradish for his advice and encouragement.

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The Effect of Nitrogen Starvation on the Activity of Nitrate Reductase and other Enzymes in Chlorella

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SUMMARY

Chlorella vulgaris grown with nitrate as nitrogen source had high nitrate reductase activity. During nitrogen starvation the rate of nitrate assimilation increased but the specific activity of nitrate reductase in extracts decreased sharply; a similar decrease occurred after transfer to an ammonium medium. Organisms grown with ammonium as nitrogen source did not assimilate nitrate but acquired the ability to do so after nitrogen starvation; nitrate reductase activity in these organisms was initially very low but increased during nitrogen starvation and then decreased. After 18 hr of nitrogen starvation ammonium-grown and nitrate-grown organisms assimilated nitrate rapidly at a rate some ten times greater than the activity of nitrate reductase in cell-free extracts would allow. The specific activity of glutamic dehydrogenase was about 40% higher in extracts from ammonium-grown organisms than in extracts from nitrate-grown organisms and its activity increased by 40–100% during nitrogen starvation. The activity of malic dehydrogenase was unaffected by 18 hr of nitrogen starvation. The activity of isocitrate lyase decreased by about one-quarter when acetate was present but decreased much more markedly when the organisms were nitrogen-starved in the presence of glucose.

INTRODUCTION

Ammonium-N is assimilated much more rapidly by nitrogen-starved *Chlorella vulgaris* than by normal organisms (Syrett, 1953; Bongers, 1956). Generally the rate of nitrate assimilation also is higher, although the difference is less marked (Bongers, 1956; Syrett, 1956). Nothing is known of the changes in cellular enzymic composition which may occur during nitrogen starvation. Following recent investigations into the control of nitrate assimilation in Chlorella (Syrett & Morris, 1963; Morris & Syrett, 1963) we have measured changes in the rate of nitrate assimilation and in nitrate reductase activity during nitrogen starvation. The effect on some other enzyme activities was also studied for comparison.

METHODS

Chlorella vulgaris (Pearsall strain) was grown under sterile conditions as described in a previous paper (Syrett & Morris, 1963). Nitrate assimilation was followed by measuring the disappearance of nitrate from the medium with a colorimetric phenol disulphonic acid method (Syrett & Morris, 1963).

Preparation of nitrogen-starved organisms. Autotrophically grown organisms were harvested by centrifugation (500*g* for 5 min.), washed and resuspended in nitrogen-

free growth medium. In some experiments, the cultures were then illuminated (tungsten-filament lamp giving about 600 foot-candles to cultures) and aerated with air containing 0·5% (v/v) carbon dioxide for 18 hr. In other experiments, glucose (2%, w/v, final concentration) was added to the cultures, which were then aerated for 18 hr in darkness. The temperature was always 25°. Comparative results showed that both methods of nitrogen starvation induced similar changes in nitrate assimilation and in nitrate reductase activity.

Preparation of cell-free extracts. Organisms suspended in 0·1 M-tris+HCl buffer (pH 7·4) were passed through a French pressure cell (Morris & Syrett, 1963), centrifuged at 10,000*g* for 20 min. and the supernatant fluid used for determination of enzyme activities. Protein contents of the extracts were determined by the method of Lowry, Rosebrough, Farr & Randall (1951) with bovine albumin dissolved in tris buffer as a standard.

Nitrate reductase activity. This was determined by measuring the rate of nitrite formation from nitrate as described by Morris & Syrett (1963).

Malic dehydrogenase activity. This was determined by measuring the oxidation of NADH in the presence of oxaloacetate and cell extract. The reaction mixture contained, in 2·5 ml., 210 μmoles tris buffer (pH 7·4); 25 μmoles oxaloacetate and 0·15 mg. NADH. Extract containing about 10 μg. protein was added at zero time and the change in light extinction at 340 mμ measured. The incubation temperature was 22–26°. The change in extinction was linear with time for 5 min. following the addition of cell extract and the rate of change was proportional to the volume of extract added.

Glutamic dehydrogenase activity. This was measured by following the oxidation of NADPH in the presence of α-oxoglutarate, ammonium and cell extract. The reaction mixture contained in 2·5 ml.: 185 μmoles tris buffer (pH 7·4); 25 μmoles $(NH_4)_2SO_4$; 25 μmoles α-oxoglutarate; 0·15 mg. NADPH. Cell extract containing about 100–200 μg. protein was added at zero time and change in extinction at 340 mμ measured. Incubation temperature was 22–26°. The rate of reaction was constant for 5 min. after the addition of extract and proportional to the volume of extract added.

Isocitrate lyase activity. This was measured by determining the rate of glyoxylate formation from isocitrate catalysed by frozen suspensions of acetate-adapted organisms (Syrett, Merrett & Bocks, 1963).

RESULTS

The effect of nitrogen starvation on the rate of nitrate assimilation

Chlorella vulgaris (Pearsall strain) grown with ammonium sulphate as sole source of nitrogen did not assimilate nitrate until after a delay of 2–3 hr, whereas organisms grown with potassium nitrate assimilated nitrate immediately (Fig. 1*a*). After incubation in nitrogen-free medium for 4 hr, ammonium-grown organisms assimilated nitrate immediately and at a rate equal to that of similarly treated nitrate-grown organisms (Fig. 1*b*). More prolonged nitrogen starvation increased the rate at which nitrate was assimilated by both types of organism (Fig. 1*c*).

Assimilation of nitrate by nitrogen-starved organisms was accompanied by rapid changes of oxygen uptake and carbon dioxide output (Syrett, 1956). Manometric

study of these gas exchanges is a sensitive method for detecting nitrate assimilation. Such studies confirmed that nitrate assimilation by nitrogen-starved organisms (whether grown on potassium nitrate or ammonium sulphate) was immediate.

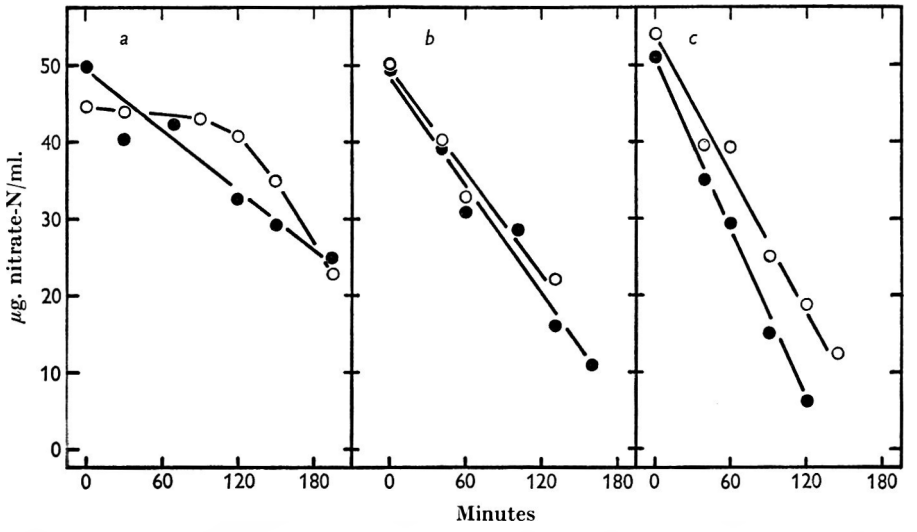

Fig. 1. The assimilation of nitrate by nitrate-grown (-●-●-) and ammonium-grown (-○-○-) *Chlorella vulgaris* after (*a*) 0, (*b*) 4, (*c*) 16 hr of nitrogen starvation. Nitrogen starvation was done by incubating cultures (3·2 mg. dry wt./ml.) at 25° with 2% (w/v) glucose. Samples were removed at suitable times, the concentration adjusted to equiv. 6·4 mg. dry wt./ml. in fresh N-free medium and nitrate added. The cultures were then incubated in darkness at 25° with 1% (w/v) glucose present and the disappearance of nitrate from the medium was followed.

Changes in nitrate reductase activity during nitrogen starvation

Rates of nitrate assimilation by Chlorella incubated in nitrogen-free medium for different periods of time, together with the accompanying changes in nitrate reductase, are summarized in Fig. 2. The nitrate reductase activity of nitrate-grown organisms was initially high and this decreased markedly during nitrogen starvation. A comparable decrease in nitrate reductase activity was found when nitrate-grown organisms were transferred to an ammonium medium (Fig. 3). The nitrate reductase activity of ammonium-grown organisms was negligible initially; it increased during the first 4 hr of nitrogen starvation and reached a value corresponding to 6–12 units of enzyme activity. After remaining constant for some time, the activity decreased and, after 18 hr of nitrogen starvation, extracts from both ammonium- and nitrate-grown organisms had the same rather low activity, namely about 1–3 units (Fig. 2). The decline in nitrate reductase shown in Figs. 2 and 3 is a decrease in *specific activity*; that is, it is a decrease in relation to general cellular protein. During these experiments there was little significant increase in number of organisms (e.g. top of Fig. 3): the decrease in nitrate reductase activity was not, therefore, a consequence of dilution due to multiplication.

The decline of specific activity may be due either to a loss of nitrate reductase protein or to loss of some essential cofactor(s). Addition to extracts of possible cofactors (e.g. flavin adenine dinucleotide, flavin-mononucleotide) had no effect

on nitrate reductase activity in extracts from normal or nitrogen-starved organisms. When an extract from normal nitrate-grown organisms was mixed with that from nitrogen-starved organisms no stimulation of activity was observed; a stimulation might be expected if some unknown cofactor was present in extracts from normal organisms but absent from extracts of nitrogen-starved organisms.

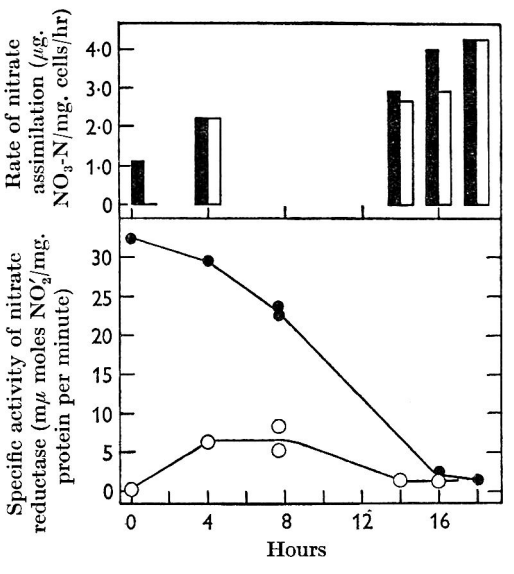

Fig. 2

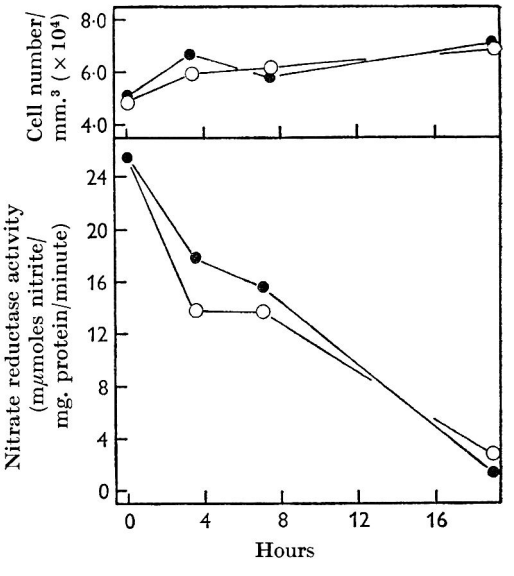

Fig. 3

Fig. 2. Changes in the rate of nitrate assimilation (upper graph) and nitrate reductase activity (lower) when ammonium-grown *Chlorella vulgaris* (open symbols) and nitrate-grown *C. vulgaris* (black symbols) were incubated in nitrogen-free medium in darkness at 25° with 2% (w/v) glucose present. Samples were removed at suitable times and after resuspending the organisms in ice-cold tris buffer cell-free extracts were made and nitrate reductase activity measured. Other samples were resuspended in fresh N-free medium, the concentration adjusted to equiv. 6·4 mg. dry wt./ml. and nitrate added. The cultures were then incubated in darkness at 25° with 1% (w/v) glucose present and the disappearance of nitrate from the medium followed for 150 min.

Fig. 3. Changes in nitrate reductase activity in cell-free extracts of *Chlorella vulgaris* when nitrate-grown organisms were transferred to nitrogen-free medium (-●-●-), or to medium containing 0·01 M-ammonium sulphate (-○-○-). Concentration of organism equiv. 2·0 mg. dry wt./ml. The suspensions were incubated at 25° in darkness with 2% (w/v) glucose present. The upper part of the figure shows little change in number of organisms during the experiment.

Morton, Dickerson & England (1960) observed an increase in proteinase activity when *Penicillium griseofulvum* was starved of nitrogen. High proteinase activity in nitrogen-starved Chlorella might destroy nitrate reductase during extraction; the extracts would then have less enzyme activity than extracts from normal Chlorella. To test this hypothesis, suspensions of normal and nitrogen-starved organisms were mixed before preparing extracts. An active proteinase might be expected to depress the activity extracted from the mixed suspension; no such depression was observed. Consequently the evidence at present available suggests that the decline in nitrate reductase activity which accompanied nitrogen starvation represented a real loss of nitrate reductase protein from the organisms.

Comparison of nitrate reductase activity in cell-free extracts with the rate of nitrate assimilation by intact Chlorella

The average nitrate reductase activity extracted from nitrate-grown organisms corresponded to the formation of about 1·8 μmoles nitrite/mg. protein/hr. After complete breakage, equiv. 1 mg. dry wt organisms gave 0·24 mg. protein in the supernatant fluid of the cell-free extract. Thus a cell-free extract from equiv. 1 mg. dry wt organism contained sufficient nitrate reductase activity to reduce about 6 μg. nitrate-N/hr. The observed rate of nitrate reduction by whole organisms was

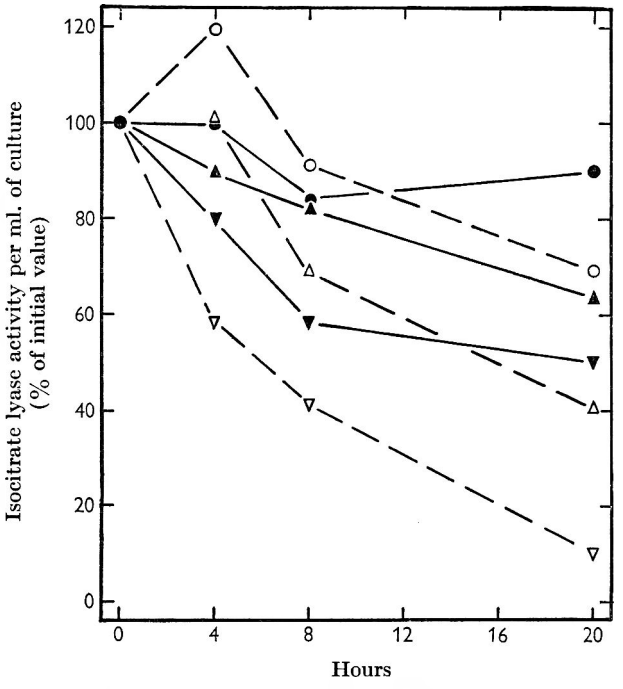

Fig. 4. The effect of nitrogen starvation of *Chlorella vulgaris* on isocitrate lyase activity. Organisms were shaken in darkness, at 25°, in 0·07 M-phosphate (pH 6·7) with the following additions: -●-●-, 0·2% acetate + N; -▼-▼-, 0·5% glucose + N; -▲-▲-, no carbon source + N. -○-○-, 0·2% acetate − N; -▽-▽-, 0·5% glucose − N; -△-△-, no carbon source − N. Nitrogen was supplied as 0·01 M-ammonium nitrate. Change in enzyme activity was stopped, at the times shown, by freezing the suspension. The number of organisms did not change markedly during the experiment except when glucose was present; in the absence of nitrogen, the number of organisms then increased two- to threefold; in its presence it increased fourfold. Isocitrate lyase activity per organism therefore declined more steeply in the glucose cultures than the graph shows.

between 1 and 2 μg. nitrate-N/mg. dry weight organism/hr. In contrast, cell-free extract from equiv. 1 mg. dry wt. nitrogen-starved organisms contained only enough activity to reduce 0·5 μg. nitrate-N/hr. The observed rate of nitrate assimilation by intact nitrogen-starved organisms was 4–4·5 μg. nitrate-N/mg. dry wt./hr. Thus, whereas extracts of nitrate-grown organisms possessed an excess of nitrate reductase in comparison with the rate at which whole organisms reduced nitrate, extracts from

nitrogen-starved organisms contained only about 10–12 % of the activity equivalent to the observed rate of nitrate reduction by these organisms.

The effect of nitrogen starvation on isocitrate lyase activity

Isocitrate lyase activity is high in Chlorella grown on acetate in darkness (Syrett *et al.* 1963). When such organisms were transferred to nitrogen-free medium for 20 hr, isocitrate lyase activity declined to about 70 % of the original value (Fig. 4). Greater decreases were observed when acetate was removed and particularly when it was replaced by glucose. The results in Fig. 4 are expressed as enzyme activity/unit volume culture. In those cultures supplied with glucose, the numbers of organisms increased during the experiment; activities expressed/organism would therefore show more marked changes in such cultures than is apparent from Fig. 4.

Table 1. *The effect of nitrogen starvation on the activity of the malic dehydrogenase of Chlorella vulgaris*

No significant change in number of organisms was observed during nitrogen starvation. The organisms were starved of nitrogen for 16 hr under autotrophic conditions. (*a*) and (*b*) denote duplicate extractions from the same culture.

Nitrogen source for growth	Expt.	Malic dehydrogenase activity (mμmoles NADH oxidized/mg. protein/min.)	
		Normal organisms	N-starved organisms
KNO_3	1	(*a*) 1560 (*b*) 2610	1650
	2	—	1340
$(NH_4)_2SO_4$	1	1720	2060
	2	2000	1940

Table 2. *The effect of nitrogen starvation on the activities of glutamic dehydrogenase and nitrate reductase in Chlorella vulgaris*

Nitrogen-starved cultures of *Chlorella vulgaris* were prepared by aeration under autotrophic conditions for 16 hr. A maximum of 40 % increase in number of organisms during nitrogen starvation was observed in some cultures. The experiment numbers denote replicate cultures; (*a*) and (*b*) denote duplicate extractions of the same culture.

Nitrogen source for growth	Expt.	Nitrate reductase activity (mμmoles NO_2'/mg. protein/min.)		Glutamic dehydrogenase activity (mμmoles NADPH oxidized/mg. protein/min.)	
		Normal organisms	N-starved organisms	Normal organisms	N-starved organisms
KNO_3	1	(*a*) 26·2 (*b*) 34·0	5·1	(*a*) 48·0 (*b*) 48·3	99·0
	2	—	4·3	—	78·5
	3	—	6·2	—	66·5
$(NH_4)_2SO_4$	1*a*	0·9	4·0	74·0	116·0
	1*b*	—	6·0	—	95·0

The effect of nitrogen starvation on malic dehydrogenase and glutamic dehydrogenase activity

Nitrogen starvation appeared to have no effect on malic dehydrogenase activity (Table 1). The activity of glutamic dehydrogenase extracted from nitrogen-starved organisms was greater than from normal organisms, the increase in activity varying between 40 and 100 % (Table 2). The increase was similar when either ammonium-grown or nitrate-grown organisms were starved of nitrogen, but generally the activity extracted from anmonium-grown organisms was about 40 % greater than from nitrate-grown organisms.

By a calculation similar to that above for nitrate reductase activity, it can be shown that the glutamic dehydrogenase activity of nitrogen-starved Chlorella was about equal to the rate at which whole organisms assimilated ammonium, while the activity in extracts of normal organisms was about three times that required to account for the rate of ammonium assimilation by whole normal organisms. However, this comparison assumes that all the ammonium assimilation by intact cells proceeds through glutamic dehydrogenase. The recent work of Sims & Folkes (1964) with yeast indicates that this may not be so; other pathways of ammonium assimilation must exist, to the guaninido group of arginine, for example. Consequently, from both normal and nitrogen-starved organisms, the extracted glutamic dehydrogenase activity probably exceeded the activity of this enzyme *in vivo*.

DISCUSSION

The effect of nitrogen starvation of *Chlorella vulgaris* on the activity of the two constitutive enzymes, malic dehydrogenase and glutamic dehydrogenase, differed from its effect on the inducible enzymes isocitrate lyase and nitrate reductase. The activity of malic dehydrogenase was unchanged after nitrogen starvation while that of glutamic dehydrogenase increased. Barratt (1963) showed that the glutamic dehydrogenase activity of Neurospora increased after nitrogen starvation and concluded that excess available nitrogen repressed the formation of this enzyme; our results are consistent with this interpretation.

In contrast, isocitrate lyase activity decreased when Chlorella was starved of nitrogen. The decrease was much greater when the inducer acetate was replaced by glucose, which represses isocitrate lyase formation in this organism (Syrett *et al.* 1963). Glucose serves as a readily available carbon source for Chlorella and, in its presence, the organisms multiplied during the experimental period even in the absence of nitrogen; thus the drain on available nitrogen in the organisms was larger when glucose was supplied. The behaviour of the other inducible enzyme, nitrate reductase, resembled that of isocitrate lyase in that the high nitrate reductase activity in nitrate-grown organisms declined rapidly when the organisms were nitrogen-starved. Transfer to an ammonium medium had a similar effect; ammonium is known to repress the formation of nitrate reductase (Morris & Syrett, 1963).

Two explanations of these effects are possible: (i) the rate of turnover of nitrate reductase and isocitrate lyase is always rather fast and, when synthesis is prevented by the removal of an inducer or the addition of a repressor, this results in a rapid loss of activity; or (ii) the conditions used, i.e. nitrogen starvation in the presence of freely available carbon, in some way stimulate the breakdown of these enzymes. The

behaviour of isocitrate lyase, in particular, resembles that of the inducible enzyme galactozymase in a yeast. Spiegelman & Reiner (1947) showed that this enzyme was lost when the substrate was removed and the loss was much faster when the yeast was incubated with glucose in the absence of a nitrogen source. They suggested that the enzyme was not inherently unstable in the absence of its substrate and that its rapid loss on incubation with glucose was a consequence of the synthesis of other enzymes. This explanation appears to imply a balance between the induced enzyme molecules and amino acids which is shifted towards breakdown when the amino acids are used for the synthesis of other proteins. It is uncertain whether this is so, but the hypothesis would explain why loss of enzyme was faster when an exogenous nitrogen source was not available.

Ammonium-grown Chlorella acquired nitrate reductase activity and the capacity to assimilate nitrate after a short period of nitrogen starvation. Evidently the presence of nitrate was not essential for the formation of this enzyme. Nitrogen-starved Chlorella, whether grown on nitrate or ammonium initially, assimilated nitrate rapidly and at a rate some ten times higher than the nitrate reductase activity recovered in cell-free extracts should allow. It appears that only a proportion of the nitrate reductase activity of whole nitrogen-starved cells was recovered in cell-free extracts. If the same is true for normal nitrate-grown organisms, which assimilate nitrate more slowly but show considerably higher nitrate reductase activities in extracts, this would imply that much of the nitrate reductase activity of normal nitrate-grown Chlorella is inhibited *in vivo*. There is evidence for an inhibition of nitrate reductase by a product of ammonium assimilation (Syrett & Morris, 1963); possibly, too, an inhibitor is formed during nitrate assimilation by normal nitrate-grown Chlorella.

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Energy Production During Nitrate Respiration by *Aerobacter aerogenes*

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SUMMARY

The molar growth yield of *Aerobacter aerogenes* growing anaerobically with glucose in a mineral medium was almost doubled when NO_3^- was added as hydrogen acceptor. About half a mole of NO_3^- was reduced to NH_4^+ per mole of glucose. The amount of ATP produced from glucose fermentation calculated from the molar growth yield and the acetate production was about 3 moles ATP/mole glucose. The total amount of ATP produced, deduced from the molar growth yield, was used to calculate that about 3 moles ATP were produced per mole of NO_3^- reduced. Comparable experiments with mannitol as carbon source gave the same results. When nitrite was used as hydrogen acceptor the anaerobic molar growth yield was the same or even smaller than in its absence, although the acetate production increased. The results suggest that during NO_2^- reduction no ATP is produced and that the ATP formed during reduction of NO_3^- to NH_4^+ is completely produced during the reduction of NO_3^- to NO_2^-.

INTRODUCTION

The relation between energy production and growth has been studied and discussed by Bauchop & Elsden (1960), Gunsalus & Shuster (1961) and Senez (1962). Bauchop & Elsden found that the growth yield per mole of ATP ($= Y^{ATP}$) was the same for different micro-organisms. Recently Hadjipetrou, Gerrits, Teulings & Stouthamer (1964) found that the yield of *Aerobacter aerogenes* per mole of ATP produced during glucose fermentation was 10·2 g. By measuring the molar growth yield under aerobic conditions and the oxygen uptake during growth the molar growth yield per atom oxygen taken up ($= Y^O$) has been determined. By dividing Y^O by Y^{ATP} the number of ATP molecules formed per atom oxygen taken up is found. In this way it was found that the P/O ratio is about three. Many facultative aerobic micro-organisms can utilize NO_3^- as ultimate hydrogen acceptor (see review by Nason, 1962). The influence of nitrate on the formation of fermentation products from glucose with *Aerobacter aerogenes* was studied by Forget & Pichinoty (1964). They concluded that under anaerobic conditions in the presence of NO_3^- the citric acid cycle did not function; that the production of hydrogen, ethanol and formate was decreased and the formation of acetate and CO_2 increased. Until now very little has been known about the production of ATP during nitrate reduction in facultative aerobic organisms. There exists some indirect evidence (increased phosphorus turnover in resting organisms during NO_3^- reduction) which suggests that in *Escherichia coli* and *Pseudomonas denitrificans* phosphorylation coupled to

electron transport from substrate to nitrate takes place (Takahaski, Taniguchi & Egami, 1957; Ohnishi & Mori, 1960). Therefore we decided to study the efficiency of nitrate respiration in *Aerobacter aerogenes* as a continuation of our previous aerobic experiments.

METHODS

The minimal medium and the growth conditions were as described previously (Hadjipetrou *et al.* 1964). NO_3^- was determined as described by Middleton (1959), NO_2^- by the method of Rider & Mellon (1946) and NH_2OH by the method of Czáky (1948). Acetate was determined with a purified preparation of acetatekinase as described by Rose, Grunberg-Manago, Korey & Ochoa (1954). Dry weight was estimated by measuring the extinction at 660 mμ in an Unicam SP 600 spectrophotometer. From the extinction the dry weight was calculated with the previously determined relation: μg. dry weight bacteria/ml. = 380 × extinction at 660 mμ (Hadjipetrou *et al.* 1964).

RESULTS

Influence of nitrate on the molar growth yield with glucose and mannitol

The molar growth yields of *Aerobacter aerogenes* growing: (1) anaerobically, (2) anaerobically with nitrate as hydrogen acceptor and (3) aerobically with glucose are given in Table 1. It is evident that NO_3^- increased the anaerobic molar growth yield with glucose. However, the growth yield was not as high as when O_2 was the

Table 1. *Molar growth yields and acetate production of Aerobacter aerogenes grown under different conditions with glucose as sole carbon source*

The glucose concentration in all experiments was 3 μmoles/ml. The nitrate concentration varied but was larger than 1·33 μmoles/ml. (see text); yields of organism are given as equivalent dry weight bacteria obtained turbidimetrically; the numbers in parentheses give the number of determinations.

Growth condition	Molar growth yield (g./mole glucose)	Acetate production (μmole/μmole glucose)
Anaerobic	26·1 ± 0·5 (17)	0·84 ± 0·04 (15)
Anaerobic + NO_3^-	45·5 ± 0·3 (10)	1·63 ± 0·08 (4)
Aerobic	72·7 ± 1·0 (21)	—

ultimate hydrogen acceptor. In the presence of NO_3^- about the same amount of fermentation occurred as in its absence (Forget & Pichinoty, 1964): pyruvate was decomposed by a thioclastic reaction to acetyl-CoA and formate, then acetyl-CoA converted to acetate by means of phosphate acetyltransferase and acetatekinase. Thus, above the net gain of 2 moles ATP from the conversion of 1 mole glucose to 2 moles pyruvate, an additional mole of ATP is generated for each mole of acetate formed. Ethanol, which is also formed in this fermentation, is produced by reduction of acetyl-CoA (Dawes & Foster, 1956) by the NADH produced during the formation of pyruvate. During this reduction the energy-rich bond present in acetyl-CoA is lost. The gross fermentation balance for this type of fermentation is: glucose → 1 acetate + 1 ethanol + 2 formate. Therefore the net gain of ATP from the fermentation can only be calculated when the amount of acetate is known. The amount of acetate produced at the moment of maximal growth is included in Table 1 for the anaerobic cultures growing in the presence or in the absence of NO_3^-. It is

evident from Table 1 that the production of acetate was much higher in the presence of NO_3^- than in its absence; this confirms the results of Forget & Pichinoty (1964). In contrast to their results, however, no pyruvate accumulation from glucose under an aerobic conditions in the presence of nitrate was detected.

Similar experiments were made with mannitol as sole carbon source. Mannitol is more reduced than glucose and one oxidation step is required to convert mannitol to the hexose oxidation level. Therefore the amount of NADH produced during the conversion of mannitol to pyruvate is larger than the amount produced during the formation of pyruvate from glucose. Thus more of the acetyl-CoA is reduced to ethanol than when glucose is the carbon source. The gross fermentation balance is: 1 mannitol $\rightarrow$ 0·5 acetate + 1·5 ethanol + 2 formate. The results of the growth experiments and of the acetate determinations are given in Table 2. The molar growth yield with mannitol under anaerobic conditions was smaller than with glucose; this is in accordance with the smaller acetate production. In the presence of nitrate as hydrogen acceptor the growth yield with mannitol was larger than with glucose.

Table 2. *Molar growth yield of Aerobacter aerogenes grown with mannitol as sole carbon source*

The mannitol concentration was 3 μmoles/ml. and the NO_3^- concentration 6 μmoles/ml.; other data as for Table 1.

Growth condition	Molar growth yield (g./mole)	Acetate production (μmole/μmole mannitol)
Anaerobic	21·8 ± 0·6 (5)	0·42
Anaerobic + NO_3^-	50·6 (3)	1·07

Products of nitrate reduction

To calculate the amount of ATP produced per mole of NO_3^- we should know how much NO_3^- is reduced per mole of glucose. Therefore experiments were performed in which we studied the growth yield with a fixed amount of glucose (3 μmoles/ml.) and different amounts of nitrate (from 0 to 35 μmoles/ml.). The growth yield increased with NO_3^- concentrations between 0 and 1·33 μmoles/ml., but did not change when higher nitrate concentrations were used. This suggests that at least 1·33/3 = 0·44 μmole NO_3^- per μmole glucose was necessary to increase the molar growth yield from the anaerobic value to the maximum value for anaerobic growth in the presence of NO_3^-. Chemical estimation of nitrate revealed that indeed 0·44 μmole nitrate was reduced per μmole glucose at the moment of maximal growth. The amount of nitrite produced from this amount of nitrate was very small: only 0·06 μmole/μmole glucose. In similar experiments with mannitol it was found that 0·93 μmole/NO_3^-/μmole mannitol were needed to obtain the maximum molar growth yield for anaerobic growth in the presence of NO_3^-. The amount of nitrite produced in this experiment was 0·42 μmole/μmole mannitol.

These results suggest that nitrate was reduced beyond the nitrite stage; this was confirmed by the observation that larger amounts of nitrite were present before maximal growth was obtained. In this aspect our strain differs from the one used by Forget & Pichinoty (1964) because their strain reduced NO_3^- only to NO_2^-.

In the supernatant fluid no hydroxylamine was detected and, as expected, NO_3^- was not reduced to such gaseous products as N_2 or N_2O. That NO_3^- was reduced to

NH_4^+ was confirmed by observing that our *Aerobacter aerogenes* grew in a minimal medium with NO_3^- as sole nitrogen source. The molar growth yields are given in Table 3. Under aerobic conditions the molar growth yield with NO_3^- as sole nitrogen source was much smaller than with NH_4^+ as nitrogen source; a similar observation was made by Pichinoty (1960). Under anaerobic conditions nearly the same yield of organism was obtained in a medium with NH_4^+ as N-source and NO_3^- as hydrogen acceptor, as in the medium with NO_3^- as combined nitrogen source and hydrogen acceptor. These results confirm that NO_3^- is reduced to NH_4^+.

Table 3. *Molar growth yields of Aerobacter aerogenes grown with NO_3^- as sole nitrogen source and glucose as sole carbon source*

The glucose concentration in these experiments was 1·5 μmoles/ml.; nitrate concentration 35 μmoles/ml. Other data as for Table 1.

Growth conditions	Molar growth yield (g./mole glucose)
Aerobic	52
Anaerobic	43

Table 4. *Molar growth yield and acetate production of Aerobacter aerogenes grown anaerobically with glucose as sole carbon source and with various concentrations of nitrite as hydrogen acceptor*

The glucose concentration in these experiments was 3 μmoles/ml. Other data as for Table 1.

Nitrite concentration (μmoles/ml.)	Molar growth yield (g./mole glucose)	Acetate production μmoles/μmole glucose
0	26·1	0·84
0·5	27	1·2
1	24·5	1·58
1·5	22·4	1·6
2·6	20·6	Not determined
5	16	Not determined
10	No growth	

The influence of nitrite on the molar growth yield with glucose

The previous results suggested that the production of some ATP by our *Aerobacter aerogenes* was coupled to the reduction of NO_3^- to NH_4^+. To know to which step in this reduction the ATP production was coupled, experiments were made in which NO_2^- was used as ultimate hydrogen acceptor. NO_2^- was very toxic to the bacteria, especially when there was no other hydrogen acceptor (O_2, NO_3^-) present. A similar phenomenon was observed for *A. aerogenes* by Lewis & Hinshelwood (1948). The growth yield and the acetate production with different amounts of nitrite are given in Table 4; acetate production was increased in presence of NO_2^- indicating that it indeed acted as hydrogen acceptor. About 0·3 μmole NO_2^- were reduced per μmole glucose, independent of the initial NO_2^- concentration. Although the acetate production increased, the molar growth yields were similar to or smaller than those obtained in the absence of NO_2^- and decreased as the NO_2^- concentration was increased. This effect was undoubtedly due to growth inhibition by NO_2^-. In experiments in which NO_2^- was added to a complete medium with NH_4^+ as N-

source and the organisms were grown under aerobic conditions, we also found a lower growth yield. A similar decrease in growth yield was obtained when, under anaerobic conditions, a mixture of 35 μmoles NO_3^- and 0·5 μmole NO_2^- was used as hydrogen acceptor. Our strain could also utilize NO_2^- as sole N-source. The yields of organisms are given in Table 5; they are much smaller than when NO_3^- was used as sole N-source (compare Table 3). In all these experiments the growth rate was decreased by the presence of NO_2^-. The results indicate that the growth yield was decreased at lower growth rates.

Table 5. *Molar growth yield of Aerobacter aerogenes grown with NO_2^- as sole nitrogen source and glucose as sole carbon source*

The NO_2^- concentration in both experiments was 5 μmoles/ml.; glucose concentration 3 μmoles/ml.

Growth condition	Molar growth yield (g./mole glucose)
Aerobic	39
Anaerobic	16

DISCUSSION

Y^{ATP} was calculated from the results of Table 1 (Hadjipetrou *et al.* 1964) to be $Y^{ATP}_{glucose} = 10{\cdot}2$. This value was used to calculate the ATP yield from growth yields under anaerobic conditions in the presence of nitrate, by dividing it into the molar growth yield with nitrate. In this way we find that $45{\cdot}5/10{\cdot}2 = 4{\cdot}46$ moles ATP were produced. Hadjipetrou *et al.* (1964) showed that the carbon content of the *Aerobacter aerogenes* is 40 %, hence 45·5 g. glucose were converted to cell material and $180 - 45{\cdot}5 = 134{\cdot}5$ g. were fermented. As the net gain of ATP/mole glucose transformed to pyruvate is 2 moles and as one extra mole is formed per mole of acetate, the amount of ATP formed during fermentation of one mole of glucose was $2(134{\cdot}5/180) + 1{\cdot}63 = 3{\cdot}12$ moles of ATP. From the growth yield we find that the total production of ATP was 4·46 moles, therefore $4{\cdot}46 - 3{\cdot}12 = 1{\cdot}34$ moles of ATP must have been produced by phosphorylation coupled to nitrate reduction. The amount of nitrate used as hydrogen acceptor during the fermentation of glucose was 0·44 μmole/μmole glucose. Thus the reduction of 1 mole of NO_3^- gave $(1/0{\cdot}44)1{\cdot}34 = 3{\cdot}0$ moles of ATP.

From the growth and acetate yields after anaerobic growth with mannitol, we can calculate that $Y^{ATP}_{mannitol} = 10{\cdot}0$. This is in good agreement with $Y^{ATP}_{glucose}$ (10·2) and $Y^{ATP}_{fructose}$ (10·6) determined by Hadjipetrou *et al.* (1964). The molar growth yield for anaerobic growth in the presence of NO_3^- was larger with mannitol than with glucose. In the same way as for glucose we can calculate that during anaerobic growth with mannitol 2·46 moles of ATP must have been produced by phosphorylation coupled to nitrate reduction, which is much more than when glucose is the growth substrate. As mannitol is more reduced than glucose more NO_3^- is needed per mole of mannitol (0·93 mole NO_3^-/mole mannitol). From the mannitol results we find that $2{\cdot}46/0{\cdot}93 = 2{\cdot}6$ moles of ATP are produced per mole of NO_3^-, which is in good agreement with the value of 3·0 found for glucose.

The growth with NO_3^- as sole nitrogen source shows that NO_3^- is reduced to NH_4^+. It is likely that ATP production is not coupled to the reduction of NO_2^- to NH_4^+, since no increased growth yield was obtained with NO_2^- as a hydrogen

acceptor (Table 5). That NO_2^- acts as a hydrogen acceptor follows from the increase in acetate production. This increase implies that an increase in growth yield might have been expected. Instead, the molar growth yields were similar to or even smaller than those obtained in the absence of NO_2^-. The explanation for this phenomenon is that in the presence of nitrite the growth rate is decreased by the growth inhibitory properties of NO_2^-. When the growth rate is decreased part of the ATP may be dissipated, which explains the lowered molar growth yields.

Thus it is most likely that the formation of three ATP molecules is coupled to the reduction of NO_3^- to NO_2^-, indicating that the hydrogen from NADH produced during fermentation is transferred to nitrate in several steps and that cytochromes are involved. Several schemes in which cytochromes are involved in nitrate reduction were given by Nason (1962). Nitrite and the other intermediates of nitrate reduction were most probably reduced directly by NADH. Several such enzymes have been reported present in a number of micro-organisms (Nason, 1962).

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The Fine Structure of Lactobacillus Bacteriophages

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SUMMARY

Twelve phages isolated from sewage and active on lactobacilli were examined by electron microscopy with a negative-staining technique. Those phages active on *Lactobacillus fermenti* (heterofermentative) possess icosahedral heads and sheathed tails which end in base-plates and pins. Those phages active on *Lactobacillus casei* (homofermentative) differ in that their heads are octahedral or icosahedral and they possess collars. The overall length of all the phages is similar and their base-plates remain attached to the sheaths when these contract. No tail fibres were seen. A temperate *Lactobacillus fermenti* phage was also examined. It has a small hexagonal head and a long unsheathed tail which ends in a star-shaped structure.

INTRODUCTION

An electron micrograph of a shadow-cast preparation of a phage active on *Lactobacillus casei* was presented in an M.S. thesis (Walter, 1958). De Klerk, Coetzee & Theron (1963) examined shadow-cast preparations of a number of phages active on lactobacilli. These phages are morphologically heterogeneous and it was decided to study their fine structure.

METHODS

Media. Media used were MRS broth and agar (de Man, Rogosa & Sharpe, 1960).

Phages. These phages were isolated from sewage and were as follows: nos. 41, 69, 206, 222, 222*a*, 315, 514 and 517 active on *Lactobacillus fermenti* strains; 300, 316, 356 and 780 active on strains of *L. casei* (de Klerk *et al.* 1963). The temperate phage 535/222*a* (Coetzee & de Klerk, 1962) was also examined.

Electron microscopy. Lysates of the sewage phages were prepared, purified and concentrated as previously described (de Klerk *et al.* 1963). High-titre lysates of the temperate phage were prepared by a double agar layer method (Hershey, Kalmanson & Bronfenbrenner, 1943). The purified phages (plaque-forming titres about 1×10^{12}/ml.) were suspended in 0·1 M-ammonium acetate (pH 7·2). The negative staining method of Brenner & Horne (1959) was used. Perforated carbon films were prepared on Veco 400 mesh/in. support grids (Sjöstrand, 1956); these were freed from formvar and oil by immersion in redistilled chloroform. The specimens were mounted by the spreading technique (Bradley, 1962) and examined with a Philips EM 200 electron microscope.

RESULTS

Phages active on Lactobacillus fermenti. Plate 1, figs. 1–5, shows *Lactobacillus fermenti* phages 206, 222*a*, 315 and 514. Their heads are composed of capsomeres. Some of the latter show a central core filled with phosphotungstate and are presumably hollow. The arrangement of the capsomeres has not been determined but the shapes of the heads are consistent with that of a regular icosahedron. The tails are composed of thin central cores surrounded by contractile sheaths composed of subunits in a helical arrangement. No collars are present and the terminal tail structures are rosette-like when the sheaths are extended. When the sheaths contract the central cores are exposed. The tail structures then show base-plates carrying no more than six tail pins, attached to the sheaths. No tail fibres were seen. The dimensions of these *L. fermenti* phages are presented in Table 1. The other sewage phages active on *L. fermenti* organisms which were examined had identical features and similar dimensions.

Table 1. *Dimensions (Ångström units) of Lactobacillus bacteriophages*

Phage	Head*	Tail length	Sheath width		Core width	Overall length
			Uncontracted	Contracted		
206	720†	1380	—	180	55	2100
222*a*	690	1380	160	200	55	2070
315	720	1480	160	—	—	2200
514	720	1380	—	180	60	2100
300	820	1230	150	190	70	2050
356	820	1270	160	—	—	2120
535/222*a*	500	1820	—	—	80	2320

* Dimension from apex to tail joint.

† Figures are the mean of 25 to 30 measurements.

Temperate phage. The temperate phage 535/222*a* possesses a different morphology (Pl. 1, figs. 6, 7). Its head is hexagonal and capsomeres were not seen. The tail is long, hollow and unsheathed and terminates in a star-shaped blob. The dimensions are presented in Table 1.

Phages active on Lactobacillus casei. Plate 2, figs. 8–10, shows *Lactobacillus casei* phages 300 and 356. The heads are composed of hollow capsomeres. The shape of the heads appears to be octahedral but few structural details have been discerned, and the head shape of phage 356 may be icosahedral. These phages also differ from those active on heterofermentative lactobacilli in that collars are present which have the same diameters as the tail sheaths. The remaining tail structures are similar to those of the phages active on heterofermentative lactobacilli. The dimensions of these phages are given in Table 1. Phages 316 and 780 revealed an identical morphology and similar dimensions. The periodicity of the tail sheaths of both groups of sewage phages in the contracted state was 36 Å and 28 Å when extended.

DISCUSSION

A clear distinction is possible between sewage phages active on heterofermentative and homofermentative lactobacilli. Not only do they differ serologically (de Klerk *et al.* 1963) but the latter have collars and probably octahedral heads while the former possess icosahedral heads and lack collars. The collars are thicker than those of coliphage T4 (Bradley, 1963) and resemble the collar of *Bacillus subtilis* phage SP3 (Eiserling & Romig, 1962). The overall length of the two groups of phages is similar, although the heads of those active on homofermentative organisms are larger. Their dimensions are similar to those of many other phages (Shirling, 1956; Bradley & Kay, 1960; Davison, 1963). The sheaths of both groups of phages, when contracted, remain attached to the base-plates. However, methods to produce contraction which may dissociate the base-plate from the contracted sheath (Kellenberger & Arber, 1955) were not used. The tail endings of these phages in the uncontracted state resemble that of *B. subtilis* phage SP8 (Davison, 1963), which is described as a mass of fibres and pins. However, no tail fibres or fibrous network around the sheaths of these phages were detected. This is not exceptional, for tail fibres and the fibrous networks detected in coliphages T2 and T4 have not been described in phages active on Gram-positive organisms (Bradley & Kay, 1960; Eiserling & Romig, 1962; Davison, 1963). The temperate lactobacillus phage differs from the sewage phages in having a smaller head and a longer and unsheathed tail which is distinctly broader than the cores of the other phages. No tail pins or fibres have been identified in the rosette-shaped tail ending of this phage, which is similar to the temperate *B. cereus* phages described by Dawson & Smillie (1962).

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EXPLANATION OF PLATES

The magnification in all figures is × 276,000. All phages in ammonium acetate and phosphotungstate.

PLATE 1

Fig. 1. Phage 206.

Figs. 2, 3. Phage 222*a*.

Fig. 4. Phage 514.

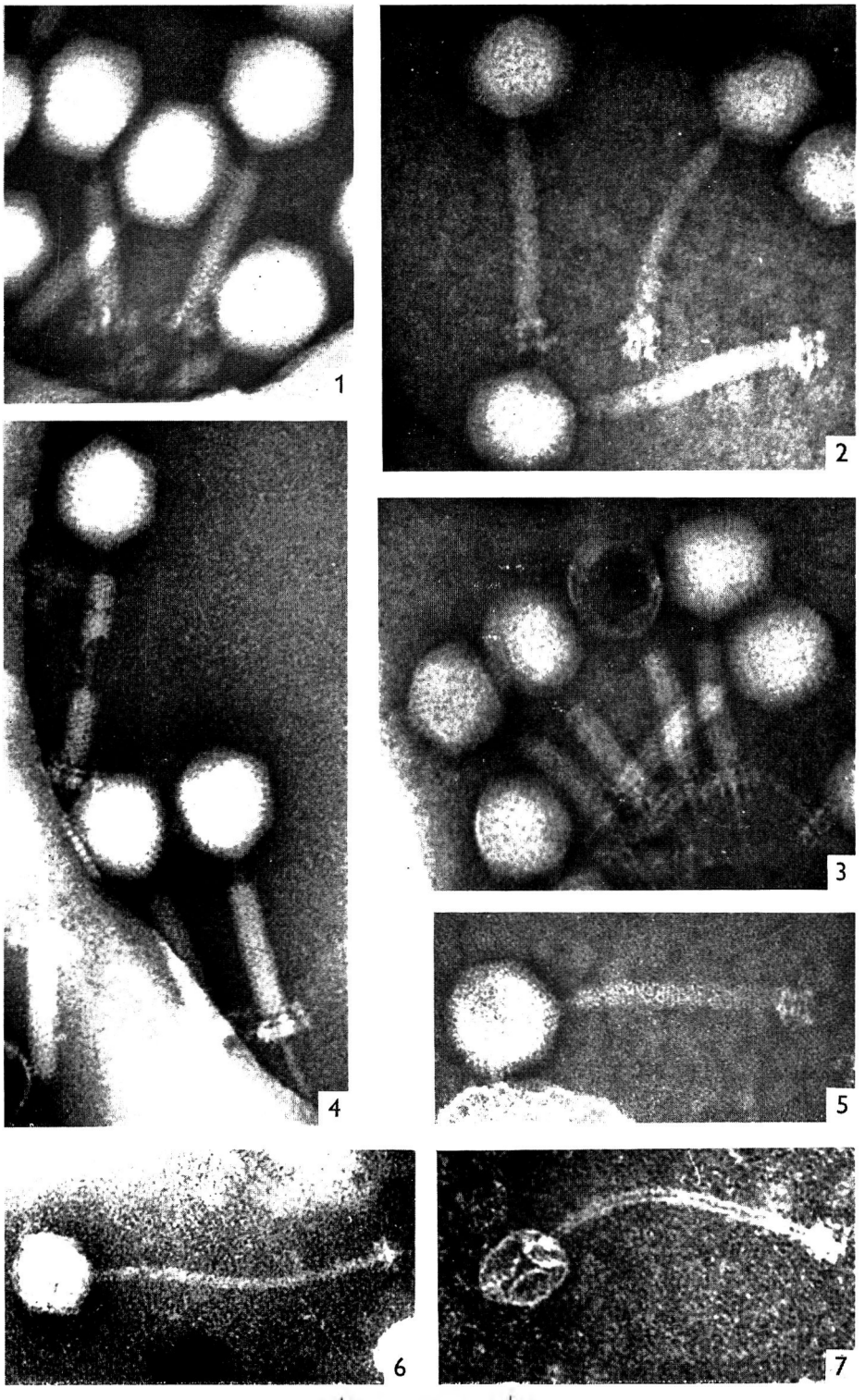
Fig. 5. Phage 315.

Figs. 6, 7. Phage 535/222*a*.

PLATE 2

Figs. 8, 9. Phage 300.

Fig. 10. Phage 356.



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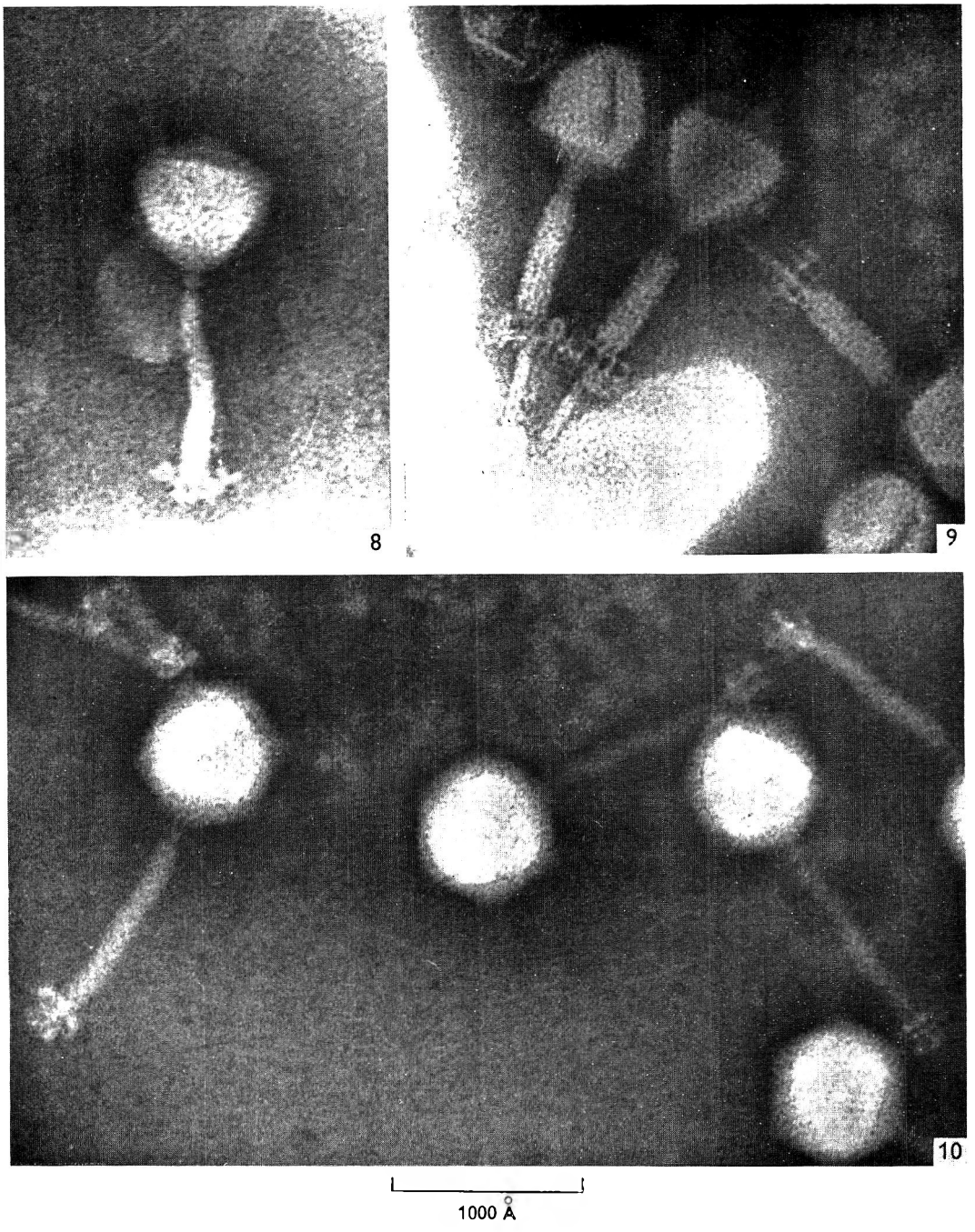

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Antigens from Vaccinia Virus Particles

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SUMMARY

Soluble material extracted from purified vaccinia virus particles produced eight precipitin lines with antiserum by immunodiffusion. The material contained about 20% of the viral nitrogen and was obtained by extraction with alkaline buffers up to pH 10·5, by prolonged autolysis or, more conveniently, by digestion with trypsin. It appeared to originate mainly from the virus surface but, as it also contained about 5% of the viral DNA and a small proportion of the virus particles were converted to ghosts, some of the precipitin lines probably represented internal components of the virus. When more material (up to 75% of the viral-N) was extracted no further precipitin lines were detected but some immunospecificities may have been destroyed. Serial extractions at increasing alkaline pH values separated the antigens from most of the viral DNA and indicated that the 'nucleoprotein antigen' of earlier workers was probably an artifact.

INTRODUCTION

The difficulty in studying the constituents of vaccinia virus was considered by Smadel & Hoagland (1942) as due to 'its extreme insolubility in all but the most drastic reagents'; 0·04 N-NaOH at 56° dissolved about half the weight of purified virus particles. The extracted material, which included the viral DNA, formed precipitates with vaccinia virus antiserum and was named 'nucleoprotein antigen' (Smadel, Rivers & Hoagland, 1942). No other method of obtaining antigens in solution from the virus particles has been reported apart from some early observations by Craigie & Wishart (1936), who detected that small amounts of antigen slowly dissociated from the particles in suspension. The effect of proteolytic enzymes was investigated by Hoagland, Ward, Smadel & Rivers (1940). Pepsin and papain partially digested the virus but trypsin, chymotrypsin and ficin did not. Later, Dumbell, Downie & Valentine (1957) noted that trypsin could decrease the infectivity of vaccinia virus.

The relatively large size and complex morphology of vaccinia virus suggest the possibility of disrupting the particles by mechanical methods. We have subjected virus suspensions to prolonged ultrasonic treatment, repeated freezing and thawing, grinding at −60°, treatment in the Hughes press and osmotic shock. As with earlier attempts by other workers, we could detect little or no effect on the virus and no antigens were released. Thus it appeared necessary to attack the virus with chemical reagents and to find conditions which caused minimal loss of antigen specificity. The principal chemical constituents of the virus are protein,

DNA and lipid. Small amounts of other substances reported in the early chemical investigations (Smadel & Hoagland, 1942) are probably not true components of the virus (Zwartouw, 1964).

METHODS

Virus purification. Vaccinia virus was obtained from the skin of infected rabbits and purified by centrifugation in a sucrose density gradient as described by Zwartouw, Westwood & Appleyard (1962). Samples of purified virus were stored as frozen aqueous suspensions at −80°.

Assay methods. Nitrogen, DNA and virus infectivity were determined as described previously (Zwartouw, 1964).

Immunodiffusion. For most tests the double-diffusion technique of Ouchterlony (1948) was used with a 2 mm. depth of gel prepared from 1% Ion-agar (Oxoid) in 0·9% NaCl solution. Reactant wells were 7 mm. diameter with 14 mm. between centres. Precipitin lines were recorded after 4 days at room temperature. The more sensitive microtechnique of Crowle (1958) was used to detect the maximum number of precipitin lines.

Antiserum. After an initial dermal vaccination, rabbits were hyperimmunized with four intravenous injections of 5×10^9 pock-forming units of vaccinia virus. The sera from six rabbits were pooled.

General extraction procedure. Purified virus was suspended in the required reagents so that the final virus concentration was 1 mg. (dry weight)/ml. After treatment, a sample was removed for infectivity assay and then the mixture centrifuged at 30,000 *g* for 15 min. to obtain a clear extract. The extract was tested for precipitin antigens by immunodiffusion and the amounts of viral-nitrogen (viral-N), and DNA determined and expressed as a percentage of the total viral-N or DNA.

Electron microscopy. Specimens were negatively stained with potassium phosphotungstate solution (0·2%, pH 6·8) as described in the text and examined in a Siemens Elmiskop 1 electron microscope operating at 80 kV.

RESULTS

Alkaline extraction

An extract was prepared by treating virus with 0·04 N-NaOH for 15 min. at 56° as described by Smadel *et al.* (1942) for the preparation of 'nucleoprotein antigen'. The extract contained all the viral DNA and about 60% of the total-N but produced only faint precipitin lines with antiserum in gel diffusion tests. Extracts prepared at less alkaline pH values and lower temperatures produced stronger precipitin lines although they contained smaller amounts of the viral material. Therefore, attempts were made to obtain the best yield of precipitins by first extracting at pH 9 and then at increasingly alkaline pH values. The material remaining insoluble after each extraction was collected by centrifugation and extracted at the next higher pH value of the series. Each extraction was carried out for 30 min. at 37° with 0·1 M-Na_2HPO_4 and sufficient NaOH to give the required pH value. For the final extraction at pH 12·8, 0·1 N-NaOH was used. Table 1 shows the amounts of material extracted from the virus and the precipitin lines produced by the extracts. Similar results were obtained with 0·1 M-glycine + NaOH buffer, but this was less convenient for measuring the extracted viral-N.

Extracts 1–4 (pH 9–10·5, Table 1) contained most of the precipitins and 20% of the viral-N. Extracts 6 and 7 (pH 11·5–12·0) contained 31% of the N and 71% of the viral-DNA but did not produce any precipitin lines. Although a small amount of DNA was obtained in the first two extracts, possibly from virus particles with defective or damaged outer structures, the precipitins were clearly separated from the main part of the DNA.

Comparison by gel diffusion indicated that the weak precipitin lines produced by 'nucleoprotein antigen' extract were also present in the lines produced by the pH 9–10·5 extracts. This was confirmed when the pH 10·5 extract, in which DNA was not detected, was treated with NaOH in the way used for 'nucleoprotein antigen' extraction. The precipitin line formation was considerably weakened and the residual weak reaction appeared similar to that of 'nucleoprotein antigen'.

Table 1. *Serial alkaline extraction of vaccinia virus*

Extract no.	pH	Soluble-N (% of total-N)	Soluble DNA (% of total DNA)	Formation of precipitin lines
1	9·0	5	7	++
2	9·5	4	2	++
3	10·0	3	0	+
4	10·5	8	0	++
5	11·0	12	6	+
6	11·5	22	35	–
7	12·0	9	36	–
8	12·5	3	7	–
9	12·8	4	0	–
Residue		26	0	

Table 2. *Tryptic digestion of vaccinia virus*

Digest no.	Trypsin conc. (%)	Infectivity (% of original)	Soluble-N* (% of total-N)	Formation of precipitin lines
1	0	109	3	Trace
2	0·00001	119	6	+
3	0·0001	122	10	++
4	0·001	30	19	+++
5	0·01	9	23	++
6	0·1	0·2	22	++

* After correction for trypsin content.

Digestion with proteases

Several proteolytic enzymes were tested at 0·01% for their ability to liberate antigens from 0·1% (dry weight) virus suspensions. Digestion with pepsin at pH 2·5 did not produce any material which formed precipitin lines by immunodiffusion. Trypsin, chymotrypsin, papain and ficin, enzymes which could be used at pH 7–8, all liberated material which formed similar precipitin lines; trypsin was selected for further study. Digestion was carried out with a range of different concentrations of crystallized trypsin (Armour & Co. Ltd.) in 0·01 M-phosphate at pH 8 for 3 hr at 37°. Table 2 shows the amounts of viral-N obtained in solution and the precipitin lines produced by the soluble materials. Both intensity of precipitin lines and amount of N released increased with the trypsin concentration

up to 0·001 % trypsin. Higher concentrations released only a little more N and caused some loss of the precipitin lines. At the lower trypsin concentrations the virus infectivity was apparently enhanced (Table 2) and in a few experiments it was increased to 150 % of the original infectivity titre. This was most likely due to the separation of virus particles from aggregates when the enzyme started to attack the surface of the particles. The higher trypsin concentrations caused loss of infectivity.

With 0·001 % trypsin similar results were obtained when the pH was varied between 7·7 and 9·0 but slightly less material was released between 7·0 and 7·4. A second digestion of the insoluble residue with trypsin released only a further 3 % of the total-N. As a standard procedure for obtaining the precipitin antigens, 0·1 % (dry weight) of virus in 0·01 M-sodium phosphate at pH 8·0 was incubated with 0·001 % trypsin for 3 hr at 37°. The material obtained in solution contained about 20 % of the total viral-N and small amounts of DNA (3–7 % of the total). The latter may have originated from damaged virus particles as suggested for the similar amounts of DNA extracted with alkaline buffer at pH 9–9·5.

Table 3. *Autolysis of vaccinia virus at pH 7·5 and 37°*

Sample no.	Time at 37° (hr)	Infectivity (% of original)	Soluble-N (% of total-N)	Formation of precipitin lines
1	4	107	4	Trace
2	8	110	6	+
3	16	102	8·5	+
4	24	93	9·5	+ +
5	48	87	12	+ + +
6	72	52	13	+ + + +
7	144	14	15	+ + + +

Autolysis of virus suspensions

Control samples of virus incubated at 37° for 3 hr without trypsin released a small amount of material which produced weak precipitin lines (see Table 2). Prolonged incubation of virus suspended in dilute buffer at pH 7·5–8·0 with 0·1 % sodium azide, to prevent growth of any contaminating organisms, resulted in a slow autolysis. The intensity of the precipitin lines produced increased with the time of incubation up to 72 hr when 13 % of the viral-N was in solution. Incubation for an additional 72 hr released only a further 2 % of N with no detectable increase in the precipitin lines produced (Table 3). The virus infectivity usually decreased slowly during the autolysis, but in some experiments almost no loss was apparent up to 72 hr. This was probably due to disaggregation of virus which masked any loss of infectivity.

Number of precipitin lines detected

Comparison of the precipitin lines produced by the materials released from virus with alkaline buffer up to pH 10·5, tryptic digestion or autolysis showed that they were similar. Some immunodiffusion patterns suggested the presence of six precipitin lines but usually the components did not separate clearly. The line patterns were not improved by altering the distance between reactant wells, adjusting the agar concentration or including buffer in the gel. However, with the more sensitive micro-immunodiffusion method of Crowle (1958) eight precipitin lines were detected.

Morphological changes accompanying the extraction of antigens

Some evidence which indicated that trypsin digested a superficial layer of protein from the surface of vaccinia virus has already been reported (Westwood, Harris, Zwartouw, Titmuss & Appleyard, 1964). Virus negatively stained with phosphotungstic acid (PTA) usually appeared to have a surface consisting of coiled threads. Digestion with trypsin had little effect on the thread layer although it allowed some increased penetration of PTA into the virus. More definite evidence for the digestion of a superficial layer external to the thread layer has been obtained as follows. Untreated virus was allowed to dry on a carbon film and negatively stained by allowing fine droplets (3–5μ diameter) of PTA solution to fall on to the particles. In areas where the PTA solution dried rapidly so that penetration of the stain was prevented, virus particles had a relatively smooth appearance and the thread layer was not revealed (Pl. 1, fig. 1). When about 20% of the viral-N had been dissolved by digestion with trypsin, no superficial smooth layer could be detected by the same staining technique and the surface of the virus appeared to consist of the thread layer (Pl. 1, fig. 2). Digestion with trypsin also converted a small proportion of the virus particles to empty ghosts. These particles presumably accounted for the 3–7% of the total viral-DNA obtained in the soluble material. Similar ghosts (Pl. 1, fig. 3) were obtained from all virus particles after the serial extraction with alkaline buffers. This final residue, insoluble in 0·1 N-NaOH, contained 26% of the total-N and no DNA.

Attempts to extract further antigens

The extracted material which formed precipitin lines represented only 20% of the total viral-N. Further material dissolved with alkaline buffer above pH 10·5 did not form precipitin lines so some other methods of extraction were examined. The small amounts of DNA obtained with the precipitin components suggested that a defective fraction of the virus particles released internal components and attempts were made to increase the damaged or defective fraction. However, no further precipitin components could be extracted by tryptic digestion or with alkaline buffer from freeze-dried virus (which had lost 90% of its infectivity on drying), virus treated by ultrasonic vibration in anhydrous suspension (freeze-dried virus suspended in carbon tetrachloride), or virus treated with 0·1 N-HCl or 0·1 N-NaOH for 3 min. at 0°.

Lipid was removed from virus by adding a 1% (dry weight) suspension to 100 volumes of acetone at −60° and the treated virus was recovered by centrifugation. The weight of lipid obtained after evaporating the acetone (5% of the original virus weight) showed that extraction of the lipid was almost complete. Although the lipid-free virus was not more soluble in alkaline buffer, it was digested to a greater extent by trypsin. The soluble digest contained 43% of the viral-N but the pattern of precipitin lines produced was not more intense and did not appear to contain any different components when compared with the digest from untreated virus. A similar result was obtained when 75% of the viral-N was dissolved by tryptic digestion of lipid-free virus which had been treated with 2-mercaptoethanol (2% for 1 hr at 20°).

After digestion of native virus with trypsin, 0·1 M-KCN (pH 11) at 37° for 1 hr extracted a further 42% of the viral-N compared with 16% extracted by 0·1 M-Na_2CO_3+$NaHCO_3$ at the same pH. The materials extracted by either reagent appeared to contain a little of the same precipitin components which had already been obtained with trypsin.

DISCUSSION

The earlier observation of Craigie & Wishart (1936) that antigen was slowly released from vaccinia virus suspensions has been confirmed. At 37° the material released contains up to 15% of the viral-N after 144 hr. Similar material was obtained more conveniently by suitable digestion with trypsin, which rapidly released about 20% of the viral-N. Serial extraction with alkaline buffers up to pH 10·5 also dissolved the same material from the virus. However, extraction above pH 10·5 dissolved the viral-DNA with further amounts of protein which did not produce precipitin lines. Thus the 'nucleoprotein antigen' extracted with NaOH by Smadel *et al.* (1942) was probably a mixture of nucleic acid and protein with partially degraded precipitin components.

Eight precipitin lines were produced by the extracted material, although only about 20% of the viral-N was dissolved. When more than 20% was obtained in solution no further precipitin lines were detected but some antigens may have been destroyed. Indirect evidence for one further antigenic component of the virus is reported in the accompanying paper (Westwood, Zwartouw, Appleyard & Titmuss, 1964), which describes a comparison of the virus particle antigens with the soluble antigens found in vaccinia virus infected tissue. Most of the immunospecific material appeared to be extracted from a surface layer of the virus. However, the conversion of a small proportion of the virus particles to empty ghosts by tryptic digestion and the presence of about 5% of the total viral-DNA in the soluble material indicated that some virus particles were completely dissolved except for the residual ghosts. Thus it seems likely that some of the eight precipitin lines represented internal components of the virus. Some experiments suggested that the surface layer could be dissolved away to leave virus which was still infective. However, the results were inconclusive since partial loss of infectivity might have been masked by the increased infectivity due to disaggregation of virus. Some observations of Morgan, Rifkind & Rose (1962) indicate a definite surface layer of antigens on vaccinia virus. These authors found that antibody labelled with ferritin combined with the surface of mature vaccinia virus but not with immature virus. The surface layer of antigens appears to be present as an envelope around the 'thread layer', and the latter is probably the most insoluble part of the virus which constitutes the ghosts left after alkaline extraction. Reagents which allow more material to be extracted from the virus, such as 2-mercaptoethanol and KCN, probably dissociate disulphide bonds in the insoluble thread layer.

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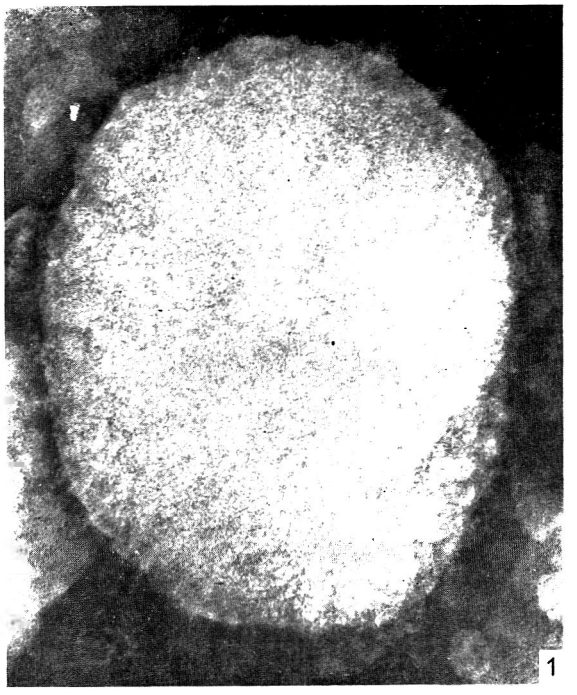

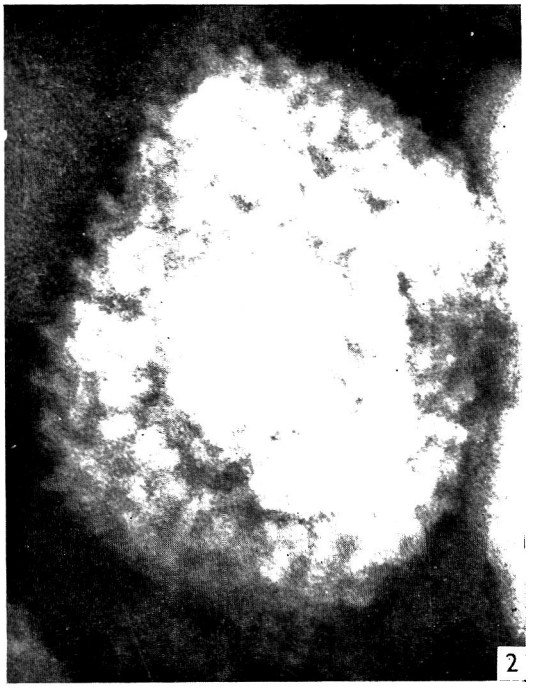

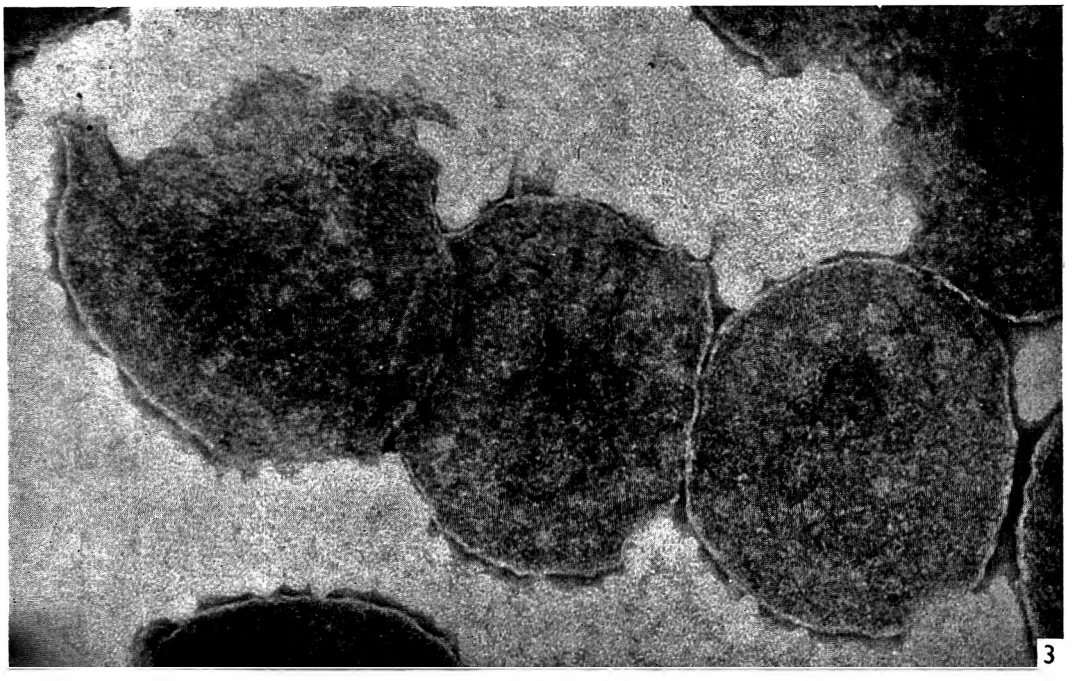

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EXPLANATION OF PLATE 1

Figs. 1–3. Electron micrographs of vaccinia virus.

Fig. 1. Untreated virus with minimal penetration of PTA stain showing the smooth surface (× 190,000).

Fig. 2. Virus after digestion with 0·001 % trypsin and stained with PTA as in Fig. 1; showing the thread layer at the surface (× 240,000).

Fig. 3. Residual ghosts left after extraction of virus with alkali (× 135,000).

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Comparison of the Soluble Antigens and Virus Particle Antigens of Vaccinia Virus

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SUMMARY

Soluble antigens extracted from rabbit skin infected with vaccinia virus produced immunodiffusion patterns containing up to 17 lines. At least 5 of the components which produced lines were labile when heated at 60°. Soluble material obtained from purified vaccinia virus particles produced 8 precipitin lines and an additional virus component was detected with antiserum prepared against inactivated virus particles. Seven of the virus particle precipitin lines were identical with soluble antigen lines, but up to 10 of the soluble antigen lines did not appear to represent components of the virus particle. It is suggested that they represent specific substances (perhaps enzymes) required for virus replication but not incorporated into the virus.

INTRODUCTION

Early studies of vaccinia virus antigens by classical methods of serology were reviewed by Smadel & Hoagland (1942). The serological activity of filtered extracts of vaccinia virus infected tissue (soluble antigen) was attributed to a single substance called LS-antigen. This antigen had two specificities, one of which was labile and the other stable when heated. Vaccinia virus particles were considered to have LS-antigen as a surface component as well as another antigen X-agglutinogen and to contain an internal nucleoprotein antigen. To account for the production of virus neutralizing antibody and immunity to infection a further antigen was also postulated. Application of immunodiffusion techniques has revealed that poxvirus soluble antigen is more complex than the earlier classical methods could show. Gispen (1955) found up to 6 precipitin lines and Rondle & Dumbell (1962) detected at least 9 precipitin lines produced by extracts from vaccinia virus infected tissue. Appleyard, Westwood & Zwartouw (1962) found that a total of about 15 precipitin lines was produced by the partially separated soluble components in extracts of tissue cultures infected with rabbit poxvirus.

The simplest explanation for the existence of the soluble antigens is that they represent excess synthesis of virus particle components which have not been incorporated into complete virus. However, some early experiments of Salaman (1937) and Downie (1939) suggest that this may not be the complete explanation. These authors found that antiserum which had been absorbed with virus particles still reacted with vaccinia soluble antigen preparations. We have investigated this problem by immunodiffusion comparison of soluble antigens with antigens obtained

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from vaccinia virus particles. The method for extracting nucleoprotein antigen from virus described by Smadel, Rivers & Hoagland (1942) destroys some viral antigens and nucleoprotein antigen is believed to be an artifact. Soluble components which produce up to 8 precipitin lines can, however, be obtained from vaccinia virus particles, as described in the previous paper (Zwartouw, Westwood & Harris, 1964).

METHODS

Antigens

Vaccinia virus soluble antigens. Infected dermal pulp was obtained from the skin of infected rabbits, as described by Hoagland, Smadel & Rivers (1940). The material from each rabbit was collected into 25 ml. of 0·004 M-McIlvaine buffer (pH 7·8) containing 0·1 % sodium azide (to inhibit growth of contaminating organisms) and dispersed by shaking with glass beads. The suspension was clarified by centrifugation at 800 *g* for 10 min. and the deposit re-extracted with half the original volume of buffer. The total suspension was then centrifuged at 35,000 *g* for 30 min. to sediment virus particles. The supernatant fluid, which contained between 5 and 7 mg. protein/ml., was the material used as soluble antigens.

Purified vaccinia virus particles. The virus sedimented from the soluble antigen solution was washed and purified by centrifugation in a sucrose density gradient, as described by Zwartouw, Westwood & Appleyard (1962). The purified virus produced no precipitin lines in immunodiffusion tests.

Extraction of antigens from vaccinia virus particles. Antigens were obtained from purified virus particle suspensions (1 mg. dry-weight/ml.) in 0·01 M-phosphate buffer (pH 8·0) by incubation with 0·001 % crystallized trypsin (Armour & Co. Ltd.) for 3 hr at 37°. Clear solutions containing about 20 % of the virus N were obtained after centrifugation at 30,000 *g* for 15 min. (Zwartouw *et al.* 1964).

Antisera

Complete vaccinia antiserum. Rabbits were initially vaccinated on the skin and then hyperimmunized with two to four intravenous injections of 5×10^9 pock forming units of virus from the skins of rabbits. Sera from nine rabbits were pooled. The method of preparation made the possibility of any reaction with rabbit tissue antigens very unlikely and this was confirmed by the absence of precipitin lines when the antiserum was tested against extracts from uninfected rabbit skin.

Soluble antigen antiserum. Soluble antigen was freed from infective virus by two Seitz filtrations followed by two filtrations through collodion membranes (average pore diameter 330 mμ). Rabbits were injected subcutaneously with 4 weekly doses of 1 ml. of the filtered soluble antigen emulsified with Freund's complete adjuvant (Difco Laboratories).

Virus particle antiserum. Two alternative methods were used to inactivate purified virus for immunization. (1) Formaldehyde was added to an aqueous virus suspension to make final concentrations of 0·4 % formaldehyde and 1·3 mg. dry weight of virus/ml. The mixture was kept at 20° for 1 hr. (2) An aqueous virus suspension (1·3 mg./ml. dry weight) was irradiated as a layer 1·7 mm. in depth and gently agitated by rocking at 34 cm. from a 15 W. Phillips ultraviolet lamp for 5 min. No infective virus was detected when either type of preparation was tested in chick embryos. The inactivated virus suspensions were emulsified with 1·5 vol.

of Freund's complete adjuvant and 2 ml. volumes of the mixtures (containing 1 mg. dry weight of virus) injected intramuscularly into rabbits. A second injection was given subcutaneously 6 weeks later.

Immunodiffusion

Double diffusion tests in gels were carried out on microscope slides as described by Crowle (1958). The diffusion medium was 1 % Ion-agar (Oxoid) in 0·9 % NaCl and antigen reservoirs were spaced in the Perspex templates either 4 or 6 mm. from a central antiserum reservoir. After 3 days at room temperature, the gel layers were washed and stained and the precipitin lines recorded photographically.

RESULTS

Soluble antigens

Immunodiffusion patterns produced by the complete vaccinia antiserum with samples of the soluble extract from infected tissue were very complex. It was difficult to resolve all the precipitin lines and the full number was not always distinguishable. The maximum number counted was 17. Samples of soluble antigen differed in that the components which produced the 4 lines nearest the antigen reservoirs were not present in all samples. These particular components, which were subsequently found to be important in the comparison with virus particle antigens, were detected in four out of the six soluble antigen preparations tested. One of the deficient samples had been Seitz filtered, a process which was generally applied by earlier workers to remove virus particles from soluble antigen, and this appeared to be a cause of the deficiency. Filtration of soluble antigen through a collodion membrane (average pore diameter 350 mμ) produced a similar loss. Although the unfiltered material was not completely free from virus particles, the small number present could not account for the extra precipitin lines. No precipitin lines were produced by purified intact virus particles at 1000-times the concentration present in soluble antigen.

In order to relate the precipitin line pattern to the earlier concept of a heat-labile and a heat-stable group on what was regarded as the single precipitating soluble antigen, the stability of the components revealed by immunodiffusion was similarly tested. A comparison was made of untreated soluble antigen with the same material after heating at 60° for 90 min. The stability of components producing some of the fainter lines could not be assessed, but the presence of five labile and six stable components was demonstrated.

Comparison of soluble antigens with virus particle antigens

The material obtained in solution by tryptic digestion of purified virus particles produced up to 8 precipitin lines with the complete vaccinia antiserum. When these lines were compared with the lines produced by filtered soluble antigen samples, no linkages could be detected between the two sets of lines. Thus, there appeared to be no immunological identity between any of the components in the two sets of antigens. However, when the comparison was made with unfiltered soluble antigen samples, linkages were detected between the extra soluble antigen lines (the 4 nearest

the antigen reservoir) and the centre 4 lines of the group produced by the virus particle antigens.

Since evidence of immunological identity with virus components was shown only by the extra soluble antigen components, the possibility was considered that their presence in soluble antigen might be due to breakdown of virus particles during preparation of the materials. Incubation of purified virus suspensions at 37° produced a slow release over 72 hr of the same antigens that were rapidly released from virus particles by tryptic digestion (Zwartouw *et al.* 1964). Preparations of soluble antigen and virus were normally obtained from batches of ten or more rabbits. On this scale, extraction of the material from infected skin and subsequent clarification extended over 1–2 days before most of the virus was separated from the soluble antigens by centrifugation. Although the material was kept at 4° for most of this time, some release of antigens from the virus into the soluble fraction could have occurred. We tried to eliminate this possibility by rapidly processing material obtained from single rabbits. In this way, virus was separated from soluble antigens within 4 hr of harvesting the material from infected rabbit skin. Two samples of rapidly processed soluble antigen both contained the components which produced lines linking with virus particle antigen lines as in the previous patterns. Further tests were carried out to detect any release of antigens from the small amount of virus remaining in unfiltered soluble antigen after centrifugation. Samples of the rapidly processed soluble antigen were incubated for 72 hr at 37°. This treatment caused no apparent increase in any of the components producing precipitin lines but some loss of one component was noted.

Components detected with soluble antigen antiserum

It was impossible to be certain that no linkages occurred between some of the fainter lines produced by soluble antigens and virus particle antigens in the complex patterns obtained with the complete vaccinia antiserum. Further evidence was sought by testing an antiserum prepared against soluble antigen. To avoid any antibodies produced directly against virus particles, the antiserum was prepared by injecting filtered soluble antigen. This preliminary filtration probably accounted for the inability of the antiserum to produce all the soluble antigen lines. In particular, it did not reveal the extra components of unfiltered soluble antigen or the stronger virus antigen lines which formed linkages in the previous tests. However, the immunodiffusion pattern obtained with the soluble antigen antiserum confirmed that some soluble antigen components were not present in the extract from virus particles. It also revealed that two weak lines produced by the virus particle antigens appeared to link with soluble antigen lines.

Components detected with virus particle antiserum

An attempt to detect any further virus particle antigens, which were not represented in the soluble extract prepared from virus particles, was made by testing antiserum against inactivated whole virus particles. Two alternative methods of inactivation, formaldehyde treatment and ultraviolet irradiation, were used to reduce the possibility of antigen destruction by the inactivation treatment. Although no infective virus could be detected in the treated preparations, addi-

tional evidence that virus replication had not occurred in the immunized rabbits was obtained by testing the antisera for anti-haemagglutinin. A vaccinia haemagglutination test system was inhibited by a 1/160 dilution of the complete vaccinia antiserum, but not by 1/10 dilutions of the virus particle antisera. On the other hand, the sera had high 50% neutralization titres (200,000–500,000) when tested with infective vaccinia virus by the method of Boulter (1957). Immunodiffusion patterns produced by antiserum prepared against virus particles inactivated by either method were similar. Only one weak precipitin line was produced by the soluble antigens and the same component was not detectable as a linking precipitin line produced by the virus particle extract. Thus the line indicated a component present in both soluble antigens and virus particles which had not been obtained in solution by tryptic digestion of the virus.

DISCUSSION

Immunodiffusion analysis has shown that soluble antigens produced in poxvirus infections contain a large number of different components. Seventeen precipitin lines were produced by extracts from rabbit skin infected with vaccinia virus and recently Appleyard & Westwood (1964) detected over 20 lines produced by extracts of tissue cultures infected with rabbitpox virus. Heating the vaccinia virus soluble antigens at 60° demonstrated five labile and six stable components. Some of the components were probably not included in the earlier investigations of filtered infected tissue extracts since losses occur during filtration. However, it seems likely that the original concept of a single LS-antigen was an expression of the collective behaviour of a number of antigens.

Table 1. *Summary of immunodiffusion tests*

Antigen	Antiserum	Precipitin lines
Soluble antigens		
Total detected	Complete	17
Linkages with extracted components of virus	Complete	4
Linkages with extracted components of virus	Soluble antigen	2
Present in virus but not in extract of virus	Virus particle	1
Total shared with virus particle		7
Virus particle antigens		
Present in extract of virus	Complete	8
Detected as antibody	Virus particle	1
Total detected		9

We have attempted to determine whether the soluble antigens represent excess synthesis of virus particle components by testing for their presence in virus particles. In direct comparisons with the lines produced by soluble material obtained from the virus particles, the appearance of immunodiffusion patterns suggests that the major components of virus particle extracts were identical with minor components of soluble antigen. Evidence for identity of two minor components in both sets of antigens was obtained with soluble antigen antiserum. However, the strongest lines produced by the soluble antigens clearly did not correspond to any component in

the solution of virus particle antigens. The antigen solution obtained from virus particles contained about 20% of the total viral N. Although no further precipitin components were detected when larger amounts of the virus were obtained in solution (Zwartouw *et al.* 1964), it was possible that some antigens were destroyed by the treatments used. If such antigens were also present in the soluble antigen mixture, they should have been detectable as precipitin lines formed by soluble antigen with antiserum prepared against inactivated virus particles. When this system was tested, only one weak precipitin line was produced by soluble antigen. A summary of the results is shown in Table 1.

Two vaccinia virus antigens could be detected by specific properties which showed that one was present in both virus particle and soluble antigen but the other only in soluble antigen. The first is the antigen reacting with virus neutralizing antibody which was demonstrated in soluble antigen by Appleyard (1961). The second is the haemagglutinin which is present in infected tissue extracts. Purified virus does not haemagglutinate (Zwartouw *et al.* 1962) and antiserum prepared against inactivated virus particles does not inhibit the specific haemagglutination. It is possible that these two antigens were not represented in the precipitin line patterns. Appleyard, Zwartouw & Westwood (1964) were not able to correlate the serum blocking activity of soluble antigen with any specific precipitin line and the relatively large size (65 mμ) of the haemagglutinin (Chu, 1948) may have prevented this antigen from diffusing in the gel system. Most of the virus particle antigens were also detected in the soluble antigen mixture. These soluble antigen components are readily explained as excess virus particle components which have not been utilized to form complete virus. However, there appear to be up to ten further components in soluble antigen which could not be detected in virus particles. A possible explanation for these is that they are specific enzymes for synthesis of new virus material and their formation in cells is initiated by the infecting virus. Increased amounts of several enzymes concerned with DNA synthesis in vaccinia virus infected cells have been reported and were reviewed by Cohen (1963). These increased amounts might be due to enzymes with virus rather than host cell specificity. Furthermore, Appleyard & Westwood (1964) have recently shown that some soluble antigens are produced in tissue cultures infected with rabbitpox before the viral DNA is replicated.

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Phospholipid Synthesis by *Rhodopseudomonas spheroides* in Relation to the Formation of Photosynthetic Pigments

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SUMMARY

The concentration of phospholipids in pigmented *Rhodopseudomonas spheroides* was higher than that in organisms lacking the photosynthetic pigments; the amounts of lipid phosphorus/mg. protein were, respectively, 157 and 90 mμmoles. Phosphatidylethanolamine comprised about 40% of the phospholipids, the remainder consisting of phosphatidic acid, phosphatidylglycerol and phosphatidylcholine. The phospholipids were confined to the membrane fraction of the organisms. As organisms adapted from the non-pigmented to the pigmented state the amount of lipid phosphorus/mg. protein increased. Experiments with radioactive phosphate showed phosphatidyl glycerol to incorporate label more rapidly than the other phospholipids. It is suggested that the formation of the photosynthetic pigments may be accompanied by modifications to the membrane structure so that it can accommodate more phospholipids.

INTRODUCTION

The pigments of photosynthetic bacteria together with enzymes and cofactors required for photophosphorylation are located in relatively homogeneous particles termed chromatophores; these can be separated by differential centrifugation of cell-free extracts (Schachman, Pardee & Stanier, 1953). In electron micrographs of sections of some photosynthetic bacteria the chromatophores appear as membrane-bound vesicles, the number of which varies with the degree of pigmentation (Cohen-Bazire, 1963). In Athiorhodaceae grown aerobically, and consequently almost devoid of the pigments, few vesicles can be seen; they become more numerous and extend more deeply into the cytoplasm as the organisms increase in pigment content (Cohen-Bazire & Kunisawa, 1963). There is now evidence that the vesicles are derived from the peripheral membrane and that the chromatophore fractions obtained from cell-free extracts arise by comminution of the membranes. Some electron micrographs of intact and lysed organisms have shown continuity between the vesicles and the peripheral membrane, suggesting that they may arise by invagination of this structure (Cohen-Bazire & Kunisawa, 1963; Drews & Giesbrecht, 1963; Boatman, 1964). Also, the photosynthetic pigments and photophosphorylation activity have been shown to reside in the 'ghost' fraction of *Rhodospirillum rubrum* disrupted by osmotic lysis (Tuttle & Gest, 1959). The possibility that chromatophores arise from the peripheral membrane raises the question of whether adaptation of Athiorhodaceae from the non-pigmented to the pigmented state involves the synthesis of new membrane which contains the photosynthetic pig-

ments incorporated into it. If so, it might be expected that pigmented organisms would contain a higher proportion of membrane than non-pigmented organisms.

In other types of bacteria there is abundant evidence that the phospholipids are largely confined to the membrane fraction (Kolb, Weidner & Toennies, 1963; Robrish & Marr, 1962) and the high content of phospholipids in chromatophore preparations again suggests their origin from the membrane (Newton & Newton, 1957; Gibson, Neuberger & Tait, 1962; Bull & Lascelles, 1963). In the present work evidence for membrane synthesis associated with the development of photosynthetic pigments has been sought by following phospholipid synthesis in relation to bacteriochlorophyll formation in *Rhodopseudomonas spheroides*.

METHODS

Organisms. Rhodopseudomonas spheroides N.C.I.B. 8253 and the non-pigmented mutant strain L-70 were used. The latter was derived from the parent organism by repeated aerobic subculture on agar slopes and had completely lost the ability to form bacteriochlorophyll and carotenoids; it was therefore unable to grow anaerobically in the light.

Stock cultures were grown on malate + glutamate + yeast extract agar; cultures of the parent organism were maintained as stabs, grown in the light (Lascelles, 1956), while the mutant was kept on slopes incubated in the dark at 30° for 24 hr.

Growth of cultures. Organisms were grown on the malate + glutamate medium aerobically in the dark or anaerobically in light, as described by Lascelles (1959). Organisms grown under the former conditions are termed 'non-pigmented' and under the latter as 'pigmented'.

Experiments with suspensions of organisms. Aerobic cultures were harvested at the end of the exponential phase and resuspended in phosphate-free medium (−P medium). This was modified from the normal malate + glutamate medium (Lascelles, 1959) by omission of potassium and ammonium phosphates and by the addition of 50 mM-tris buffer (pH 7·5) and 10 mM-NH_4Cl. The initial protein concentration of the suspensions was 0·8–1·2 mg./ml. After addition of [^{32}P]KH_2PO_4 (mM; about 3 μc./ml.), suspensions were incubated under low aeration at 34° as described previously (Lascelles, 1959). Samples were removed at intervals for determination of protein, lipid phosphorus and bacteriochlorophyll.

Determination of protein, bacteriochlorophyll and lipid phosphorus. Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951) after digestion of the organisms with 0·5 N-NaOH at 100° for 7 min. The standard used was crystalline bovine plasma albumin, treated in the same way. Bacteriochlorophyll was estimated by the method of Cohen-Bazire, Sistrom & Stanier (1957).

Lipid phosphorus was determined after extraction of lipids from acid-treated organisms or lysates with chloroform + methanol as follows. Samples of suspension or lysate equivalent to 10–20 mg. protein were treated with trichloroacetic acid (5%, w/v, final) and centrifuged. The pellet was suspended in 5% trichloroacetic acid (10 ml.) and recentrifuged. The supernatant fluid was completely sucked off and the pellet homogenized with 5 ml. chloroform + methanol (2 + 1, by vol.). After centrifugation, the supernatant fluid was removed and the pellet re-extracted with 5 ml. chloroform + methanol. The combined supernatant fluids were made to a

known volume (10–15 ml.) and represented the extracted lipids. Total phosphorus was determined on 0·5–1·0 ml. samples of this extract by the method of Bartlett (1959).

Distribution of phosphorus in phospholipids. The proportion of the various phospholipids in the extracted lipids was determined by methods based on those used by Dawson (Dawson, 1960; Dawson, Hemington & Davenport, 1962) and by Gray & MacFarlane (1964). In principle, the phospholipids were deacylated by mild alkaline hydrolysis; the water-soluble deacylated compounds were separated by paper chromatography and estimated by determination of phosphorus after elution.

Samples (4–6 ml.; 0·25–1·0 μmole phosphorus) of the chloroform + methanol extract prepared as above were shaken twice with an equal volume of 2 M-KCl and once with water; after each treatment the aqueous layer was removed. The washed extract was taken to dryness under an air stream at 60°. The residue was dissolved in 0·4 ml. chloroform and 0·6 ml. methanolic NaOH (0·1 N) added. The mixture was incubated at 37° for 20 min., neutralized with N-HCl, and taken to dryness under an air stream. The residue, consisting of unsaponifiable lipid, free fatty acids and deacylated phospholipids, was shaken with 1 ml. of the upper phase and 2 ml. of the lower phase of a mixture containing chloroform + methanol + water (27 + 13 + 10 by vol.). The aqueous phase, containing the deacylated phospholipids, was removed and freeze-dried. The product was dissolved in 0·2 ml. water and 0·04 ml. applied to Whatman paper No. 1 which had been washed with 2 N-acetic acid. Descending chromatography was used, the solvents being either water-saturated phenol + acetic acid + ethanol (100 + 10 + 2 by vol., Dawson, 1960) or water-saturated phenol containing 0·1 % (v/v) ammonia sp.gr. 0·88 (Kanfer & Kennedy, 1963).

The developed chromatograms were sprayed with ninhydrin to reveal the compound corresponding to phosphatidylethanolamine. In samples labelled with ^{32}P the phosphorus-containing compounds were made visible by radioautography on Kodak 'Blue Brand' X-ray film (4–24 hr exposure). Non-radioactive samples were run beside labelled samples and the phosphorus-containing areas delineated by extrapolation.

Elution was as follows. The areas containing phosphorus were cut out and rolled in aluminium foil allowing about 1 mm. of paper to show at one end; the rolls were fixed to the top of conical centrifuge tubes with adhesive tape and about 5 drops of water applied to the top edge of the paper. The tubes were centrifuged for about 1 min., the eluate collecting at the bottom of the tube; this procedure was repeated twice more. The total phosphorus of the eluates was determined by the method of Bartlett (1959).

Measurement of radioactivity. The colour developed after treatment of the eluates by the Bartlett method was measured spectrophotometrically. The solutions were then transferred to a thin glass-walled liquid counter, Type M6M (Panax Equipment Ltd., Surrey) and the radioactivity determined using an Ekco automatic scaler Type N530D (Ekco Electronics Ltd., Essex). Results are expressed as counts/100 sec. (minus background)/mμmole phosphorus.

Materials. ^{32}P was obtained from the Radiochemical Centre, Amersham, Buckinghamshire; it was in the form of carrier-free orthophosphate.

RESULTS

Phospholipid content of pigmented and non-pigmented organisms

Rhodopseudomonas spheroides 8253 grown anaerobically in the light and therefore containing the photosynthetic pigments had 50–70% more phospholipid than did non-pigmented organisms grown with high aeration. The mutant organism, which does not form photosynthetic pigments, contained about the same amount of lipid phosphorus as the parent strain grown in the non-pigmented state (Table 1).

The difference in phospholipid content between the pigmented and non-pigmented forms of the parent organism was reflected in the rate of phospholipid synthesis by cultures growing photosynthetically and aerobically (Fig. 1). The differential rate (ratio of increase in phospholipid to increase in protein) was about 1·8 times higher in the photosynthetic culture, and this difference was maintained throughout the growth curve.

The proportion of individual phospholipids was determined on the deacylated products as described in the methods. When samples containing ^{32}P were subjected to radioautography four major radioactive spots were detected. The R_F values in the phenol + acetic acid + ethanol solvent were: 0·35, 0·51, 0·66 (ninhydrin-positive) and about 0·9. These values corresponded to the deacylation products of, respectively, phosphatidic acid, phosphatidylglycerol, phosphatidylethanolamine and phosphatidylcholine (Dawson *et al.* 1962). Phosphatidylethanolamine predominated, comprising about 40% of the total phospholipids; this pattern of phospholipids was found in both pigmented and non-pigmented *R. spheroides* 8253 and in the mutant organism (Table 1). Identification of these phosphatides was also confirmed by other methods (Szilágyi & Lascelles, to be published).

Location of lipid phosphorus in membrane fraction

Treatment of *Rhodopseudomonas spheroides* 8253 with lysozyme in the presence of EDTA resulted in rapid lysis and within 5 min. few if any intact organisms were visible microscopically. Centrifugation of the lysates, after treatment with DNA-ase to render them less viscous, resulted in a pellet fraction containing all the photosynthetic pigments. This pellet presumably is derived largely from the cytoplasmic membrane though the presence of cell-wall fragments cannot be excluded. Analysis of lipid phosphorus in the washed pellet fraction showed that more than 90% was located in the pellet derived from both pigmented and non-pigmented organisms (Table 2). It can therefore be concluded that the phospholipids of *R. spheroides*, as in other bacteria, are largely confined to the particulate or membrane fraction of the organisms.

Phospholipid synthesis by suspensions of Rhodopseudomonas spheroides

Phospholipid synthesis was studied with suspensions of aerobically grown *R. spheroides* during adaptation to form photosynthetic pigments under conditions of low aeration (Lascelles, 1959). Over a period of 3 hr the lipid phosphorus content/mg. protein increased by about 1·7-fold, while the bacteriochlorophyll content increased by about 200-fold (Fig. 2*a*). Suspensions of the mutant strain of *R. spheroides*, which does not form pigments, were incubated under the conditions

Table 1. *Phospholipid content of Rhodopseudomonas spheroides* 8253 *and L-70*

The mean values for lipid phosphorus are from analyses of four different batches of organisms grown under the various conditions; the range is shown in brackets. The distribution of lipid phosphorus in the various phospholipid fractions is the result of one analysis in each case.

Strain no.	Growth conditions	Bacteriochlorophyll (mμmoles/mg. protein)	Lipid phosphorus (mμmoles/mg. protein)	Distribution of lipid phosphorus* (%)			
				Phosphatidyl-ethanolamine	Phosphatidic acid	Phosphatidyl-choline	Phosphatidyl-glycerol
8253	Anaerobic in light	44 (36–48)	157 (144–172)	40	24	22	14
	Aerobic in dark	0·48 (0·44–0·56)	90 (73–105)	39	28	20	13
L-70	Aerobic in dark	n.d.	82 (69–97)	41	24	21	14

n.d. = not detectable.
* = as % of total phosphorus recovered after chromatography of the deacylated lipids.

Table 2. *Distribution of lipid phosphorus in lysed preparations of Rhodopseudomonas spheroides* 8253

Suspensions of organisms containing 140–200 mg. protein were incubated for 15 min. at 34° in the presence of (final conc.): tris buffer (pH 8·6), 0·1 M; EDTA, 5 mM; lysozyme, 0·5 mg./ml. $MgSO_4$ (10 mM) and DNA-ase (5 μg./ml.) were then added and the mixture incubated for 5 min. longer. Samples (2 ml.) were removed for determination of lipid phosphorus on the whole lysate. The remainder was centrifuged for 1 hr at 100,000*g*; the pellet was resuspended in 20 ml. water and centrifuged again. The washed pellet was suspended in 20 ml. water and samples taken for determination of lipid phosphorus.

Expt.	Type of organisms	Total protein of suspension (mg.)	Total lipid phosphorus (μmoles)		Lipid phosphorus recovered in pellet (%)
			Whole lysate	Pellet fraction	
1	Pigmented	162	24·2	22·4	93
2	Pigmented	139	24·6	24·7	100
3	Non-pigmented	202	22·2	20·3	92

which promoted pigment formation by the parent organism. Though phospholipid synthesis occurred it was matched by the increase in protein and consequently the amount of lipid phosphorus/mg. protein did not change (Table 3).

To determine whether there were differences in the rate of synthesis of the various phospholipids under these conditions, experiments were done with [^{32}P]phosphate. The specific activity of the deacylated lipids was determined after separation by

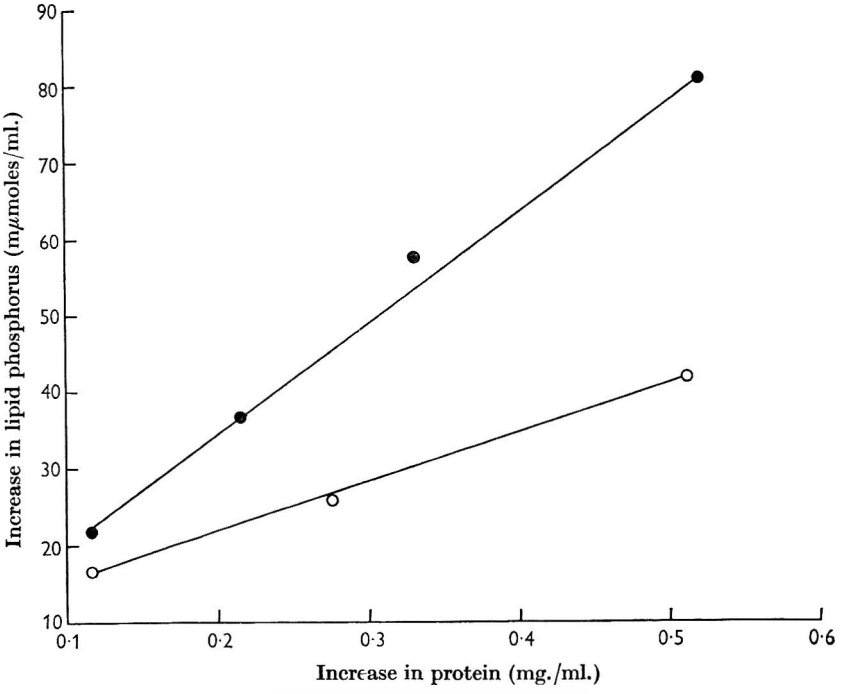

Fig. 1. Differential rate of phospholipid synthesis by cultures of *Rhodopseudomonas spheroides* 8253 growing anaerobically in the light (●) and aerobically in the dark (○).

paper chromatography. Phosphatidylglycerol increased in radioactivity at a more rapid rate than the other phospholipids; phosphatidylcholine was formed at the slowest rate (Fig. 2*b*). This pattern of incorporation of ^{32}P into the phospholipids was, however, not uniquely associated with pigment synthesis. The same rapid incorporation into phosphatidylglycerol occurred with suspensions of the parent organism incubated under high aeration which represses pigment synthesis. Also, the mutant strain showed the same pattern of labelling as the parent organism when incubated under comparable conditions (Table 3).

DISCUSSION

The concentration of phospholipid in *Rhodopseudomonas spheroides* is of the same order as that found in other Gram-negative organisms; values varying from about 90 to 180 mμmoles lipid phosphorus/mg. protein have been found in a variety of non-photosynthetic organisms (MacFarlane, 1964; Kaneshiro & Marr, 1962). The pattern of phospholipids found in *R. spheroides* is also not unique; phosphatidyl-

ethanolamine is the predominant phosphatide in many bacteria, though the occurrence of phosphatidylcholine is less common (MacFarlane, 1964). The experiments with suspensions of *R. spheroides* with [^{32}P]-phosphate suggest that phosphatidylglycerol is the most metabolically active of the phospholipids. In growing cultures

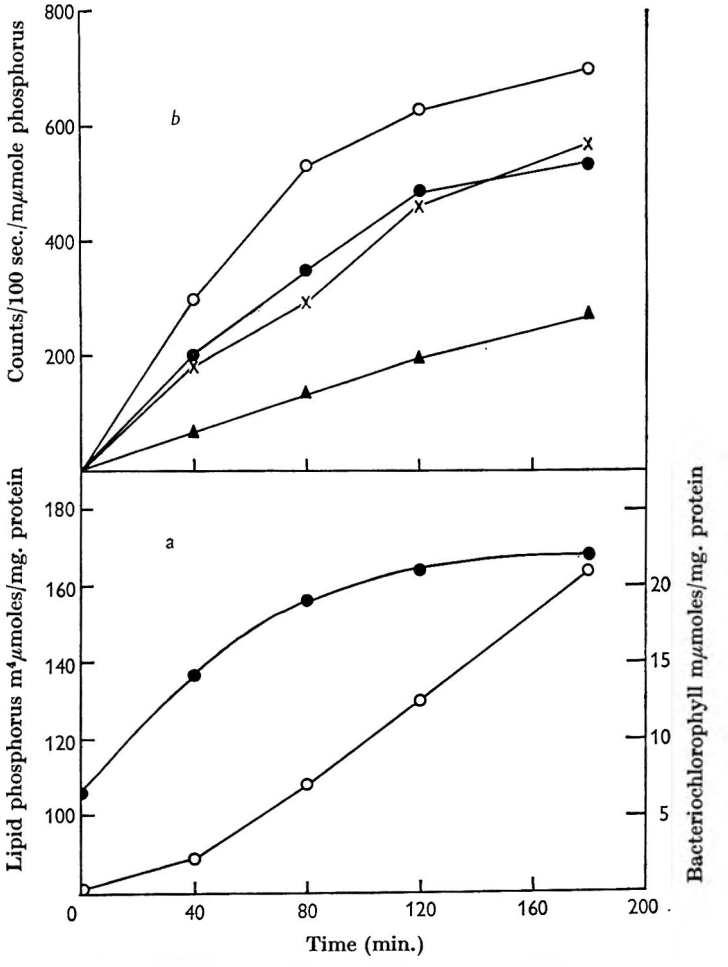

Fig. 2*a*, *b*. Synthesis of phospholipids and bacteriochlorophyll by suspensions of *R. spheroides* 8253 under low aeration. Aerobically grown organisms were suspended in P-medium supplemented with [^{32}P]KH_2PO_4 (1 mM; 3 μc./ml.) The suspensions were incubated under low aeration and samples removed at intervals for the determination of lipid phosphorus (●) and bacteriochlorophyll (○) (Fig. 2*a*). The deacylated phospholipids were separated by paper chromatography and the specific radioactivity determined as described in the Methods (Fig. 2*b*). Phosphatidylglycerol, ○; phosphatidic acid, ●; phosphatidylethanolamine, × ; phosphatidylcholine, ▲. Initial protein conc. = 0·8 mg./ml.; after 120 min., 1·4 mg./ml.

of *Escherichia coli* this phosphatide is subject to rapid turnover suggesting again that it plays some dynamic metabolic role, which has yet to be established (Kanfer & Kennedy, 1963).

Since the phospholipids are located in the membrane fraction of *Rhodopseudo-*

Table 3. *Phospholipid synthesis by suspensions of Rhodopseudomonas spheroides* 8253 *and L-70*

Suspensions of aerobically grown parent (8253) and mutant (L-70) organisms were incubated under low or high aeration in −P medium supplemented with [^{32}P]KH_2PO_4 (1 mM; 2·4 μc/ml.) Analyses were made as described in Fig. 2.

Strain no	Incubation conditions	Protein (mg./ml.)	Bacteriochlorophyll (mμmoles/mg. protein)	Lipid phosphorus (mμmoles/mg. protein)	Specific activity (counts/100 sec./mμmole phosphorus			
					Phosphatidylglycerol	Phosphatidic acid	Phosphatidylethanolamine	Phosphatidylcholine
8253	Nil	1·25	0·22	73	—	—	—	—
	High aeration; 40 min.	1·3	0·34	82	367	91	93	63
	High aeration; 120 min.	1·8	0·32	95	496	387	342	171
	Low aeration; 120 min.	1·5	6·3	122	515	370	375	213
L-70	Nil	0·85	0	81	—	—	—	—
	Low aeration; 40 min.	0·95	0	81	345	90	80	50
	Low aeration; 120 min.	1·4	0	87	676	294	282	179

monas spheroides the higher concentration found in pigmented organisms might suggest that pigment formation is associated with increased membrane formation. Such an interpretation would be consistent with the results of electron microscopy. However, there are other observations that do not support this. In both *R. spheroides* and *Rhodospirillum rubrum* the proportion of protein in the particulate fraction (assumed to be derived from the membrane) is similar in extracts from pigmented and non-pigmented organisms (Cohen-Bazire & Kunisawa, 1960; Sistrom, 1962; Bull & Lascelles, 1963). As suspensions of non-pigmented *R. spheroides* adapt to form bacteriochlorophyll protein synthesis occurs, but the distribution of protein between the soluble and particulate fractions remains the same (Bull & Lascelles, 1963). Thus, there is no evidence for a higher proportion of membrane protein in pigmented organisms, though the membrane fraction is apparently richer in phospholipid. Possibly, the pigmented organisms have a membrane structure differing from that in non-pigmented organisms in that it accommodates more phospholipid; this may serve as a medium for orientation of the hydrophobic pigment molecules. The problem of how the extra phospholipids and the photosynthetic pigments become incorporated into the membrane structure as organisms adapt from the non-pigmented to the pigmented state now requires to be answered. Perhaps the preferential turnover of protein in the particulate fraction that accompanies the adaptation (Bull & Lascelles, 1963) may represent some form of differentiation of the membrane into a lipid-enriched form.

Phospholipids may play an important role in all structures associated with electron transport. They comprise about 20% of the dry weight of preparations of respiratory particles from bacteria and their importance in mitochondrial electron transport is well established (Green & Fleischer, 1963). Studies of mitochondrial substructure have suggested that phospholipids are bound to a structural protein matrix as well as to the electron transport particles which are thought to be attached to the matrix (Richardson, Hultin & Green, 1963). Whether this concept can be extended to the photosynthetic and electron transport systems of bacteria requires fractionation of the complexes combined with cytological studies.

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The Metabolism of Polyphosphates in *Chlorobium thiosulfatophilum*

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SUMMARY

The synthesis and breakdown of long chain polyphosphate in *Chlorobium thiosulfatophilum* was studied. An enzyme is present which reversibly transfers phosphate groups from polyphosphate to adenosine diphosphate to give adenosine triphosphate. A second enzyme, an adenosine triphosphatase probably located in the cytoplasmic membrane, hydrolyses the adenosine triphosphate so formed to adenosine diphosphate and orthophosphate.

INTRODUCTION

Hughes, Conti & Fuller (1963) characterized long chain 'acid-insoluble' polyphosphate from the green sulphur bacterium *Chlorobium thiosulfatophilum*, and showed that this was associated with granules which were electron dense in thin sections in the electron microscope and stained metachromatically when treated with basic dyes such as toluidine blue. Experiments designed to measure photophosphorylation in cell-free extracts demonstrated a release of orthophosphate (P) which was stimulated by adenosine diphosphate (ADP), but was absent from cells depleted of polyphosphate. It was suggested that polyphosphate in Chlorobium is degraded in the presence of ADP to give adenosine triphosphate (ATP), and that this is hydrolysed by an adenosine triphosphatase (ATPase) to release orthophosphate and regenerate ADP according to the following scheme:

$$\begin{array}{c}(\text{Polyphosphate})_n + \text{ADP} \rightleftharpoons (\text{Polyphosphate})_{n-1} + \text{ATP} \\ \text{ATP} \rightarrow \text{ADP} + \text{orthophosphate} \\ \hline \text{Polyphosphate} + \text{ADP} \rightarrow \text{orthophosphate} + \text{ADP}\end{array}$$

In the present paper, it is shown that the orthophosphate does arise from polyphosphate breakdown under the control of ADP; ATP is formed as an intermediate and can be utilized for other energy-consuming processes such as the phosphorylation of glucose.

METHODS

Growth of organism. The growth medium used for the strict anaerobe *Chlorobium thiosulfatophilum*, strain 8346, was that described by Larsen (1953). The mixed medium was poured into sterile Pyrex reagent bottles (1, 2, 5 l. with ground-glass stoppers), inoculated, and the bottles then filled completely to avoid trapping air bubbles with the stopper. The freshly inoculated bottles were stored overnight in

complete darkness to remove any traces of oxygen by interaction with the hydrogen sulphide in the medium. They were then transferred to the photosynthesis room, which was illuminated with and maintained at 30° by a thermostatically controlled bank of 100 W. tungsten lamps. The inoculum consisted of organisms grown for 3–5 days and its volume was 20–30% (v/v) of the medium into which it was inoculated, this high volume being necessary to achieve satisfactory growth of dark-green to yellow-green organisms. Elementary sulphur was deposited in variable amounts in the medium during growth; very high light intensities appeared to increase the deposition of sulphur and led to low yields of organisms yellow in colour instead of green.

Preparation of cell-free extracts. Cultures were harvested after 3–6 days, depending on the rate of growth, by centrifugation at 14,000 *g* in 250 ml. containers (MSE Speed 17 centrifuge). Organisms were washed by suspending them in 0·1 M-tris + maleate buffer (pH 7·0) and centrifuging again at 14,000 *g*. Organisms together with fine particles of sulphur collected from the medium were crushed in a Hughes (1951) bacterial press at −25°. The disintegrated organisms were collected and resuspended in 0·1 M-tris + maleate buffer (pH 7·0) at 0° by using a Kontés type glass homogenizer. Next, 0·1 mg. deoxyribonuclease (DNAase) in distilled water was added for each 5·0 ml. of cell extract, and the preparation left at 0° for 30 min. Digestion of the deoxyribonucleic acid (DNA) was indicated by a marked decrease in the viscosity which facilitated further fractionation by differential centrifugation.

Electron microscopy. Whole organisms or fractions obtained by centrifugation were mounted by the 'blot dry' method on formvar-coated grids, the preparations being unfixed or fixed in 1% osmic acid. A mixture of 10% Pd and 90% Au was used for shadowing. Grids were examined in the Akashi TSR 50 Tronoscope at magnifications from ×500 to ×17,000.

Extraction of polyphosphate. Polyphosphate was extracted from organisms in two fractions as described by Muhammed (1959). First the organisms were shaken with 5% trichloroacetic or perchloric acid at 0°. This procedure was repeated, the flask being shaken for 1 hr each time. The residue was collected by centrifugation, and the extract contained 'acid-soluble' low molecular weight polyphosphate. The residue was then washed with distilled water and extracted with 10% trichloroacetic or perchloric acid, previous work (Muhammed, 1959) having shown that all the remaining polyphosphate is extracted by 10 vol. of acid within 4 hr at 20°. Both extracts were neutralized with alkali, brought to pH 3·5 with 5 M-acetate buffer, and treated with saturated $BaCl_2$ solution. Barium polyphosphate was allowed to separate out at 0° overnight and then collected by centrifugation. The barium salt was washed twice with acetate buffer (pH 3·5) and once with acetone, and the polyphosphate content estimated as orthophosphate liberated after hydrolysis with N-HCl for 7 min. at 100°.

Chemicals. Sodium polyphosphate was prepared by heating KH_2PO_4 at 780° as described by Pfanstiel & Iler (1952). When uniformly ^{32}P-labelled material was prepared, up to 1 mc. of carrier-free orthophosphate was added.

Adenosine triphosphate (ATP) was labelled in the terminal position by the method of Glynn & Chappel (1964). 1 mc. of ^{32}P-orthophosphate yielded 0·8 mc. of radioactive ATP, 98% of the ^{32}P being in the terminal position; small amounts of ADP were also present.

All other chemicals were commercial specimens, and were used without further purification.

Chemical estimations. Inorganic orthophosphate was estimated by the method of Fiske & SubbaRow (1925). Polyphosphate was estimated as orthophosphate after hydrolysis at 100° for 7 min. Any orthophosphate present was determined before hydrolysis.

Chromatography. Nucleotides were isolated by the method of Krebs & Hems (1953) in which orthophosphate is separated by ascending chromatography in the Hanes & Isherwood (1949) solvent. The paper was dried, phosphate removed, and nucleotides resolved by descending chromatography in isobutyric acid + ammonia. Orthophosphate or nucleotide spots were located either by their ^{32}P activity by using an end-window counter, or colorimetrically by a modification of the method of Hanes & Isherwood (1949) in which the nucleotides were hydrolysed by heating in an oven at 80° after the ammonium molybdate spray; the blue colour was developed by illumination from an ultraviolet lamp for 10 min.

Samples expected to contain ^{32}P-labelled glucose-6-phosphate (G-6-^{32}P) were chromatographed by the descending technique with the isobutyric acid + ammonia solvent described above to separate other phosphorus compounds from the sugar phosphates. The paper was dried, examined under the u.v. lamp and segments between the origin and the ATP spot cut off. These were eluted with water for 2 hr to bring the bulk of the ^{32}P activity in this segment into solution. The eluate was then chromatographed by descending chromatography with solvent 80 vol. methanol + 15 vol. 90% (w/v) formic acid + 5 vol. of 0·002M-EDTA as solvent (Bandurski & Axelrod, 1951). After 6 hr the chromatogram had run 30 cm. and a separation of G-6-P from glucose-1-phosphate and other sugar phosphates was obtained. The paper was dried, and the position of the G-6-^{32}P determined by an end-window counter, and confirmed by comparing it with the position of unlabelled marker G-6-P after development of both by spraying with the Hanes & Isherwood (1949) reagent described previously.

Measurement of ^{32}P radioactivity. The end-window counter used was the Panax Scaling Unit type D657 (Redhill, Surrey) the end-window counter operating at a voltage of 1625 V.

Liquid ionization counting was done in a 10 ml. counting tube type M6 (supplied by 20th Century Electronics Ltd., Croydon, Surrey) operating at 1090 V. in a lead castle from E.R.D. Engineering Co. Ltd. (Slough, Middlesex). The scaling unit with 'autocounts' and 'Autotime' refinements was type N 530 F obtained from Ecko Electronics Ltd. (Southend-on-Sea, Essex).

Estimation of enzymic activity. Cell extracts and other components to be incubated were put into 5 ml. Pyrex tubes standing in ice at 0°, made up to standard volume and mixed. Before and after incubation, 0·1–1·0 ml. samples were transferred to tubes containing the same volume of 1% (w/v) solution, and allowed to stand at 0° for several hours in the cold. Trichloroacetic acid (0·5 ml., 10%, w/v, TCA) was then added to precipitate protein and polyphosphate as a protein–polyphosphate complex and the tubes centrifuged at 6000 *g* for 2 min. Samples of the clear supernatant fluid were then immediately analysed for orthophosphate. Control experiments to study the efficiency of this procedure for removing polyphosphate showed that the method finally adopted was satisfactory.

RESULTS

Phosphate content of whole organisms and extracts. Approximately 5 g. (wet wt.) organisms were collected by centrifugation, washed with 0·9% saline and extracted twice with 5% and once with 10% trichloroacetic acid. Acid-labile phosphate present in the 10% TCA extract was taken as the polyphosphate content of the organisms, and amounted to 40% of the cell total-phosphorus.

Cell-free extracts from such organisms were centrifuged at various speeds and the polyphosphate content of supernatant fluids and solid residues estimated (Table 1). Both supernatant fluids and solid residues contained polyphosphate in sufficient amounts to account for the orthophosphate formation studied in the subsequent sections. This widespread distribution of polyphosphate in fractions separated from disintegrated organisms is similar to that found by Muhammed (1959) and is probably due to the fragmentation of the metachromatic granules.

Table 1. *Polyphosphate content of cell-free extracts of Chlorobium thiosulfatophilum prepared by centrifugation*

Crude extract (12·0 ml.) was centrifuged at 25,000 *g* for 30 min.; then at 10,000 *g* for 1 hr and then 20 hr. Polyphosphate was extracted and estimated in the solid residues and supernatant fluids as described in the text.

Fraction	Supernatant fluids. Polyphosphate content (μmole)	Residues. Polyphosphate content (μmole)
25,000 *g* extract	389	84
100,000 *g* (1 hr) extract	355	34
100,000 *g* (20 hr) extract	162	193

Table 2. *ADP dependence of orthophosphate release from crude cell-free extracts of Chlorobium thiosulfatophilum*

Crude extract (1·0 ml. containing 0·1 g wet weight organism/9 μmole synthetic polyphosphate) and ADP in a total vol. of 2·0 ml. were incubated at 27° for 4 hr. 0·5 ml. 1% (w/v) bovine serum albumin solution was used to precipitate polyphosphate. Samples were analysed for orthophosphate before and after incubation.

	ADP added (μmole)	Orthophosphate released (μmole)
Expt. 1	0	0·4
	3·5	2·7
	7·0	2·9
	14·0	0·1
Expt. 2	0	3·3
	1·6	4·9
	9·5	6·9
	38·0	1·6

Phosphate formation by cell-free extracts. The crude cell-free extract was incubated with and without added ADP. Low concentrations of ADP stimulated, but high concentrations inhibited the release of orthophosphate (Table 2). After centrifuging

at 6000 *g* for 10 min. to remove uncrushed organisms and larger pieces of cell debris, the supernatant fluid was incubated with 3·2 μmoles uniformly ^{32}P-labelled synthetic polyphosphate, with and without ADP. Release of orthophosphate, measured either colorimetrically or by radioactivity, was higher in the presence of ADP (Table 3). The ratio of orthophosphate release with and without ADP (1·4) was approximately equal to the corresponding ratio of the increase in radioactivity (1·26). This agreement supports the idea that the bulk of the orthophosphate formed arose from polyphosphate and that added synthetic polyphosphate was metabolized at the same rate as the endogenous material.

Table 3. *Metabolism of radioactive synthetic polyphosphate by crude extracts of Chlorobium thiosulfatophilum*

The cell-free extract was incubated with 3·2 μmole ^{32}P-labelled synthetic polyphosphate, and with and without added ADP.

ADP added (μmole)	Orthophosphate released (μmole)	Increase in radioactivity after incubation (counts/100 sec.)
7	14·0	592
0	9·1	463

Table 4. *Effect of centrifugation on orthophosphate release from Chlorobium extracts*

1·0 ml. samples of cell-free extracts obtained after different degrees of centrifugation (see text) were incubated at 27° for 5 hr with and without added ADP and polyphosphate. Both the increase in radioactivity in the orthophosphate fraction, and the orthophosphate released during the incubation period were assayed.

25,000 *g* residue (ml.)	25,000 *g* supernatant fluid (ml.)	100,000 *g* residue (ml.)	100,000 *g* supernatant fluid (ml.)	ADP added mole	Poly ^{32}P added mole	P_i* released mole	^{32}P† released (counts/100 sec.)
1·0	—	—	—	—	3·5	0·1	24
1·0	—	—	—	7	3·5	0·1	44
—	1·0	—	—	—	3·5	4·8	405
—	1·0	—	—	7	3·5	9·3	591
—	—	1·0	—	—	3·5	0·3	36
—	—	1·0	—	7	3·5	0·5	182
—	—	1·0	—	—	—	0·6	—
—	—	1·0	—	7	—	0·5	—
—	—	—	1·0	—	3·5	4·6	472
—	—	—	1·0	7	3·5	9·0	620
—	—	—	1·0	—	—	5·3	—
—	—	—	1·0	7	—	10·8	—

* P_i + inorganic P.

† These results give the increase in ^{32}P activity (counts/100 sec.) in the orthophosphate samples assayed. Polyphosphate had been removed as the protein complex with TCA.

Effect of centrifugation. The crude extract was centrifuged at 25,000 *g* for 30 min. and separated into supernatant fluid and residue fractions. The supernatant fluid contained most of the dark green pigment and the particles measuring about 200 Å in diameter which appeared to be similar to those previously found to photophosphorylate (Hughes *et al.* 1963). An attempt was made to isolate these particles

free from contaminating cytoplasmic membrane by further centrifugation at 100,000 *g* for 1 hr and then for 20 hr at this relative centrifugal force (RCF). Electron micrographs showed that all fractions obtained contained small pieces of cell membrane, and that these tended to associate in clumps with other organelles and cell fragments. The stimulation of orthophosphate release was greater in the supernatant fluids of these centrifuged fractions. They also were found to yield labelled orthophosphate from added synthetic ^{32}P-labelled polyphosphate (Table 4).

Some preliminary experiments were made in which a bacterial ATPase prepared from *Lactobacillus arabinosus* was added to the extracts. Stimulation of orthophosphate release from polyphosphate was found, but the experiments were unsatisfactory because the ATPase preparation was found to have polyphosphatase and a marked ADPase activity which gave rise to high blanks.

Table 5. *Formation of ^{32}P-orthophosphate from radioactive ATP*

Supernatant fluids from centrifuged extracts of *Chlorobium thiosulfatophilum* were incubated with ^{32}P-labelled ATP. Samples were chromatographed before and after incubation to separate orthophosphate and nucleotide phosphates, and the release of ^{32}P-orthophosphate from ATP determined.

	25,000 *g* supernatant fluid	100,000 *g* supernatant fluid
μmole P_i released	9·7	2·9
Increase in ^{32}P-activity in P_i/10 l. incubation mixture/400 sec.	1160	436
Corresponding decrease in ATP radioactivity	920	404

Most fractions formed ^{32}P-orthophosphate from added AT^{32}P (Table 5). The activity was greatest in the crude fractions and was decreased in the supernatant fluids of fractions centrifuged at highest speeds (100,000 *g*). This is consistent with the idea that the bulk of the bacterial ATPase activity is associated with particulate fractions which in some bacteria are derived wholly from the cytoplasmic membrane (Hughes, 1962). As stated above, it has not yet been found possible to prepare purified membrane fractions from Chlorobium.

The supernatant fluids were incubated with ATP (5 μM) and ADP. Phosphate formation was decreased from 9·7 to 6·0 μmole for the 25,000 *g* supernatant fluid when extra ADP was present, and from 2·9 to 2·0 μmole for the 100,000 *g* (1 hr) extract. This suggests that the ATPase is inhibited by ADP.

Formation of ATP from polyphosphate. The 25,000 *g* supernatant fluid extract was incubated with ^{32}P-labelled polyphosphate and ADP, and samples were chromatographed to separate nucleotide phosphates and orthophosphates from polyphosphate. Spots corresponding in position to ATP, ADP, orthophosphate, were found to be labelled together with an unidentified spot. The ATP spot was the most active; the orthophosphate had about 60 % of the ATP activity, and both the ADP and the unidentified spot approximately 25 % of that of the ATP. These proportions suggest that ADP and the unidentified fraction arose from secondary reactions of orthophosphate and ATP. The unidentified spot had an R_F value, relative to marker AMP, of 0·4, the value characteristic of sugar phosphates. These results are consistent with the presence of an enzyme similar to that in *Escherichia coli*

(Kornberg, Kornberg & Sims, 1956) which can form ATP from polyphosphate and ADP.

To establish further that polyphosphate gave rise to ATP directly, glucose and hexokinase were added to the usual incubation mixture. After incubation, 3% (v/v) bovine serum albumin and 10% (w/v) TCA were added to remove polyphosphate, cell debris and protein, and the supernatant fluid after centrifugation was chromatographed to separate G-6-P from nucleotide phosphates and other sugar phosphates. The sugar phosphate spot was eluted and concentrated by evaporation. Standard marker G-6-P was chromatographed alone and with the test sample, and in each case the standard and the radioactive spot had identical R_F values. The ^{32}P-activity found in G-6-P after incubation was 70% of the activity originally added as polyphosphate. In previous experiments with synthetic polyphosphate in which orthophosphate release was followed, about 10% of the added polymer only was metabolized.

The reversibility of the enzyme which catalysed the phosphorylation of ADP by polyphosphate was tested by incubating the 25,000 *g* supernatant fluid extract with ^{32}P-labelled ATP, and isolating the polyphosphate formed. After 5 hr at 31°, carrier Kurrol's salt, 5% (v/v) albumin and 10% (w/v) TCA were added to the incubation mixture. The resulting precipitate was collected by centrifugation, extracted with 10% (w/v) TCA for 4 hr and barium polyphosphate precipitated at pH 3·5. This was washed with two volumes of water and two of ethanol to ensure complete removal of ATP. The precipitate was then spread on a filter paper, air dried, and the radioactivity counted with the end-window apparatus; 12·9% of the original activity added as AT^{32}P was found in this polyphosphate.

DISCUSSION

The presence of two enzymes, one reversibly forming ATP from polyphosphate in the presence of ADP, and the other hydrolysing ATP to ADP + orthophosphate, would account for these and all previous observations of ADP-stimulated phosphate release in cell-free extracts of *Chlorobium thiosulfatophilum*. The polyphosphate metabolic pathway may be summarized as:

$$(\text{Polyphosphate})_n + \text{ADP} \rightleftharpoons \text{ATP} + (\text{polyphosphate})_{n-1} \quad (1)$$

$$\text{ATP} + \text{H}_2\text{O} \rightarrow \text{ADP} + \text{H}_3\text{PO}_4 \quad (2)$$

The inhibition of orthophosphate release by high concentrations of ADP, the decrease in ATPase activity in the presence of added ADP, and the high rate of formation of G-6-P when excess glucose + hexokinase are present, all suggest that reaction (2) is the rate-limiting one and that polyphosphate breakdown is controlled by the concentration of ADP. The reversibility of enzyme (1) shows that it is similar to that found in *Escherichia coli* by Kornberg *et al.* (1956) and Kornberg (1957). Both enzymes reversibly transfer terminal 'high energy' phosphate units from polyphosphate to ADP to form ATP. It would appear that, in this phototrophic organism *Chlorobium thiosulphatophilum*, when ATP is formed in excess of other requirements by photophosphorylation (Arnon, 1959), polyphosphate is synthesized. Under conditions where ATP utilization is high, net breakdown of polyphosphate could occur. This would be similar to the mechanism proposed for chemohetero-

trophs (Hughes & Muhammed, 1962) where the disappearance of polyphosphate and metachromatic granules has been associated with rapid growth (Harold, 1963). Polyphosphate in this organism, by its ability to give rise to ATP, may be regarded as an 'energy store' or 'phosphagen'. No evidence has been found in the present work for transference of phosphate from polyphosphate to other acceptors directly without the intermediation of ATP (Szymona, 1962).

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Hexokinase of *Escherichia coli*. Assay of Enzyme Activity and Adaptation to Growth in Various Media

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SUMMARY

Hexokinase was determined in extracts of *Escherichia coli* by measurement of the reduction of nicotinamide-adenine dinucleotide phosphate by glucose-6-phosphate dehydrogenase, and the glucose-6-phosphate formed by the action of hexokinase. This method gave reproducible results. Extracts of *E. coli* contained low hexokinase activity when the organism had been grown in the absence of glucose and this enzyme activity was increased after adaptation of the organism to growth on glucose. The *E. coli* needed 2–2·5 divisions to reach the degree of hexokinase activity of glucose-grown bacteria when transferred to salts + glucose from media not containing glucose.

INTRODUCTION

Previous studies (Scott & Chu, 1959) showed that glucose-6-phosphate (G-6-P) dehydrogenase activity was higher in extracts of *Escherichia coli* when the bacteria had been grown in the presence of glucose than when grown in media containing other sources of carbon. Phosphogluconate (PG) dehydrogenase activity was relatively constant in the extracts from bacteria grown in different media. In further studies of the uptake and utilization of glucose by *E. coli*, the activity of hexokinase was determined to see whether it behaved like G-6-P or PG dehydrogenase (see below). It was found that certain changes in the media involving carbon sources or other additions produced changes in the degree of activity of the hexokinase in extracts which were not, however, as great as the changes in G-6-P dehydrogenase.

$$\text{Glucose} + \text{ATP} \xrightarrow[Mg^{2+}]{\text{hexokinase}} \text{glucose-6-phosphate} \xrightarrow[NADP^{+},\ Mg^{2+}]{\text{G-6-P dehydrogenase}}$$

$$\text{phosphogluconate} \xrightarrow[NADP^{+},\ Mg^{2+}]{\text{PG dehydrogenase}} \text{ribulose-5-phosphate}$$

METHODS

The method for cultivation of 'weekly' and 'overnight' cultures was described previously (Scott & Chu, 1959). The cultures of *Escherichia coli* strain B were grown at 37° in an aerated medium of Bacto-beef extract and Bacto-peptone (Difco

Laboratories, Detroit, Michigan, U.S.A.) or salts media (Cohen & Arbogast, 1950), with glucose 2 mg./ml. medium. Casein hydrolysate (an acid hydrolysate of casein; General Biochemicals, Inc., Chagrin Falls, Ohio, U.S.A.) 4 mg./ml. medium, or Difco yeast extract (the water-soluble portion of autolysed fresh yeast) 2 mg./ml. medium, was added to the salts media instead of glucose as indicated.

The 'weekly' culture, which was a salts + glucose culture, was used to inoculate two 'overnight' cultures of either one form of salts medium or nutrient broth. One culture was harvested to be extracted. The other was centrifuged and the bacteria were resuspended in salts medium to inoculate the final cultures, which were allowed to grow 1, 1·5 or 2 divisions as noted in Table 1. The harvested organisms were washed in saline, recentrifuged, the bacterial pellet weighed, ground with alumina (2·5 × wet weight) in the cold and extracted with 0·05 M-tris buffer adjusted to pH 7·4 with 2 N-HCl. The extracts were centrifuged 1 hr at 10,000*g* at 4° in an International Refrigerated Centrifuge PR-2. The amounts of protein in the extracts were determined by light extinction after precipitation with 10% (w/v) perchloric acid.

The methods of measurement of G-6-P and PG dehydrogenases were as reported previously (Scott & Cohen, 1953). The direct method for the hexokinase reaction was done in 1 ml. of a mixture containing 0·05 M-tris chloride buffer (pH 7·4), 1 μmole glucose, 10 μmoles $MgSO_4$, 0·02 N-nicotinamide and extract containing 0·05–1 mg. protein. $NADP^+$ was added and the endogenous reaction recorded. With the addition of 1 μmole ATP, the G-6-P formed was oxidized by the G-6-P dehydrogenase, permitting the rate of NADPH formation to be measured at 340 mμ at 37° in a Beckman DU spectrophotometer. Two other methods were also used for comparison; these were measurement of glucose removed and measurement of G-6-P formed. The mixture described above that was used for the direct measurement was incubated in a 37° water bath and 1 ml. samples removed at selected times into cold HCl (0·04 mM). After centrifugation at 4°, the supernatant fluid was neutralized with NaOH (0·04 M). Glucose and G-6-P concentrations were measured in the samples. The glucose was determined by the 'Glucostat', a preparation of glucose oxidase (Worthington Biochemical Corp., Freehold, New Jersey, U.S.A.). The assay for G-6-P was done in 1 ml. of a mixture of 0·05 M-tris chloride buffer (pH 7·4), 10 μmoles $MgSO_4$, 0·4 μmole $NADP^+$ and the sample. Purified G-6-P dehydrogenase was added and the total formation of NADPH was measured in a Beckman DU spectrophotometer at 340 mμ at 37°. The G-6-P dehydrogenase was obtained either from Sigma Chemical Co. (St Louis, Missouri, U.S.A.) or was purified in our laboratory from extracts of *Escherichia coli* by methods previously reported (Scott & Cohen, 1953).

RESULTS

Figure 1 shows the rate of reduction of $NADP^+$ by G-6-P formed when ATP was added to mixtures of glucose and an extract of *Escherichia coli* grown in salts + glucose medium. The rate of reaction with G-6-P indicated that G-6-P dehydrogenase was present in large excess in the extract and was not limiting in the measurement of the rate of the hexokinase reaction. In the hexokinase assay during the first few minutes the G-6-P formed was limiting; then a constant rate was attained which was taken as the measure of the hexokinase activity. There was no NADPH oxidase activity in these extracts when magnesium ions were present unless oxidized

glutathione was added. There was slight or no activity with ATP or glucose omitted. The presence of nicotinamide inhibited $NADP^+$ase. It was not necessary to make corrections for NADPH produced by PG dehydrogenase since the NADPH was in high enough concentration to inhibit PG dehydrogenase activity. The presence of some activator in the extracts was indicated by the lack of proportionality of activity to protein content at low dilutions of extracts, as is illustrated in Fig. 2.

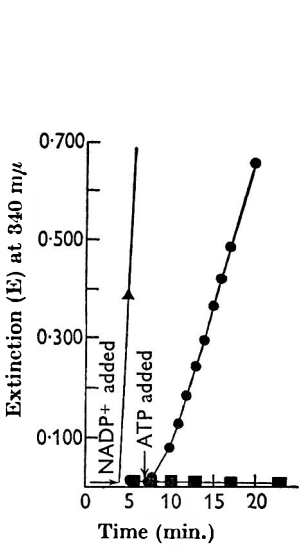

Fig. 1

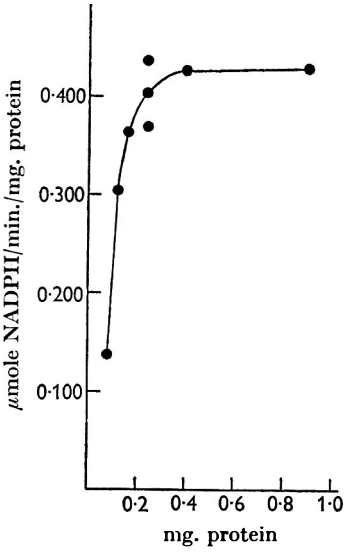

Fig. 2

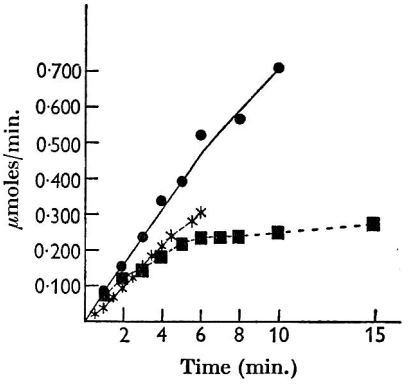

Fig. 3

Fig. 1. Hexokinase of extracts of *Escherichia coli* grown in salts + glucose medium. Each 1·5 ml. cuvette contained 10 μmoles $MgSO_4$, 0·01 ml. extract (equiv. 81 μg. protein). ▲, G-6-P dehydrogenase reaction: 1 μmole G-6-P, 0·05 M-tris chloride buffer (pH 8·7). ●, Hexokinase reaction: 0·05 M-tris chloride buffer (pH 7·4), 1 μmole glucose, 1 μmole nicotinamide; 0·03 ml. $NADP^+$ (10 mg./ml.) was added to each cuvette. After 3 min. 1 μmole ATP was added. Total volume was 1 ml. ■, Hexokinase reaction with either ATP or glucose omitted. Extinction, E, at 340 mμ was read on a Beckman DU spectrophotometer at 37°.

Fig. 2. Effect of sample size on hexokinase activity. The sample used was an extract from a salts + glucose culture of *Escherichia coli* that contained equiv. 8·1 mg. protein/ml. The other contents of the cuvette were as in Fig. 1.

Fig. 3. Comparison of three methods to determine hexokinase of *Escherichia coli* extracts. The extract used was from bacteria grown on salts + glucose medium and contained equiv. 9·82 mg. protein/ml. The methods are explained in the Method section. The direct method (*) was a measurement of μmole NADPH/ml. reaction mixture. The glucose uptake method (●) was a measurement of μmole glucose removed/ml. reaction mixture and the G-6-P measurement (■) was a measurement of μmole G-6-P found/ml. reaction mixture.

The comparison of the three methods used for determination of hexokinase is shown in Fig. 3. The initial rates of glucose disappearance and appearance of G-6-P were the same for the first 2 min. After 2 min. the G-6-P was metabolized by other pathways, so that it became unavailable for measurement. The glucose was taken up at a decreasing rate because of the inhibition of hexokinase by G-6-P formed and not removed (Crane & Sols, 1954).

Table 1. *Effect of transfer of Escherichia coli to different media on enzyme activities**

	Medium	Number of divisions	Hexokinase Units/ mg. protein	Hexokinase Units/ 10^9 bacteria	G-6-P dehydrogenase Units/ mg. protein	G-6-P dehydrogenase Units/ 10^9 bacteria	PG dehydrogenase Units/ mg. protein	PG dehydrogenase Units/ 10^9 bacteria
I.	Inoculum nutrient broth (overnight)	—	100	100	100	100	100	100
	A. ½ vol. nutrient broth + ½ vol. salts + glucose	1	285	400	110	137	92	124
		2·5	232	164	116	76	104	70
	B. Salts + glucose	1	92	86	112	112	93	86
		1	100	212	132	274	96	200
		1	52	59	218	227	97	105
		1·5	206	271	132	158	78	100
		1·5	150	350	120	313	78	186
II.	Inoculum salts + glucose	2	100	100	100	100	100	100
	A. ½ vol. nutrient broth + ½ vol. salts + glucose	1	74	—	56	—	88	—
	B. Nutrient broth	1	34	6	32	6	109	19
		2	44	—	57	—	82	—
		2	52	10	76	14	106	19
		3	54	—	60	—	—	—
		3	56	12	54	10	96	19
III.	Inoculum ½ vol. nutrient broth + ½ vol. salts + glucose	1	100	100	100	100	100	100
	Nutrient broth	1	67	—	62	—	108	—
IV.	Inoculum salts + casein hydrolysate (overnight)	1	100	100	100	100	100	100
	Salts + glucose	1	191	300	190	314	84	132
		1	89	94	157	170	114	120
		1	207	300	207	278	79	106
		1·5	228	238	228	245	100	102
		1·5	126	144	184	206	102	174
		1·5	230	550	322	733	72	171
V.	Inoculum salts + casein hydrolysate + yeast extract (overnight)	1·5	100	100	100	100	100	100
	Salts + glucose	1	162	150	182	170	105	100
		1	170	200	126	158	91	113
		1·5	218	225	300	290	110	107
		1·5	168	230	167	239	114	160
VI.	Inoculum salts + yeast extract (overnight)	1·5	100	100	100	100	100	100
	Salts + glucose	1	107	325	186	590	118	338
		1	67	125	114	216	108	219
		1·5	110	400	161	620	106	375
		1·5	106	350	163	426	118	333

* The enzyme activities of the inoculum were set at 100.

Table 1 lists the results from experiments testing the effects of growth in different media on values of hexokinase activity. The activity of the original inoculum is taken as 100 %. In the first series (I), the overnight inoculum was grown in nutrient broth. When the bacteria were transferred to media of equal parts of nutrient broth and salts + glucose, in only one division striking increases (3-fold) were found in hexokinase activity. The *Escherichia coli* grown on nutrient broth were smaller in size than those organisms grown on defined media with glucose. This difference in size was reflected in differences in enzyme activity/10^9 bacteria as compared to activity/mg. protein. The G-6-P dehydrogenase showed some fluctuation but the PG dehydrogenase (/mg. protein) remained in the same range for all the series with a mean

Table 2. *Average division times of growing cultures of Escherichia coli*

Inoculum	Medium	Division	Time (min.)
Salts + glucose	Salts + glucose	1	73
		2	60
		3	60
Nutrient broth	Salts + glucose	1	175
		1·5	48
Salts + casein hydrolysate	Salts + glucose	1	108, 78*
		1·5	48
		2	67*
Salts + yeast extract	Salts + glucose	1	105
		1·5	40
Salts + casein hydrolysate + yeast extract	Salts + glucose	1	142
		1·5	52
Salts + glucose	Nutrient broth	1	62
		2	58
		3	46
½ vol. salts + glucose + ½ vol. nutrient broth	Nutrient broth	1	30
Nutrient broth	Nutrient broth	1	65
Nutrient broth	½ vol. nutrient broth + ½ vol. salts + glucose	1	70
		2	88
Salts + glucose	½ vol. nutrient broth + ½ vol. salts + glucose	1	62
Salts + casein hydrolysate	Salts + casein hydrolysate	1	95
		2	70
Salts + yeast extract	Salts + yeast extract	1	75
		2	60
Salts + yeast extract + casein hydrolysate	Salts + yeast extract + casein hydrolysate	1	90

* With 8 mg. casein hydrolysate/ml.

of 97·5 % ± 11 (S.D.). When the overnight inoculum grown in nutrient broth was put into salts + glucose containing no nutrient broth, the first division was delayed. Division occurred at 175 min. instead of 73 min. when the inoculum was grown in salts + glucose. The hexokinase ranged from 50 to 100 % of control but, at ½ division time (48 min.) later, the activity increased sharply. Table 2 shows a comparison of division times in different media.

In series II (Table 1) where the bacteria were grown in salts + glucose for two divisions and then were transferred to media made up of equal parts salts + glucose

and nutrient broth for 1 division, the hexokinase activity was decreased 25% and the G-6-P dehydrogenase was decreased 50%. When bacteria grown in salts + glucose + nutrient broth media for one division (Table 1, series III) were transferred to nutrient broth for 1 division, the activity of both enzymes fell 40% from their previous values or 80% from the original salts + glucose values. Series II (Table 1) shows the greater decreases when bacteria grown on salts + glucose (2 divisions) were transferred to nutrient broth for 1 division. Both hexokinase and G-6-P dehydrogenase activities were only 30% of original values/mg. protein. When the *Escherichia coli* organisms were allowed to grow 2 or 3 divisions in nutrient broth, the activity of both enzymes levelled off at 50% of original activity of the salts + glucose inoculum. The activity of both enzymes/10^9 bacteria decreased to 15% of those grown in glucose because the bacteria were so much smaller.

Bacteria grown in salts + casein hydrolysate overnight and transferred to salts +

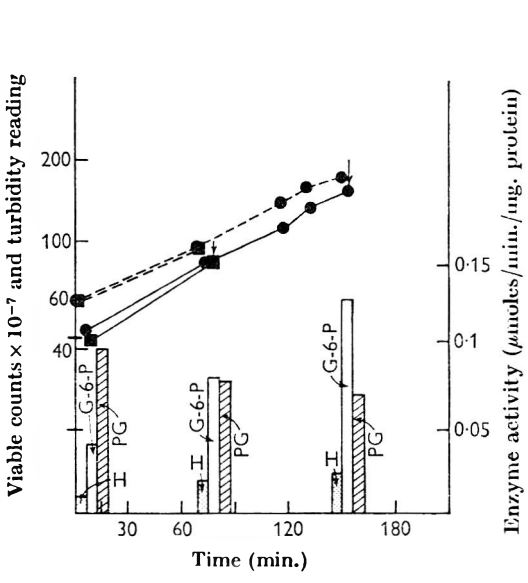

Fig. 4

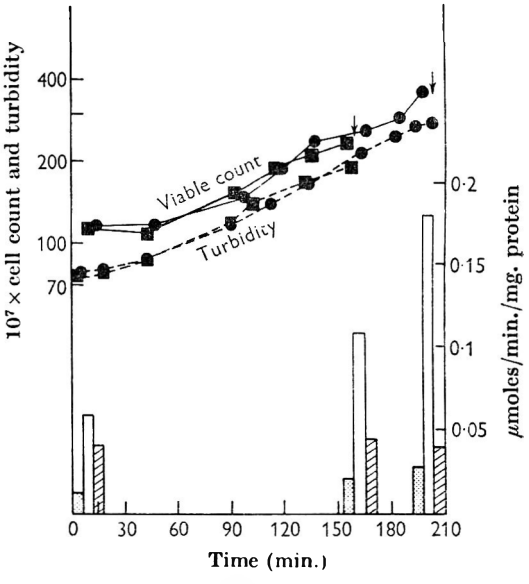

Fig. 5

Fig. 4. Growth and enzyme values of *Escherichia coli* grown in salts + casein medium and transferred to salts + glucose medium. Two towers each containing 200 ml. of salts + casein medium were grown overnight. One was harvested to be extracted; the other was centrifuged, the organisms washed and suspended in salts medium. This suspension was used to inoculate two towers each containing total 200 ml. salts medium + glucose (2 mg./ml.). Turbidity curves are shown as - - - and viable cell counts as —. Arrow ↓ indicates time of harvesting. Enzyme activities of the extracts of the washed pellets expressed in μmole $NADP^+$/min./mg. protein are shown in the histograms, shaded for hexokinase (H), clear for G-6-P dehydrogenase and cross-hatched for PG dehydrogenase. The turbidity was measured with a 420 mμ filter in the Klett photometer at the time of harvest is indicated by $\log_2 T/T_0$.

Fig. 5. Growth and enzyme values of *Escherichia coli* grown in salts + casein + yeast-extract medium and transferred to salts − glucose medium. Two towers each containing 200 ml. of salts + casein + yeast-extract were grown overnight. One was harvested for extraction and the other was centrifuged, washed and suspended in salts medium. This was used to inoculate two towers each containing total 200 ml. salts medium with glucose (2 mg./ml.). Symbols are as in Fig. 4.

glucose showed a two- to threefold increase in activity of hexokinase and G-6-P dehydrogenase as seen in series IV (Table 1). When the overnight cultures also had yeast extract added (Table 1, series V), the same pattern resulted. However, when the overnight inoculum was grown in salts + yeast extract (Table 1, series VI), there was essentially no gain in hexokinase activity/mg. protein and a 1·5–4-fold gain/10^9 bacteria. The G-6-P dehydrogenase showed a gain of 1·5-fold/mg. protein and of 2–6-fold/10^9 bacteria. Figures 4 and 5 show typical experiments with turbidity and enzyme activities plotted.

DISCUSSION

The experiments showed that the spectrophotometric method for determining hexokinase was reproducible. Cochrane, Peck & Harrison (1953) showed with extracts of a Streptomyces a reduction of $NADP^+$ by glucose and ATP and by G-6-P dehydrogenase and PG dehydrogenase, indicating the presence of hexokinase. Changes in hexokinase activity in *Escherichia coli* could be induced by changes in media. The hexokinase activity was increased after changes from media not containing glucose to media containing glucose. It was reported by Damodaran, Jagannathan & Singh (1955) for an Aspergillus that most of the enzymes of the glycolytic pathway and the hexosemonophosphate shunt, including hexokinase, decreased as the culture aged while growing on glucose and accumulating citrate. Hamilton & Dawes (1959, 1960, 1961) reported that *Pseudomonas aeruginosa*, when grown on medium containing half glucose and half citric acid from an inoculum grown on citric acid, used first the citric acid and then the glucose, so that a 'diauxie' was shown. However, when the inoculum organism was grown on glucose, both citric acid and glucose were metabolized simultaneously. In our experiments when *Escherichia coli* grown on nutrient broth was used to inoculate a medium containing half salts + glucose medium and half nutrient broth, there was a lag while the hexokinase built up, and then growth began. The *E. coli* needed 2·5 divisions to reach the degree of hexokinase activity of glucose-grown bacteria when transferred to salts + glucose from any medium tested, except casein hydrolysate + yeast extract, which needed only 2 divisions. The mechanism of adaptation must be the same as or similar to that reported by Hamilton & Dawes. There does not seem to be a substance in the casein hydrolysate, yeast extract or nutrient broth which acts as a repressor of hexokinase because glucose induced hexokinase in their presence. In an attempt to find the activator in the extracts a whole series of amines and other compounds were tested in our system. A report of these results is in preparation.

A preliminary report of a part of this work was presented at the Fifty-Ninth General Meeting of the Society of American Bacteriologists, May, 1959. This study was supported by grants C 4170 and CY 2189 from the National Cancer Institute, National Institutes of Health, United States Public Health Service.

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Further Studies on Cocoa Yellow Mosaic Virus

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SUMMARY

Cocoa yellow mosaic virus from Sierra Leone is readily transmitted by sap to many woody and herbaceous dicotyledonous plants. It is serologically related to wild cucumber mosaic virus and to turnip yellow mosaic virus, and has similar chemical and physical properties. It is stable when frozen or lyophilized, inactivated when kept for 10 min. at 65°, and is precipitated without loss of infectivity in a half-saturated ammonium sulphate solution. The particles appear to be 25 mμ across when mounted in neutral phosphotungstate, and 29 mμ when shadowed. All preparations contained infective nucleoprotein particles which sedimented at 108 S, and non-infective protein shells which sedimented at 49 S.

INTRODUCTION

In 1958 cocoa plants with unusual symptoms were found in Sierra Leone, from which Blencowe, Brunt, Kenten & Lovi (1963) obtained a virus apparently different from any virus previously recognized in cocoa in West Africa. In the present paper we report the results of further experiments with this virus, which we propose shall be called cocoa yellow mosaic virus (CYMV).

METHODS

A stock culture of CYMV from Giehun, Sierra Leone, was kept in graft-infected cocoa seedlings (*Theobroma cacao* L.) and in *Chenopodium amaranticolor* Coste & Reyn and *C. quinoa* Willd. seedlings infected originally by inoculation with cocoa leaf extracts.

The infectivity of different virus suspensions was compared by counting either the numbers of lesions that developed on the inoculated leaves of *Chenopodium amaranticolor* or the proportion of inoculated cocoa beans which later produced seedlings showing symptoms (after about 80 days). Cocoa beans were used in preference to cocoa seedlings, not only for convenience, but also because they showed symptoms sooner, usually on the first leaves produced. In the first experiments one cotyledon was removed from each bean before it was inoculated, but later whole beans were used because, as with cocoa swollen shoot virus (Brunt, Kenten & Nixon, 1964), they were more susceptible: in nine tests a total of 149

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out of 197 (75%) inoculated whole beans, but only 76 out of 192 (40%) inoculated half beans, were infected. Cocoa beans and plants were inoculated by using a small hog's bristle brush (Reeve's no. 3) that had been dipped in inoculum; *C. amaranticolor* and other plants were inoculated by finger. An abrasive was usually added to the inocula: Celite 545 (Johns Manville Ltd.) for cocoa beans and hard-leaved plants and Hyflo Super Cell (Johns Manville Ltd.) or 600-mesh carborundum for other plants.

Some purified preparations of the virus were made from infected cocoa leaves. The leaves were mechanically macerated in twenty times their weight of water or a neutral buffer containing either 0·05 M-Na_2HPO_4 + 0·005 M-diethydithiocarbamate (DIECA) or 0·05 M-Na_2HPO_4 + 0·01 M-thioglycollic acid + 0·005 M-DIECA. The slurry was then filtered through muslin, and the resulting extract clarified by centrifugation for 20 min. at 8000 *g*. The virus was then either sedimented from the clarified extract by centrifugation at 75,000 *g* for 2 hr or precipitated by half-saturating the extract with ammonium sulphate and centrifuging at 8000 *g* for 20 min. The pellet was then resuspended in $\frac{1}{50}$th to $\frac{1}{200}$th of the original volume of water, or neutral 0·03 M-phosphate, or in normal saline when used to inject rabbits. Other preparations of the virus were made from leaves of infected *Chenopodium amaranticolor*. Infected leaves were macerated mechanically in at least five times their weight of a solution containing 0·05 M-ascorbic acid + 0·1 M-disodium hydrogen phosphate (pH 7·0). Chloroform (a quarter of the volume of buffer) was then added, and, after the mixture had been macerated further to form an emulsion, it was centrifuged at 8000 *g* for 10 min. and the aqueous phase removed. The virus was then purified and concentrated from the aqueous extract by two or more cycles of differential centrifugation, with 0·03 M-phosphate (pH 7·5) as the suspending fluid. Finally the preparations were dialysed against 0·03 M-phosphate to remove traces of ascorbic acid. The other viruses used in the serological tests were purified in the same way.

Preparations were fractionated by centrifuging them in sucrose density gradients, and were examined in a Siemens Elmiskop I electron microscope by the methods described by Nixon & Harrison (1959).

Antisera were prepared by injecting rabbits intravenously with partially purified virus preparations obtained from artificially infected cocoa seedlings by ultracentrifugation. The virus was injected into the rabbits either intravenously or intramuscularly, after being emulsified in an equal volume of Freund's complete adjuvant. The rabbits were bled about 10 days after the last injections. One rabbit produced a serum with a titre of 1/512 after three intravenous injections at 3-day intervals, whereas another gave a titre of 1/1280 after one intravenous injection followed by two intramuscular injections at fortnightly intervals, with a final intravenous injection 1 week later. Serological tests were done by the tube precipitation method (Bawden, 1958).

RESULTS

Host-range and symptoms

The two plant species we found to be most useful for work with cocoa yellow mosaic virus (CYMV) were cocoa and *Chenopodium amaranticolor*. Blencowe *et al.* (1963) described the mosaic symptoms shown by infected cocoa (Pl. 1, fig. 2).

C. amaranticolor was readily infected by inoculation with water extracts of infected cocoa or *C. amaranticolor*; numerous necrotic lesions developed on inoculated leaves 1–2 weeks after inoculation, and tip leaves produced later showed chlorotic and necrotic flecks and many were distorted (Pl. 1, fig. 3).

The susceptibility of other plant species to CYMV was tested. Most species were

Table 1. *Hosts of cocoa yellow mosaic virus*

(*a*) Species susceptible to CYMV (*those infected systemically)

Apocynaceae	*Vinca rosea* L.*
Begoniaceae	*Begonia*
Bombacaceae	*Adansonia digitata* L.*, *Ceiba pentandra* (L.) Gaertn.*, *Pachira oleagina* Decne.
Chenopodiaceae	*Beta vulgaris* L. (sugar beet var. Klein E.)†, *Chenopodium amaranticolor* Coste & Reyn.*†, *C. quinoa* Willd.‡
Cucurbitaceae	*Cucurbita ficifolia* Bouche, *C. melo-pepo* L. (Delicious Golden), *C. pepo* L. (Orange Gourd), *Cucumis melo* L. (Cantaloup Charantais), *C. sativus* L. (Lockies Perfection, Tender and True, Gherkin), *Luffa cylindrica* Roem., *Momordica charantia* L.
Papillionaceae	*Voandezia subterranea*, *Vigna sinensis* L.†
Solanaceae	*Nicotiana tabacum* L. (White Burley), *N. clevelandii* Gray†, *Nicandra physaloides* L.*
Sterculiaceae	*Abroma augusta* L.*, *Cola lateritia* K. Schum. var. *maclaudi* (A. Chev.) Brenan & Keay*, *Leptonychia pubescens* Keay, *Sterculia foetida*, *Theobroma cacao* L.*, *T. bicolor* Humb. & Bonpl.*, *T. grandiflora* K. Schum.*, *T. speciosa* Spreng*

(*b*) Species inoculated with CYMV, but which showed no symptoms, and from which CYMV was not recovered

Amaranthaceae	*Gomphrena globosa* L.†
Bombacaceae	*Bombax brevicuspe* Sprague
Caesalpiniaceae	*Caesalpinia pulcherrima* Sw.
Compositae	*Calendula officinalis* L., *Lactuca taraxifolia*, *Zinnia elegans* Jacq.
Cucurbitaceae	*Momordica foetida*
Cruciferae	*Brassica chinensis* L.‡, *Raphanus sativus* L.
Euphorbiaceae	*Ricinodendron heudelotii* (Baille) Pierre ex Pax.
Gramineae	*Avena sativa* L. (var. Blenda)†, *Dactylis glomerata* L.†, *Hordeum vulgare* L. (var. Proctor)†, *Lolium perenne* L. (var. S22)†, *Zea mays* L.
Musaceae	*Musa balbisiana* L.†
Nyctaginaceae	*Boerhavia diffusa*
Papillionaceae	*Arachis hypogea* L., *Phaseolus lunatus* L., *P. vulgaris* L. (Canadian Wonder Improved, Kentucky Wonder, Masterpiece, Prince‡), *Pisum sativum* L. (Laxton's Wonder Superb), Soja max†, *Trifolium incarnatum* L.†
Polemoniaceae	*Phlox drummondii* Hook
Portulaccaceae	*Montia perfoliata* (Willd.) Howell.†
Solanaceae	*Capsicum frutescens* L. (Tabasco), *Datura stramonium* L., *Lycopersicum esculentum* Mill., *Nicotiana glutinosa* L., *Petunia hybrida* Villm.‡, *Physalis floridana* L.†, *P. peruvia* L., *P. pruinosa* L., *Solanum melongea* L., *S. verbascifolium*
Sterculiaceae	*Cola gigantea* A. Chev., *Guazama ulmifolia* Lam., *Sterculia tragacantha* Lindl.
Tiliaceae	*Corchorus aestuans* L.
Umbelliferae	*Daucus carota* L.†

All plants inoculated with CYMV from, and back tested to *Theobroma cacao*; except those marked †, which were inoculated with partially purified CYMV and back tested to *Chenopodium amaranticolor*, and those marked ‡, which were tested in both ways.

inoculated with infective cocoa leaf extracts but some were inoculated with preparations of the virus purified from *Chenopodium amaranticolor*. All were tested for infection after about 1 month by inoculating sap from the leaves which had been inoculated and from the uninoculated tip leaves to either cocoa beans or *C. amaranticolor*. Table 1 shows that CYMV has many hosts, which include woody and herbaceous species of different dicotyledonous families. The failure to infect some species may have been caused by inhibitors of infection in the cocoa leaf extract or preparations from *C. amaranticolor*; however, some species known to be influenced by such inhibitors, including *Cucumis* and *Nicotiana* spp., were readily infected in these tests.

Properties of the virus

In these tests water extracts of the first flush leaves of cocoa seedlings, infected as beans, were used. Since cocoa leaf extracts are very mucilaginous, they were diluted to contain the equivalent of 1 g. leaf in 20 ml. distilled water. Such diluted extracts were at about pH 6·3. Extracts were made only from leaves showing clear symptoms, for these were more infective than extracts of symptomless leaves or other parts of infected plants. Such extracts sometimes caused infection when diluted to 10^{-4} with water and inoculated with Celite (Table 2) which increased the infection end-point a thousand-fold.

Table 2. *Thermal inactivation of cocoa yellow mosaic virus*

	Cocoa leaf extracts heated for 10 min. at					
	65°	60°	55°	50°	45°	Unheated
Dilution of extract	% infected beans (about 25 beans per treatment)					
1/1	0*	5	25	80	67	74
1/10	—	0	3	41	63	87
1/100	—	0	0	3	23	30
1/1000	—	0	0	0	0	7

* 1 g. leaf extracted in 20 ml. distilled water.

Stability at different temperatures. When 2 ml. samples of extracts were heated to different temperatures, some infectivity remained after 10 min. at 60°, but not at 65° (Table 2). At 25–30° the extracts were infective after 16 days, but not after 32 days; at 0–4° they were still infective after 76 days; other extracts containing 0·2 M-phosphate buffer (pH 8·0) were highly infective after 100 days at 0–4°. Infected cocoa leaves and water extracts of the leaves were frozen and thawed with no apparent effect on the infectivity of the virus they contained. Infectivity was also retained in cocoa leaves dried and stored over anhydrous alumina at 25°, and in lyophilized cocoa leaf extracts or *Chenopodium amaranticolor* sap.

Precipitation of the virus. CYMV precipitated from water extracts when these were half saturated with ammonium sulphate. Precipitates were suspended in distilled water, dialysed for 24 hr against 0·003 M-phosphate (pH 8·0), and centrifuged briefly at 8000 *g* to sediment insoluble material. The resulting suspensions were almost colourless, slightly turbid, and contained most of the infectivity of the original extract. By contrast, when extracts of infected cocoa leaves were mixed with different amounts of ethanol or acetone in the cold to give concentrations of

30–75% and 25–60% (v/v), respectively, the sparse precipitates which formed were not infective when resuspended in water.

The virus was not precipitated when extracts of infected cocoa leaves were adjusted to different pH values between pH 3 and pH 6, though infectivity was apparently destroyed when the extract was acidified to pH 3·0.

Partially purified preparations

Partially purified preparations made by differential centrifugation were slightly turbid and usually colourless or slightly yellow. The virus crystallized when ammonium sulphate was added slowly to these preparations as described by Markham & Smith (1949) for crystallizing turnip yellow mosaic virus (TYMV); these crystals were similar to but smaller than those of TYMV. They were kept in the ammonium sulphate solution for 12 months at 4°, without increasing in size; they dissolved when the ammonium sulphate was removed by dialysis.

Electron microscopy. Extracts of infected cocoa or *Chenopodium amaranticolor* leaves contained many approximately spherical particles, not found in comparable preparations from uninfected plants. The particles appeared to be 25·2 mμ across when negatively stained (Pl. 1, fig. 5) and 29·0 mμ when shadowcast after fixation with 2% (v/v) formalin for 1 hr. The first figure is presumably an underestimate because stain penetrates into the particles, and the second an overestimate because the particles flatten on drying. The apparent size of negatively stained CYMV particles is almost the same as that recorded by Nixon & Gibbs (1960) for similarly treated turnip yellow mosaic virus particles, but fixed and shadowed CYMV particles appear to be slightly larger than untreated shadowed TYMV at 26 mμ (Cosentino, Paigen & Steere, 1956). CYMV particles shadowcast without fixation with formalin always appear very flattened, unlike TYMV particles, which retain their shape well. Some of the particles in negatively stained preparations of CYMV seemed empty because they were penetrated by the phosphotungstate and were visible only as outlines (Pl. 1, fig. 4). Others were partly penetrated by the stain and were an intermediate grey tone on the plate (Pl. 1, fig. 5, near the upper edge).

In the best electron micrographs, particles were indistinguishable from those of turnip yellow mosaic virus (Huxley & Zubay, 1960; Nixon & Gibbs, 1960), and showed the same arrangement of morphological subunits.

Centrifugation. Purified CYMV preparations, suspended in neutral 0·1 M-KCl, were examined in a Spinco Model E analytical centrifuge. All showed two boundaries, with sedimentation coefficients of 49S and 108S (corrected to infinite dilution in water at 20°), which we have called the top component and whole virus, respectively (Markham & Smith, 1949). The top component absorbed much less ultraviolet (u.v.) radiation than the whole virus, and during preparation by differential centrifugation some of it was inevitably lost. When the virus in extracts from infected *Chenopodium amaranticolor* leaves was concentrated and purified by precipitation with ammonium sulphate and examined in the analytical centrifuge the area of the schlieren peak for the top component was about two-thirds of that from whole virus.

The two components were separated by centrifugation in sucrose density gradients. Electron microscopy showed that the whole virus fraction contained mostly intact virus particles, unpenetrated by phosphotungstate. It had the u.v.-absorption spectrum of a nucleoprotein (Fig. 1), and was highly infective. By

contrast, the top component contained mostly particles penetrated by phosphotungstate, had the u.v.-absorption spectrum of a protein (Fig. 1), and was only slightly infective. We have assumed that the few intact particles and slight infectivity of the top component was due to incomplete separation, and that apparently empty particles seen in the whole virus fraction were either due to incomplete separation or to damage during mounting for electron microscopy. The

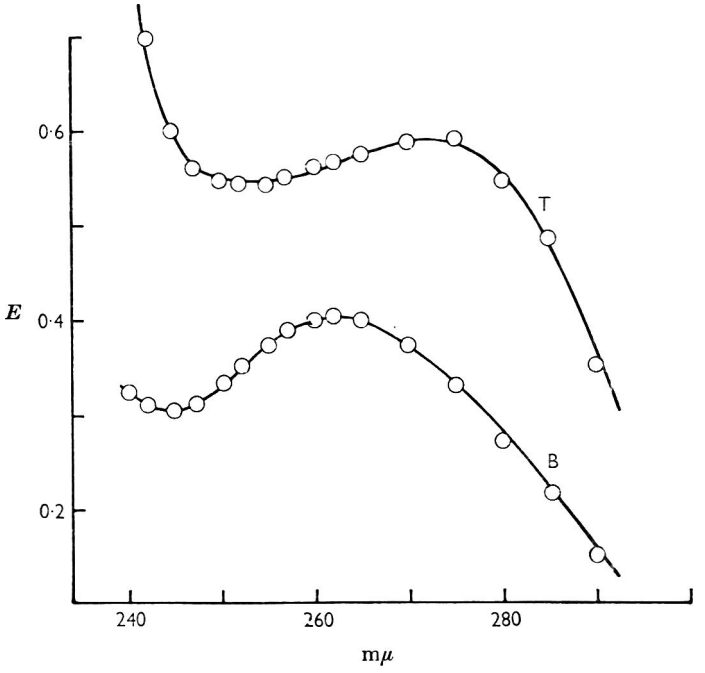

Fig. 1. Ultraviolet absorption spectra of separated cocoa yellow mosaic virus components. Upper line is spectrum of top component, lower line that of the bottom component (whole virus).

separated components each reacted with an antiserum made by injecting a rabbit with an unfractionated CYMV preparation. In gel diffusion tests each component gave only one line of precipitate, and in suitable tests these lines were confluent.

Thus CYMV preparations contain both fast sedimenting infective nucleoprotein particles and more slowly sedimenting non-infective protein particles, which have the shape and size of and are antigenically similar to the nucleoprotein particles.

Electrophoresis. CYMV preparations were dialysed against neutral 0·06 M-phosphate buffer and then examined in a Perkin-Elmer Model 38A electrophoresis apparatus with a schlieren optical system.

All the preparations examined separated into two components, the major one migrating at $-11{\cdot}8 \times 10^{-5}$ cm./sec./V./cm. and the minor at $-11{\cdot}3 \times 10^{-5}$ cm./sec./V./cm. Because of the small difference in the mobilities of these electrophoretic components it was impossible to separate them and study their composition. They seem not to correspond to the protein and nucleoprotein components of the virus preparation, for in one experiment the separated whole virus contained both electro-

phoretic components in the same relative amounts as in the unfractionated preparations.

Serology. Our results show that CYMV resembles turnip yellow mosaic (TYMV) and wild cucumber mosaic (WCMV) viruses in many respects. TYMV and WCMV are serologically related (MacLeod & Markham, 1963), so tests were made to find whether CYMV is serologically related to them and to cowpea mosaic virus, which is also transmitted by beetles (Dale, 1949; Chant, 1959). Table 3 shows the sources of the viruses and antisera used in the tests.

Table 3. *Hosts and sources of virus antigens*

Virus	Host species for virus for injection	Host species for virus used as antigen	Source
Cocoa yellow mosaic	*Theobroma cacao*	*Chenopodium amaranticolor*	Sierra Leone
Wild cucumber mosaic	*Cucurbita pepo*	*C. pepo*	MacLeod & Markham
Turnip yellow mosaic	*Brassica chinensis*	*B. chinensis*	Scotland
Cowpea mosaic			
Nigeria	*Phaseolus vulgaris*	*P. vulgaris*	Nigeria
Trinidad	*Phaseolus vulgaris*	*P. vulgaris*	Trinidad
Surinam	?	—	Hari Agrawal

Table 4. *Antiserum titrations*

Antiserum titre (reciprocal of dilution end-point) after 4-hr incubation at 37°. In parentheses least dilution tested, when no reaction.

	Antigen						
				Cowpea mosaic virus		Healthy sap	
Antiserum	Cocoa yellow mosaic virus	Wild cucumber mosaic virus	Turnip yellow mosaic virus	Nigeria	Trinidad	*C. amaranticolor*	*P. vulgaris*
Cocoa yellow mosaic	512	0 (2)	0 (2)	0 (2)	—	0 (2)	—
Wild cucumber mosaic	8–16	1024	16	0 (2)	—	—	—
Turnip yellow mosaic	0 (2)	8	1024	0 (2)	—	—	—
Cowpea mosaic							
Nigerian	0 (1)	0 (2)	0 (2)	64	64	—	0 (2)
Surinam	—	—	—	0 (16)	128	—	—

The serological relationships between the viruses were estimated by determining the precipitation end-points of the antisera when titrated against purified preparations of the different viruses. First the precipitation end-point of each virus preparation was found using the homologous antiserum, then the preparation was used as antigen in the antiserum titration tests at approximately four times the concentration at the precipitation end-points.

The tests (Table 4) confirmed the results of MacLeod & Markham (1963) and showed a distant serological relationship between TYMV and WCMV. The WCMV antiserum, but not the TYMV antiserum, precipitated CYMV, whereas the CYMV antiserum did not precipitate either TYMV or WCMV. The failure of CYMV antiserum to precipitate WCMV, when the reciprocal test was positive, may be because it had a lower titre than the WCMV antiserum.

Thus CYMV is apparently serologically related to WCMV but not to TYMV, even though TYMV and WCMV are related. However, further tests showed that WCMV has some antigenic groups common to both TYMV and CYMV, for when

WCMV antiserum was mixed with excess CYMV it did not precipitate TYMV, and when mixed with excess TYMV it did not precipitate CYMV.

Neither CYMV, WCMV, TYMV nor their antisera gave precipitates in tests with the cowpea mosaic viruses or their sera. Limited tests showed that the different cowpea mosaic viruses were serologically related.

All the precipitates formed in these tests were white and granular, as usually obtained with isometric virus antigens, and unlike the less-dense microgranular and often coloured precipitates formed when plant proteins react with their antisera.

Dorner, Kahn & Wildman (1958) and van Regenmortel (1963) found that the 'Fraction 1' proteins of different plant species were serologically related. Van Regenmortel (1963) has suggested that reactions with such proteins, which are often present in plant-virus preparations, might be responsible for the reports of distant serological relationships between viruses, such as those reported by MacLeod & Markham (1963). However, our results confirm those of MacLeod & Markham (1963) and provide further evidence to suggest that plant proteins are not responsible for the observed serological reactions. Dorner *et al.* (1958) and van Regenmortel (1963) apparently found one cross-reacting plant protein antigen in all species. However, our results (Tables 3, 4) show the presence of at least three distinct antigens; one in *Phaseolus vulgaris* only, one in both *Brassica chinensis* and *Cucurbita pepo* only, and another in *C. pepo* and *Chenopodium amaranticolor* only. Furthermore, the preparations of CYMV, WCMV, and TYMV used as antigens in our tests were highly purified and concentrated, and they were therefore diluted 1/32, 1/100, and 1/150 respectively for use in serological tests. The WCMV and TYMV preparations showed no components attributable to plant proteins when examined in the analytical centrifuge. By contrast, the cowpea mosaic preparation was relatively impure. It was diluted only to 1/10 for serological tests, and contained more plant protein than the other virus preparations. Out of all those tested, it should therefore have been the most readily precipitated by antibodies to plant proteins, and yet it reacted only with its homologous antiserum. Dorner *et al.* (1958) found that the precipitation end-point of untreated tobacco sap was only 1/320 when tested in ring interface tests with an antiserum specifically prepared against 'Fraction 1' protein. Thus it is unlikely that enough plant protein to give a precipitate was present in the diluted antigen preparations we used except with cowpea mosaic virus preparations.

DISCUSSION

An interesting feature of cocoa yellow mosaic virus (CYMV) is its similarity to turnip yellow mosaic virus (TYMV) and wild cucumber mosaic virus (WCMV). Their particles have similar shape, size, external morphology, and sedimentation rates; moreover, MacLeod & Markham (1963) and MacLeod (personal communication) found that the nucleoprotein particles of all three viruses contain similar amounts of nucleic acid, of similar composition (with 37–40% cytidylic acid). Therefore it is perhaps not surprising that these viruses are serologically related; however, the relationship is unusual in that WCMV antiserum reacted with both CYMV and TYMV, though there was no reaction between TYMV and CYMV antiserum or CYMV and TYMV antiserum.

The natural vector of CYMV in Sierra Leone is not known. Blencowe *et al.* (1963)

failed to transmit the virus by three species of mealy-bug, and attempts in Sierra Leone to transmit the virus by soil and seed also failed; 60 pots of soil collected from around the roots of naturally infected cocoa plants were each sown with 5 cocoa beans, none became infected, and 150 beans from naturally infected cocoa plants all produced apparently healthy seedlings. However, the similarity of CYMV to TYMV and WCMV, both of which are transmitted by chrysomelid beetles, suggests that CYMV may also have a beetle vector.

Other viruses have been found to be beetle transmitted, including cowpea mosaic viruses in Trinidad (Dale, 1949) and in Nigeria (Chant, 1959). Our tests, and those of Shepherd (1963) and Agrawal & Maat (1964) have shown that the two cowpea viruses are closely serologically related, but that they are apparently unrelated to CYMV, TYMV or WCMV. Cowpea mosaic virus also differs from CYMV in other ways, for although preparations of it have two components, like those in CYMV preparations, the particles appear different in the electron microscope (Chant, 1959) and do not show clearly defined subunits when mounted in phosphotungstate. Furthermore, bean pod mottle virus, which has recently been shown to be beetle-transmitted (Ross, 1963; Walters, 1964) and is serologically closely related to cowpea mosaic virus (Shepherd, 1963), has a nucleic acid composition (Semancik & Bancroft, 1964) quite different from that of CYMV.

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EXPLANATION OF PLATE 1

Fig. 1. Necrotic local lesions on leaf of *Chenopodium amaranticolor* inoculated with a purified cocoa yellow mosaic virus (CYMV) preparation.

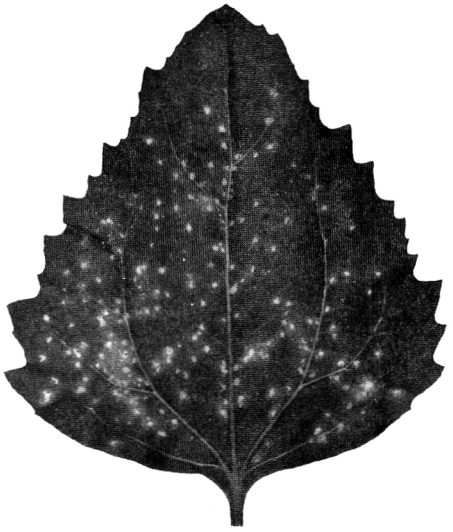
Fig. 2. Tip leaf of *C. amaranticolor* plant systemically infected with CYMV, showing distortion and necrotic flecks.

Fig. 3. Chlorotic patterns on systemically infected leaf from a mechanically inoculated cocoa seedling.

Fig. 4. Electron micrographs showing empty protein particles of the top component of CYMV, separated from a purified preparation in a sucrose density gradient, and mounted in phosphotungstate; about ×240,000.

Fig. 5. Nucleoprotein particles from the bottom component. Details as for fig. 4.

Fig. 6. Schlieren pattern from a purified preparation of CYMV sedimenting in the analytical ultracentrifuge. Sedimentation is from left to right.



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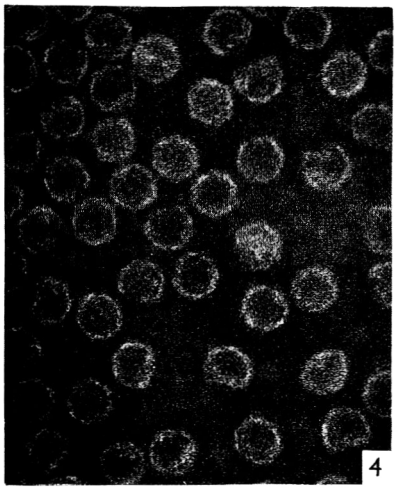

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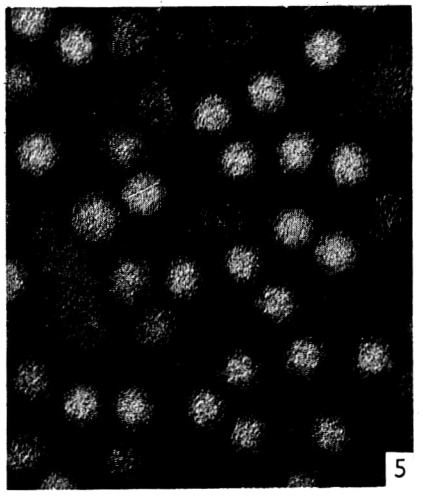

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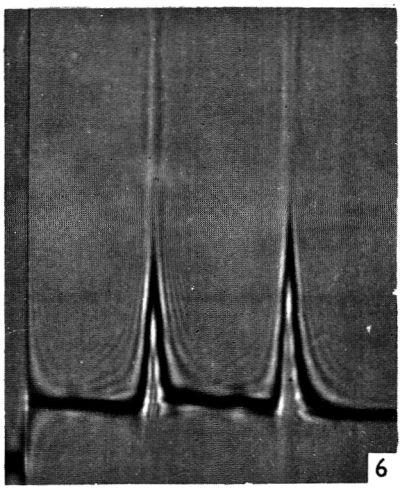

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A Serological Comparison of Various Species of Mycoplasma by an Agar Gel Double-Diffusion Technique

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SUMMARY

Sixteen serological types of Mycoplasma previously distinguished by complement-fixation tests were compared by means of a gel precipitin (Ouchterlony) technique. Each of the sixteen gave a consistent pattern of three to seven precipitation lines with its homologous antiserum. Cross-reactions occurred between heterologous strains, especially with antisera produced with the aid of an adjuvant. Homologous reactions were complex and sufficiently distinctive for the technique to be used to identify unknown strains. Two of the sixteen types, *Mycoplasma mycoides* var. *mycoides* and *M. mycoides* var. *capri*, had certain antigenic components in common. The appearance of other cross-reactions suggested that the majority were due to the presence in different species of partly related rather than identical antigens.

INTRODUCTION

A previous communication (Lemcke, 1964*a*) reported the results of using a complement-fixation (CF) test to characterize mycoplasmas from a variety of sources. Among 82 strains, 17 serological types were distinguished, although two of these, *Mycoplasma arthritidis* and strain R38, both from rats, were partly related. Although these serological types were easily distinguishable by complement-fixation tests, cross-reactions of the order of 1/16 to 1/32 of the homologous titres occurred between all of them. This phenomenon was also noted in complement-fixation tests by Card (1959) and Coriell, Fabrizio & Wilson (1960). Non-specific reactions with certain constituents of the medium were responsible at least in part, but it was not clear whether certain antigenic components common to different species were also involved (Lemcke, 1964*a*). To clarify this point, an agar gel double-diffusion technique was used to examine the reactions of various Mycoplasma strains with homologous and heterologous antisera.

METHODS

Organisms. Details of the organisms used are given in Table 1. The sources of all the strains except BRA were described in earlier reports (Lemcke, 1964*a*, *b*). Strain BRA was freshly isolated from the rectum of a woman with vaginal cervicitis.

Media and growth conditions. The liquid medium used for cultures from which the gel diffusion antigens were prepared consisted of Hartley's digest broth (Oxoid)

supplemented with: unheated horse serum 20% (v/v); yeast extract (Oxoid), 0·5% (w/v); deoxyribonucleic acid, 0·002% (w/v); penicillin, 50 units/ml. For the growth of cultures for immunizing rabbits, yeast extract was omitted and unheated pooled normal rabbit serum was substituted for horse serum. All strains were incubated at 37°, *Mycoplasma salivarium*, *M. orale* and *M. fermentans* anaerobically and the rest aerobically with continuous shaking.

Table 1. *Mycoplasma strains*

Designation of strain	Source	Serological type or species
H34,* H33, MJW, 4387P, H27 H68, BRA	Man (genital tract)	*Mycoplasma hominis* type 1
B1, B3,* B5	Man (mouth)	*M. salivarium*
837,* E	Tissue culture	*M. orale*†
S, BM, 898/2	Man (throat)	*M. orale*†
G2*	Man (genital mucosa)	*M. fermentans*
Navel*	Man (umbilicus)	Umbilical
FH	Man (primary atypical pneumonia)	*M. pneumoniae*
Campo (PG27)*, O7	Man (genital tract)	'*M. hominis* type 2'
Jasmin*	Rat (polyarthritis)	*M. arthritidis*
R38	Rat (rhinitis)	Partly related to *M. arthritidis*
M1*	Mouse (lung)	*M. pulmonis*
KSA*	Mouse ('rolling disease')	*M. neurolyticum*
PG11*	Cattle (genital tract)	*M. bovigenitalium*
Gladysdale*	Cattle (contagious pleuropneumonia)	*M. mycoides* var. *mycoides*
G1/61*	Goat (contagious pleuropneumonia)	*M. mycoides* var. *mycoides*
pp. goat*	Goat (contagious pleuropneumonia)	*M. mycoides* var. *capri*
Agalactia*	Goat (agalactia)	*M. agalactiae*
Fowl*	Chicken (upper respiratory tract)	*M. gallinarum*
A36*	Chicken (tracheal exudate)	Non-pathogenic avian
T*	Embryonated egg	*M. gallisepticum* (Nelson's coccobacilliform bodies)
Laidlaw A*	Sewage	*M. laidlawii*

* Precipitating antisera available against these strains.

† Name assigned to new oral type (Dr D. Herderscheê, personal communication; Taylor-Robinson, Canchola, Fox & Chanock, 1964).

Preparation of antigens. Cultures were grown for 2–4 days since it was found that fewer precipitation lines were obtained with strain H34 when incubation was continued for 6–14 days. Organisms were harvested by centrifugation at 10,000*g* for 30 min. at 4°, washed twice in 0·85% (w/v) saline and finally suspended in saline to a concentration 100 times that of the original culture. Although some precipitation lines were formed with suspensions of whole organisms, more were obtained with suspensions which had been cooled in ice and water and treated for 10 min. with a Branson S-75 Sonifier (Branson Instruments Inc., Stamford, Connecticut, U.S.A.) at 20 kcyc./sec. Suspensions in de-ionized water, which were frozen and thawed ten times in solid carbon dioxide+ethanol at −25°, gave precipitation patterns similar to those given by organisms disrupted ultrasonically. The freeze/thaw method was suitable for small quantities of culture. In most of the work reported below

suspensions of organism from 250 or 500 ml. medium were disrupted ultrasonically. Suspensions were stored at −25° to −30° in volumes of 0·5–1·0 ml. Storage in large volumes was impracticable because some antigens deteriorated during the repeated thawing and freezing required for the removal of samples for test. Suspensions preserved at 4° with the addition of thiomersalate (the procedure for storing complement fixation test antigens) gave fewer precipitation lines than those stored frozen. To detect non-specific reactions with the medium, a control 'antigen' consisting of the resuspended residue obtained by centrifugation of sterile broth was prepared.

Antisera. Most of the antisera used had been prepared for use in complement-fixation tests (Lemcke, 1964*a*). They were selected for their ability to give precipitation lines with a suspension of homologous organism and were tested for reactivity with the medium and the control 'antigen'.

Antisera induced by antigen+Freund's adjuvant gave the best results in gel diffusion tests and new antisera were prepared by this method. The adjuvant consisted of a mixture of 9 parts Drakeol 6-VR (Pennsylvania Refining Co., Butler, Pa., U.S.A.) and 1 part Arlacel (Atlas Chemical Industries Inc., Wilmington, Del., U.S.A.). The organisms from 500 ml. of a 3- to 4-day culture were harvested by centrifugation, washed, resuspended in 2 ml. normal saline and homogenized with an equal volume of adjuvant. Rabbits were inoculated subcutaneously in the flank. Those receiving samples of strains B 3, G 2 and Campo were given one injection, those receiving strains 837 and Navel two injections separated by an interval of 3 weeks. Three weeks after the last subcutaneous inoculation, all the rabbits were given a course of six intravenous injections of the appropriate organism suspended in saline. The best antisera were obtained 5–7 days after the last intravenous injection.

Double-diffusion tests. The medium and pattern of the reservoirs used were those described by Lemcke (1964*a*). Precipitation was allowed to take place at room temperature; at 4° fewer lines developed and at 30–37° the lines became very diffuse. Optimal development occurred between 2 and 5 days, depending on the antiserum.

RESULTS

Of the stock antisera tested for ability to give precipitation lines with homologous antigen preparations, only those against *Mycoplasma pneumoniae* and strain R 38 did not react. Thus, precipitating antisera were available against 15 of the 17 serological types previously described (Lemcke, 1964*a*) and against *M. gallinarum*, another 'avian' species which proved to be serologically distinct by the complement-fixation test (Table 1). Of these 16 type antisera, 14 gave no sign of precipitation with the medium or control 'antigen'. The remaining two, an antiserum against strain KSA (*Mycoplasma neurolyticum*) and one against strain Gladysdale (*M. mycoides* var. *mycoides*) gave diffuse zones of precipitation with both the medium and the control 'antigen'. With both, the reaction was eliminated by absorption of the sera with an equal volume of medium. The absorbed sera, even though diluted 1/2 by this procedure, gave several clear precipitation lines with the homologous antigen; only the rather diffuse non-specific band disappeared. In the case of strain KSA, the absorbed antiserum did not react with suspensions of any other strains.

Adjuvant-induced antisera gave more precipitation lines with their homologous antigens than did antisera produced by the intravenous inoculation of saline suspensions (Table 2).

The results of cross-testing suspensions with 16 antisera against strains representing different serological types are shown in Table 3. When two antisera were available against any one strain, as was the case for strains H34, B3, 837, G2, Navel and Campo, the results obtained with the adjuvant-induced serum are given. Although heterologous cross-reactions were frequent, the homologous reaction in each case was always more complex and characteristic of the particular strain. To make sure that none of the cross-reactions represented non-specific reactions, all

Table 2. *Comparison of antisera produced by suspensions of mycoplasmas, with and without adjuvants*

	No. of precipitation lines with homologous suspensions		Cross-reactions with heterologous suspensions	
Mycoplasma strain	With adjuvant	No adjuvant	With adjuvant	No adjuvant
H34	6	1–2	+	–
B3	4–5	2	+	–
837	6–7	2	+	–
G2	5–7	2	+	–
Navel	3	1	–	–
Campo (PG27)	5–6	2	+	+

cross-reacting sera were tested against concentrations of the complete medium up to twenty-five times and against two-fold dilutions down to 1/4096 of the complete medium, Hartley's digest broth, undiluted horse serum, yeast extract (10 %, w/v), and deoxyribonucleic acid (0·2 %, w/v). No precipitation lines were detected in any of these tests.

There was a substantial degree of cross-reaction between the strains H34 (*Mycoplasma hominis* type 1), B3 (*M. salivarium*), 837 (*M. orale*), G2 (*M. fermentans*) and Navel, which represented five different serological types of human origin (Table 3). Of these, only antiserum to strain Navel was entirely monospecific, despite the use of adjuvant in its preparation; suspensions of this strain did, however, react with antisera to the other serotypes of human origin. Table 3 also shows extensive cross-reactions between the five strains of human origin and Campo, a strain of the so-called '*Mycoplasma hominis* type 2'. Although strain Campo and another 'type 2' strain, O7, were isolated from the human genital tract, complement-fixation tests have shown them to be indistinguishable from strains of *M. arthritidis* associated with polyarthritis in rats (Lemcke & Csonka, 1962; Lemcke, 1964*a*). Gel diffusion tests confirmed that strains Campo and O7 were very closely related to *M. arthritidis* strain Jasmin. With antiserum to strain Campo, strain Jasmin shared at least four precipitation lines with strain Campo, but lacked another line (A) given by the latter (Fig. 1*a*). With antiserum to strain Jasmin, strains Campo, O7 and Jasmin shared three lines of precipitation (Fig. 1*b*). In addition strains Campo and Jasmin had a fourth line, nearest the antigen well, which formed a spur at the point of junction (B) and was therefore not quite identical in the two organisms; this component was apparently missing from O7.

Table 3. *Number of gel precipitation lines obtained in homologous and heterologous reactions with Mycoplasma strains*

Antigens	Antisera from strains: H34*	B3*	837*	G2*	Navel*	Campo* (PG27)	M1	KSA*	PG11*	Gladysdale*†	pp. goat	Agalactia	Fowl	A36*	T	Laidlaw A*
H34	6	2	2	2	—	2	—	—	—	—	—	—	—	—	—	—
B3	2	4–5	2	1	—	2	—	—	—	1	—	—	—	—	—	—
837	1	2	6–7	1	—	1	—	—	—	—	—	—	—	—	—	—
G2	1	2	2	5–7	—	2	—	—	—	—	—	—	—	—	—	—
Navel	1	1	2	3	3	1	—	—	—	—	—	—	—	—	—	—
Campo	2	1	2	2	—	5–6	—	—	—	—	—	—	—	—	—	—
M1	—	—	1	—	—	—	3	—	—	—	—	—	—	—	—	—
KSA	—	—	1	1	—	—	1	5–6	—	—	—	—	—	—	—	—
PG11	—	—	2	2	—	—	—	—	4	—	—	—	—	—	—	—
Gladysdale	—	—	—	—	—	—	—	—	1	4–5	2	—	—	—	—	—
pp. goat	—	—	2	—	—	—	1	—	1	3	3	—	—	—	—	—
Agalactia	—	—	3	3	—	—	1	—	—	—	—	4	—	—	—	—
Fowl	2	1	2	3	—	1	—	—	1	—	—	—	4	—	—	—
A36	—	—	2–3	1	—	—	—	—	—	—	—	—	—	4–5	—	—
T	—	—	—	—	—	—	—	—	—	—	—	—	—	—	3	—
Laidlaw A	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	3–4

* Adjuvant used in antiserum production.
† Antisera aborbed with medium.
— = no precipitation lines.

The technique thus revealed a slight difference, not apparent from the results of complement-fixation tests, between the 'type 2' strains from man and *M. arthritidis* from a rat. Nevertheless, since strains from both sources shared the majority of their antigens, there seems to be no justification for regarding '*M. hominis* type 2' as a species distinct from *M. arthritidis* (Lemcke, 1964*a*).

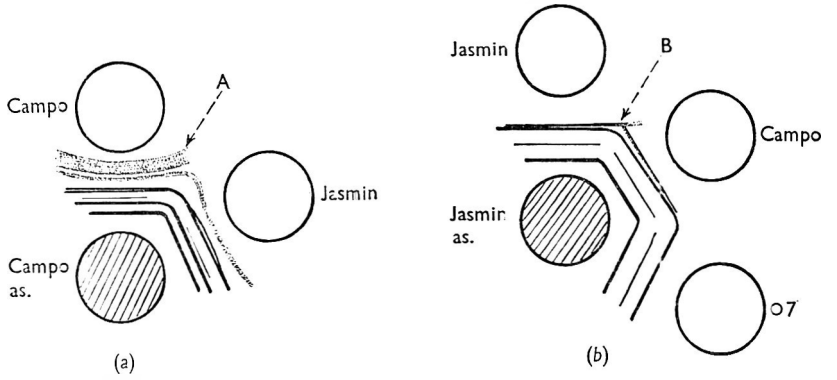

Fig. 1. Agar gel precipitin reactions of (*a*) antiserum to '*Mycoplasma hominis* type 2' strain Campo with homologous strain and *M. arthritidis* strain Jasmin (A, extra band formed by homologous strain), and (*b*) antiserum to *M. arthritidis* strain Jasmin with homologous strain and '*M. hominis* type 2' strains Campo and o7 (B, partial intersection of lines from strains Jasmin and Campo). as. = antiserum well.

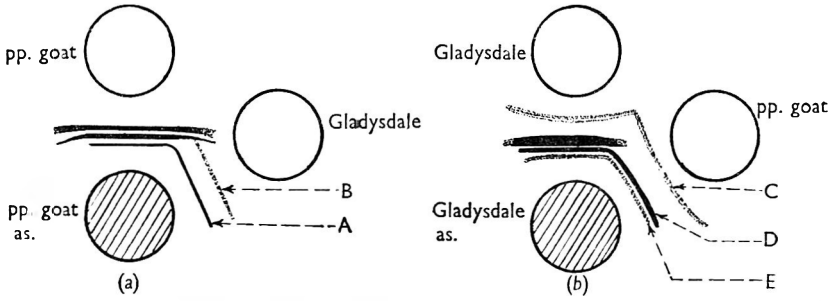

Fig. 2. Agar gel precipitin reactions of (*a*) antiserum to *Mycoplasma mycoides* var. *capri* strain pp. goat with homologous strain and *M. mycoides* var. *mycoides* strain Gladysdale (A, line common to both strains; B, line partly intersecting with one of homologous lines), and (*b*) antiserum to *M. mycoides* var. *mycoides* strain Gladysdale with homologous strain and *M. mycoides* var. *capri* strain pp. goat (C, D, E, lines common to both strains). as. = antiserum well.

With regard to the nature of the cross-reactions between the sixteen serological types, two of them, *Mycoplasma mycoides* var. *mycoides* from contagious bovine pleuropneumonia and *M. mycoides* var. *capri* from contagious pleuropneumonia in a goat, clearly had common antigens. With antiserum to *M. mycoides* var. *capri* strain pp. goat, *M. mycoides* var. *mycoides* strain Gladysdale gave one precipitation line which fused completely with one produced by the homologous strain (Fig. 2*a*, line A). A second line partially intersected with one of the other two homologous lines giving spur formation (Fig. 2*a*, line B). This suggested the presence of one identical and one partly related component in the two organisms. The reciprocal

test with antiserum to *M. mycoides* var. *mycoides* strain Gladysdale (Fig. 2*b*), showed that three of the precipitation lines (C, D, E) formed by 'mycoides' and 'capri' were identical. Tests with an antiserum to *M. mycoides* var. *mycoides* strain G1/61 (a strain from goat pleuropneumonia but indistinguishable from strain Gladysdale) showed that 'mycoides' and 'capri' had one antigenic component in common and one partly related. The rather more complex picture obtained with antiserum to strain Gladysdale was probably due to the use of adjuvant in its production; strains pp. goat and G1/61 antisera were prepared without adjuvant. 'Mycoides' and 'capri' also had an identical line in their cross-reaction with antiserum to *M. bovigenitalium*, suggesting an antigenic component common to both.

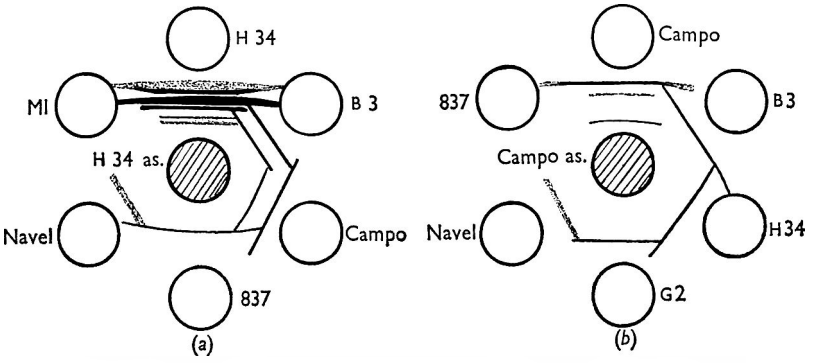

Fig. 3. Agar gel precipitin reactions of (*a*) antiserum to *Mycoplasma hominis* type 1 strain H34 with homologous strain, *M. salivarium* strain B3, '*M. hominis* type 2' strain Campo, M. orale strain 837, *M. pulmonis* strain M1 and strain Navel, and (*b*) antiserum, obtained after one subcutaneous inoculation, to '*M. hominis* type 2' strain Campo with homologous strain, *M. salivarium* strain B3, *M. hominis* type 1 strain H34, *M. fermentans* strain G2, *M. orale* strain 837 and strain Navel. as. = antiserum well.

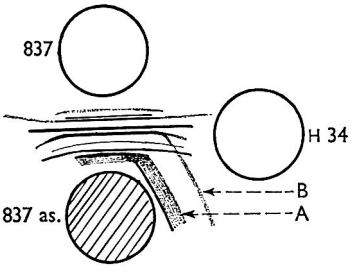

Fig. 4. Agar gel precipitin reactions of antiserum to *Mycoplasma orale* strain 837 with homologous strain and *M. hominis* type 1 strain H34 (A, diffuse band apparently fusing with one of homologous lines; B, line partly intersecting with one of homologous lines). as. = antiserum well.

In most of the other cross-reactions spur formation occurred where the heterologous precipitation lines joined one or other of the homologous lines (Fig. 3*a*, *b* and Fig. 4, line B). In some cases, the complexity of the homologous pattern tended to obscure the junction of homologous and heterologous lines. Nevertheless, reactions with less complex sera obtained after a single subcutaneous injection suggested that spur formation did indeed occur (Fig. 3*b*). Both Figs. 3*a* and *b*

illustrate reactions with several different heterologous strains. Spur formation occurred not only between the homologous and the adjacent heterologous systems, but also between different heterologous strains.

Besides the partly related antigens indicated by spur formation, a few strains may have identical antigens in common. With *Mycoplasma orale* strain 837 antiserum, *M. hominis* type 1, *M. salivarium*, *M. fermentans*, Navel, '*M. hominis* type 2' and *M. gallinarum* all showed a rather diffuse precipitation band which apparently fused with the homologous line nearest the antiserum well. The width and diffuseness of the bands may however give a false appearance of fusion (Fig. 4, line A). Similar reactions were observed between *M. salivarium* strain B3 antiserum and *M. orale*, and between *M. fermentans* strain G2 antiserum, and *M. orale* and *M. gallinarum*.

The technique was also used to compare strains which were indistinguishable by the complement-fixation test. Thus, with any one antiserum to *Mycoplasma hominis* type 1, strains from a post-operation wound infection (H34), non-gonococcal urethritis (H33, MJW, 4387P), Reiter's disease (H27), Bartholin's abscess (H68) and the rectum (BRA) gave similar reactions. No difference was detectable between the freshly isolated strain BRA and the others, which had been maintained in the laboratory for some time. The only differences noted between strains were in the intensity of certain of the weaker precipitation lines. Such differences were probably due to the deterioration during storage of the more labile antigenic components, since the same lines were found to be missing when suspensions of strain H34 were stored at 4° with thiomersalate and when cultures to be used as antigens were incubated for 6–14 days instead of 2–4 days.

Apart from differences in intensity, the precipitation reactions of these seven human genital strains were indistinguishable and the results thus confirm those obtained by the complement-fixation test (Lemcke, 1964*a*). Some earlier observations (Nicol & Edward, 1953; Card, 1959; Oates, Whittington & Wilkinson, 1959; Lemcke & Csonka, 1962 and Taylor-Robinson, Somerson, Turner & Chanock, 1963) suggested that there might be slight antigenic differences between strains of *Mycoplasma hominis* type 1. There was, however, no evidence from the strains examined here that some possessed antigenic components not present in others.

Three strains from the human mouth (B1, B3, B5; Card, 1959) appeared identical when tested with antiserum to strain B3, just as in complement-fixation tests.

The similarity of certain tissue culture contaminants isolated by Hayflick to a new oral type (Herderscheê, Ruys & van Rhijn, 1963) was reported previously (Lemcke, 1964*b*). This type has been named *Mycoplasma orale* (Dr D. Herderscheê, personal communication; Taylor-Robinson, Canchola, Fox & Chanock, 1964). The precipitation patterns of the strains from tissue cultures (strains 837, E) and from the human throat (strains S, BM, 898/2) were closely similar, the only difference being in the intensity of the precipitation band nearest the antigen wells. This may have been due to the better preservation of a labile antigenic component in some suspensions than in others or it may have reflected differences in the amount of a particular antigen present in different strains.

DISCUSSION

It is clear that in many of the Mycoplasma species examined here there was a complexity of antigenic components not revealed in previous immunodiffusion studies (e.g. Villemot & Provost, 1959; Taylor-Robinson *et al.* 1963). Clearly, much depends on the quality of the antiserum used; some of the best antisera were produced with the aid of adjuvant in the primary subcutaneous inoculation of rabbits, followed by a secondary course of intravenous injections. The disrupted cell suspensions need to be concentrated, and the contained antigens (some of which are labile) preserved from deterioration. Homologous reactions were always clearly distinguishable from the cross-reactions. The gel diffusion technique therefore constitutes a relatively simple method of identification, provided the appropriate antisera are available. It has been successfully used in this laboratory for identifying mycoplasmas from human genital infections.

However, the results are probably of greater significance as a basis for further work on the separation and purification of the various antigenic components. Immunochemical studies of this kind have so far been concerned mainly with the contagious bovine pleuropneumonia organism *M. mycoides* var. *mycoides* (Dafaalla, 1957; Yoshida, 1961; Plackett, Buttery & Cottew, 1963). Similar work on species of human origin would be of value in improving serological methods for diagnosing mycoplasma infections in man.

There was no evidence from the present work, after exhaustive tests, that the cross-reactions observed between different species in gel diffusion tests were non-specific. Cross-reactions in the complement-fixation test were thought to be associated, at least in part, with the broth medium (tryptic digest of horse meat) used to grow cultures for both the complement-fixation test antigen and for immunizing rabbits (Lemcke, 1964*a*). In fact, a different broth medium (Hartley's digest of beef heart muscle) was used to grow the suspensions used in gel diffusion tests. It seems clear from these tests that cross-reactions between species of *Mycoplasma* are due to specific antigenic components. More cross-reactions between species would probably have been detected if potent sera against each strain had been available.

The cross-reacting antigenic components were sometimes identical. *Mycoplasma mycoides* var. *mycoides* and *capri* probably possessed three antigens in common. Because they can be distinguished serologically, the author previously thought that there was little justification for regarding the goat pleuropneumonia organism as a variant of the bovine pleuropneumonia organism and for naming them both as varieties of *M. mycoides* (Lemcke, 1964*a*). However, the precipitation patterns show that these two organisms are more closely related than any of the other serological types examined. Most of the other cross-reactions appeared to be due to partly related rather than identical antigens. There was no evidence of the widespread distribution of the same antigens among very diverse species of Mycoplasma, as suggested by Villemot & Provost (1959). Nevertheless, the use of complex mixtures of antigens which comprise the disrupted suspensions makes it difficult to determine the exact relationship between some of the cross-reacting components. These need further study, with cross-absorbed antisera and with, if possible, the separate individual components.

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The Inhibition of the Growth of Brucellas *In Vitro* and *In Vivo* by Analogues of Erythritol

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SUMMARY

The growth of *Brucella abortus*, *B. melitensis* and *B. suis* in laboratory media was inhibited by several analogues of erythritol. The use of erythritol analogues in this manner was suggested by previous work on the localization of brucellas in brucellosis. The growth of *B. abortus* within bovine phagocytes was inhibited by analogues of erythritol under conditions in which streptomycin was ineffective. Although selected erythritol analogues did not eliminate brucellosis in guinea pigs, they produced a significant diminution in infection and showed no toxicity to the host.

INTRODUCTION

The treatment of human brucellosis with antibiotics and other drugs suffers from the disadvantage that large doses must be used and the relapse rate is high (Eisele, 1950; Spink, 1956; Stableforth, 1959). Undoubtedly the tendency for relapse is due to the intracellular nature of this chronic disease, which results in latent infection at sites inaccessible to the drugs. Hence, therapy against brucellosis might benefit from a drug which could attack the intracellular organism even if this drug were not as active as other antibiotics against freely growing brucellas. The possibility that analogues of erythritol might have some inhibitory action on intra- as well as extracellular brucellas was suggested by a recent investigation into the cause of the localization of brucellas in susceptible tissues of susceptible animals (cattle, goats, sheep, swine). It was shown, first in cattle, that localization of *Brucella abortus* in infected pregnant animals was due to the presence, in foetal but not in adult tissues, of erythritol, a growth stimulant for *B. abortus*, which was concentrated in tissues (placenta, foetal fluids, chorion) most prone to heavy infection (Smith *et al.* 1961, 1962*a*; Pearce *et al.* 1962; Williams, Keppie & Smith, 1962). Later, similar observations were made relating to *B. melitensis* and *B. suis* infections in sheep, goats and pigs (Williams *et al.* 1963; Keppie, Williams, Witt & Smith, 1965). On the other hand, no detectable erythritol was found in the placenta of humans, rats, rabbits or guinea pigs, which do not suffer intense placentitis in brucellosis (Keppie *et al.* 1965). In relation to the present paper, the main points of previous work

were: (i) the peculiar preference of brucellas for erythritol in the presence of glucose which has been further emphasized by recent work in a defined medium (Anderson & Smith, 1963; Anderson & Smith, 1965); (ii) that stimulation by erythritol of the growth of *B. abortus* occurred both *in vivo* and within phagocytes in a system where extracellular growth of the organism had been inhibited by streptomycin (Pearce *et al.* 1962). These points, coupled with the relative insusceptibility of erythritol to metabolism by mammalian tissue (Batt, Dickens & Williamson, 1960), suggested that suitable analogues of erythritol might act as antimetabolites and inhibit the growth of extra- and possibly intracellular brucellas, without being particularly toxic to the hosts, such as man, laboratory animals and non-pregnant ungulates. This paper describes the examination of a few erythritol analogues to see whether any experimental support could be gained for these hypotheses.

METHODS

Erythritol analogues. 2-Deoxy-2-fluoro-DL-erythritol and -threitol were synthesized by the methods of Taylor & Kent (1956) and Barnett & Kent (1963). 2-Deoxy-DL-erythritol ((±)-butane-1,2,4-triol) and (±)-butane-1,3-diol were obtained as described by Barnett & Kent (1963), 1-deoxy-1-fluoro-DL-glycerol by the method of Gryskiewicz-Trochimowski (1947) and 6-deoxy-6-fluoro-D-galactose according to Taylor & Kent (1958). 1,4-Dimesyl-DL-threitol, 1,4-dimesylerythritol, 1,6-dimesyl-D-mannitol (Mannitol Myleran), 2,3-diacetyl-1,4-dibromo-1,4-dideoxy-DL-threitol, 2,3-dibenzoyl-1,4-dimesyl-DL-threitol, 1,2,3,4-tetramesyl-erythritol and 1,2,3,4-tetramesyl-DL-threitol were prepared by the methods of Brown & Timmis (1961). 1,2-3,4-Dianhydro-DL-threitol (DL-butane diepoxide) was obtained by the method of Bose, Foster & Stephens (1959), 1,4-dibromo-1,4-dideoxy-DL-threitol, 1,4-dibromo-1,4-dideoxy-erythritol and 2,3-dibromo-2,3-dideoxy-DL-threitol by the method of Feit (1960), 1,6-dibromo-1,6-dideoxy-D-mannitol by the method of Overend, Montgomery & Wiggins (1948) and 2,6-dimesyl-α-D-glucopyranose by the method of Mitra, Ball & Long (1962).

1,4-Dibromo-1,4-dideoxy-2,3-dipalmitoyl-DL-threitol was prepared by Drs G. M. Timmis and S. S. Brown by the following method. Palmitoyl chloride (11 g.) was added dropwise, during 45 min., to a stirred solution of 1,4-dibromo-1,4-dideoxy-DL-threitol (5 g.) dissolved in pyridine (100 ml.) and kept between 5° and 10°. When the addition was complete a substantial precipitate formed. The mixture was kept at 0° for 18 hr and then mixed with water (300 ml.) at 0°. The product was collected, washed and dried in a desiccator to yield 13 g. of crude material. Recrystallization from an equal volume mixture of methanol and acetone (approx. 150 ml.) yielded 10 g. of the pure material, m.p. 70–75° (Found: C, 59·8; H, 9·8; Br 22·4. $C_{36}H_{68}Br_2O_4$ requires C, 59·7; H, 9·5; Br, 22·1).

1,4-Dimesyl-D-threitol and 1,4-dimesyl-L-threitol was generously supplied by Dr F. W. Feit (Leo Pharmaceutical Products, Denmark).

Inhibition of growth of Brucella abortus in tryptic meat broth. Sterile concentrated solutions of analogues (0·1 ml.; containing amounts equivalent to 100, 50, 25 and 10 μg./ml. in the final medium) were added to tryptic meat broth (5 ml.) containing $1–10 \times 10^6$ *Brucella abortus*/ml. These cultures, together with controls, were shaken at 37° in 5% CO_2 in air for 24 hr and the concentrations of organisms determined

photometrically (570 mμ) at 16 and 24 hr. When an analogue inhibited growth, the lowest concentration of analogue which produced 50% inhibition as compared with the population of the control at 24 hr was noted. Any material, 100 μg./ml. of which produced no inhibition at 24 hr, was scored as inactive.

Inhibition of growth of Brucella abortus in a defined medium containing glucose. Sterile solutions of the inhibitors (1 ml., 5 μmole/ml.) and the inoculated defined medium (4 ml. containing $2{\cdot}5 \times 10^7$ *Brucella abortus*/ml.) were mixed in flasks (25 ml.) and shaken at 37° in 5% CO_2 in air as described by Anderson & Smith (1965). At various time intervals, the extinctions of the contents of duplicate flasks were compared as described by Anderson & Smith (1965) with those of similar control samples.

Inhibition of growth of Brucella abortus within bovine phagocytes. The test was that described by Pearce *et al.* (1962) for testing substances which affect the growth of *Brucella abortus* (strain 544) within bovine phagocytes. Sterile solutions of the analogues in Locke solution (0·5 ml.; the concentrations were such that analogue at 200, 100, 50, 25 and 10 μg./ml. was present in the final mixture at incubation) were the test materials (see Pearce *et al.* 1962; under the heading 'Addition of test material and incubation for 40 hr'). They were mixed with a suspension (1·3 ml.) of bovine white blood cells in which intracellular *B. abortus* (strain 544) would grow on incubation despite the fact that the suspending fluid was a mixture of fresh (bactericidal) bovine serum containing streptomycin (2 μg./ml.). After incubation for 40 hr, the growth of intracellular *B. abortus* was estimated as described by Pearce *et al.* (1962). When the analogue inhibited growth, the lowest concentration of analogue which produced 50% inhibition as compared with growth in control samples was noted.

Action of analogues against Brucella abortus infection in the guinea pig. Guinea pigs were infected with *Brucella abortus* (strain 544; 1×10^4 organisms intramuscularly). After 7 days, the guinea pigs were treated daily with the test material injected subcutaneously and at intervals of 3, 6, 9 and 12 weeks groups of guinea pigs were withdrawn from the main batch, weighed, left 1 week without treatment, and then the number of organisms in their spleens estimated by the method described by Smith *et al.* (1962*b*). Comparisons were made with the infections in control animals.

Inhibition of growth of Brucella melitensis and B. suis in a laboratory medium. Various concentrations of 2-deoxy-2-fluoro-DL-erythritol and 1,4-dimesyl-DL-threitol were dissolved in a mixture of tryptic meat broth (1 ml.) and saline (2 ml.) in flasks (25 ml.). After inoculation (100 organisms/ml.) the flasks were shaken at 37° for 27 hr. Viable counts (see Pearce *et al.* 1962) indicated the inhibition of growth as compared with control samples. The lowest concentration of analogue which produced 50% inhibition was recorded.

RESULTS

The action of erythritol analogues and related compounds on the growth of Brucella abortus in tryptic digest meat broth

The results in the first column of Table 1 show that certain fluoro-, mesyl- and bromo- derivatives of erythritol and threitol inhibited the growth of *Brucella*

abortus in tryptic meat broth. The lack of activity shown by fluoro-, mesyl- and bromo-derivatives of other sugars and polyols indicated the specificity of the inhibitory effect. The high activity of 1,2–3,4-dianhydro-DL-threitol was not surprising in view of the known cytotoxic action of epoxides. Apart from this epoxide, the most active materials were the 1,4-dimesyl-threitols especially the L compound.

Table 1. *Action of erythritol analogues and related compounds on the growth of Brucella abortus in tryptic meat broth and within bovine phagocytes*

Compound	Concentration of compound producing 50 % inhibition (μg./ml.)	
	In tryptic meat broth*	In bovine phagocytes*
Erythritol derivatives		
2-Deoxy-2-fluoro-DL-erythritol	100	50–100
1,4-Dimesyl-erythritol	100	50–100
1,4-Dibromo-1,4-dideoxy-erythritol	Inactive†	—
2-Deoxy-DL-erythritol	Inactive	—
Threitol derivatives		
2-Deoxy-2-fluoro-DL-threitol	Inactive	Inactive
1,4-Dimesyl-DL-threitol	25–50	25
1,4-Dimesyl-D-threitol	50	100
1,4-Dimesyl-L-threitol	25	25
1,2-3,4-Dianhydro-DL-threitol	5	5
1,4-Dibromo-1,4-dideoxy-DL-threitol	100	—
2,3-Dibromo-2,3-dideoxy-DL-threitol	Inactive	—
Related compounds		
1,6-Dimesyl-D-mannitol	Inactive	—
1,6-Dibromo-1,6-dideoxy-D-mannitol	Inactive	—
1-Deoxy-1-fluoro-DL-glycerol	Inactive	—
6-Deoxy-6-fluoro-D-galactose	Inactive	Inactive
(±)-Butane-1,3-diol	Inactive	Inactive
2,6-Dimesyl-α-D-glucopyranoside	Inactive	—

— = not tested.

* For details of tests see Methods. In the test with intracellular *B. abortus*, 2-deoxy-2-fluoro-DL-erythritol produced no toxic effects on the phagocytes (for evidence of toxicity see Pearce *et al.* 1962); at high concentrations the mesyl compounds and anhydro-threitol showed some toxicity, but at the concentrations used for growth inhibition no significant toxic effects were observed.

† No significant inhibition at 100μg./ml.

The action of erythritol analogues and related compounds on the growth of Brucella abortus within bovine phagocytes

The results in the second column of Table 1 show that the compounds which inhibited the growth of *Brucella abortus* in a laboratory medium also inhibited the growth of intracellular *B. abortus*. This means that, unlike the streptomycin which was present in the suspending fluid (see Methods), the inhibitory analogues penetrated into the bovine phagocytes. In the intracellular test, the relative activity of the different inhibitors was the same as their relative activity in the laboratory medium. At the concentrations tested, the compounds were without apparent deleterious effect upon the phagocytes.

The action of erythritol analogues and related compounds on the growth of Brucella abortus in a defined medium containing an optimal amount of glucose

The results in Table 2 show that in a defined medium a representative selection of analogues produced inibition of the growth of *Brucella abortus* comparable with that found in tryptic meat broth and in bovine phagocytes. Furthermore, the inhibitory effects of 2-deoxy-2-fluoro-DL-erythritol and 1,4-dimesyl-DL-threitol were annulled by mixing them with an equimolar quantity of erythritol.

Table 2. *The effect of erythritol analogues on the growth of Brucella abortus in a defined medium containing glucose; the effect of mixing certain inhibitors with erythritol*

Analogue (1 μmole/ml.)	Population as % of control after incubation for		
	16 hr	24 hr	40 hr
2-Deoxy-2-fluoro-DL-erythritol (A)	70	62	37
1,4-Dimesyl-erythritol	95	85	69
1,4-Dibromo-1,4-dideoxy-erythritol	82	69	77
1,4-Dimesyl-DL-threitol (B)	95	65	53
1,4-Dibromo-1,4-dideoxy-DL-threitol	85	73	56
2,3-Dibromo-2,3-dideoxy-DL-threitol	98	98	95
1,2-3,4-Dianhydro-DL-threitol	30	19	13
1,Deoxy-1-fluoro-DL-glycerol	96	97	89
1,6-Dimesyl-D-mannitol	100	95	96
Erythritol	232	271*	180
A + erythritol	184	206	119
B + erythritol	222	204	139

* All erythritol was used by 24–30 hr.

The effect of erythritol analogues on Brucella abortus infections in guinea pigs

The results in Table 3 show that 2-deoxy-2-fluoro-DL-erythritol, 1,4-dimesyl-DL-threitol and 1,4-dibromo-1,4-dideoxy-DL-threitol had no dramatic effect on brucellosis in guinea pigs. However, 1,4-dimesyl-DL-threitol and 1,4-dibromo-1,4-dideoxy-DL-threitol produced a significant decrease (statistical analysis by our colleague Mr S. Peto) in infection and showed no evidence of toxicity for the host despite the prolonged administration. The epoxide, 1,2-3,4-dianhydro-DL-threitol, was too toxic for use as a therapeutic agent. The analogues shown in Table 3 would have been excreted rapidly, but attempts to produce an effective depot effect by using less-soluble analogues (2,3-dibenzoyl-1,4-dimesyl-DL-threitol, 2,3-diacetyl-1,4-dibromo-DL-threitol, 1,2,3,4-tetramesyl-DL-threitol, 1,2,3,4-tetramesyl erythritol, 1,4-dibromo-1,4-dideoxy-2,3-dipalmitoyl-DL-threitol) were without success.

The inhibitory effect of 2-deoxy-2-fluoro-DL-erythritol and 1,4-dimesyl-DL-threitol on the growth of Brucella melitensis and B. suis in vitro

The growth in tryptic meat broth of three strains of *Brucella melitensis* (6015, 1374, B 115) and two strains of *B. suis* (KG 25, 1330) was inhibited to the extent of 50 % by 1,4-dimesyl-DL-threitol (50 μg./ml.) and 2-deoxy-2-fluoro-DL-erythritol (100 μg./ml.).

Table 3. *The effect of erythritol analogues on Brucella abortus injection in guinea pigs*

Examination of animals†	Controls	Treatment with analogues*		
		2-Deoxy-2-fluoro-DL-erythritol	1,4-Dimesyl-DL-threitol	1,4-Dibromo-1,4-dideoxy-DL-threitol
No. of animals examined after each period of treatment	15–20	5	10	10
At 3 weeks				
% animals with spleen count				
< 300	Nil	Nil	Nil	Nil
> 300 and < 1×10^6	60	80	80	80
Average body weight (g.)	575	570	540	540
At 6 weeks				
% animals with spleen count				
< 300	Nil	—	30	Nil
> 300 and < 5×10^5	40	—	40	40
Average body weight (g.)	650	—	580	600
At 9 weeks				
% animals with spleen counts				
< 300	Nil	Nil	Nil	20
> 300 and < 5×10^4	45	60	70	60
Average body weight (g.)	700	720	620	650
At 12 weeks				
% animals with spleen count				
< 300	25	—	50	50
> 300 and < 1×10^4	5	—	30	40
Average body weight (g.)	690	—	750	730

* Guinea pigs, infected 7 days previously with 1×10^4 *B. abortus* given intramuscularly, were treated daily for the periods stated with 10 mg. of the analogue given subcutaneously.

† The extent of infection in the spleen was obtained by methods described by Smith *et al.* (1962*b*).

DISCUSSION

Although only relatively few erythritol analogues have been examined in this work, the results support the hypotheses stated in the introduction to this paper. Some erythritol analogues inhibit the growth of *Brucella abortus*: (1) *in vitro* in two media; (2) within bovine phagocytes in a test in which extracellular streptomycin has no bactericidal action intracellularly; (3) in infected guinea pigs without being toxic to the host. It appears also that, like the stimulatory effect of erythritol, the inhibitory effect of erythritol analogues extends to growth of *B. melitensis* and *B. suis*. This work adds weight to the evidence of a specific relation between the carbohydrate metabolism of Brucella and erythritol. The inhibitory action of at least two of the analogues is reversed by addition of erythritol; the analogues inhibit growth, just as erythritol stimulates growth, in the presence of large and optimal amounts of glucose and usage of glucose by *B. abortus* is decreased by addition of erythritol or 2-deoxy-2-fluoro-DL-erythritol (Anderson & Smith, 1965). The significance of these observations will only become clear when we know more about the metabolism of erythritol by *B. abortus*.

Since relatively few analogues were available for examination, little can be said about the reasons for the relative activity of different analogues, e.g. the differences

in activity between the 1,4- and 2,3-dibromo- derivatives of DL-threitol, the fluoro-derivatives of erythritol and DL-threitol and the D- and L- forms of 1,4-dimesyl-threitol. Perhaps the superior activity of the threitol over erythritol compounds is due to the fact that threitol, liberated by spontaneous hydrolysis or after alkylation of enzymes, does not, in contrast to erythritol, stimulate the growth of *Brucella abortus*. Also, the molecular conformation of 2-deoxy-2-fluoro-DL-erythritol bears a very close resemblance to that of erythritol so as to make a competitive mechanism likely.

It was hoped that the dimesyl- and dibromo-derivatives might be capable of some degree of irreversible inhibition. 1,6-Dimesyl-D-mannitol appears to inhibit fructose diphosphate irreversibly, an effect which was speculatively attributed to the alkylating ability of this compound (Timmis, Weber & Singhal, 1963), which perhaps suggests a stable bond between the drug and the enzyme. Although it is not suggested that this particular enzyme is concerned in the inhibition of Brucella described here, the fact that 1,4-dimesyl-L-threitol was more active than the D isomer indicates that the enzyme concerned has a type of specificity dependent upon optical isomerism of polyols, similar to fructose diphosphatase, which is not inhibited by 1,6-dimesyl-L-mannitol. Although some erythritol analogues will inhibit the intracellular growth of Brucella, no analogue so far examined is sufficiently active to be considered as a therapeutic agent. Nevertheless, sufficient work has been done to indicate that such an agent might emerge from further exploration along these lines.

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The Metabolism of Erythritol by *Brucella abortus*

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SUMMARY

The growth of a virulent strain of *Brucella abortus* was stimulated by low concentrations of erythritol in a medium containing high concentrations of glucose and a wide range of amino acids. During growth in this medium the organisms used about $1\frac{1}{2}$ times their weight of erythritol as a carbon and energy source. The effect of erythritol was specific, since several C_3 to C_6 homologues had no growth-stimulating activity. Radiotracer studies showed that a large proportion of the carbon of the erythritol was excreted as carbon dioxide; that remaining in the organisms was fairly uniformly distributed over all components. Erythritol and 2-deoxy-2-fluoro-DL-erythritol inhibited incorporation of glucose by the organism.

INTRODUCTION

In bovine brucellosis the preferential growth of *Brucella abortus* in certain foetal materials (placenta, chorion, the fluids) appeared to be due to the presence in these tissues of erythritol, a growth stimulant for *B. abortus* which is absent from maternal tissues (Smith *et al.* 1962; Pearce *et al.* 1962; Williams, Keppie & Smith, 1962). This polyol had already been shown to be the best of nine carbohydrates tested as a sole carbon source for the growth of Brucella in a simple medium (McCullough & Beal, 1951). Further work showed that the course of brucellosis in other animal species might also be explained by the presence of erythritol in susceptible tissues (Keppie, Williams, Witt & Smith, 1965). The importance of erythritol metabolism by *B. abortus* in animal infections prompted the present *in vitro* investigation and influenced the choice of experimental conditions. The effect of erythritol has been studied during the growth of a virulent strain (544) of *B. abortus* in a complex defined medium containing adequate carbohydrate in addition to erythritol. Kinetic and radiotracer studies soon showed that, despite the presence of an optimum amount of glucose, *B. abortus* used erythritol as a carbon and energy source. The growth-stimulating effect of small quantities of erythritol (0·001 μmole/ml.) previously reported (Smith *et al.* 1962; Pearce *et al.* 1962) had occurred because only correspondingly small populations were studied. Hence, the uptake and subsequent fate of erythritol have been investigated together with the influence of erythritol on glucose metabolism. This work was reported briefly elsewhere (Anderson & Smith, 1963) and is complemented by parallel work on the effect of erythritol analogues on the growth of *B. abortus* (Smith *et al.* 1964).

METHODS

Erythritol. Commercial erythritol was recrystallized from aqueous acetone to yield a chromatographically pure sample.

Organism. The virulent, CO_2-dependent strain 544 of *Brucella abortus* was freeze-dried for storage. Cultures from the freeze-dried stock on slopes of tryptic meat agar (37°; 5% CO_2 in air) were stored at 4° for up to one month to provide seed. A subculture (37°; 5% CO_2 in air, overnight) on a tryptic meat agar slope was washed off with the defined medium (see below), centrifuged, resuspended in fresh defined medium and then grown overnight (shake culture; 37°; 5% CO_2 in air) to provide inoculum for the growth experiments.

Table 1. *Composition of a defined medium for studies of the metabolism of erythritol by Brucella abortus*

	g./l.		mg./l.
Sodium chloride	7·5	Glycine	100
Dipotassium hydrogen phosphate	1·0	DL-α-Alanine	100
Magnesium sulphate heptahydrate	0·1	DL-Valine	100
Sodium thiosulphate pentahydrate	0·1	DL-Leucine	50
Glucose	7·0	DL-*iso*-Leucine	50
Ammonium sulphate	1·35	DL-Aspartic acid	200
	mg./l.	DL-Serine	25
		DL-Threonine	25
Ferrous sulphate heptahydrate	0·10	L-Proline	100
Manganous sulphate tetrahydrate	0·10	L-Cystine	5
Thiamine	0·20	DL-Methionine	50
Nicotinic acid	0·20	DL-Phenylalanine	25
Calcium pantothenate	0·04	L-Tyrosine	25
Biotin	0·001	L-Tryptophan	25
		L-Arginine	100
		L-Lysine	100
		L-Histidine	100

Adjusted (NaOH) to pH 6·8 at 37°.

Defined media. The medium shown in Table 1 is a modification of that of Rode, Oglesby & Schuhardt (1950); the large glutamic acid component (3000 μg./ml.) was replaced by ammonium sulphate and additional glucose. The stimulatory effect of erythritol could not be demonstrated in the original medium and, although smaller amounts of glutamic acid (10–1000 μg./ml.) did not interfere with the erythritol effect, all glutamic acid was excluded from the medium. A stock medium (4× strength, without biotin) was stored at −25°. When required, biotin was added to the diluted stock medium adjusted to pH 6·8, and the medium sterilized by filtration through a double layer of Oxoid membrane filter (Grade AP; Oxoid Ltd.) Any modifications of the chosen medium were stored and sterilized in a similar manner.

Growth of Brucella abortus in a liquid medium, with and without erythritol. Apparatus was washed successively in detergent, chromic+sulphuric acid, water, nitric acid and distilled water, then sterilized at 160° for 2 hr. Inoculated medium (4·9 ml.) and various concentrations of erythritol in Locke's solution (0·1 ml.) were placed in flasks (25 ml.) plugged with gauze-covered cottonwool. The flasks were shaken (5 cm. throw, 100 oscillations/min.) at 37° in gas-tight aluminium boxes filled with humidified 5% CO_2 in air. Flasks were removed for examination at intervals up to 60 hr.

Growth occurred consistently from inocula of 2×10^7 bacteria/ml. or more but with small inocula (1×10^4 bacteria/ml.) erratic results were obtained unless gelatin (Simeon & Co. Ltd, Luton; solution sterilized at 120° for 15 min.) was added to the medium (10 μg./ml.; see ZoBell & Meyer, 1932).

Measurement of bacterial growth. Most bacterial populations were estimated by optical extinction measurements at 430 mμ (Coleman spectrophotometer, Model 14, for populations greater than 1×10^8 bacteria/ml.; the Unicam (S.P. 500) spectrophotometer with the 4 cm. cell for populations down to 2×10^7 bacteria/ml.) after treatment with formalin (3%, v/v; 30 min.). A linear relation between extinction readings and Helber chamber counts was obtained for the range 2×10^7 to 5×10^9 bacteria/ml. (statistical analysis by Mr S. Peto); and this relation, originally determined on cultures without erythritol, held for cultures of *Brucella abortus* grown with various concentrations of erythritol and examined before and after the erythritol had been depleted. Comparison of Helber chamber counts and viable counts (on tryptic meat agar—with 5% peptic sheep blood) showed the bacteria to be greater than 85% viable.

$2{\cdot}05 \times 10^{10}$ organisms weighed 1 mg. (log-phase bacteria treated with formalin 3%, v/v, washed twice with water, then dried at 105°). This relation did not change significantly when the bacteria were grown with erythritol. Populations below 2×10^7 bacteria/ml. were estimated by viable counts.

Examination of culture filtrates for erythritol, erythrose and erythrulose. Cultures were filtered through Oxoid (grade A.P.) membranes, de-ionized by passage through a mixed bed of Amberlite IR-120 (H) and Permutit deacidite-G (HCO_3) ion-exchange resins, then submitted to paper chromatography. Chromatograms were treated with silver nitrate and sodium hydroxide reagents (Trevelyan, Proctor & Harrison, 1950) or *p*-anisidine and periodate (Bragg & Hough, 1958) reagents. Control experiments showed that, despite the large concentration of glucose in the medium, erythritol, erythrose or erythrulose at 0·1 μmole/ml. could be detected in culture filtrates.

Erythritol homologues. 'AnalaR' grade glycerol was supplied by British Drug Houses Ltd (Poole, Dorset). DL-threitol was prepared from 1,2-3,4-dianhydro-DL-threitol supplied by Dr G. M. Timmis. Ribitol (adonitol), D-glucitol (sorbitol), D-galactitol (dulcitol) and D-mannitol were supplied by George T. Gurr Ltd, London, and D- and L-arabitol by Mann Research Laboratories Inc. New York. For growth studies, solutions of the homologues (1 ml. containing 5 μmole) in the defined medium were mixed with the inoculated medium (4 ml. in 25 ml. flasks) then treated as described previously for studies with erythritol.

[^{14}C]-*Labelled compounds.* [U-^{14}C]-Erythritol (2 mC/mmole) from the Radiochemical Centre, Amersham, Buckinghamshire, was diluted (25 μmole/ml.; 1 μC/ml.) and stored at −20°. [$^{14}C_1$]-Erythritol (0·9 mC/mmole), synthesized by Dr P. W. Kent of the University of Oxford, was treated similarly. [U-^{14}C]-D-Glucose (8·5 mC/mmole) was also diluted (10 μC/ml.; negligible weight of glucose) and stored similarly.

Measurement of radioactivity. Radioactivity was measured by a 'coincidence' scintillation counting technique (Apparatus by Isotope Developments Ltd, Reading, Berkshire: liquid measuring head No. 2022, coincidence control unit No. 2032; and a decade counter) at room temperature in disposable polythene tubes (25 ml. with

lids; catalogue No. XT 1580; X-Lon Products Ltd, London, S.W. 1). 'Scintillation Grade' dioxane, formamide, and a dioxane-based scintillator (NE 220) were obtained from Nuclear Enterprises (G.B.) Ltd, Edinburgh. The solvents were stored under oxygen-free nitrogen but the polythene tubes were not flushed with nitrogen during assay. Replicate counts agreed within $\pm 3\%$, but counting efficiencies (about 30–60 %) varied with the nature of the solvent mixture in which the experimental sample was examined. Thus, comparisons of experimental samples with standard amounts of radiotracer were made in identical solvent mixtures. Since some mixtures were non-aqueous, standards were dissolved in dioxane, which could be included in every solvent system. More than 10^4 counts were determined for each sample and appropriate background and coincidence corrections applied.

The radioactivity of aqueous solutions or suspensions (0·5 ml.) was measured after mixture with formamide (0·5 ml.), dioxane (4 ml.) and scintillator (5 ml.) The formamide and dioxane decreased the possibility of phase separation in saline solutions. Any suspensions which were assayed in these aqueous systems were insufficiently dense to affect the counting efficiency. Samples in organic solvents (1 ml.) were mixed with dioxane (4 ml.) and scintillator (5 ml.) for assay. Insoluble protein residues (5–20 mg.) from the solvent extraction of bacteria (see below) were dispersed in hot 6 N-HCl (1–5 ml. at 100°). Portions (0·5 ml.) of this suspension were mixed with water (1 ml.) and 1·0 M-hyamine hydroxide (1 ml.; see below). Portions (1 ml.) of this mixture with added dioxane (4 ml.) and scintillator (5 ml.) were assayed as described above for organic solutions.

[^{14}C]-Carbon dioxide (see below) was absorbed in a methanolic solution (0·3 M) of hyamine hydroxide (2-methyl-4-(1, 1, 3, 3-tetramethylbutyl)-phenoxy-ethoxy-ethyl-dimethylbenzyl ammonium hydroxide; Passmann, Radin & Cooper, 1956) for assay as described above for organic solvents.

Estimation of radiotracer in components of protein hydrolysates. Two-dimensional chromatograms of protein hydrolysates were placed against X-ray film (Ilford, Industrial G, 14 × 17 in.) in a suitable holder. After an appropriate exposure, the developed film was used to locate the individual spots; these were cut out and placed with water (0·5 ml.) in assay tubes. The tubes were gently agitated for a few minutes and the radiotracer determined after addition of formamide (0·5 ml.), dioxane (4 ml.), and scintillator (5 ml.). Duplicate chromatograms sprayed with ninhydrin reagent were used to identify the spots by comparison with standard mixtures of amino acids.

Collection of $^{14}CO_2$ produced during growth of Brucella abortus on [U-^{14}C]-erythritol. Narrow-necked conical flasks (100 ml.) with rubber stoppers and provided with inlet and outlet tubes were joined to cottonwool filters by thick-walled rubber tubing. Gas flow through this tubing could be blocked with double screw clips. The length of the tubing was kept as short as possible to minimize loss of CO_2 by diffusion through the rubber. Suspensions (10 ml., containing 2×10^7 bacteria/ml.) of the organism in medium (containing 0·5 μmole/ml. and 0·02 μC/ml. of [U-^{14}C]-erythritol) were placed in the flasks. A stream of 5 % CO_2 in air (0·5 l./min. for 2 min.) was introduced and the clips on the inlet and outlet tubes were then closed. The whole apparatus was then shaken at 37° in the aluminium boxes already described.

At intervals, flasks were removed and gently agitated whilst their atmospheres were flushed out with a stream of air (0·5 l. over 2 min.). To remove remaining

traces of $^{14}CO_2$, carbon dioxide (5 ml.) was flushed in with a little air and after agitation for a further minute the flasks were again flushed out with air (1·0 l. over 2 min.). Carbon dioxide displaced from the flasks was trapped for measurement in a bubbler containing 0·3 M-methanolic hyamine hydroxide (25 ml.). This procedure removed all $^{14}CO_2$ from the apparatus. After removal of the $^{14}CO_2$ the extinction of the bacterial suspension was measured photometrically and bacteria and supernatant fluid separated by centrifugation (5000*g* for 20 min.) for radio-assay.

Examination of fractions of Brucella abortus for the presence of radiotracer derived from [U-^{14}C]-erythritol

Growth of organisms. Cultures (30 × 100 ml. flasks each containing 10 ml. medium) of *Brucella abortus* (inoculum 5×10^7 bacteria/ml.) containing [U-^{14}C]-erythritol (1 μmole/ml., 0·04 μC/ml.) were grown overnight in shake flasks as described previously until about 80 % of the erythritol had been used. The bacteria were harvested by centrifugation, washed twice with water, then fractionated as described below and the weights and radiotracer content of the various fractions determined.

Method of fractionation. A convenient method for the solvent fractionation of *Brucella abortus* was developed from that of Roberts *et al.* (1957). A portion (equiv. about 10 mg. dry weight) of the washed bacterial suspension was dried (100°, 30 min. to halt enzyme action; then as described below) and the remainder (equiv. about 20 mg. dry weight) was treated with 5 % (w/v) trichloroacetic acid (10 ml.) in a tared centrifuge tube. After 10 min. insoluble material was centrifuged down, washed twice with water, dried and weighed. The residue from this extraction was treated successively with methanol (10 ml.) and methanol + chloroform (1 + 2 by vol.; 10 ml.) then centrifuged down, dried, and weighed. The methanol + chloroform mixture (Folch *et al.* 1951) appeared to extract all lipid, since no appreciable amounts of bound lipid were removed by a mixture of ether + ethanol + conc. HCl (450 + 300 + 13, by vol.; Stahl, Pennell & Huddleson, 1939), or by extraction with ether after refluxing with methanol + water (19 + 1, by vol.; Reichert, 1944). The residue from the lipid extraction was treated with hot trichloroacetic acid (5 %, w/v; 10 ml. at 90° for 15 min.) then centrifuged down, washed twice with water, dried and weighed.

All fractions were dried to constant weight under reduced pressure over phosphoric oxide and paraffin chips. The lipid solvents were removed in a stream of warm nitrogen before the fraction was dried. To obtain preliminary information on the chemical nature of the fractions a large quantity (3 g. dry weight) of bacteria was grown on a defined medium (Rode *et al.* 1950) without erythritol and extracted and examined in a similar way.

Analysis of fractions. Hydrolyses in sealed tubes were carried out with N-H_2SO_4 (100°, 18 hr, neutralized with $BaCO_3$), N-NaOH (100°, 3 hr, neutralized with Amberlite IR-120 (H) ion exchange resin) and 6 N-HCl (100°, 18 hr, dilute, filter, then concentrated to dryness to remove excess acid) as appropriate. Sugars and polyols were identified by single dimension chromatography in several solvents. Free sugars were detected with *p*-anisidine spray reagent (Hough, Jones & Wadman, 1950) and polyols with periodate and anisidine (Bragg & Hough, 1958) and periodate–Schiff's reagents (Baddiley, Buchanan, Handschumacher & Prescott, 1956). Phosphates

were detected with molybdate + perchloric acid reagent (Hanes & Isherwood, 1949). Our colleague Mr A. G. Ness identified amino compounds by two-dimensional paper chromatography and confirmed the identity of α,ϵ-diaminopimelic acid (Powell & Strange, 1956); amino compounds were detected with ninhydrin reagent (Consden & Gordon, 1948). Similar protein hydrolysates were examined by auto-radiography, as described above.

The [^{14}C]-content of purine ribose was determined by dilution analysis. A portion (5 ml.) of the hot trichloroacetic acid extract (10 ml.) with ribose carrier (100 mg.) was refluxed with N-HCl (1 hr at 100°), de-ionized, and concentrated to yield ribose on crystallization from aqueous acetone. The specific activity of this ribose remained unchanged on further recrystallization.

Further analyses were made on samples from the large scale preliminary fractionation which could not be performed on the smaller available quantities of radioactive fractions. These analyses included: nitrogen (Nesslerization after Kjeldahl digestion); total phosphorus (after perchloric acid digestion; King, 1951); protein (Lowry *et al.* 1951; against human serum albumin as a standard after dispersion in N-NaOH at 100° for 5 min.); polysaccharide as glucose (Dische, 1955); DNA (diphenylamine reaction against purified calf thymus DNA as standard) and RNA (orcinol reaction against purified ribose as a standard) by the methods of Morse & Carter (1949); nucleotides spectrophotometrically (assuming $\epsilon_{260\,m\mu}$ M for nucleotides = 10^4) by our colleague Mr H. E. Wade.

Preparation of cell walls from Brucella abortus which had metabolized [$^{14}C_1$]-, *and* [*U*-^{14}C]-*erythritol.* The organism was grown in the presence of the two forms of [^{14}C]-erythritol as previously described, then harvested by centrifugation and diluted with carrier bacteria to form a sufficient quantity for disintegration (100 mg.). Cell walls were then prepared by disintegration (apparatus by H. Mickle, Hampton, Middlesex), treatment with heat (60° for 1 hr), DNAase and RNAase, and purification by centrifugation (Dr D. C. Elwood; private communication).

Effect of erythritol and 2-deoxy-2-fluoro-DL-erythritol on glucose incorporation by Brucella abortus. To obtain a measurable uptake of radioactivity by the comparatively small amount of bacteria (this is limited by the available erythritol), the normal glucose content (39 μmole/ml.) of the defined medium had to be decreased and [U-^{14}C]-D-glucose of high specific activity had to be used. The modified medium contained [U-^{14}C]-D-glucose (5 μmole/ml., 0·2 μC/ml.) with additional NaCl (1 mg./ml.). This medium (1 ml.) alone, or containing either erythritol (10 μmole/ml.) or 2-deoxy-2-fluoro-DL-erythritol (10 μmole/ml.), was dispensed into flasks (100 ml.). Additional medium (9 ml.) inoculated with the organism ($1{\cdot}0 \times 10^8$ bacteria/ml.) was then added and growth under the standard conditions in shake culture determined as described previously. Heat-killed bacteria (60°, 1 hr, 3·5 mg. dry wt) were added as carrier to portions (9 ml.) of the suspensions of radioactive bacteria. After centrifugation (5000*g*; 20 min.) the bacteria were washed (original medium containing [^{12}C]-D-glucose instead of the radiotracer; 10 ml.) and suspended in formol saline (3%, v/v) for radio-assay. The heat-killed bacteria did not absorb radiotracer when added alone to the labelled medium.

Short-term uptake of [U-^{14}C]-erythritol and [U-^{14}C]-D-glucose by dense suspensions of Brucella abortus

Apparatus. 'Medical flat' bottles (8 oz.) were provided with metal screw tops in which were punched several small holes so that, although the punched cap with its intact rubber washer formed a secure seal, samples of the contents could be removed with a hypodermic syringe. A slight vacuum was maintained in the bottles and a tight rubber cap was fitted over the top to prevent contamination during sampling. Bottles were filled and handled at 37° and during the uptake studies were rotated end over end (60 rev./min.).

Uptake of [U-^{14}C]-erythritol. The virulent organism (*Brucella abortus* strain 544) was grown on standard medium supplemented with erythritol 1 μmole/ml. and harvested by centrifugation before the erythritol was depleted. After re-suspension in fresh medium, the suspension was rotated in the 'medical flat' (5% CO_2 in air) for 10 min. before injection of [U-^{14}C]-erythritol through the cap. A sample (4 ml.) of the bacterial suspension (initially 60 ml. containing equiv. 200 μg. dry weight bacteria/ml. and [U-^{14}C]-erythritol 0·5 μmole/ml., 0·020 μC/ml.) was withdrawn immediately and further samples were taken at convenient intervals over 40 min. The samples were filtered quickly through a double 'Oxoid' membrane (grade A.P.) to separate the culture medium for determination of radiotracer loss.

Uptake of [U-^{14}C]-D-glucose in the presence and absence of erythritol. Cells were grown as described above for the virulent strain of *Brucella abortus* and the uptake of [U-^{14}C]-D-glucose was studied in a similar manner. The quantity of bacteria required for this work (i.e. those harvested during log phase before erythritol depletion) was limited. Hence, to obtain a measurable loss of radiotracer from the medium by the relatively small quantity of bacteria, the concentration of glucose in the normal medium (39 mole/ml.) had to be drastically decreased. One 'medical flat' contained bacteria (equiv. 200 μg. dry weight/ml.) and the defined medium with the glucose content decreased to 0·5 μmole/ml. (0·02 μC/ml.). A second 'medical flat' contained the same system with added erythritol (0·5 μmole/ml.).

Examination of a hot water extract of Brucella abortus for erythritol. The organisms were grown in the presence of [U-^{14}C]-erythritol as described previously. The washed bacteria (equiv. about 20 mg. dry weight) were treated with water (10 ml.) at 100°; the extract was de-ionized, treated with Na_2CO_3 (2%, w/v; 100°; 30 min.) and then de-ionized again (Adcock, 1957). The concentrated extract was examined chromatographically for radioactive erythritol.

Attempts to obtain broken-cell preparations which would metabolize erythritol. Organisms were grown on the standard medium supplemented with erythritol (1 μmole/ml.) and harvested by centrifugation before depletion of the erythritol. The bacteria were disrupted by disintegration of a suspension with glass beads (apparatus by H. Mickle, Hampton, Middlesex), by alternate freezing and thawing (Gerhardt *et al.* 1953) and by disintegration of a frozen suspension in a suitable press (Hughes, 1951).

The various preparations (final concentrations equal to whole bacteria, 1 mg./ml. were incubated (37°) with, and without, glucose (7 mg./ml.) in a buffer (K_2HPO_4, 0·10%; $NaH_2PO_4.2H_2O$, 0·33%; NaCl, 0·10%; $MgSO_4.7H_2O$, 0·07%; pH 7·0) containing NAD, NADP, ATP (0·02 μmole/ml. each) and [U-^{14}C]-erythritol (0·5μmole/

ml., 0·02 μC/ml.) Samples (1 ml.) withdrawn at intervals up to 30 min. were heated (100°, 2 min.) to halt enzyme activity then diluted to 5 ml. and filtered through Oxoid membranes (grade AP). The filtrate was treated (100°, 2 hr) with Biodeminrolite (Permutit Co. Ltd, London, W. 4) mixed-bed ion-exchange resin (2 g.) to remove ionic compounds and sugars from polyols (Anderson, Andrews & Hough, 1961). After dilution to 10 ml. the radiotracer content of the supernatant fluids were determined as a measure of their erythritol content, on the assumption that products of erythritol metabolism would be associated with the cell debris or be removed by the mixed-bed resin.

RESULTS

The metabolism of erythritol by Brucella abortus during growth in the presence of an optimum (high) concentration of glucose

In previous work with small inocula (see references in Introduction) minute quantities of erythritol (less than 0·01 μmole/ml.) stimulated the growth of *Brucella abortus* in bovine phagocytes and in laboratory media. Similarly, in the present work with a defined medium, small quantities of erythritol (0·005 μmole/ml.) had a significant effect on the growth of *B. abortus* provided that the inoculum was small (about 1×10^4 bacteria/ml.). However, larger quantities of erythritol were needed if the inoculum was to be sufficiently large (about $2{\cdot}5 \times 10^7$ bacteria/ml.) to allow increases in growth to be followed turbidimetrically.

Fig. 1 shows the effect of various concentrations of erythritol on the growth of *Brucella abortus* strain 544. Stimulation of growth continued for a period which was determined by the original concentration of erythritol. After this period, the growth rate approached that of the control. These results suggest that, despite the presence of an optimum high concentration of glucose, erythritol was a main carbon or energy source and had been exhausted at the points of inflexion of the curves. Examination of culture filtrates obtained in similar growth experiments with various concentrations of erythritol up to 5 μmole/ml. confirmed that at the points of inflexion of the growth curves all detectable erythritol had disappeared. At these inflexion points, the ratios of erythritol used to dry weight of bacteria formed were from 1:1·4 to 1:2·0. A similar large usage of erythritol by *B. abortus* was confirmed in experiments with small inocula (about 1×10^4 bacteria/ml.).

Although a large quantity of erythritol was needed to produce the growth stimulation of *Brucella abortus* in this system containing glucose, too high an initial concentration of erythritol (about 10–50 μmole/ml. for an inoculum of 5×10^7 bacteria/ml.) was inhibitory. For this reason, the initial concentration was related to the size of the original inoculum to produce the maximum stimulation of growth. Erythritol concentrations up to 5 μmole/ml. have been used in this work.

The specificity of the effect of erythritol on Brucella abortus in a medium containing glucose

A wide range of erythritol homologues had little effect on the growth of the organism in this medium containing glucose (Table 2). This specificity of growth enhancement by erythritol was supported by the inhibition of the growth of *Brucella abortus*, *in vitro* and *in vivo*, by analogues of erythritol (Smith *et al.* 1964). The effect of one of these analogues (2-deoxy-2-fluoro-DL-erythritol) on glucose utilization by

B. abortus will be discussed later; its inhibitory effect on growth and the annulment of this inhibition by addition of erythritol is shown in Table 2.

The fate of erythritol when metabolized by Brucella abortus in the presence of glucose

The results of radiotracer studies on the growth of *Brucella abortus* with [U-^{14}C]-erythritol (0·5 μmole/ml.), shown in Fig. 2, indicated that all erythritol had been depleted at 19–20 hr; this was confirmed by analysis of the culture filtrates. At this time, the ratio of erythritol used to weight of organisms formed was 1 : 1·4 (see above). At 21 hr, the distribution of original activity was: bacteria 23 %, medium 37 %,

Table 2. *The action of erythritol homologues and analogues on the growth of Brucella abortus in a medium containing glucose*

Substrates (1 μmole/ml.)	Population at 16 hr as % of population of a control
Glycerol	100 (±5)*
DL-Threitol	
Ribitol	
Xylitol	
D-Arabitol	
L-Arabitol	
D-Glucitol	
D-Galactitol	
D-Mannitol	
D-Erythrose	125*
Erythritol	225
2-Deoxy-2-fluoro-DL-erythritol	64
2-Deoxy-2-fluoro-DL-erythritol + erythritol	195

* At 40 hr similar ratios were obtained.

Table 3. *The incorporation of [U-^{14}C]-erythritol by Brucella abortus during growth in the presence of glucose*

Period of growth (hr)	Carbon ex erythritol / Total cell carbon
12	20
14	26
16	24
19	22
21	22
Erythritol depleted after 21 hr	
39	5

carbon dioxide 40 %; thus indicating that erythritol was used as a general carbon and energy source.

Material in the medium containing radiotracer has not been identified but paper chromatography has excluded the presence of significant amounts of erythrose or erythrulose which might have been formed by the partial oxidation of erythritol as occurs with *Acetobacter suboxydans* (Whistler & Underkofler, 1938). Only small

amounts of radiotracer were present in glucose and glycerol regions of autoradiograms, although large amounts of glycerol were excreted into the medium.

The results in Table 3 show that up to the time when the polyol was depleted only about one-quarter of the carbon content of the organisms (assumed to be 55% of the dry weight) was derived from erythritol despite the fact that the bacteria had used approximately 1½ times their own weight of erythritol during growth.

The incorporation of [U-^{14}C]-erythritol into cell fractions of Brucella abortus

Table 4 summarizes the distribution of activity and the nature and yield of fractions obtained from *Brucella abortus* 544 grown in the presence of [U-^{14}C]-erythritol until about 80% of the erythritol had been depleted. Evidence on the chemical nature of fractions shown in Table 4 was supplemented by the following

Table 4. *The incorporation of [U-^{14}C]-erythritol into cell fractions of Brucella abortus*

Fraction	Chemical composition	Weight as % of dry weight of organism	Radioactivity as % of radioactivity in organism
Material soluble in cold trichloroacetic acid	Diffusible material (about 70%) with a non-diffusible residue containing glucose, glycerol, ethanolamine and amino acids	17·4	11·6
Material soluble in methanol and in methanol + chloroform	Lipids containing fatty acids, glycerol, ethanolamine, phosphate and traces of amino acids	15·7	14·3
Material soluble in hot trichloroacetic acid	Nucleic acids	12·2	15·8
Insoluble cell residue	Protein containing large amounts of valine, alanine, the leucines, glutamic and aspartic acids with lesser amounts of serine, glycine, threonine, arginine, lysine and tyrosine and with traces of histidine, proline, phenylalanine, methionine and α,ε-diaminopimelic acid	56·3	58·0

analyses (expressed as % of dry weight of original bacteria) of the fractions obtained from a large quantity of *B. abortus* in a preliminary experiment: material soluble in trichloroacetic acid, 14% (containing diffusible material 70% including 12% nucleotides, and 30% of non-diffusible material including 30% glucose and 10% protein); the lipid fraction, 11·4% (containing 53% acetone-insoluble material, P, 2·6%; N, 4·2%; and 47% of an acetone-soluble material, P, 0·9%; N, nil); the nucleic acid fraction, 10·5% (containing 66% RNA and 34% DNA); and the insoluble residue, 66% (containing 98% protein).

The even distribution of radiotracer over all fractions is apparent. Further evidence that radiotracer was evenly distributed within the solvent fractions was provided by the study of two selected cases, namely, the purine-bound ribose of the nucleic acids and the amino acids obtained on hydrolysis of the protein residue. The purine-bound ribose contained 17% of the total radioactivity and made up 18% of

the total carbon in this fraction (assuming the fraction was 67 % RNA and 26 % of the RNA carbon was in purine-bound ribose). The distribution of radioactivity in the amino acids of the protein fraction (see Table 5) roughly correlated with the relative amounts of the individual components (estimated by visual comparison of spots on paper chromatograms). No single amino acid appeared to contain an undue proportion of the radio-activity.

Table 5. *Radiotracer content of the amino acids in the protein of Brucella abortus grown in the presence of glucose and [U-^{14}C]-erythritol*

Amino acid	[^{14}C]-Content of individual amino acids as % of [^{14}C] in hydrolysate	Amino acid	[^{14}C]-Content of individual amino acids as % of [^{14}C] in hydrolysate
Aspartic acid	15	Arginine	5
Leucines	15	Threonine	5
Alanine	14	Phenylalanine	2
Glutamic acid	11		(approx.)
Valine	8	Methionine	1·4
Lysine	8	Histidine	0·3
Tyrosine	8	$\alpha\varepsilon$-diaminopimelic acid	0·3
Serine + glycine	6	Unidentified spots	4·35

The incorporation of [^{14}C]-erythritol into the cell wall of Brucella abortus

A cell-wall preparation of *Brucella abortus* 544 formed 29·7 % of the dry weight of organisms which had been grown in the standard medium in the presence of erythritol. Similar preparations from bacteria grown in the presence of [$^{14}C_1$]- and [U-^{14}C]-erythritol contained 29 and 31 %, respectively, of the total activity of the bacteria, thus confirming the random distribution of carbon from erythritol among cell components.

The effect of erythritol and 2-deoxy-2-fluoro-DL-erythritol on the incorporation of [U-^{14}C]-D-glucose by Brucella abortus

The results summarized in Table 6 show that erythritol and the fluoro analogue depressed the percentage incorporation of glucose by the organism but that once the erythritol had been depleted the incorporation approached that of the control.

Table 6. *The action of erythritol and 2-deoxy-2-fluoro-DL-erythritol on the incorporation of [U-^{14}C]-D-glucose by Brucella abortus*

	% of cell carbon derived from [U-^{14}C]-D-glucose		
Period of growth (hr)	Control	1 μmole/ml. 2-deoxy-2-fluoro-DL-erythritol	1 μmole/ml. erythritol
16	22	16	16
20	26	16	19
23	29	19	19
40	49	35	46*

* The erythritol had been used up at about 30 hr.

The short-term uptake of [U-^{14}C]-erythritol and [U-^{14}C]-D-glucose by dense suspensions of Brucella abortus

One reason why erythritol was used preferentially by *Brucella abortus* in the medium containing glucose might have been that erythritol entered the bacteria more rapidly than did glucose. Hence short-term uptake experiments were done, but, for reasons described in Methods uptake of glucose could not be studied at the high concentration normally present in the medium used in most of this work (39 μmole/ml.). Nevertheless, the curves in Fig. 3 show that, at a glucose concentration (0·5 μmole/ml.) equal to that of the erythritol, the rate of loss of glucose from the medium was only about half that of erythritol.

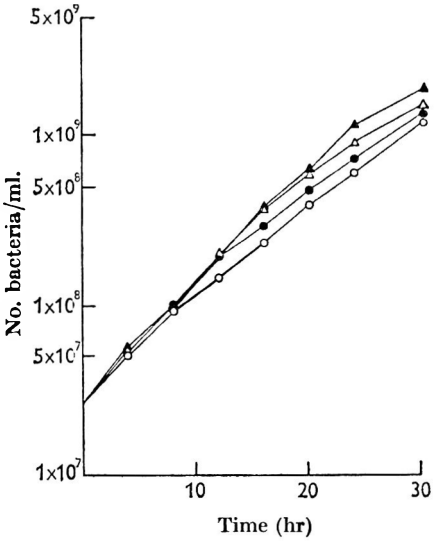

Fig. 1

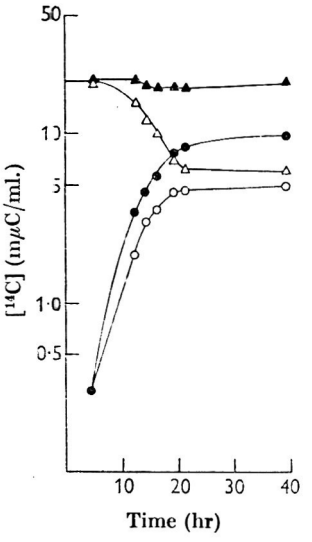

Fig. 2

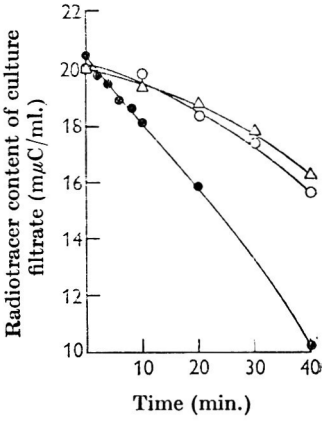

Fig. 3

Fig. 1. Growth of *Brucella abortus* in the presence of glucose (39 μmole/ml.) and initial erythritol concentrations of: 0 (-○-○-), 0·1 (-●-●-), 0·33 (-△-△-), and 1·0 (-▲-▲-) μmole/ml.

Fig. 2. The distribution of radiotracer in bacteria (-○-○-), medium (-△-△-), and carbon dioxide (-●-●-) during growth of *Brucella abortus* in the presence of glucose and [U-^{14}C]-erythritol; total recovery of radiotracer shown thus: -▲-▲-.

Fig. 3. The uptake of [U-^{14}C]-erythritol and [U-^{14}C]-D-glucose by dense (200 μg./ml.) suspensions of *Brucella abortus*. -●-●- uptake of [U-^{14}C]-erythritol (0·5 μmole/ml.; 0·020 μC/ml.) in the presence of a large amount of glucose (39 μmole/ml.); -○-○- uptake of [U-^{14}C]-D-glucose (0·020 μC/ml.) in the same medium containing only a small amount (0·5 μmole/ml.) of glucose; and -△-△- uptake of [U-^{14}C]-D-glucose (0·5 μmole/ml.; 0·020 μC/ml.) in the presence of erythritol (0·5 μmole/ml.).

The curves in Fig. 3 show that there was no initial rapid uptake of erythritol (or glucose), indicating no significant accumulation of free polyol and that the rates of uptake and metabolism were balanced. This was supported by the absence (less than 0·0005% of the dry weight) of free erythritol from hot water extracts of bacteria which had been grown with [U-^{14}C]-erythritol and from the cold trichloroacetic acid extract of the solvent fractionation procedure. Erythritol had no appreciable effect on the total uptake of glucose (see Fig. 3) but since the growth rate was increased

by erythritol the % incorporation of glucose into the organism was decreased (see above).

Attempts to demonstrate metabolism of erythritol by broken-cell suspensions. The three broken-cell preparations described in Methods did not appear to metabolize [U-^{14}C]-erythritol under the experimental conditions.

DISCUSSION

The stimulatory effect described previously of very small quantities of erythritol on the growth of *Brucella abortus* was observed because small populations were used in those preliminary investigations (references in Introduction). Work with larger populations has now shown that, in fact, *B. abortus* utilizes about 1½ times its own weight of erythritol. This utilization occurred during growth in a medium which contained a large excess of amino acids (1180 μg./ml.) and an optimum concentration of glucose (7000 μg./ml.). In most experiments initial glucose: erythritol ratios were approximately 115:1; in experiments with small inocula these ratios were much higher (10,000:1) and rose even further as the erythritol was preferentially consumed. Obviously erythritol is a main carbon and energy source for *B. abortus*, which appears to have a mechanism for the preferential uptake or metabolism of erythritol in the presence of an excess of glucose and other potential metabolites. Sorbitol and fructose are found along with glucose in bovine foetal fluids and it was interesting that the stimulatory effect of erythritol still took place when glucose was replaced by either of these carbohydrates.

The effect of erythritol appears to be highly specific since several of C_3 to C_6 homologues had no stimulatory effect and Smith *et al.* (1964) showed that erythritol annulled the inhibition of the growth of Brucella by analogues of erythritol.

Radiotracer studies confirmed that erythritol was used as a general carbon and energy source. A large proportion of the radiotracer was converted to carbon dioxide and the tracer in the cell substance was fairly evenly distributed over all cell components; erythritol is thus probably broken down to small units during metabolism.

Efforts to show that the preferential usage of erythritol was due to a more rapid entry than glucose into the cell were inconclusive. As yet no cell-free system has been obtained which will metabolize erythritol and the influence of permeability cannot therefore be assessed. Furthermore, comparison of the short-term uptake of erythritol and glucose could not be conducted with the normal very high concentration of glucose; experiments with low glucose concentrations indicated that the rate of uptake of the two carbohydrates was not very different. The absence of detectable amounts of erythritol within the bacteria suggests that the rate of metabolism was not slower than that of permeation, which might well be the rate-determining factor.

A connexion between erythritol metabolism and glucose metabolism was indicated by the inhibitory effect of erythritol and 2-deoxy-2-fluoro-DL-erythritol on glucose incorporation. This might have been an effect on carbohydrate transport, but another possible mechanism is the inhibition of phosphoglucose isomerase or transketolase by erythrose-4-phosphate, a possible product of erythritol metabolism (Grazi, Deflora & Pontremoli, 1960; Dische & Igals, 1961).

No evidence was obtained about the mechanism of erythritol metabolism in *Brucella abortus*. Oxidation to erythrose-4-phosphate would be a possible entry point into the pentose phosphate cycle (Horecker, Smyrniotis, Hiatt & Marks, 1955). On the other hand, the degree of incorporation of radiotracer into ribose and the wide distribution of activity in *B. abortus* suggests that the immediate products of erythritol metabolism enter the tricarboxylic acid cycle by a more direct route. A more selective pattern of incorporation of the radiotracer would probably have occurred if much carbon dioxide from erythritol metabolism had been re-utilized (Newton, Marr & Wilson, 1954; Tepper & Wilson, 1958). Metabolism of erythritol might occur by a reversal of mechanisms known to produce erythritol or its immediate oxidation products, e.g. the aldol condensation of formaldehyde and dihydroxyacetone to an erythrulose-1-phosphate (Charalampous, 1954; Peanasky & Hardy, 1958), the metabolism of hydroxypyruvic acid to L-erythrulose (Dickens & Williamson, 1956), the metabolism of dihydroxymaleic acid to D-erythrose and D-erythrulose (Akabori, Uehara & Muramatsu, 1952), and the dismutation of D-erythrose into D-erythronic acid and erythritol (Uehara, Sugeno & Mizoguchi, 1963).

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Nutritional Control of Cellular Morphology in an Aerobic Actinomycete from the Hamster

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SUMMARY

An aerobic actinomycete isolated from a type of periodontal disease in hamsters showed a characteristic filament/diphtheroid dimorphism. Incubation under carbon dioxide favoured development of the diphtheroid form, whereas aerobic incubation favoured the mycelial form. Iron depletion also induced filament formation. Although magnesium concentration directly affected the rate and amount of growth, no effect on morphology was observed. Critical concentrations of streptomycin sulphate and chloramphenicol also induced mycelium formation.

INTRODUCTION

The aetiological relationship of an aerobic filamentous organism to a type of periodontal disease in hamsters was recently reported (Jordan & Keyes, 1963; Keyes & Jordan, 1964; Jordan & Keyes, 1964). The morphology and physiology of this organism were described in detail (Howell, 1963; Howell & Jordan, 1963) and its inclusion in the family Actinomycetaceae suggested. The tendency of the filaments to be replaced by diphtheroid organisms under certain cultural conditions was noted. Hurst (1950) had previously described this change of morphology in a similar organism from the hamster. Diphtheroid formation by *Actinomyces bovis* was also reported by Pine, Howell & Watson (1960). Preliminary observations led us to believe that certain growth conditions might regulate these changes in morphology. For example, aerobic incubation seemed to favour the filamentous stage of growth. In the present work we have tested further the effects of the atmosphere of incubation as well as the influence of metals and antibiotics on the diphtheroid/filament dimorphism of this actinomycete.

METHODS

The inoculum was derived from strains which had been serially transferred in Trypticase Soy broth (Baltimore Biological Laboratories, Baltimore, Md.) and incubated at 37° in 'sealed' tubes in an atmosphere of CO_2 produced from 10% (w/v) Na_2CO_3 + M-KH_2PO_4 (Pine & Howell, 1956). This was to ensure that the inoculum would be essentially in the diphtheroid stage at the start of all experiments. The only exception to this was in experiments comparing the effect of atmosphere of incubation or to determine the effects of certain metals on morphological

changes. In these cases, the inoculum was grown in Trypticase Soy broth incubated either aerobically or under 10% (w/v) Na_2CO_3 + M-KH_2PO_4 'seals'. Incubation was at 37° in all experiments.

To determine the effect of atmosphere of incubation upon cellular and colony morphology, a direct comparison was made of the growth of 6 strains grown aerobically and under an atmosphere of 5% CO_2 + 95% N_2 (v/v). Streak plates were prepared on brain heart infusion agar (Difco Laboratories, Detroit, Michigan) with one loopful of a 24–48 hr Trypticase Soy broth culture. Identical plates were incubated at 37° in air and under an atmosphere of 5% CO_2 + 95% N_2 (v/v). These were examined for cellular and colonial morphology under ×100 and ×980 magnification after 16–24 hr incubation.

In a separate experiment growth of 3 strains was compared by incubation in air, 5% CO_2 + 95% N_2 (v/v) and also in an atmosphere of CO_2-free nitrogen.

The effects of certain metals on the growth and morphology of two strains of this organism were determined in a Casitone + yeast extract medium (Medium I), incubated with CO_2 'seals' as used in previous studies (Howell & Jordan, 1963).

Each of the metals normally added to the medium (g./l.: $MgSO_4.7H_2O$, 0·2; $CaCl_2.2H_2O$, 0·02; $FeSO_4.7H_2O$, 0·04; $MnSO_4.2H_2O$, 0·0015; $NaMoO_4.2H_2O$, 0·0015) was omitted separately to observe any change in growth or morphology due to metal deprivation. One loopful from a culture grown in Medium I with decreasing concentrations of magnesium (0·200 g./l. down to none added) was plated on the same medium containing 1·5% washed Noble's agar (Difco Laboratories). In similar experiments the various other metals were separately omitted. The plates were incubated at 37° in an atmosphere of 5% CO_2 + 95% N_2 (v/v). It was considered beyond the scope of these experiments to do any further metal purification of the medium itself.

A possible influence of sub-inhibitory concentrations of antibiotics on the dimorphism of this organism was also considered. One strain (T 6) was first exposed to a range of concentrations of sodium penicillin G, bacitracin, chloramphenicol and streptomycin sulphate in Trypticase Soy broth incubated under CO_2 'seals', to establish inhibitory concentrations for each antibiotic. The organism was then exposed to a narrow range of concentrations where growth would occur but where the morphology of the organisms might be influenced. Streak plates of Trypticase Soy agar containing appropriate concentrations of the antibiotic were incubated under 5% CO_2 + 95% N_2 (v/v). These concentrations were as follows: sodium penicillin G, 0·01–0·1 units/ml.; bacitracin, 0·2–1·5 units/ml.; chloramphenicol, 0·2–0·75 μg./ml. (w/v); streptomycin sulphate, 1·0–10·0 μg./ml. (w/v). Micro-colonies on the plates were examined at ×100 and ×980 magnifications at intervals from 16 to 96 hr.

RESULTS

The atmosphere of incubation had a marked effect on cellular and colony morphology. Smooth cultures of 5 of 6 strains (and one of these in repeated experiments) on plates incubated under nitrogen + carbon dioxide continued to develop diphtheroid micro-colonies (Pl. 1, fig. 1) or micro-colonies bearing a short, refractile mycelial fringe with little or no branching, whereas those incubated in air reverted to branching mycelial micro-colonies (Pl. 1, fig. 2) so characteristic of these strains when first isolated from animals. The smooth cultures of three strains incubated

under CO_2-free nitrogen became predominantly filamentous, though occasional diphtheroid-like micro-colonies were found.

In contrast, filamentous cultures of one strain maintained by serial transfer in Trypticase Soy broth and incubated aerobically, continued to produce filamentous spider-like micro-colonies when inoculated on brain heart infusion plates, regardless of the atmosphere of incubation. Plates inoculated from initially filamentous cultures which had been carried by serial transfer in the Trypticase Soy broth with $KH_2PO_4 + Na_2CO_3$ 'seals', however, invariably showed predominantly diphtheroid colonies when the plates were incubated under $CO_2 + N_2$. Thus, it is obvious that increased CO_2 tension was in large part responsible for the cultural dimorphism exhibited by these organisms.

In repeated experiments with strain HS 1 and rough and smooth forms of strain T 6, when less than 50–70 mg. $MgSO_4.7H_2O$ were added per l. Medium I, both the rate and amount of growth were affected. Suboptimal concentrations had no effect upon cellular or colony morphology. Although the omission of iron, calcium, or manganese from Medium I did not influence significantly the rate or amount of growth, omission of iron, in some experiments, resulted in filamentous micro-colonies (Pl. 1, fig. 3) in place of the diptheroid type found in controls (similar to Pl. 1, fig. 1). Omission of calcium or manganese had no effect on growth or morphology. Lack of consistency in these experiments with iron, and failure to find any effect of calcium or manganese may have been due to near adequate or adequate amounts of these elements already present in the medium.

Strain T6 used in the experiments in which antibiotics were incorporated in the medium was markedly sensitive to each of the four antibiotics tested. In addition, at a concentration of chloramphenicol 0·2 μg./ml., only spider-like micro-colonies were observed after incubation for 66 hr whereas control plates, after 16 hr of incubation, showed predominantly diphtheroid colonies, or diphtheroid colonies bearing very short refractile filaments around the margin. Spider colonies were also found in plates containing chloramphenicol 0·3 μg./ml. after incubation for 90 hr. Low concentrations of sodium penicillin G (0·05–0·075 units/ml.) and bacitracin (0·6–1·0 units/ml.), although somewhat inhibitory, did not induce the formation of filamentous micro-colonies, but somewhat atypical heaped dense diphtheroid colonies were found after incubation for 40–90 hr. Filamentous colonies alone were produced on plates containing streptomycin sulphate 5 μg./ml (Pl. 1, fig. 4) after 48 hr incubation, whereas diphtheroid colonies alone were found on control plates. This effect of streptomycin on morphology was not found consistently in subsequent experiments.

DISCUSSION

Variations in the morphology of a variety of micro-organisms under the influence of different metabolic regulators have been described by numerous investigators. Winder & Denneny (1959) reported that the growth of *Mycobacterium smegmatis* in a medium deficient in iron and zinc resulted in elongated filament-like organisms containing a low concentration of deoxyribonucleic acid as compared with normal organisms of the same age. In *Nocardia opaca* a manganese deficiency caused a marked filamentous growth and failure to fragment (Webley, 1960). Filamentous forms of *Escherichia coli* were observed by Ratledge & Winder (1964) when this

organism grew in an iron or zinc deficient medium. The tendency of Gram-positive bacteria to grow as long filaments under conditions of magnesium deficiency was described by Webb (1953). Although magnesium was required for growth of the 'hamster organism' in a medium containing other metals, no effect on morphology was observed. The addition of certain amino acids and other organic acids to a basal medium which supported growth of coccoid forms only of *Arthrobacter crystallapoietus* resulted in the formation of rod forms (Ensign & Wolfe, 1964).

Similar effects may result from exposure to bacterial inhibitors. Elongated forms of *Escherichia coli* and *Bacillus subtilis* induced by penicillin were described by Pratt & Dufrenoy (1948). The same effect was obtained by the use of sulphonamides and bacitracin. Fleming, Voureka, Kramer & Hughes (1950) reported very long filaments and bizarre branching by *Salmonella typhi*, *Vibrio cholerae*, *Pseudomonas pyocyanea*, and *Escherichia coli* in the presence of sub-inhibitory concentrations of penicillin.

Carbon dioxide has been shown to exert a controlling influence on the morphology of *Coccidiodies immitis* (Lones & Peacock, 1960), *Sporotrichum schencki* (Drouhet & Mariat, 1952) and *Histoplasma farciminosum* (Bullen, 1949). Of additional interest in this respect is a report by Chan (1964) of aberrant forms of *Arthrobacter globiformis* induced by a biotin deficiency. Nickerson & Mankowski (1953) reported that *Candida albicans* was maintained in the yeast phase in a simplified basal medium containing glucose and biotin.

Regulation of mould/yeast dimorphism of *Mucor rouxii* by adjustment of the carbon dioxide and oxygen tensions of the growth atmosphere was discussed by Bartnicki-Garcia (1963). The hypothesis was advanced that carbon dioxide fixation proceeds through the well-known malate pathway leading to aspartate formation. This may stimulate the formation and accumulation of mannan-protein in the cell wall of the yeast form. The inhibitory effect of oxygen on development of the yeast form was explained on the basis that under aerobic growth conditions operation 'of the tricarboxylic acid cycle may prevent any excessive accumulation of carboxylic acids resulting from CO_2 fixation'.

Thus it is obvious that the factors which were shown to influence the morphology of our actinomycete have been adequately demonstrated to exert their morphogenic effects on a variety of other microbial forms. Demonstration of a nutritional control of morphology in cultures of an actinomycete raises questions about the relationship of various filamentous and diphtheroid organisms commonly described from the oral cavity, particularly in surveys which are based on visual examination of dental plaque material. Hurst (1950) pointed out the difficulties of a quantitative estimation of actinomycetes from tooth scrapings, based on morphology alone. The confusion surrounding the true identity of oral 'diphtheroids' which have been described in the literature was also mentioned by Morris (1954). Our work illustrates the fact that the morphology of these organisms *in vivo* can be significantly different from their appearance as cultivated *in vitro*. It is possible that the relatively high concentrations of available CO_2 in the oral cavity would favour the diphtheroidal form of the organism.

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EXPLANATION OF PLATE

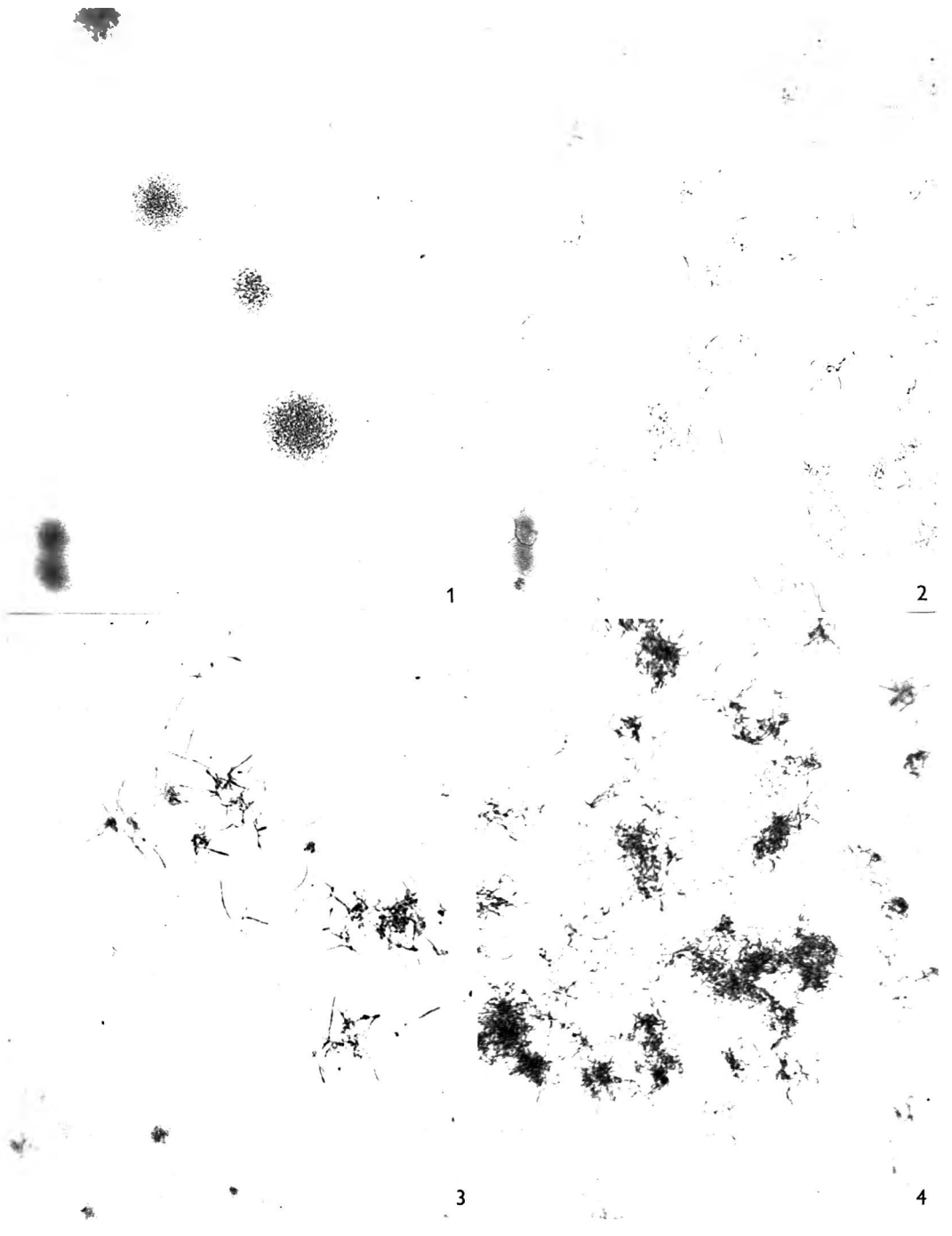
PLATE 1

Fig. 1. Micro-colonies of actinomycete strain T 6, on brain heart infusion agar incubated for 16 hr at 37° in 5% CO_2 + 95% N_2 (v/v). ×440.

Fig. 2. Micro-colonies of actinomycete strain T 6, on brain heart infusion agar incubated for 24 hr at 37° in air. ×440.

Fig. 3. Micro-colonies of actinomycete strain T 6, on medium I, containing 1·5% (w/v) washed Noble's agar without added iron. Incubated for 20 hr at 37° in 5% CO_2 + 95% N_2 (v/v). ×440.

Fig. 4. Micro-colonies of actinomycete strain T 6, on Trypticase Soy agar containing streptomycin sulphate 5 μg./ml.; incubated for 48 hr at 37° under 5% CO_2 + 95% N_2 (v/v). ×440.



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Studies on the Biochemical Basis of the Minimum Temperatures for Growth of Certain Psychrophilic and Mesophilic Micro-organisms

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SUMMARY

Growth, respiratory activity and the ability to accumulate D-glucosamine were examined in three psychrophilic and three related mesophilic micro-organisms at temperatures between 0° and 20°. Each of the psychrophils (strains of Arthrobacter, Candida and *Corynebacterium erythrogenes*) grew well at 0° and also respired exogenous glucose and accumulated glucosamine at this temperature. The minimum temperatures for growth (5–10°) of the mesophilic strains of Arthrobacter and *Candida utilis* were approximately the same as those at which the organisms ceased to respire glucose and accumulate glucosamine. But the mesophil *Corynebacterium xerosis* respired exogenous glucose and accumulated glucosamine at temperatures as low as 10°, which is well below the minimum temperature for growth of this bacterium in freshly inoculated culture (20°). Cultures of *C. xerosis* transferred from 30° to 15° in the mid-exponential phase of growth were capable of a limited amount of growth at the lower temperature, corresponding approximately to a doubling in the size of the population; late-exponential phase cultures when transferred to 15° did not have this ability. Although cultures of *C. xerosis* transferred from 30° to 10° did not grow, the bacteria were able to respire exogenous glucose and accumulate glucosamine even after 48 hr at the lower temperature. The psychrophilic organisms did not differ from their mesophilic counterparts in their sensitivity to 2,4-dinitrophenol, uranyl ions and nystatin, three metabolic inhibitors which affect respiratory activity and sugar uptake in micro-organisms.

INTRODUCTION

Many micro-organisms fail to multiply when incubated at temperatures below 5–10°. These organisms have been described as mesophilic to distinguish them from psychrophilic micro-organisms which grow well at temperatures as low as, and sometimes lower than, 0°. The biochemical factors which determine the minimum temperatures for the growth of micro-organisms have only recently been explored although the problem was stated quite succinctly by Rahn many years ago when he observed that, since growth and respiration are chemical reactions, they should continue though at a greatly decreased rate until the medium in which the micro-organisms are suspended freezes solid (Rahn, 1932). Although this behaviour is found with psychrophilic micro-organisms, it is not characteristic of mesophils.

Ingraham (1958) compared the effect of incubation temperature on the generation times of a psychrophilic and a mesophilic bacterium, and showed that the psychrophil had a lower temperature characteristic (a value which is synonymous with μ

of the Arrhenius equation) as compared with the mesophil. This difference in temperature characteristic prompted Ingraham & Bailey (1959) to examine the effect of heat on the activities of glucose 6-phosphate-, isocitrate-, and malate-dehydrogenases in cell-free extracts of these bacteria, to discover whether the enzymes in the psychrophil had lower activation energies than the corresponding enzymes from the mesophil. It was found, however, that the activation energies were very similar if not identical for the enzymes examined. But, in other experiments, it was shown that the temperature coefficient (i.e. Q_{10} value) of glucose oxidation was less for the psychrophil than for the mesophil, and these findings led Ingraham & Bailey (1959) to suggest that the differences in temperature response between psychrophils and mesophils might be the result of differences in some aspect of the biochemical organization of the organisms rather than in the properties of individual enzymes. The first indication of an aspect of cell organization which might form the basis of the difference in response to near-zero temperatures came from a report by Baxter & Gibbons (1962). These workers compared the respiratory activities of a psychrophilic strain of Candida, a yeast, with a mesophilic strain of *Candida lipolytica* and found that the psychrophil respired endogenous reserves at a greater rate than the mesophil at all temperatures up to 30° and that, although the psychrophil oxidized glucose at an appreciable rate even at 0°, virtually no exogenous substrate was oxidized by the mesophil at temperatures below 5°. Baxter & Gibbons suggested that the main factors determining the minimum temperature for growth of a micro-organism are the temperature characteristics of the mechanisms for transporting solutes into the organisms.

The present paper describes experiments on the effect of temperatures between 0° and 20° on the growth, respiratory activity and glucosamine-accumulating capacity of three psychrophilic and three related mesophilic micro-organisms. These experiments were done to assess the extent to which the temperature characteristics of the solute transport mechanisms determine the minimum temperatures for growth of these organisms.

METHODS

Organisms. The psychrophilic micro-organisms used were Arthrobacter strain No. R 22/3A (provided by Mr S. B. Thomas, National Agricultural Advisory Service, Ministry of Agriculture, Fisheries and Food, Trawscoed, Aberystwyth, Wales), Candida strain No. A 3E-2 (Straka & Stokes, 1960; obtained through the courtesy of Dr R. P. Straka, Western Utilization Research and Development Division, U.S. Department of Agriculture, Albany, California, U.S.A.) and *Corynebacterium erythrogenes* strain NCMB 5. The mesophilic counterparts of these organisms were Arthrobacter strain No. 16 (Veldkamp, Van Den Berg & Zevenhuizen, 1963; supplied by Professor H. Veldkamp, Bacteriology–Serology Laboratory, University of Groningen, Holland), *Candida utilis* strain NCYC 321, and three strains of *Corynebacterium xerosis*, one (strain NCL) from the culture collection in this department, and the other strains NCTC 7243 and 9755.

The strains of Candida were maintained on slopes of malt wort agar (10 %, w/v, spray-dried malt extract, 'Muntona', Munton & Fison, Ltd., Stowmarket, Suffolk, +2 %, w/v, agar) and the bacteria on nutrient agar. Stock cultures of the psychrophils were stored at 0° and the mesophils at room temperature (18°–20°).

Experimental cultures. The glucose + salts + vitamins medium (pH 4·5) of Rose & Nickerson (1956) supplemented with D-biotin (2·0 μg./l.) was used for growing the yeasts. The bacteria were grown in a medium (pH 6·7) of the following composition: glucose, 20·0 g.; Casamino acids, vitamin-free (Difco), 10·0 g.; Na_2HPO_4, 6·0 g.; KH_2PO_4, 3·0 g.; NaCl, 3·0 g.; NH_4Cl, 2·0 g.; $MgSO_4.7H_2O$, 0·1 g., L-cysteine, 0·1 g.; DL-tryptophan, 0·1 g.; nicotinic acid, 4·0 mg.; thiamine, 1·0 mg.; calcium-D-pantothenate, 1·0 mg.; pyridoxin HCl, 1·0 mg.; riboflavin, 0·25 mg.; D-biotin, 2·0 μg.; distilled water, 1 l.

Portions (100 ml.) of yeast medium or of glucose-free bacterial medium (90 ml.) were dispensed into 350 ml. conical flasks plugged with cottonwool. Yeast medium was sterilized by autoclaving momentarily at 110° and bacterial medium at 115° for 15 min. After cooling, each portion of bacterial medium was supplemented with 10 ml. of sterile 20 % (w/v) glucose solution. In certain experiments, cultures (6 ml.) were grown in Samco tubes covered with anodized aluminium caps (Oxo Ltd., Queen Street Place, London, E.C. 4; Northam & Norris, 1951). Yeast inocula were prepared by suspending sufficient organisms from a slope culture into 6 ml. phosphate buffer (M/15 KH_2PO_4; pH 4·5) to give a concentration equivalent to 0·07–0·09 mg. dry wt./ml. With bacterial inocula, the organisms were suspended in 6 ml. 0·85 %(w/v) NaCl to give a concentration equivalent to 0·05–0·07 mg. dry wt./ml. The organisms in suspensions were washed twice with sterile buffer or saline and resuspended in a final 6 ml. portion. Each flask of sterile medium was inoculated with 0·8 ml. of washed suspension and each tube (6 ml.) with 0·05 ml. suspension. Growth of the organisms was measured turbidimetrically in Samco tubes with the Hilger 'Spekker' absorptiometer (Model H 760) and neutral green-grey H 508 filters, and a medium blank. Turbidity readings were related to dry weight of organism by using a calibration curve for each organism. It was shown, with each of the organisms used in this study, that the temperature of incubation did not alter the relationship between the turbidity of the culture and the equivalent dry weight of organisms present or their deoxyribonucleic acid content as determined by the method described by Ahmad, Rose & Garg (1961).

Cell suspensions. After growth had been measured, organisms were separated from culture fluid by centrifugation. Yeast crops were washed three times with phosphate buffer (pH 4·5) and bacteria with phosphate buffer (pH 7·0; Gomori, 1955). During centrifugation and washing, the organisms were maintained at the same temperature as that at which they had been incubated.

Respirometry. Determinations of the rates of respiration of endogenous reserves ($Q_{O_2\,end.}$) and of exogenous glucose ($Q_{O_2\,gluc.}$) at different temperatures were made by the direct Warburg method (Umbreit, Burris & Stauffer, 1957) by using the Braun Warburg apparatus (Model V 85; B. Braun, Melsungen, West Germany) fitted with cooling coils. Each flask contained the equivalent of between 2 and 12 mg. dry-weight washed organism in 2·0 ml. of phosphate buffer (pH 4·5) with the yeasts or phosphate buffer (pH 7·0) with the bacteria. The centre well contained 0·2 ml. 20 % (w/v) KOH and the side arm 0·3 ml. 10 % (w/v) glucose or 0·3 ml. water. Air was the gas phase. The $Q_{O_2\,end.}$ and $Q_{O_2\,gluc.}$ values are expressed as the number of μl. oxygen consumed/mg. dry wt. organism/hr at the temperature stated.

Measurement of glucosamine uptake. Transport of sugar into the micro-organisms

was studied by measuring their capacity to accumulate D-glucosamine. It was possible to use this amino sugar since none of the organisms utilized it as a carbon or nitrogen source for growth. Portions of a suspension containing the equivalent of 40–50 mg. dry wt. organism were centrifuged at the appropriate temperature and the organisms resuspended in 15 ml. of phosphate buffer (pH 4·5 or 7·0) containing 1·2% (w/v) D-glucosamine HCl. Control suspensions were prepared in phosphate buffer lacking glucosamine. In certain experiments, 2,4-dinitrophenol (DNP; mM) was included in the suspending buffer. The portions of buffer were equilibrated at the appropriate temperature before being used. The suspensions were placed in 50 ml. conical flasks and stirred with a magnetic stirrer for 2 hr at a constant temperature. A portion (6 ml.) of each suspension was removed after 2 hr, and these portions were rapidly transferred to ice-cold tubes and centrifuged on a high-speed centrifuge at 0°. The organisms in the pellets were washed three times with ice-cold water, and finally suspended in 3 ml. water and placed in a boiling water bath for 10 min. On cooling, the debris in the boiled suspensions was removed by centrifugation and, after being supplemented by washings from the debris, the supernatant fluid was made up to 5 ml. and assayed for glucosamine by the method of Rondle & Morgan (1955). It is possible that some of the glucosamine transported into the organisms was phosphorylated, but Burger & Hejmova (1961) and Cirillo, Wilkins & Anton (1963) showed that this does not affect the chemical estimation of the sugar in extracts of the micro-organisms. The results are expressed as the number of μg. D-glucosamine accumulated/mg. dry-weight organism/hr at the temperature stated.

RESULTS

Effect of incubation temperature on growth, respiration and glucosamine accumulation

Initially an examination was made of the effect of temperatures of incubation between 0° and 20° on growth of each of the six organisms and on their ability to respire endogenous reserves ($Q_{O_2\ end.}$) and exogenous glucose ($Q_{O_2\ gluc.}$). The results (Fig. 1) showed that with all six organisms the values for $Q_{O_2\ end.}$ decreased as the temperature was lowered, but, at 0°, the value was finite and ranged from 0·5 for *Corynebacterium xerosis* to 4·7 for *C. erythrogenes*. Over the range 10°–20°, temperature coefficient (Q_{10}) values for endogenous respiration were higher for the mesophils (ranging from 2·3 for *Candida utilis* to 3·2 for *C. xerosis*) than for the psychrophils (which ranged from 1·3 to 1·8). The values for $Q_{O_2\ gluc.}$ also decreased as the temperature was lowered. With each of the psychrophilic organisms, there was a detectable respiration of exogenous glucose at 0°, and the $Q_{O_2\ gluc.}$ values at this temperature ranged from 0·7 for Arthrobacter R22/3A to 3·2 for the psychrophilic Candida strain. But, with the three mesophilic organisms, there was negligible respiration of exogenous glucose at temperatures below about 5°. The temperature coefficient for the $Q_{O_2\ gluc.}$ values of the mesophils over the range 10–20° varied between 6·3 for *C. utilis* to 12·6 for Arthrobacter 16 but were much lower (between 1·5 and 2·7) for the psychrophils. These differences in the temperature coefficients of the Q_{O_2} values were therefore in general agreement with the data reported by Ingraham & Bailey (1959). The rate of growth also declined as the temperature of incubation was lowered. All of the psychrophilic organisms grew well at 0° but the minimum temperature for growth of the mesophils was higher. With *C. utilis* and

Arthrobacter 16, the minimum temperature was approximately the same as that at which the $Q_{O_2\ gluc.}$ values approached zero. With *C. xerosis*, however, the minimum temperature for growth (about 20°) was much higher than the temperature at which the $Q_{O_2\ gluc.}$ values for this organism fell to zero.

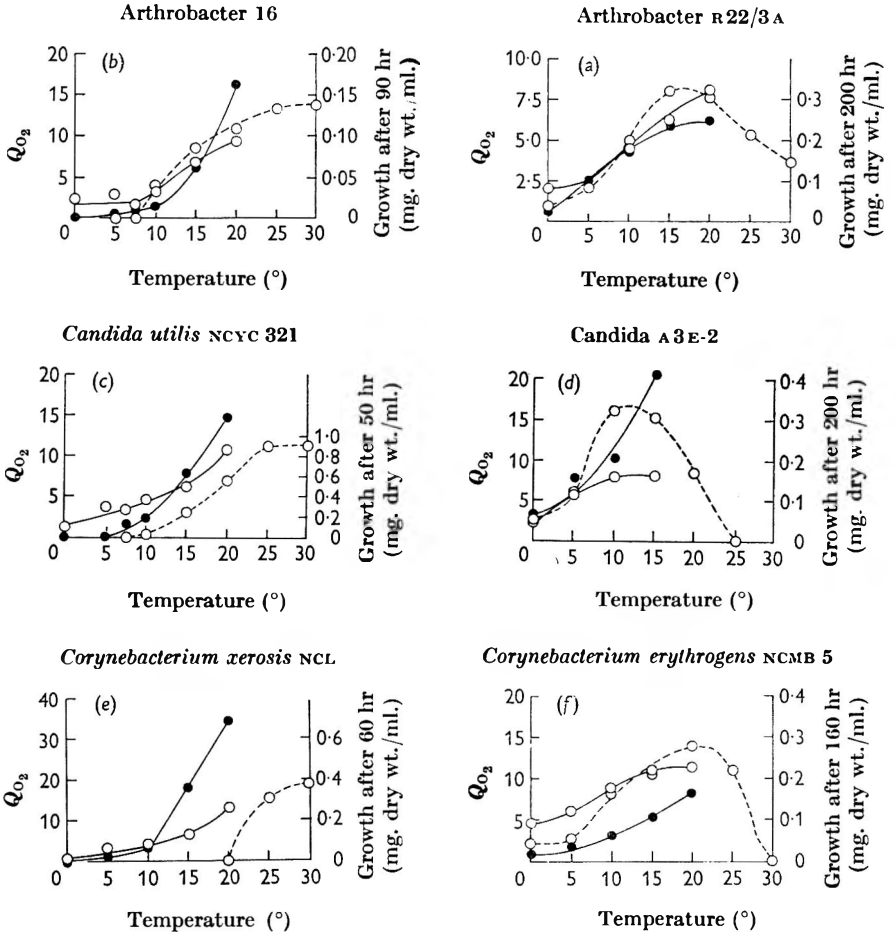

Fig. 1(*a*) to (*f*). Effect of temperature of incubation on growth and on the respiration of endogenous reserves and of exogenous glucose by certain psychrophilic and mesophilic micro-organisms. Growth (○- - -○) at the different temperatures was measured at a time at which cultures incubated at the optimum temperature for growth were in the late-exponential phase. Measurements of respiratory activity were made on organisms grown at the following temperatures which are at or near the optimum for growth; psychrophils: (*b*) Arthrobacter R22/3A, 20°; (*d*) Candida A3E-2, 10°; (*f*) *Corynebacterium erythrogenes*, 20°; mesophils: (*a*) Arthrobacter 16, 30°; (*c*) *Candida utilus*, 25°; (*e*) *Corynebacterium xerosis* strain NCL, 30°. When cultures at these temperatures had reached the mid-exponential phase of growth, organisms were harvested and washed in phosphate buffer (pH 4·5 for the yeasts; pH 7·0 for the bacteria). During washing, the organisms were maintained at the temperature at which they had been incubated. Portions of the washed suspensions, containing known concentations of organisms (mg. dry wt. equivalent/ml.) were placed in Warburg flasks and the Q_{O_2} values (μl. oxygen consumed/mg. dry wt. organism/hr) were determined as described under Methods for the respiration of endogenous reserves (○—○) and of exogenous glucose (●—●).

When an examination was made of the effect of temperatures between 0° and 20° on the capacity of each organism to accumulate glucosamine, it was found that this property was affected in the same way as the ability to respire exogenous glucose. This suggested that the inability of the mesophilic micro-organisms to respire exogenous glucose at temperatures below about 5° was a result of their not being able to transport sugars into the organisms at these temperatures.

Table 1. *Effect of treatment with aqueous butanol on the respiratory activity of Candida utilis at 5°*

The yeast was harvested from 40 hr cultures grown at 25°, washed three times in phosphate buffer (pH 4·5) and suspended in this buffer to equiv. 18 mg. dry wt. yeast/ml. Portions of this suspension were centrifuged at room temperature and the supernatant fluids removed. The yeast pellet was then suspended in a volume of phosphate buffer, or of 2 % or 5 %, v/v, *n*-butanol in phosphate buffer, equal to that of the original portion of suspension. The suspensions were centrifuged immediately at room temperature and the organisms resuspended in an equal volume of buffer. Portions of these suspensions were then placed in Warburg flasks and the respiratory activity of the organisms measured at 5° as described in Methods.

Concentration of *n*-butanol in phosphate buffer (%, v/v)	Respiratory activity	
	$Q_{O_2\ end.}$	$Q_{O_2\ gluc.}$
0·0	3·6	0·0
2·0	3·2	1·1
5·0	1·6	0·7

The correlation between the effect of low temperatures on the ability of the mesophilic strains of Arthrobacter and Candida to grow in freshly inoculated culture and their capacity to respire exogenous glucose and to accumulate glucosamine lent support to the hypothesis of Baxter & Gibbons (1962) that the minimum temperatures for growth of micro-organisms are determined by the temperatures at which the mechanisms for transporting solutes into the organisms are inactivated. Further evidence to support this hypothesis came from experiments in which *Candida utilis* was treated with aqueous butanol before measurements were made on its respiratory activity. Treatment with aqueous butanol (2–5 %, v/v) breaks the osmotic barrier in micro-organisms and permits the free diffusion into organisms of solutes permeation of which normally requires the intervention of specific transport mechanisms (Mitchell & Moyle, 1956; Rose, 1963). When *C. utilis* was washed, rapidly suspended in 3 % or 5 % (v/v) *n*-butanol in phosphate buffer (pH 4·5), centrifuged, resuspended in buffer and added to a Warburg vessel, there was a marked depression in the $Q_{O_2\ end.}$ values. However, when 2 % (v/v) *n*-butanol in phosphate buffer (pH 4·5) was used, the respiration of endogenous reserves by the yeast was much less affected, and the yeast respired glucose at 5°, at which temperature there was no detectable respiration of exogenous glucose in untreated yeast (Table 1).

Effect of inhibitors on sugar transport

Because of the observed differences in the effect of incubation temperature on the activity of the mechanisms for transporting sugars into mesophilic and psychro-

philic micro-organisms, further experiments were made to discover any other differences in the properties of the sugar transport processes in these two groups of micro-organisms. 2,4-Dinitrophenol (DNP), which uncouples respiration and oxidative phosphorylation, has been shown to inhibit 'uphill' transport of many sugars into micro-organisms (Cirillo, 1961). An examination of the effect of DNP on respiration and on glucosamine accumulation by each of the six organisms showed that the $Q_{O_2\ gluc.}$ values and the ability of each organism to accumulate glucosamine were inhibited in the presence of DNP (mM). The extent of the inhibition varied with each organism but was not markedly different with the psychrophils as compared with the mesophils.

Uranyl ion, in the form of uranyl nitrate at mM concentration, inhibits transport of sugars (Cochrane & Tull, 1958) and amino acids (Gale, 1954) into bacteria, yeasts and filamentous fungi. The ion, which is presumed to act by combining with phosphate groups on molecules in the cytoplasmic membrane, does not affect the respiration of endogenous reserves by micro-organisms. The effect of UO_2^{2+} (mM) on the respiration of each of the six organisms was examined at several temperatures above and below the minimum for growth. Respiration of exogenous glucose was completely suppressed by UO_2^{2+} in all organisms but the ion had no detectable effect on the endogenous respiration at any of the temperatures employed.

The polyene antibiotic nystatin acts on yeasts and filamentous fungi and causes changes in the permeability properties of the cytoplasmic membranes (Marini, Arnow & Lampen, 1961). The antibiotic acts by combining with sterols in the membrane (Lampen, Arnow, Borowska & Laskin, 1962); bacteria, which do not contain sterols, are therefore not sensitive to polyene antibiotics. Respiration of the mesophilic and psychrophilic strains of Candida was depressed by nystatin (Squibb 'Mycostatin'; 0·22 mg./ml. in dimethyl sulphoxide) at temperatures above and below the minimum for growth. But the antibiotic was without effect on the respiratory activities of any of the bacteria at temperatures above or below the minimum for growth.

Growth, respiration and glucosamine accumulation by Corynebacterium xerosis at various temperatures

The inability of two of the three mesophilic micro-organisms to respire exogenous glucose and accumulate glucosamine at temperatures below the minimum for growth in freshly inoculated culture, suggested that inactivation of the sugar transport mechanisms occurred rapidly following the transfer of these organisms to temperatures between 10° and 0°. On the other hand, the NCL strain of *Corynebacterium xerosis*, which was unable to grow in freshly inoculated culture at temperatures below about 20°, respired exogenous glucose and accumulated glucosamine at temperatures as low as about 8°. This behaviour was not peculiar to the NCL strain of *C. xerosis* since two other strains of this bacterium (NCTC 7243, 9755) were shown to have minimum temperatures for growth of about 20° in defined medium and to behave similarly to the NCL strain with regard to the effect of temperature on their ability to respire endogenous reserves and exogenous glucose. *Bergey's Manual* (1957) reports that high minimum temperatures for growth are characteristic of *C. xerosis*. It was conceivable that this behaviour of *C. xerosis* was due to a comparatively slow inactivation of the sugar transport mechanisms at

Table 2. *Respiratory activity and glucosamine-accumulating capacity of Corynebacterium xerosis strain* NCL *following transfer from* 30° *to* 10°

Cultures of the *C. xerosis* were grown at 30° and after 40 hr, when they had reached the mid-exponential phase of growth, certain of the cultures were transferred to 10°. There was no detectable growth of the cultures at 10°. At the time of transfer and after a further 24 and 48 hr, cultures were removed from 30° and 10° and the bacteria harvested by centrifugation, during which they were maintained at the temperature at which they had been incubated. The bacteria were then washed with phosphate buffer (pH 7·0) and portions of the washed suspension containing the equivalent of a known dry weight of bacteria were placed in Warburg flasks and the respiratory activity of the bacteria determined at 30° or at 10° as described in Methods. The organisms in the remaining suspension were used to measure the glucosamine-accumulating capacity of the bacteria as described in Methods.

Duration of incubation following transfer of cultures from 30° to 10° (hr)	Temperature of incubation (°)	Respiratory activity		μg. glucosamine accumulated/ mg. dry wt. bacterium/hr
		$Q_{O_2\ end.}$	$Q_{O_2\ gluc.}$	
0	10	3·0	1·9	3·7
	30	6·4	35·6	9·8
24	10	3·0	3·7	3·9
	30	2·0	6·1	4·9
48	10	2·3	2·2	3·2
	30	1·6	5·7	4·2

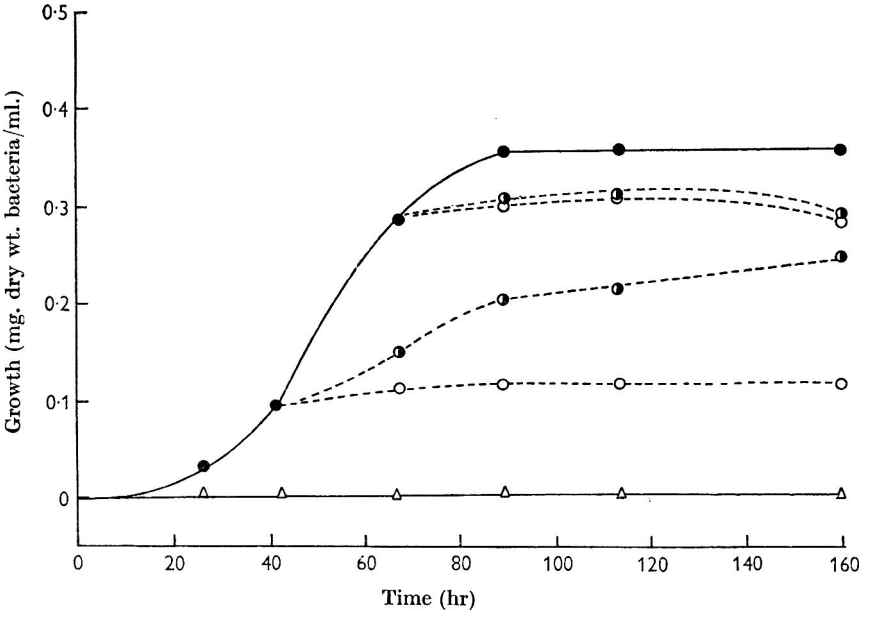

Fig. 2. Effect of change in incubation temperature from 30° to 10° or 15° on the growth of *Corynebacterium xerosis* strain NCL. Cultures (6 ml.) of the bacterium were grown at 30° in Samco tubes (●—●). After 41 hr and 67 hr, cultures were transferred to 10° (○- - -○) or 15° (◑- - -◑). The graph also shows (△—△) growth of cultures incubated at 10° or 15° immediately after inoculation.

temperatures below the minimum for growth; experiments were therefore made to test this hypothesis. Cultures of the NCL strain of *C. xerosis* were grown at 30° and, when the cultures reached the mid-exponential phase of growth (after 40 hr incubation) they were transferred to 10°. Bacteria in cultures transferred to this temperature did not multiply but continued to respire exogenous glucose and to accumulate glucosamine even after 48 hr at 10° (Table 2). Although cultures of *C. xerosis* NCL transferred from 30° to 10° did not multiply at the lower temperature, mid-exponential phase cultures of this bacterium transferred from 30° to 15° were capable of a limited amount of growth which corresponded approximately to a doubling in the size of the population (Fig. 2). Bacteria transferred in the late-exponential phase of growth did not have this ability to grow at 15°. The inability, or limited ability, of the bacterium to grow after transfer to a lower temperature was probably not caused by an increased nutritional demand by the bacteria at 10° or 15° since the same behaviour was observed in cultures grown in nutrient broth.

DISCUSSION

The minimum temperatures for growth of the mesophilic strains of Arthrobacter and *Candida utilis* examined in this study are probably determined, in part at least, by the inactivation of the mechanisms for transporting sugars into the organisms at temperatures between about 5° and 10°. All of the psychrophils, on the other hand, grew well at 0° and continued to transport sugars into the organisms at this temperature. Clearly the sugar transport mechanisms in these mesophils and psychrophils differ in their sensitivity to near-zero temperatures, but the results of experiments on the effect of metabolic inhibitors did not reveal any other differences between the transport mechanisms in the two groups of organisms. The similarity in the effect of 2,4-dinitrophenol on the sugar-accumulating capacity of the psychrophils and mesophils showed the need for metabolic energy for this process in both groups of organisms, while the sensitivity to uranyl ion indicated that the carrier molecules involved in the transport of sugars into both groups of organisms probably need to have phosphate groups free in order to function. The sensitivity of the mesophilic and psychrophilic Candida strains to the polyene antibiotic nystatin showed that sterols are present in the cytoplasmic membranes in both of the strains examined; by the same token, none of the psychrophilic or mesophilic bacteria would appear to contain sterols. The only previous report of a comparison of the properties of the sugar transport mechanisms in mesophilic and psychrophilic micro-organisms is by Cirillo *et al.* (1963), who showed that, in mesophilic and psychrophilic strains of Candida, these had the properties of a carrier-mediated transport.

In view of the lack of knowledge about the chemical nature of the solute carriers in cytoplasmic membranes, it is difficult to suggest a biochemical basis for the difference in the response to low temperatures shown by the sugar transport processes in these mesophilic and psychrophilic organisms. Kates and his colleagues (Kates & Baxter, 1962; Kates & Hagen, 1964) reported that the triglycerides and phospholipids from certain psychrophilic micro-organisms differed in composition from those extracted from related mesophilic organisms particularly with regard to the degree of saturation in the fatty acids and suggested that these differences in

composition may be in components of the solute transport mechanisms (Baxter & Gibbons, 1962). However, there is little evidence for the participation of triglycerides or phospholipids in transport processes in micro-organisms, and it is more likely that the carrier molecules are composed mainly, if not entirely, of proteins (Cohen & Monod, 1957) which undergo conformational changes while effecting the transport of solutes. It is conceivable, therefore, that the protein carriers in mesophils become hyperfolded at low temperatures and are then unable to combine with the solute. Very little is known about the hyperfolding of proteins at low temperatures (Linderstrøm-Lang & Schellman, 1959) although the phenomenon has been invoked to explain the inactivation of certain enzymes at temperatures just above and below 0° (Kavanau, 1950; Maier, Tappel & Volman, 1955).

It is clear however that, in the strains of *Corynebacterium xerosis* examined here, the minimum temperature for growth is not determined by the temperature at which the sugar transport processes in these organisms are inactivated, since these bacteria accumulated sugars at temperatures well below the minimum for growth. At present, it is only possible to speculate regarding the factors that determine the minimum temperatures for growth of these bacteria. One possible explanation is that, at temperatures below about 20°, they are unable to synthesize one or more compounds (e.g. nucleotides) that are essential for growth, but that, when transferred down from 30° to 15° in the mid-exponential phase, they are capable of a limited amount of growth at the expense of the stored reserves of these compounds. But the bacteria are unable to grow on being transferred down to 10°, and it is possible that, at this temperature, they are not able to use these stored reserves. Clearly, the biochemical basis of the minimum temperature for growth of *C. xerosis* is complex and probably involves the operation of a number of different factors. It is conceivable that one of the factors involved is a repression of enzyme synthesis which Ingraham and his colleagues (Ng, Ingraham & Marr, 1962; Marr, Ingraham & Squires, 1964) have demonstrated at low temperatures in *Escherichia coli*. It would also be interesting to discover to what extent the type of biochemical behaviour observed in *C. xerosis* at low temperatures is found in other micro-organisms.

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The Anti-viral Action of Rutilantin A

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SUMMARY

Rutilantin A has been tested for antiviral activity *in vitro*. This antibiotic had no demonstrable effect on the growth of poliomyelitis or influenza viruses, but it completely inhibited the multiplication of rabbitpox virus. It was effective against rabbitpox virus when added to tissue-cell cultures as late as 4 hr after infection. The lowest concentration of rutilantin that prevented the formation of infective rabbitpox virus did not apparently affect the synthesis of viral DNA or soluble antigens. It is concluded that rutilantin inhibits a late stage of virus development. The toxicity of rutilantin for both cultured tissue cells and animals makes it unlikely to be of value in the treatment of virus disease.

INTRODUCTION

Asheshov, Strelitz, Hall & Flon (1954) made a survey of about 1000 strains of actinomycetes in a search for antibiotics with anti-viral activity. Bacteriophages were used as the test organisms, and some 140 strains of actinomycetes were found which produce substances inhibitory of phage replication. From one of these, strain A 220, rutilantin A was isolated and purified by the Medical Research Council Antibiotics Station at Clevedon. The chemical structure of rutilantin, which is related to the anthraquinones, has been intensively studied by Ollis and his co-workers (Ollis & Sutherland, 1961). While the anti-phage activity of rutilantin was clear-cut (Asheshov & Gordon, 1961), its activity against animal viruses was more doubtful. Tests on unpurified material were made through the courtesy of Dr J. Spizizen (Merck, Sharp and Dohme, Inc.) and of Dr P. K. Smith (George Washington School of Medicine, Washington, D.C., U.S.A), but the results were inconclusive and varied from batch to batch. Accordingly, the present work was undertaken with pure crystalline rutilantin A kindly supplied by Dr W. D. Ollis of Bristol University.

METHODS

Rutilantin. Crystalline rutilantin A was dissolved in 0·01 N-HCl at a concentration of 5 mg./ml. and the solution then adjusted to pH 5·4. This stock solution was stored at −80°, and was diluted appropriately in medium as required. Alternatively, a solution of 1 mg./ml. was made in acetone immediately before use, and then diluted in medium.

Tissue culture cells. RK 13. A cell line derived from rabbit kidney was kindly supplied by Mr G. Christofinis (Glaxo Laboratories Ltd.). The cells were cultured in medium '199' containing 10% (v/v) calf serum. *HeLa* (*ERK*). This cell line was

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maintained in Porton calf serum medium as previously described (Appleyard & Westwood, 1964). *CE/P.* Primary chick cultures were prepared by trypsinization of 10-day embryos followed by growth of the cells in Porton calf serum medium. All three types of tissue culture were normally grown as monolayers in 8 cm. Carrel flasks. For the few experiments that entailed microscopic examination of RK 13 cultures for viral DNA, the cells were inoculated onto coverslips in the bottom of Petri dishes.

Viruses. Influenza virus. The WS strain of virus was used. It was propagated in the allantoic cavity of fertile hen eggs. *Poliovirus.* This was the Brunhilde strain of type 1 virus. It was maintained by passage in HeLa (ERK) cells. *Rabbitpox virus.* The Utrecht strain of virus was grown in HeLa (ERK) cells. Virus was liberated from the infected cells by ultrasonic treatment, to produce a stock suspension with a titre of 10^8 plaque forming units (pfu)/ml.

Inhibition of rabbitpox virus

Virus growth. For reasons to be discussed under 'Results', the effect of rutilantin on the growth of rabbitpox virus was studied in RK 13 cells. These were infected at 4° by the inoculation of 4 ml. virus suspension of titre 10^8 pfu/ml. onto confluent Carrel flask cultures containing about 6×10^6 cells. After an adsorption period of 1 hr the excess virus was removed by two washes with phosphate buffered saline, fresh medium was added, and cultures incubated at 36°. Since rabbitpox virus adsorbs to but does not penetrate cells at 4° (Appleyard & Westwood, 1964), the amount of infecting virus could be determined directly by titration of sample cultures at the end of the adsorption period; it was usually 2 to 3 pfu/cell. All virus growth periods were measured from the time at which the cultures were warmed to 36°.

Virus titration. For the assessment of virus yield from individual cultures, cells were scraped from the glass and then disintegrated by ultrasonic treatment with a 500 W. 'Soniclean' generator at 40 kcyc./sec. (Dawe Instruments, Ltd., London). Virus was titrated as pfu in HeLa (ERK) monolayers by the method of Appleyard & Westwood (1964).

Detection of viral antigens. Cells from infected cultures were concentrated to 4×10^7/ml. by centrifugation, and then disrupted by ultrasonic treatment. The resulting extracts were examined for soluble viral antigens by micro-immunodiffusion (Crowle, 1958) against hyperimmune rabbitpox serum.

Detection of viral DNA. Coverslip cultures of RK 13 cells were infected by the adsorption of 0·2 ml. rabbitpox virus suspension of titre 10^8 pfu/ml. for 1 hr at 4°. They were then washed, and incubated in medium at 36°. Sample cultures were withdrawn at intervals, washed, fixed in absolute ethanol and stained with a solution of acridine orange in methanol (Randles, 1960). The coverslips were mounted in liquid paraffin, and examined under dark-ground ultraviolet illumination for the presence of green cytoplasmic inclusions.

RESULTS

Effect of rutilantin on tissue cells

Toxicity. The toxicity of rutilantin was tested on RK 13, HeLa (ERK) and CE/P cell cultures in order to select the most suitable system for further experiments. A range of concentrations of rutilantin, from 0·1 to 100 μg./ml., was added to actively growing cultures of each cell type, and incubation was continued for 24 hr. At a concentration of 100 μg./ml., crystalline rutilantin was deposited both intra- and extracellularly; in all three cell types gross abnormalities appeared, and the cells became rounded and stripped from the glass. The toxic effect was decreased with decreasing concentrations of rutilantin, but in HeLa (ERK) and CE/P cultures it was still evident at 0·1 μg./ml. Cultures of RK 13 on the other hand were less susceptible. Those treated with concentrations of 0·1 and 1 μg./ml. appeared morphologically normal, although cell division either ceased or was considerably reduced. From these results it was decided to use where possible the RK 13 cell line as the test tissue culture system, with concentrations of rutilantin not exceeding 1 μg./ml.

Irreversibility of toxic effect. Rutilantin was clearly a highly cytotoxic material. From the point of view of its possible value as a chemotherapeutic agent, it was desirable to know whether the toxic effect could be annulled by removal of the drug from the cellular environment. The concentration of rutilantin chosen for this test was 0·2 μg./ml. since this was found, as discussed below, to be the minimum concentration that inhibited the growth of rabbitpox virus.

Table 1. *Failure of RK 13 cells to recover from the toxic effect of rutilantin*

	Number of living cells* per culture ($\times 10^{-6}$)		
Incubation period (hr)	Control cultures	Test cultures (rutilantin 0·2 μg./ml.)	Test cultures after further 48 hr without rutilantin
0	6·5	6·5	—
24	7·5	3·9	3·3
72	14·5	2·9	2·0

* Cells unstained by 1 % trypan blue.

Actively growing cultures of RK 13 cells were incubated in medium with or without rutilantin 0·2 μg./ml. for 24 hr or 72 hr at 36°. After this exposure period the rutilantin-containing medium was replaced by normal medium, and incubation was continued for a further 48 hr 'recovery' period. Cell counts were made on control and test cultures at 0, 24 and 72 hr and again on the test cultures after the recovery period. The results are shown in Table 1. It can be seen that cell replication stopped on addition of rutilantin to the cultures, and growth was not re-established even when the drug was removed after only 24 hr.

Effect of rutilantin on the growth of influenza virus

The possible inhibitory effect of rutilantin on the WS strain of influenza virus was tested in the allantoic cavity of 10-day chick embryos. In several experiments,

infectivity titrations were carried out simultaneously in groups of untreated eggs and in eggs inoculated with up to 10 μg. rutilantin. No evidence of protection was obtained. In one experiment, three groups of ten eggs were inoculated with 1, 5 or 10 μg. rutilantin/egg and then challenged with approximately 200 EID50 of virus; five eggs, treated with saline only, served as controls. All eggs, except for five that died, were found to be infected when tested after 48 hr incubation. Haemagglutinin titrations on the allantoic fluids from individual eggs showed further that the virus yield was not significantly reduced by treatment with rutilantin.

Effect of rutilantin on the growth of poliovirus

Because of the toxicity of rutilantin for HeLa (ERK) cells, the only available cell line that was susceptible to infection with poliovirus, satisfactory tests on the inhibition of poliovirus were difficult to perform. However, a single experiment was set up to indicate whether further work might usefully be done. Six Petri dishes were seeded with a suspension of HeLa (ERK) cells in 1% agar. Three of the cultures were infected by the inclusion of about 500 p.f.u. poliovirus in the suspension, according to the plaque technique of Cooper (1955). Rutilantin at concentrations of 10 or 500 μg./ml. was added to 9 mm. diameter wells cut in the centre of each of two cultures, one infected and the other uninfected. The cultures were incubated for 3 days at 36°, and then stained with a tetrazolium derivative to differentiate living from dead cells. In the uninfected cultures, the toxicity of rutilantin was shown by an inner zone of complete clearing around the central well and a wider outer zone of partial clearing which extended to a diameter of 25 mm. in the plate with the higher dose of rutilantin. In the infected cultures, poliovirus plaques of apparently normal size and number could be seen extending right up to the inner zone of complete clearing of the cells. The results of this experiment seemed to indicate that rutilantin was more toxic to the cells than to the virus, and therefore no further tests were carried out.

Effect of rutilantin on the growth of rabbitpox virus

In contrast to its apparent lack of action against the other two viruses, rutilantin was found to have a definite inhibitory effect on the growth of rabbitpox virus. Experiments were therefore made to define its effectiveness and indicate its mode of action.

Dose-response curve. The effect of rutilantin on rabbitpox virus replication was tested over a range of concentrations from 0·008 to 1·0 μg./ml. Medium containing the inhibitor was added to RK 13 cultures after infection with virus at 4°, and the virus yield was determined after incubation for 22 hr at 36°. The results of three experiments are shown together in Fig. 1. At a concentration of 0·4 μg./ml., rutilantin completely suppressed virus multiplication, so that less than 1 p.f.u./cell could be recovered. In presence of rutilantin 0·2 μg./ml. virus growth was either greatly decreased or completely prevented.

Irreversibility of inhibitory action. It had been found that the toxic effect of rutilantin on RK 13 cells was not annulled by withdrawal of the drug from the culture medium. An experiment was therefore set up to determine whether the inhibition of virus replication was similarly irreversible. Two test cultures were

incubated for 3 hr in medium containing rutilantin 1 μg./ml. After a further 3 hr in normal medium, the cultures were infected with rabbitpox virus at 4°. One culture was harvested immediately and the other incubated for 22 hr at 36° to allow virus growth. Three control cultures, which had not been exposed to rutilantin, were infected in parallel with the test cultures. One was harvested immediately after virus adsorption, one after incubation for 22 hr in normal medium, and, to check the inhibitory action of the rutilantin, one was incubated for 22 hr in medium containing rutilantin 1 μg./ml. The results (Table 2) showed that pretreatment of cells with rutilantin was completely effective in suppressing virus growth. It therefore seems that the inhibition of virus replication was brought about by some alteration in the cells which, like the toxic effect of rutilantin, was not annulled by removal of the drug. The fact that the same amount of virus was adsorbed by control and test cultures indicates that pretreatment of cells with rutilantin did not interfere with virus adsorption.

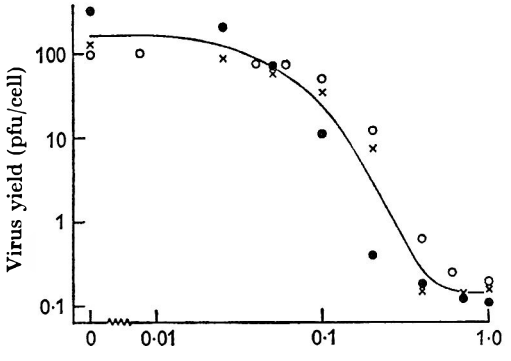

Fig. 1

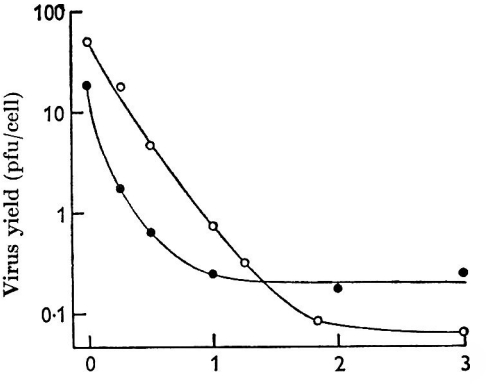

Fig. 2

Fig. 1. Effect of rutilantin concentration on the yield of rabbitpox virus from RK 13 cultures. The symbols ○, ● and × represent the results of three separate experiments.

Fig. 2. Effect of duration of pretreatment with rutilantin on the yield of rabbitpox virus from RK 13 cultures. Rutilantin concentration: ○, 0·75 μg./ml.; ●, 1·0 μg./ml.

Table 2. *Inhibition of rabbitpox virus growth by pretreatment of RK 13 cultures with rutilantin*

Culture	Time of test: 0 hr	Time of test: 22 hr
	Virus titre (p.f.u. per cell)	
Control	2·5	247
Rutilantin 1 μg./ml. during virus growth	2·5	0·11
Rutilantin 1 μg./ml. before virus growth	2·4	0·24

Two further experiments were made to determine the duration of exposure to rutilantin that was necessary to prevent virus growth. Cultures were treated with rutilantin, either 0·75 or 1·0 μg./ml., for various lengths of time, and 2 hr after removal of the drug they were infected with rabbitpox virus. The virus yields after

growth for 18 hr are shown in Fig. 2. Almost complete inhibition was caused by exposure to rutilantin 1 μg./ml. for $\frac{1}{2}$ hr or 0·75 μg./ml. for 1 hr. It is not known whether these times represent mainly the time taken for the drug to penetrate the cells or that required for it to exert its action when it is already intracellular.

Mode of action of rutilantin

Direct action on virus infectivity. Samples of a rabbitpox virus suspension of titre $1{\cdot}5 \times 10^6$ p.f.u./ml. were incubated for 1 hr at 36° with various concentrations of rutilantin up to 100 μg./ml. Each sample was then diluted by a factor of 10^4 and inoculated on to Petri dish cultures of HeLa (ERK) cells for the titration of surviving virus. There was no significant difference between the titres of the treated virus and that of control virus incubated without rutilantin. Rutilantin therefore exerted no direct viricidal effect even at concentrations far in excess of those required to suppress virus replication.

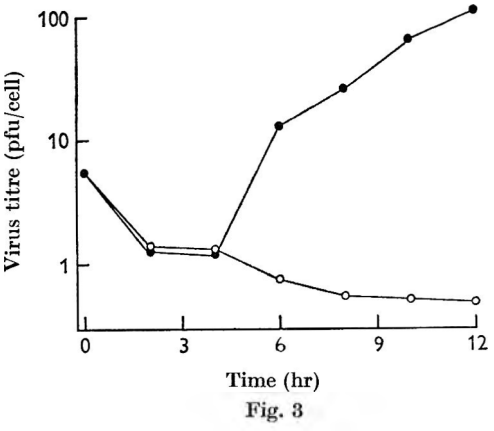

Fig. 3

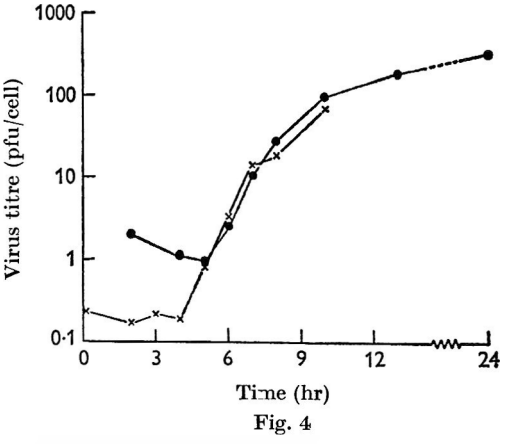

Fig. 4

Fig. 3. One-step growth curves of rabbitpox virus in RK 13 cells. ●, normal growth; ○, growth in the presence of rutilantin 0·2 μg./ml.

Fig. 4. Effect of time of addition of rutilantin on the yield of rabbitpox virus from RK 13 cultures. ●, normal growth curve; ×, virus yield at 24 hr after addition of rutilantin 0·4 μg./ml. at times shown.

Action on virus penetration and eclipse. One-step virus growth curves were made in presence or absence of rutilantin 0·2 μg./ml. (Fig. 3). The results suggest that virus penetration and eclipse took place normally in the presence of rutilantin, but that no new infective virus was formed.

Action on intracellular replication. To determine the sensitive stage of virus growth, rutilantin 0·4 μg./ml. was added to cultures at various times after infection, and the final yields of virus from the cultures were titrated after incubation for 24 hr. In addition, other infected cultures were harvested at intervals and titrated to show the normal virus growth curve (Fig. 4). Rutilantin was completely effective in preventing the formation of new virus when added as late as 4 hr. after infection of the cultures, and only a small amount of virus was produced when it was added at 5 hr. Rutilantin therefore exerts its inhibitory effect very late in the virus growth cycle.

Action on synthesis of viral protein. The development of soluble viral antigens

during the course of infection was taken as an indication of the synthesis of viral protein. Infected cell cultures were incubated for 24 hr in normal medium or in medium with concentrations of rutilantin from 0·1 to 1·0 μg./ml. The yields of virus from the cultures were those shown by the solid circles in Fig. 1; the titre was decreased to less than 10% of the control value by rutilantin 0·1 μg./ml. and to less than 1% by 0·2 μg./ml. In gel diffusion tests against rabbitpox antiserum, extracts of cells infected in the presence of rutilantin 0·1 or 0·2 μg./ml. gave a pattern of precipitin lines indistinguishable from that produced by the control, though higher concentrations of rutilantin did prevent or decrease the formation of many antigens. The results suggested that, while minimum inhibitory concentrations of rutilantin did not suppress soluble antigen synthesis, this was progressively affected as the concentration of drug was increased. It seemed also that the inhibition was selective for certain antigens.

Action on synthesis of viral DNA. Cell cultures on coverslips were infected with virus of titre 10^8 p.f.u./ml. and then incubated in normal medium or medium containing rutilantin 0·2, 0·4 or 0·8 μg./ml. All three concentrations of drug caused complete inhibition of virus multiplication as judged by the yield of infective virus at 24 hr. Sample coverslips were withdrawn at intervals, stained with acridine orange, and examined under ultraviolet illumination for green-fluorescing inclusions of DNA. Almost all cells of the control cultures developed multiple small DNA foci; these increased in size, and by 24 hr had diffused through most of the cytoplasm. In the presence of rutilantin 0·2 μg./ml., the development of foci was indistinguishable from that in normal medium. Higher concentrations of rutilantin did decrease DNA synthesis, though even in the presence of 0·8 μg./ml. some inclusions were seen in many of the cells. It is therefore unlikely that rutilantin inhibits the growth of rabbitpox virus by preventing the synthesis of viral DNA.

Table 3. *The toxicity of rutilantin for mice*

No. of mice	Dose of rutilantin (mg.)		Days after inoculation			
			1	2	3	7
	Per mouse	Per kg.	Total deaths			
10	0·2	10	0	5	5	7
10	0·4	20	0	7	8	9
10	0·8	40	6	10	.	.
10	1·2	60	10	.	.	.

In vivo toxicity of rutilantin

To determine whether the toxicity of rutilantin for cells in culture was reflected by a similar toxicity *in vivo*, four groups of ten mice (20 g. body weight) were inoculated intraperitoneally with doses of rutilantin ranging from 0·2 to 1·2 mg. The mice were observed for 7 days, and moribund animals were killed and autopsied. The results of this experiment are presented in Table 3. Histological examination of autopsy material showed that the main damage occurred in liver and kidney. The liver showed acute toxic hepatitis, marked by dilation and congestion of the hepatic sinusoids. There was diffuse parenchymatous degeneration with fatty changes and

a variable degree of necrosis. The kidneys showed acute toxic nephritis with widespread fatty changes, and frank tubular degeneration, with necrosis, was present mainly in the proximal convoluted tubules. The glomerular capillaries were dilated and congested, and there were focal haemorrhages mainly in the papillary zone.

In a second experiment, ten albino rabbits (2 kg. body weight) were each inoculated intraperitoneally with 20 mg. rutilantin (10 mg./kg.). Seven of the animals developed severe diarrhoea, and died within 48 hr. At autopsy these animals showed acute toxic lesions very similar to those seen in mice, the liver and kidney being severely damaged. The remaining three rabbits survived without evident ill-effects. They were killed after 7 days, and all organs appeared healthy. Rutilantin was therefore extremely toxic to mice and rabbits at doses only about 20 times greater than those needed to inhibit virus growth under the ideal conditions of tissue culture.

DISCUSSION

Rutilantin A was selected as a possible antiviral agent because of its proved activity against bacteriophage. The work reported here was undertaken initially to resolve conflicting reports about its inhibitory action against various animal viruses. In view of the extreme and apparently irreversible toxicity of the compound, the investigation was not extended to include therapeutic trials in animals.

Although the therapeutic use of rutilantin is not practicable, the substance possesses considerable interest as an inhibitor of rabbit pox virus replication. In minimal inhibitory concentrations, rutilantin apparently does not affect the formation of viral DNA nor that of soluble viral antigens. This indicates that the inhibition is directed against a late stage of the replication cycle, a suggestion which is confirmed by the fact that the drug was completely effective even when added late in the eclipse phase. The site of action of rutilantin is probably different from that of other known inhibitors of rabbit pox virus growth, none of which has been shown to act so late in the growth cycle. At higher concentrations, rutilantin also affected other processes occurring during virus development; the formation of many soluble antigens was prevented, and there was considerable inhibition of DNA synthesis.

The biochemical mode of action of rutilantin is unknown. The toxicity of the compound suggests that its effect on virus replication may be merely a reflexion of the irreversible damage that it causes to cell metabolism. This idea is supported by the observation that the capacity of cells to support virus growth was abolished by temporary exposure to rutilantin before infection. On the other hand, the relatively great sensitivity of the stage of growth occurring shortly before virus maturation suggests that minimal concentrations of the drug may have a more specifically antiviral action. In view of the advanced state of knowledge concerning the structure of rutilantin (Ollis & Sutherland, 1961), this possibility invites further investigation. If the antibiotic could be detoxified without destroying its antiviral action, not only would its value in the laboratory and as a possible chemotherapeutic agent be enormously enhanced, but valuable information might be obtained about its mode of action relative to chemical structure.

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Cholesterol and Cholesterol Esters in Mycoplasma

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SUMMARY

Free and esterified cholesterol were determined in the lipid extract of nine parasitic and saprophytic Mycoplasma strains. The saprophytic strains were usually free from esterified cholesterol. Esterified cholesterol was found in the parasitic strains at a ratio of 1:8 to 1:3 to free cholesterol; in the growth medium the ratio was about 4:1. [4-^{14}C]-cholesterol added to the growth medium was incorporated by all Mycoplasma strains without esterification or any other chemical change. [4-^{14}C]-cholesteryl oleate was incorporated into the lipid of the parasitic strains and was not hydrolysed to yield free cholesterol. The small amounts of radioactive oleic acid incorporated by several Mycoplasma strains during growth was not detected in the cholesteryl ester fraction. Our results show that under the growth conditions examined, the Mycoplasma organisms tested were unable to esterify cholesterol or hydrolyse cholesteryl esters.

INTRODUCTION

Most Mycoplasma strains have been found to require cholesterol or closely related sterols for growth (Edward & Fitzgerald, 1951; Smith & Lynn, 1958; Rodwell & Abbot, 1961; Rodwell, 1963; Smith, 1964). Only the saprophytic *Mycoplasma laidlawii* strains do not have this requirement (Edward & Freundt, 1956; Razin & Knight, 1960; Rothblat & Smith, 1961; Razin & Cohen, 1963). So far no other group of bacteria is known to require sterols for growth (Razin, 1962). Cholesterol in the growth medium was found to be incorporated in appreciable quantities into the lipid fraction of the Mycoplasma cell (Smith & Rothblat, 1960; Rothblat & Smith, 1961; Smith, 1962; Smith, 1963*a* Rodwell, 1963; Tourtellotte, Jensen, Gander & Morowitz, 1963). Most of the incorporated cholesterol was found in the cell membrane (Rothblat & Smith, 1961; Razin, Argaman & Avigan, 1963). When grown in a cholesterol-deficient medium *Mycoplasma mycoides* showed severe morphological changes, apparently due to faulty synthesis of cell membrane (Rodwell & Abbot, 1961). Thus, it is plausible that cholesterol is essential for the integrity and regular functioning of the delicate cell membrane of Mycoplasma (Razin, 1963*a*). Smith (1963*a*) suggested that cholesterol fulfils a bifunctional role: maintenance of the structural integrity of the cell membrane, and as a carrier of substrates and metabolic end-products across the cell membrane. To elucidate the role played by cholesterol in the physiology of Mycoplasma, it seemed worth while to investigate the fate of cholesterol incorporated by various Mycoplasma strains. The problems dealt with were: (*a*) is the incorporated

cholesterol esterified or metabolized in any way? (*b*) are Mycoplasma organisms able to incorporate cholesteryl esters from the growth medium? (*c*) are Mycoplasma organisms able to hydrolyse cholesteryl esters to yield free cholesterol?

METHODS

Organisms. *Mycoplasma mycoides* var. *mycoides* (PG 1), *M. mycoides* var. *capri* (PG 3), *M. agalactiae* (PG 2), *M. neurolyticum* (PG 28), *M. laidlawii* strain A (PG 8) and *M. laidlawii* strain B (PG 9) were obtained from Dr D. G. ff. Edward (The Wellcome Research Laboratories, Beckenham, Kent). A strain of *M. gallisepticum* named (R) was provided by Mrs Ruth Bernstein (Faculty of Agriculture, The Hebrew University, Rehovoth, Israel). Strain S6 of *M. gallisepticum* was obtained from Dr H. E. Adler (School of Veterinary Medicine, University of California, Davis, U.S.A.). *M. laidlawii* (oral strain) was isolated in our laboratory from the human oral cavity; this strain was serologically related to *M. laidlawii* strain A (Argaman, unpublished observations).

Growth medium and cultivation of organisms. The organisms were grown in a modified liquid Edward medium (Razin, 1963*b*) which contained 2% (v/v) Difco-PPLO serum fraction. The radioactive compounds incorporated into the growth medium included [4-^{14}C]-cholesterol with a specific activity of 24 mC/mmole; [4-^{14}C]-cholesteryl oleate with a specific activity of 20 mC/mmole and [U-^{14}C]-oleic acid with a specific activity of 94 mc/mmole (all products obtained from the Radiochemical Centre, Amersham, England). A benzene solution containing 1 μC of the radioactive compound was evaporated to dryness under a stream of nitrogen and immediately re-dissolved in a few drops of ethanol. The ethanolic solution was then thoroughly mixed with 20 ml. Difco-PPLO serum fraction and added to 1 l. of growth medium. Fifty ml. of a young culture of the organisms in Edward medium were inoculated into 500 ml. medium in 1 l. Roux flasks, which were incubated at 37° for 24–48 hr in an inclined position to improve aeration. The organisms were harvested by centrifugation at 16,000 *g* for 5 min., washed twice in 0·25 M-NaCl and the sedimented packed organisms freeze-dried.

Extraction of lipids. Lipids were extracted from the freeze-dried organisms by chloroform + methanol (2 + 1, by vol.). Extraction was carried out at 60° for 30 min. under reflux in a nitrogen atmosphere. The extract was separated from the organisms by centrifugation and washed with water according to Folch, Lees & Sloane-Stanley (1957). The solvent was then evaporated to dryness by heating under a stream of nitrogen, and the lipid residue freeze-dried and weighed. To test the efficiency of the lipid-extraction procedure the extracted organisms were hydrolysed in 2 N-HCl at 90–100° for 15 hr in sealed ampoules. The hydrolysate was extracted three times with diethyl ether. The solvent was evaporated and radioactivity of the residue determined. Lipids were also extracted from freeze-dried Difco-PPLO serum fraction, and from the other dry constituents of Edward medium for the determination of their cholesterol content. Extraction was carried out by chloroform + methanol as described above.

Determination of cholesterol and its esters

Chromatographic separation. Cholesterol and cholesteryl esters in the lipids from the Mycoplasma organisms and in the constituents of the growth medium were separated by thin-layer chromatography (Razin *et al.* 1963; Avigan, Goodman & Steinberg, 1963). Chromatoplates were prepared by using a slurry containing 50 ml. of a 0·1 % (w/v) aqueous solution of Rhodamine 6 G and 25 g. silica gel G (E. Merck A. G., Darmstadt, Germany). The lipid to be chromatographed was dissolved in a small volume of benzene and applied to the plates as bands. Chromatography was carried out at room temperature with developing solvent a mixture of benzene + ethyl acetate (5 + 1, by vol.). Running time was about 1 hr, during which the solvent front had advanced 12–15 cm. Cholesterol and cholesteryl ester zones were visible after chromatography under ultraviolet radiation as pink-yellow fluorescent areas on a pale green background. Cholesteryl esters migrated with the solvent front, whereas the R_F value of cholesterol was about 0·5.

The same chromatoplates were used for separation of free oleic acid from cholesteryl esters, but the solvent was *n*-hexane + diethyl ether + acetic acid + methanol (90 + 20 + 3 + 2, by vol.; Brown & Johnson, 1962). Oleic acid had a lower R_F value than cholesteryl esters.

The zones of cholesterol, cholesteryl esters and oleic acid to be recovered, and the zones in between were scraped off with a spatula into test tubes and the lipids eluted by extracting twice with 2 ml. chloroform. Two-thirds of the chloroform extract were taken for radioactivity measurements, while the remaining part was used for the colorimetric determination of cholesterol and itsesters.

The purity of the cholesterol fraction separated on the chromatoplate by benzene + ethyl acetate (5 + 1, by vol.) was proved by re-chromatography of this fraction, with benzene + ethyl acetate (20 + 1, by vol.) as solvent (Avigan *et al.* 1963).

Colorimetric determination of cholesterol. The chloroform from the extracts of free and esterified cholesterol zones of the chromatoplates was evaporated under a stream of nitrogen and the dry material dissolved in glacial acetic acid. The amount of cholesterol in solution was determined by the $FeCl_3$ reaction according to Wycoff & Parsons (1957). By this colorimetric method the recoveries of chromatographically separated cholesterol and its esters averaged 90 %. The non-polar lipids migrating with the cholesteryl esters had little influence on the results of the $FeCl_3$ reaction. This was proved by saponification of the lipid in the cholesteryl ester zone, re-chromatography and determination of the amount of free cholesterol produced. The liberated cholesterol produced practically the same colour intensity as the chromatographic fraction before saponification.

Radioactivity measurements. The chloroform extracts placed in scintillation vials were evaporated by heating under a stream of air. Seven ml. of 0·3 % (w/v) 2,5-diphenyloxazole (PPO, scintillation grade) and 0·1 % (w/v) 1,4-bis-2-(5-phenyl-oxazolyl)-benzene (POPOP, scintillation grade, Packard Instrument Co., Inc., Illinois, U.S.A.) in toluene were added to each vial by use of a syringe. ^{14}C-counting was done in a liquid scintillation counter model 6012A (Isotope Developments Limited, Reading, England).

Determination of cholesterol esterase activity. A slight modification of the method described by Smith (1959) was used. Mycoplasma organisms grown in Edward

medium for 24–48 hr were harvested and washed twice with 0·25 M-NaCl as above. The washed organisms were suspended in 0·06 M-phosphate buffer (pH 6·5) to give cell protein 5–10 mg./ml. The organisms suspension was distributed in 2·5 ml. quantities into test tubes. Cholesteryl acetate (5 or 10 μmoles) dissolved in 0·1 ml. acetone was injected into the suspension of mycoplasmas. The resulting emulsion was stable for at least 24 hr at 37° without the aid of an emulsifying agent. Controls without substrate or organisms were run simultaneously. The reaction mixtures and controls were incubated at 37° and 1·2 ml. samples drawn after 5 and 24 hr of incubation. To each sample 4 ml. chloroform + methanol (2 + 1, by vol.) were added, well mixed and centrifuged. The lower chloroform phase was separated, evaporated to dryness under nitrogen, the residue dissolved in benzene and chromatographed. Free and esterified cholesterol appearing on the chromatoplate were quantitatively determined as described above.

RESULTS

Free and esterified cholesterol in Mycoplasma organisms

Free and esterified cholesterol in lipid extracts of various Mycoplasma strains were separated by thin-layer chromatography. The carotenoid pigments of *Mycoplasma laidlawii* migrated with the solvent front. The lipids of all three *M. laidlawii* strains contained less free cholesterol than the lipids of the parasitic strains and were usually free from esterified cholesterol (Table 1). The parasitic strains contained various amounts of cholesteryl esters, usually $\frac{1}{9}$–$\frac{1}{4}$ of the total cholesterol, except *M. mycoides* var. *capri* which consistently contained more esterified than free cholesterol.

Table 1. *Free and esterified cholesterol in lipid of Mycoplasma*

Cholesterol and cholesteryl esters were separated by thin-layer chromatography (Avigan *et al.* 1963), and determined by the $FeCl_3$ reaction (Wycoff & Parsons, 1957).

	% (w/w) of total lipid					
	Total cholesterol		Free cholesterol		Esterified cholesterol	
Organism	Mean	Range	Mean	Range	Mean	Range
Mycoplasma agalactiae	30·2	24·1–36·0	26·1	21·4–31·5	3·4	2·7–4·5
M. gallisepticum (R)	19·5	13·8–23·0	14·7	13·2–16·0	3·5	0·6–7·0
M. gallisepticum (S6)	21·3	16·2–23·4	16·0	14·5–18·0	5·3	2·0–6·4
M. neurolyticum	12·2	9·7–12·8	9·3	5·8–10·5	3·4	2·3–3·9
M. mycoides var. *mycoides*	17·9	17·7–18·0	13·4	10·1–15·0	4·8	3·0–7·6
M. mycoides var. *capri*	19·7	18·1–20·8	6·2	5·0–8·1	14·4	10·0–15·8
M. laidlawii strain A	2·9	1·7–3·1	2·8	1·7–3·1	0·1	0–0·4
M. laidlawii strain B	3·5	2·2–4·6	3·4	2·2–4·6	0·1	0–0·4
M. laidlawii oral strain	3·6	2·4–4·4	3·4	2·4–4·4	0·2	0–0·7

The appearance of cholesteryl esters in the lipids of Mycoplasma prompted us to examine the amount of free and esterified cholesterol in the growth medium. The only component of Edward medium found to contain appreciable amounts of cholesterol and its esters was the Difco-PPLO serum fraction. In several analyses free cholesterol 90–150 μg. and esterified cholesterol 360–600 μg./ml. were found. In all other ingredients only negligible amounts of cholesterol were present.

Incorporation of [4-^{14}C]-cholesterol into the lipids of Mycoplasma

Radioactive cholesterol added to Edward medium was incorporated by all Mycoplasma strains grown in this medium. Radioactivity appeared almost exclusively in the free-cholesterol fraction of the Mycoplasma lipids (Table 2). No appreciable amount of radioactivity was found in the cholesteryl ester zone. Essentially all radioactivity was removed from the organisms by chloroform + methanol extraction. The radioactivity of the defatted organisms, as determined by hydrolysis and ether extraction, was negligible.

Table 2. *Incorporation of [4-^{14}C]-cholesterol into lipids of Mycoplasma*

The organisms were grown in Edward medium containing 1 μC [4-^{14}C]-cholesterol/l. The data given in the table represent typical results obtained in several experiments using different batches of organisms.

Organism	Quantity of lipid examined (mg.)	Radioactivity (counts/min × 10^{-3}) Total lipid	Free cholesterol fraction	Total radioactivity in cholesterol fraction (%)
Mycoplasma agalactiae	4·2	459·0	457·3	99·6
M. gallisepticum (R)	12·4	399·7	397·6	99·3
M. gallisepticum (S6)	9·6	490·0	488·0	99·4
M. neurolyticum	10·4	489·0	487·0	99·4
M. mycoides var. *mycoides*	12·0	686·2	683·6	99·6
M. mycoides var. *capri*	48·0	318·5	315·0	99·0
M. laidlawii strain A	11·1	263·0	261·0	99·2
M. laidlawii strain B	20·2	182·0	180·5	99·1
M. laidlawii oral strain	21·4	232·3	231·2	99·4
[4-^{14}C]-cholesterol 0·1 μC	—	204·0	202·0	99·0

Table 3. *Incorporation of [4-^{14}C]-cholesteryl oleate into lipids of Mycoplasma*

The growth medium contained 1 μC [4-^{14}C]-cholesteryl oleate/l.

Organism	Quantity of lipid examined (mg.)	Radioactivity (counts/min × 10^{-3}) Total lipid	Cholesterol fraction	Cholesteryl ester fraction	Total radioactivity in cholesteryl ester fraction (%)
Mycoplasma agalactiae	4·0	72·4	0·7	71·0	98·0
M. gallisepticum (R)	6·0	107·9	0·93	106·6	98·7
M. mycoides var. *mycoides*	13·0	291·1	0·6	289·5	99·2
M. mycoides var. *capri*	13·7	153·7	1·0	152·2	99·0
M. laidlawii strain A	11·6	4·5	0·3	3·9	86·6
M. laidlawii strain B	12·6	4·8	0·0	4·7	98·3
M. laidlawii oral strain	11·5	0·9	0·0	0·5	62·2
[4-^{14}C]-cholesteryl oleate, 0·1 μC	—	239·4	0·6	238·0	99·4

Incorporation of [4-^{14}C]-cholesteryl oleate into the lipids of Mycoplasma

Radioactive cholesteryl oleate, added to the growth medium, was incorporated into the lipids of the parasitic strains; the saprophytic strains incorporated only minute amounts of the radioactive compound. Radioactivity was limited to the cholesteryl ester fraction of the Mycoplasma lipids; no radioactivity was detected in the free-cholesterol fraction (Table 3). This showed the inability of the Mycoplasma strains tested to hydrolyse cholesteryl oleate. Washed suspensions of *Mycoplasma mycoides* var. *mycoides*, *M. mycoides* var. *capri*, *M. gallisepticum* (R) and *M. laidlawii* strains were unable to hydrolyse cholesteryl acetate. Extracts of *M. gallisepticum* strains did not hydrolyse cholesteryl acetate or cholesteryl stearate when tested at several values from pH 6·5 to 9·0 (see Rottem & Razin, 1964).

Table 4. *Incorporation of [U-^{14}C]-oleic acid into lipids of Mycoplasma*

The growth medium contained 1 μC [U-^{14}C]-oleic acid/l.

Organism	Quantity of lipid examined (mg.)	Radioactivity (counts/min)			
		Total lipid	Chromatographic fractions		
			Cholesteryl ester fraction	Free oleic acid fraction	All other fractions
Mycoplasma mycoides var. *capri*	7·2	11,670	120	10,800	740
M. laidlawii oral strain	17·6	3,050	20	430	2,600
M. laidlawii oral strain*	23·0	8,150	100	2,850	5,200
[U-^{14}C]-oleic acid 0·1 μC	—	175,250	200	172,000	3,050

* Grown in Edward medium without Difco PPLO-serum fraction.

Incorporation of [U^{14}C]-oleic acid into the lipids of Mycoplasma

The saprophytic *Mycoplasma laidlawii* and the parasitic *M. mycoides* var. *capri* were tested for ability to incorporate radioactive oleic acid from the growth medium. Table 4 shows that these organisms incorporated small amounts of the radioactive compound. Radioactivity was not found in the cholesteryl ester fraction, indicating the inability of these strains to esterify cholesterol. A portion of the radioactive oleic acid incorporated by the organism was found at the starting line of the chromatoplates, indicating its presence in polar lipids, probably phospholipids. Omission of Difco-PPLO serum fraction from the growth medium of *M. laidlawii* did not have a significant effect on the incorporation of the radioactive oleic acid (Table 4).

DISCUSSION

The much lower cholesterol content of saprophytic than parasitic Mycoplasma strains noted previously (Razin *et al.* 1963) has been confirmed in the present study. The saprophytic mycoplasmas were also found to differ from the parasitic strains in being practically unable to incorporate cholesteryl esters from the growth medium. The saprophytic *Mycoplasma laidlawii* strains which do not require

sterols for growth (Razin & Knight, 1960; Rothblat & Smith, 1961; Razin & Cohen, 1963) synthesize appreciable amounts of carotenoids localized in the cell membrane, (Razin *et al.* 1963; Smith, 1963*b*). The hydrocarbon structure of the carotenoids bears some resemblance to the structure of cholesterol (Smith, 1963*a*). It is a reasonable speculation that the carotenoids occupy the same sites in the membranes of the saprophytic mycoplasmas as does cholesterol in the membranes of parasitic strains. The carotenoids may thus interfere with the uptake of cholesterol and its esters by the saprophytic strains (see also Smith, 1963*b*).

Our experiments with [4-^{14}C]-cholesterol confirmed previous findings (Smith & Boughton, 1960; Rodwell, 1963; Tourtellotte *et al.* 1963) which suggested that the cholesterol found in Mycoplasma lipids originates in the growth medium. Our results show, however, that the incorporated cholesterol does not undergo esterification, glycosidation or any other chemical change. This is in accordance with the results reported by Rodwell (1963) for the incorporation of cholesterol by two *Mycoplasma mycoides* strains.

The cholesteryl esters found in the lipids of parasitic Mycoplasma are apparently incorporated as such from the growth medium and are not formed *de novo* by the organisms. The ability of mycoplasmas to incorporate cholesteryl esters is much lower than their ability to incorporate free cholesterol. Thus, in spite of the much higher concentration of cholesteryl esters in the growth medium, the amount of free cholesterol in the lipids of the parasitic Mycoplasma, except *Mycoplasma mycoides* var. *capri*, exceeded that of esterified cholesterol (see also Morowitz *et al.* 1962). The incorporated labelled-cholesteryl oleate was not hydrolysed by any of the Mycoplasma strains tested. Neither could we demonstrate cholesterol esterase activity in washed organisms or their extracts, by using cholesteryl acetate or stearate as substrates (see also Rottem & Razin, 1964). These results are in accordance with the inability of several sterol-requiring Mycoplasma strains to utilize the acetate, stearate or oleate esters of cholesterol for growth (Edward & Fitzgerald, 1951).

Smith (1959) described both cholesterol ester-hydrolysing and ester-synthesizing activities in several Mycoplasma strains which require cholesterol for growth. The enzymic formation of a cholesteryl glucoside by several strains capable of glucose utilization was also described (Rothblat & Smith, 1961). Smith (1963*a*) suggested that the function of these enzymic activities might be to mediate the transfer of glucose and short-chain fatty acids across the cell membrane. Our results indicate that cholesterol may fulfil its still unknown physiological function without being esterified or modified in any other way.

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