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THE
JOURNAL OF GENERAL MICROBIOLOGY

THE PREPARATION OF PAPERS

'The more words there are, the more words there are about which doubts may be entertained.'
JEREMY BENTHAM (1748–1832)

'What can be said at all can be said clearly; and whereof one cannot speak thereof one must be silent.'
LUDWIG WITTGENSTEIN: *Tractatus Logico-philosophicus* (tr. C. K. Ogden)

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(3) Authors should state the objective when the work was undertaken, how they did it and the conclusions they draw. A section labelled 'Discussion' should be strictly limited to discussing the results if this be necessary, and not to recapitulation.

(4) Figures and tables should be selected to illustrate the points made if they cannot be described in the text, to summarize, or to record important quantitative results.

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Nomenclature of Enzymes. The system published in *Report of the Commission of Enzymes of the International Union of Biochemistry*, Oxford: Pergamon Press, 50*s*., is used.

Nomenclature in Bacterial Genetics. The proposal by M. Demerec, E. A. Adelberg, A. J. Clark and P. E. Hartman published in *Genetics* (1966), **54**, 61 has been reprinted in *J. gen. Microbiol.* (1968), **50**, 1 as a useful guide to authors. The general principles laid down should be followed wherever practicable.

Nomenclature and Description of Micro-organisms. The authors' preference in naming micro-organisms is at present accepted provided that the designation is unambiguous and conforms with international rules of nomenclature; if desired, synonyms may be added in brackets when a name is first mentioned. Names of bacteria must conform with the Bacteriological Code of the International Committee on Bacteriological Nomenclature and the opinions issued by this International Committee (*Bacteriological Code* (1958), edited by the Editorial Board of the International Committee on Bacteriological Nomenclature and published by the Iowa State College Press, Ames, Iowa, U.S.A.). Names of algae and fungi must conform with the International Rules of Botanical Nomenclature which are considered and revised at each International Botanical Congress (published by the International Bureau of Plant Taxonomy and Nomenclature, 106 Lange Nieuwstraat, Utrecht, Netherlands (1952)). Names of protozoa must conform with the International Code of Zoological Nomenclature adopted by the XVth International Congress of Zoology (published for the International Commission on Zoological Nomenclature by the International Trust for Zoological Nomenclature, London (1961)). The 1913 rules will be found in C. M. Wenyon, *Protozoology*, (1926), vol. 2 (London: Baillière Tindall and Cox). One or two small changes have been made to these rules at later International Congresses.

Useful commentaries on the taxonomy of micro-organisms will be found in the articles of the Twelfth Symposium of the Society for General Microbiology, *Microbial Classification*, edited by G. C. Ainsworth and P. H. A. Sneath (Cambridge University Press).

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.

Bergey's Manual of Determinative Bacteriology, 7th ed. (1957), edited by R. S. Breed, E. G. D. Murray and A. P. Hitchens. London: Baillière, Tindall and Cox.

V. B. D. Skerman, *A Guide to the Identification of the Genera of Bacteria with Methods and Digest of Generic Characteristics*, (1959). Baltimore, Ma., U.S.A.: The Williams and Wilkins Company.

S. T. Cowan & K. J. Steel, *Manual for the Identification of Medical Bacteria*, (1965). Cambridge University Press.

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

List of Common British Plant Diseases, 4th ed. (1944), compiled by the Plant Pathology Committee of the British Mycological Society. Cambridge University Press.

Medical Research Council: Memorandum No. 23. 3rd ed. (1967). *Nomenclature of Fungi Pathogenic to Man and Animals*. London: H.M.S.O.

Latin Names. The species name is in italics (underlined once in typescript) and is used in full at first mention in each paragraph, but in subsequent mention with the name of the genus abbreviated, single letter abbreviations being used where they are not ambiguous. The genus name is in italic when the whole genus is referred to. When used trivially, genus names are in Roman (not underlined). Anglicized versions are not underlined and are used without capitals. Strain names or numbers are in small capitals (underlined twice in the typescript).

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

J. gen. Microbiol. (1968), **53**, 1–7
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Microbicidal Action of Compounds Generated by Transient Electric Arcs in Aqueous Systems

By L. EDEBO*, T. HOLME AND I. SELIN

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(*Accepted for publication* 29 *February* 1968)

SUMMARY

Submerged electrical discharges between copper-containing electrodes rendered the treated liquid microbicidal. Part of this activity was unstable and decreased rapidly during the first few minutes. It might have been caused by cuprous ions or substances with oxidative activity. The stable microbicidal activity was due to copper released from the electrodes. This copper existed only partly in ionic form. Inorganic salts and most organic substances tested decreased the bactericidal effect of discharge-treated water. Such substances also diminished the protracted killing effect which was observed when bacteria suspended in water of relatively high purity were subjected to transient electric arcs.

INTRODUCTION

With high-voltage discharges through water, several physical and chemical effects occur, and micro-organisms present in the discharge liquid are killed. In studies on the mechanism of the killing effect it was shown that the pressure shock-wave alone had no effect (Edebo & Selin, 1968), while the photon radiation was of great significance (Edebo, 1968). It has also been observed that the viable (colony) count decreased considerably with time, when a discharge-treated bacterial suspension was kept at 4°, and that water which had been subjected to discharges had a strong bactericidal effect (Brandt *et al.* 1962).

METHODS

The experiments reported in this paper were done with *Escherichia coli* B 17 (u.v.-resistant) in order to show the killing effect(s) not caused by direct u.v.-radiation. Similar results were obtained with *E. coli* B 15 (u.v.-sensitive) and the *E. coli* B used in earlier experiments (Edebo & Selin, 1968; Edebo, 1968).

The electrical equipment and the techniques used for cultivation and making viable (colony) counts were as described by Edebo & Selin (1968). The same electrical set-up was used in all experiments, namely $C = 0{\cdot}6\ \mu$F., $L = 46\ \mu$H., $U = 45$ kV., $s = 11$ mm. This gives an energy content of 620 J. per discharge. The volume of liquid treated was 1200 ml. The material of the electrodes was different in different experiments.

Determination of the bactericidal effect of water in which discharges had taken place. Samples (0·05 ml.) of a bacterial suspension were pipetted into a series of test-tubes and placed in ice-water. Immediately (40–60 sec.) after the discharge(s) 5 ml. of the

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treated liquid were added to each test-tube, and after 1 hr at room temperature (about 18°) viable counts were made. The influence of different substances on the bactericidal effect of discharge-treated liquid was studied by adding 5 ml. of the liquid immediately after the discharge(s) to 0·05 ml. of different solutions in test-tubes. After 10 min. at room temperature 0·05 ml. of a suspension of bacteria in distilled water was added and after 1 hr at room temperature viable counts were made.

Other analytical procedures. Copper determinations were done by the anodic stripping method (kindly performed by Dr B. Nygård, Pharmacia, Uppsala). Ozone was produced after passing oxygen through an ozonizer (Mark 2, British Oxygen Company, Ltd). Ozone and hydrogen peroxide were determined by iodometry according to Liebknecht & Katz (1953). Thyodene (Purkis, Williams, London) was used as indicator for iodine.

RESULTS

When bacteria were suspended in 10^{-4} M-KCl to a concentration of 3×10^7 viable bacteria/ml. and subjected to one discharge with copper-tungsten electrodes immersed in the suspension (Fig. 1), samples taken less than 1 min. after the discharge showed approximately 99 % inactivation. When the discharge-treated suspensions were kept in ice-water a slow protracted killing effect was observed which was regular as time proceeded. This effect was much greater than the almost inconspicuous spontaneous death in untreated suspensions. However, the magnitude of the protracted killing effect varied considerably between individual experiments, in which equal volumes of the same bacterial suspension were subjected to one discharge shortly after each other with the same electrical arrangement. After 24 hr one sample gave a viable count of $1{\cdot}3 \times 10^5$ bacteria/ml. while another gave 7×10^3 bacteria/ml. and similar experiments another day gave less than 10 bacteria/ml.

Aqueous solutions (10^{-4} M-KCl) subjected to electrical discharges had bactericidal activity which increased with the number of discharges. A decrease in the killing effect was observed with lapse of time between discharge and mixing with bacteria (Fig. 2). This decrease was most rapid during the first few minutes after the discharge. After 30 min. the killing effect remained fairly constant for 24 hr. In these experiments copper was released from the electrodes into the discharge-treated liquid. One discharge gave the following concentrations (three experiments) (μg. Cu/ml.): 1·3, 1·5, 1·7; two discharges 1·4, 2·3, 2·4; three discharges 1·8, 2·8, 3·8. Also in these experiments there was considerable variation in the bactericidal effect between solutions treated with the same number of discharges. Each point in Fig. 2 is the average of three experiments; for each experiment viable counts were made in triplicate.

The influence of the electrode material on the killing effect of the discharge-treated liquid was tested with some metals and alloys (Table 1). Discharges with electrodes containing copper (copper–tungsten, copper, brass) and silver gave considerable bactericidal activity which increased with increasing numbers of discharges. The average quantity of metal released at one discharge of 620 J. was: with electrodes made from copper, 3·5 mg. Cu; from copper-tungsten alloy, 1·3 mg. Cu (mainly as copper tungstate). The bactericidal effect of aqueous solutions treated with electrodes from iron, steel, aluminium and platinum was inconspicuous.

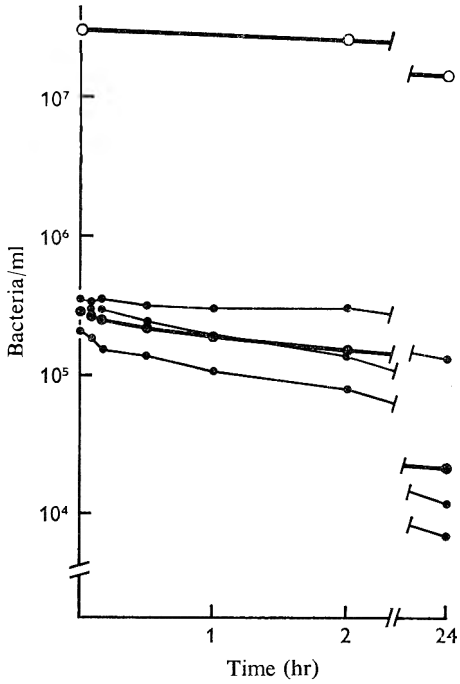

Fig. 1

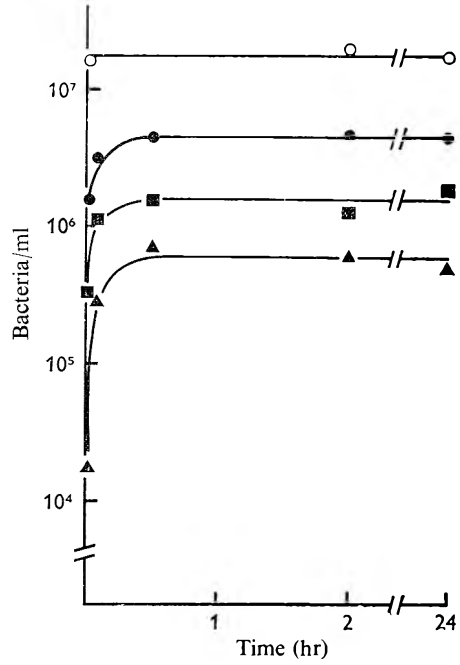

Fig. 2

Fig. 1. Survival of *Escherichia coli* B17 present in the discharge-liquid (10^{-4} M-KCl) at different intervals after one discharge with copper–tungsten alloy electrodes. Immediately after the discharge part of the suspension was cooled in ice water, and samples for viable count taken from it at different times. ○, No discharge; ●, one discharge representing the average of three different discharge-treated batches (fine lines).

Fig. 2. Bactericidal effect of 10^{-4} M-KCl treated with discharges with copper–tungsten electrodes on *Escherichia coli* B17 at different times after the discharge(s). ○, No discharge; ●, one discharge; ■, two discharges; ▲, three discharges.

Table 1. *Effect of the electrode material on the bactericidal effect on Escherichia coli B17 of 0·0001 M-KNO_3 subjected to discharge(s)*

No discharge: $2{\cdot}0 \times 10^7$ bacteria/ml.

Electrode material	Viable count (bacteria/ml.)	
	One discharge	Five discharges
Copper–tungsten	$7{\cdot}7 \times 10^6$	$1{\cdot}8 \times 10^4$
Copper	$7{\cdot}1 \times 10^5$	$1{\cdot}9 \times 10^3$
Brass	$4{\cdot}3 \times 10^6$	$8{\cdot}3 \times 10^5$
Silver	$1{\cdot}9 \times 10^5$	$< 1{\cdot}0 \times 10^1$
Iron, steel, platinum, or aluminium	$1{\cdot}7 \times 10^7$–$2{\cdot}0 \times 10^7$	

Addition of different organic and inorganic substances

Certain organic compounds added to a liquid discharge-treated with copper electrodes influenced its bactericidal effect (Table 2). Cysteine and Na EDTA decreased, while ascorbic acid increased the killing effect. Glucose slightly decreased the killing effect.

Inorganic salts inhibited the killing effect. Depending on their neutralizing capacity at 0·01 M, one series of tested salts were arranged in the following order:

$$\text{Na-phosphate} > K_2SO_4 > KCl = NaNO_3 > CH_3COONH_4.$$

As mentioned above, the addition of ascorbic acid increased the killing effect of discharge-treated water when the electrodes contained copper. Also the killing effect

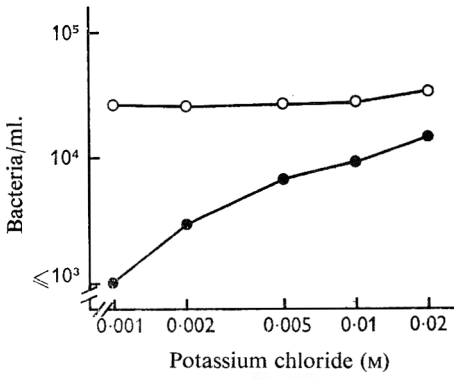

Fig. 3

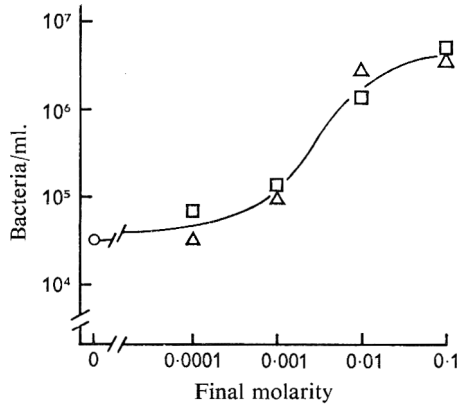

Fig. 4

Fig. 3. Bactericidal effect on *Escherichia coli* B17 suspended in different concentrations of KCl and subjected to one discharge with copper–tungsten electrodes. ○, Immediately after the discharge; ●, after 24 hr at 4°. Untreated suspension = $1·9 \times 10^6$ bacteria/ml.

Fig. 4. *Escherichia coli* B17. The influence of different concentrations of salts (final conc.) on the bactericidal effect of 10^{-4} M-NaCl subjected to three discharges with copper–tungsten electrodes. □, KCl; △, $NaNO_3$; ○, no salt added. Untreated suspension = $1·3 \times 10^7$ bacteria/ml.

Table 2. *Effect of organic substances on the bactericidal effect on Escherichia coli B17 of 0·001 M-KCl subjected to one discharge with copper electrodes*

Solution	Final conc. (M)	Viable count (bacteria/ml.) Untreated liquid	Discharge-treated liquid
Control (distilled water)		$2·4 \times 10^7$	$4·2 \times 10^6$
Ascorbic acid	0·001	$2·1 \times 10^7$	$2·0 \times 10^3$
	0·0001	$2·0 \times 10^7$	$5·0 \times 10^3$
Glucose	0·0001	$2·5 \times 10^7$	$1·1 \times 10^7$
Na EDTA	0·00001	$2·7 \times 10^7$	$2·8 \times 10^7$
Cysteine	0·001	$2·7 \times 10^7$	$2·5 \times 10^7$

of liquid discharge-treated with iron, aluminium or platinum electrodes, which in itself was inconspicuous, was increased, in some cases to more than 99%, when the liquid was mixed with ascorbic acid immediately after the discharge. Ascorbic acid alone had little killing effect.

The influence of salts on the microbicidal effects on bacteria present at the very discharge was tested by subjecting bacteria suspended in a series of concentrations of KCl to one discharge. Samples were transferred into the diluent containing nutrient

broth immediately after the discharge and after 24 hr at 4° (Fig. 3). The instantaneous killing effect was little influenced by concentrations of KCl of 0·02 M or less. However, at 0·02 M-KCl the viable counts immediately after the discharge and after 24 hr were nearly identical, while at lower salt concentrations the counts were lower after 24 hr. In 0·001 M-KCl and below no colonies were recovered after 24 hr, even when non-diluted suspensions were tested. A similar relation to the concentration was shown for the neutralization of the microbicidal effect of discharge-treated water (Fig. 4). At a concentration of 0·001 M or less of KCl or $NaNO_3$ there was a marked bactericidal effect, while at 0·01 M and above it was nearly extinguished.

The production of oxidizing substances by discharges was tested by subjecting 0·0033 M-KI in 0·036 M-H_2SO_4 (pH 1·7) or in 0·001 M-sodium phosphate (pH 7) to discharges with iron electrodes. The iodine in the discharge-treated liquid was titrated with $Na_2S_2O_3$. Immediately before the titrations 5 ml. of 3·6 M-H_2SO_4 and 3 drops of

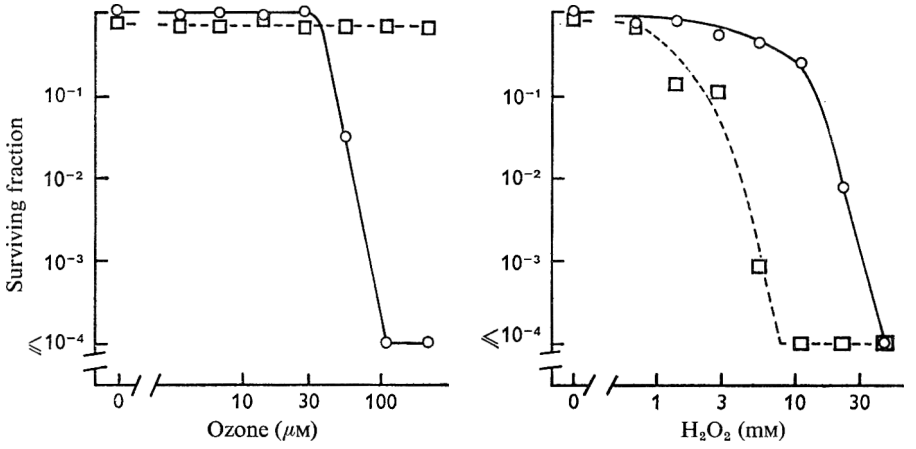

Fig. 5. The bactericidal effect of ozone and hydrogen peroxide on *Escherichia coli* B17; ○, oxidant alone; □, mixed with ascorbic acid which had a final conc. of 0·001 M.

N-ammonium molybdate were added to samples (30 ml.) of the discharge-treated liquid to accelerate the production of iodine. After 40 discharges at pH 1·7 a concentration of iodine of $3{\cdot}7 \times 10^{-5}$ M was found. This meant that each discharge caused the production of approximately 10^{-6} moles iodine. At pH 7 almost no iodine was found. Bubbling of air at 2 l./min. through a fritted glass filter into the same solutions did not produce iodine at either hydrogen-ion concentration.

The bactericidal effect of different concentrations of ozone and hydrogen peroxide was tested alone and in the presence of ascorbic acid (Fig. 5). Ozone was most active alone, while the activity of hydrogen peroxide was enhanced in the presence of ascorbic acid.

DISCUSSION

Several unstable compounds are produced by the plasma of an electric arc (Martin, 1960; Joncich & Vaughn, 1965; de Mayo, 1965). The main products are metallic atoms in various levels of excitation and ionization and reactive oxyhydrogen compounds. Directly or indirectly they may have a microbicidal effect. Some of this effect and

its decrease with time are illustrated in Fig. 4. Other compounds might have such a short life that only bacteria present at the discharge would be affected.

The fact that the bactericidal effect of discharge-treated liquids was markedly dependent on the electrode metal (Table 1) indicated that the electrode material participated in the electrochemical reaction, giving the bactericidal effect. The short-lived highly bactericidal activity displayed by the liquids immediately after the discharge-treatment (Fig. 2), when the electrodes contained copper, might be produced by an activated state of liberated copper or by other unstable compounds generated by the discharge. Liquid discharge-treated with any kind of electrode material tested and then mixed with ascorbic acid had bactericidal activity. Since ascorbic acid mixed with an oxidant has high microbicidal activity, particularly in the presence of copper (Ericsson & Lundbeck, 1955), it was supposed that the unstable microbicidal effect of discharge-treated water was caused by substances with oxidative activity generated at the discharge. This supposition was supported by the oxidation of KI by discharges. This activity was more like that of hydrogen peroxide than of ozone in being capable of oxidizing KI at pH 1·7 but not at pH 7 (Liebknecht & Katz, 1953) and in increasing its bactericidal effect in the presence of ascorbic acid (Fig. 5). However, it seemed to be more bactericidal at the same oxidative capacity and more unstable than hydrogen peroxide. Another possible explanation of the rapid decrease of the bactericidal activity after a discharge in water with copper-containing electrodes is that cuprous ions which are formed during the discharge (Martin, 1960) are oxidized to cupric ions or disproportionate to give cupric ions and metallic copper (Hemmerich, 1966) which both are of lower bactericidal activity (Cramp, 1967). Ascorbic acid regenerates cuprous ions (Zimmerman, 1966). The two proposed mechanisms do not exclude each other; nor can we exclude that trace amounts of non-recognized substances play a role.

The protracted bactericidal effect, which remained fairly constant for at least 24 hr, was probably due to metal released from the electrodes during the discharge. At the same concentration of copper, 24-hr old discharge-treated liquid had less bactericidal activity than solutions of $CuCl_2$. Presumably not all the copper released by the discharges was ionized; electric arcs have been used to produce colloidal suspensions of metals and metal oxides (Svedberg, 1909).

Inorganic salts and some organic compounds decreased the bactericidal effect of liquid discharge-treated with electrodes containing copper (Table 2). Both inorganic and organic substances decrease the oligodynamic activity of silver (Süpfle & Werner, 1954). EDTA forms a strong chelate, and cysteine forms mercaptides with copper ions, which might explain their neutralizing effect. Since the neutralizing effect of the inorganic salts seemed to be dependent on their ionic strength, and bacteria behave as ion exchangers (Edebo, 1961), the effect of inorganic salts might be a consequence of competition for and screening of vital groups by the innocuous ions to keep toxic copper ions away. The bactericidal effect of ozone and hydrogen peroxide obtained in our experiments (Fig. 5) agreed with earlier published data (Ingram & Haines, 1949; Ingram & Barnes, 1954; Wallhäuser & Schmidt, 1967). Many organic substances neutralize both compounds, and inorganic salts increase the velocity of decomposition of ozone (Ingram & Barnes, 1954). Consequently, organic substances and inorganic salts might also counteract the oxidizing effect of discharge-treated water. At salt concentrations where no bactericidal activity was found in liquids discharge-treated with electrodes containing copper (Fig. 4), little or no protracted killing effect was

observed (Fig. 3). This makes it likely that most if not all of the protracted bactericidal effect was caused by compounds generated at the discharge. This might also explain why generally little or no protracted killing effect was found after discharge-treatment of tap water.

After submitting this communication two papers (Gilliland & Speck, 1967*a*, *b*) were seen which showed the presence of copper in the discharge-liquid after discharges with copper electrodes and the oxidation of several biological substances by submerged discharges with aluminium electrodes. These investigators were using higher capacitances (6–24 μF.) and lower voltage (10 kV.). Their results indicate, however, that similar or identical mechanisms of killing operate.

This work was made possible by grants from Grängesbergsbolaget, Sweden. The assistance provided by Miss Lillemor Svensk and Mr Mahendra Pal Singh is gratefully acknowledged.

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Ploidy Level in the True Slime Mould *Didymium nigripes*

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SUMMARY

Estimation of chromosome numbers in approximately 1000 nuclei of amoebae and plasmodia of the true slime mould, *Didymium nigripes*, has shown that in the C6 and S3 strains the ploidy level of amoebae and plasmodia is the same. Nuclei with differing chromosome numbers were found in the populations studied. The data collected here are consistent with the hypothesis that as a culture ages during serial subculture of one stage of the life-cycle, nuclei with higher than normal chromosome numbers accumulate.

INTRODUCTION

During the course of its life-cycle the true slime mould *Didymium nigripes* exhibits several strikingly different morphological phases. In routine laboratory culture spores plated on agar in the presence of a food source, *Aerobacter aerogenes*, germinate to form uninucleate amoebae. After several days of growth and division members of the amoeboid population differentiate to form multinucleate coenocytic plasmodia. If subjected to conditions of starvation a plasmodium differentiates to form fruiting bodies. Cleavage within the sporangium then leads to production of uninucleate spores, thus completing the life-cycle. Heterothallism or a mating-type incompatibility system is absent in this isolate. Fusion of amoebae to form a 'plasmodial precursor cell' and meiosis during sporulation have not been confirmed by either genetic or cytological means.

Recent studies of mitosis in *Didymium nigripes* (S. Kerr, 1967) have shown that mitotic behaviour and the time course of division differ in the amoeboid and plasmodial stages of the life-cycle. The work reported here was undertaken to determine whether or not these differences in behaviour could be correlated with a change in ploidy level, which historically has been implicated as the initiating step in plasmodial differentiation. Accurate chromosome counts for the myxomycetes are nearly impossible to obtain because the chromosomes of this group of organisms are extremely small and fairly numerous. Nevertheless, estimation of ploidy level, based on chromosome counts, is feasible. This technique has been used previously by Ross (1966) to determine approximate chromosome number in three other species of slime moulds: *Physarum polycephalum*, *P. flavicomum* and *Badhamia curtisii*.

In the present study ploidy level was estimated in amoebae and plasmodia of two clonally derived strains of a single isolate of *Didymium nigripes* grown on living bacteria. One strain was a wild-type clone, C6 (N. Kerr, 1965), and the other was a minute mutant, S3 (N. Kerr, 1967), known to undergo plasmodium formation without

plasmogamy. Chromosome numbers were also estimated in four strains (also derived from the same isolate) of 'axenically grown' amoebae which had been subcultured for varying lengths of time on formalin-killed bacteria.

METHODS

Amoebae were grown as described elsewhere (N. Kerr, 1960, 1961, 1963) and in mid-log phase of growth were harvested in Schaudinn fixative (66 ml. water saturated with $HgCl_2$ + 33 ml. 95% EtOH + 1 ml. glacial acetic acid) and stored for 12 hr. The tube was then centrifuged, the fixative decanted and 65% acetic acid added. After 30 min. a drop of cell suspension was placed on a slide, 2% aceto-orcein was added, the coverslip was pressed slightly to remove excess liquid and the preparations were sealed with paraffin. In all cases the time elapsed between inoculation of the medium with spores and harvesting of the amoebae was noted; this elapsed time has been designated as the 'age' of the culture.

Young plasmodia develop after 2 days (C6) and 3 days (S3) of growth on 0·01 M-phosphate buffered agar, pH 6·5, (S. Kerr, 1967) in cultures seeded with spores from a single sporangium. Amoebae and plasmodia from these cultures were harvested and incubated as described above and slides were prepared with glycerine as the mounting medium. Microplasmodia were obtained by scraping pieces of plasmodia which had grown several days on agar, suspending them in phosphate buffer and breaking them up with a Vortex junior mixer. The resulting microplasmodia were incubated for 12 hr on phosphate-buffered agar to allow asynchrony of mitosis to develop and then harvested and treated as above. Microplasmodia formed in this manner grew normally and completely covered an agar plate in 2 days; the technique simply facilitated obtaining small pieces of plasmodium which were easy to handle and increased the probability of finding dividing nuclei.

All observations were made by phase-contrast microscopy using a Zeiss GFL microscope with a × 100 oil apochromat objective. Duplicate counts of chromosomes either in prometaphase or in metaphase were taken on nuclei where the disposition of chromosomes was favourable. Counts from a small number of nuclei were discarded because the two counts were not within 5% of each other. Photographs through several focal levels of representative nuclei were taken with a 35 mm. camera on Kodak high-contrast copy film. Enlargements of these photographs were used for a second estimate of chromosome number (Pl. 1.) Although chromosomes can be readily distinguished from bacteria enclosed in food vacuoles after a little practice, chromosome counts were made only when the presence of a spindle could be confirmed. The presence of a nuclear membrane was used to distinguish plasmodial nuclei from those of amoebae.

RESULTS

Eight populations of amoebae were surveyed: C6 wild-type cells grown on SS agar (N. Kerr, 1967) which permits plasmodial differentiation and on GPY-B agar (N. Kerr, 1960) which inhibits such differentiation; S3 cells grown on SS and GPY-B agar, both of which permit differentiation of this strain; and four strains of cells grown in 'axenic' culture (101 wild-type, C6 wild-type, S3 and A20; see N. Kerr, 1963). Chromosome number estimates were made for 100 metaphase cells of each population.

Young plasmodia containing 1–4 nuclei were examined in C6 and S3 populations grown from spores on SS agar. Nuclei of older plasmodia were examined in microplasmodia derived from C6 and S3 plasmodia of several ages.

Pooled data from all C6 and S3 amoebae (600 cells) are shown in Fig. 1. On the basis of these counts, one ploidy level (class A) contains between 31 and 38 chromosomes (Pl. 1, fig. 1, 8, 9). A second ploidy level (class C) exists which contains twice this number or about 61–76 chromosomes (Pl. 1, fig. 2, 3). Some nuclei were found which could not be placed in either of the above categories. For purposes of the statistical analysis numbers from 39 to 60 (172 nuclei) were grouped in class B and numbers over 100 (17 nuclei)

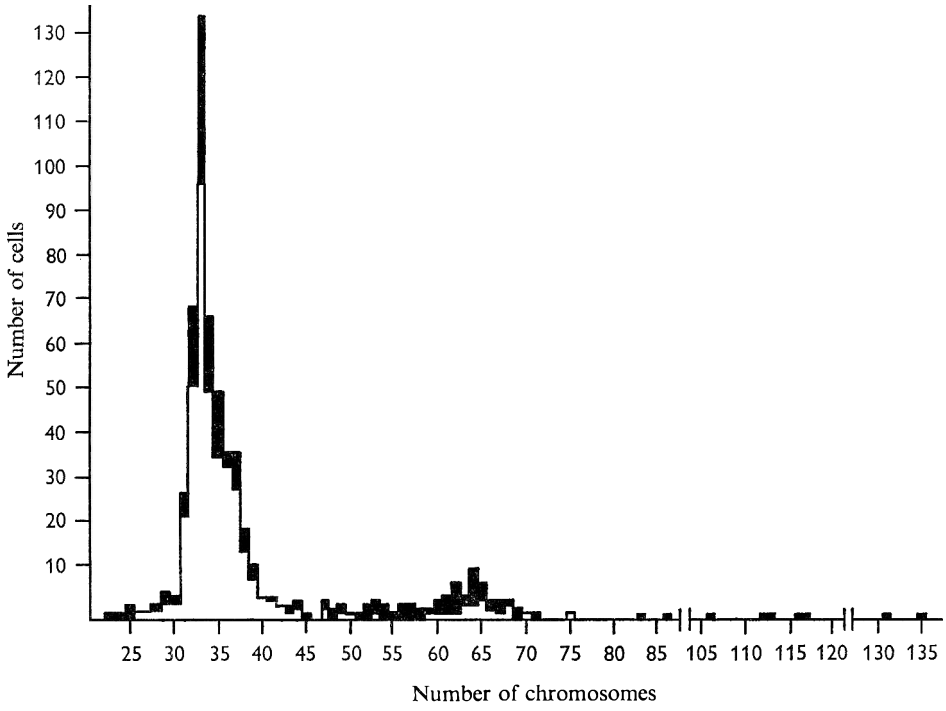


Fig. 1. Chromosome number distribution in C6 and S3 amoebae. ■, Axenically grown; □, bacterial-grown.

Table 1. *Comparison of ploidy level in amoebae and plasmodia*

Strain and culture conditions	Approximate number of chromosomes < 31	31–38	39–60	61–76	77–100	> 100	Total nuclei examined
C6 amoebae SS grown; 45 hr	1	94	2	3	0	0	100
C6 amoebae GPY-B grown; 65 hr	6	71	20	2	1	0	100
C6 young plasmodia SS grown; 45 hr	1	43	5	2	0	0	51
C6 microplasmodia various ages (30–200 days)	1	24	4	4	2	0	35
S3 amoebae SS grown; 45 hr	0	93	1	6	0	0	100
S3 amoebae GPY-B grown; 65 hr	3	81	8	8	0	0	100
S3 young plasmodia SS grown; 96 hr	0	46	1	3	0	0	50
S3 microplasmodia 60 days	0	24	0	2	0	0	26

were placed in Class D (Pl. 1, fig. 4, 5). Fewer than 31 chromosomes were counted in 30 cells. The distribution of nuclei by chromosome number classes is shown in Tables 1 and 3.

If class A represents the haploid chromosome number and class C represents the diploid, a comparison of these two classes in amoebae and plasmodia would tell whether karyogamy precedes plasmodial differentiation. Visual inspection of Table 1 shows that the majority of nuclei of both amoebae and plasmodia belong to class A. Thus in C6 and S3 karyogamy can not be a prerequisite for plasmodium formation.

The presence in the populations of nuclei with chromosome numbers which do not fall in class A remains unexplained. Some variation in numbers of nuclei which belong to classes B, C and D is apparent when different populations are compared. Data shown in Table 1 were analysed by means of χ^2 contingency tables. When the distribution of all chromosome number classes for C6 and S3 bacterially grown populations was analysed, S3 showed no significant difference in total distribution among classes ($P = 0{\cdot}33$) whereas the difference in distribution in C6 was significant ($P < 0{\cdot}001$).

Table 2. *χ^2 evaluation of class A : class C distributions*

Populations compared	Level of significance
S3 amoebae, young plasmodia and microplasmodia	0·89
C6 amoebae, young plasmodia and microplasmodia	0·08
C6 amoebae and young plasmodia	0·83

Table 3. *Comparison of ploidy level in axenic amoebae*

	Approximate number of chromosomes						Total nuclei
Strain and age	< 31	31–38	39–60	61–76	77–100	> 100	examined
C6; 9 months	2	66	4	24	0	4	100
S3; 2 years	9	42	34	10	2	3	100
A20; 3 years	7	20	41	21	7	4	100
101; 6½ years	0	8	52	31	3	6	100

In an attempt to determine the source of variability of total distribution, the data in Table 1 were regrouped and analysed as above. Class A (31–38):class C (61–76) distributions were compared to determine whether the proportions of cells in these classes differed in the various populations. The results of this analysis are presented in Table 2. Note that, as above, S3 populations showed no significant difference in distribution. When C6 amoebae, young plasmodia and microplasmodia were compared the difference was nearly significant, whereas if only amoebae and young plasmodia were compared the populations showed similar distributions. Thus the C6 microplasmodia represent one major source of variability.

One possible explanation for this difference in ploidy level is that the C6 microplasmodia were derived from plasmodia which had 'aged' up to 200 days whereas S3 microplasmodia were derived from fairly young plasmodia. In an attempt to test the hypothesis that chromosome number may vary with ageing, axenically grown amoebae which had been serially subcultured for varying lengths of time were examined (see Table 3). Comparison of the numbers of nuclei with 31–38 chromosomes in these populations shows that the youngest culture (C6 axenic) possessed a majority of nuclei with this number of chromosomes but that as the age of these cultures increased,

the proportion of cells containing chromosome numbers outside the 31–38 class also increased. A parallel trend can be noted when bacterial-grown amoebae are compared after 45 and 65 hr of growth.

DISCUSSION

Ancestry of the strains studied

It is necessary, before proceeding further, to clarify the relationship of the strains under study. Strains C6, A20 and S3 represent separate clonal lines derived from a single isolate from nature, the wild-type 101. C6 was isolated as a wild-type clone with stable morphological characteristics. A20 was selected from a series of cycloheximide-resistant mutants (N. Kerr, 1965) and S3 was isolated as a minute mutant which appeared after selection on cycloheximide (N. Kerr, 1967). Variations in chromosome number reported here therefore represent alterations of number within and not between isolates.

Sources of ploidy variation

Variation in chromosome number of myxomycete nuclei within an amoeboid population or within a single plasmodium is not a new observation. Such variation has been documented recently in plasmodia of *Physarum polycephalum* (Ross, 1966; Koevenig & Jackson, 1966) and in amoebae of *Badhamia curtisii* (Ross, 1966). The variation reported by Ross occurred in strains grown in pure culture whereas variation reported in the present study was found both in 'axenically grown' and bacterial-grown strains.

Phase-contrast observations made on C6 and S3 living amoebae growing on SS thin agar suggest how ploidy level variation may originate in these strains (S. Kerr, 1968). In brief, fusion of amoebae has been observed in cases where subsequent observations for 24 hr have shown that the resulting cell did not give rise to a plasmodium but continued to divide as an amoeba (class A to class C transition). That products of such fusions sometimes do differentiate to plasmodia was demonstrated by isolation of fusing pairs (N. Kerr, 1961). Some cells, generally larger than most in the population, may undergo tripolar divisions (N. Kerr, 1967). Occasionally two of these tripolar daughter cells may fuse. One tripolar metaphase plate was counted and contained in excess of 130 chromosomes. Division of this cell would represent a class D to class B transition. If a class C cell divided tripolarly and then two of the daughter cells fused, one of the resulting cells would belong to class B whereas the other would contain less than 30 chromosomes. Fusions and tripolar divisions occur in less than 1 % of the cells of a population if they are detected at all (S. Kerr, 1968). Perhaps it is not unreasonable to suppose that such behaviour could account for variations in chromosome number and that over a period of time the number of cells with chromosome numbers outside the 31–38 range should increase.

The origin of plasmodia

The classical myxomycete life-cycle dictates that fusion of gametes, either amoebae or flagellates, serves as an initiating step in the differentiation of the plasmodial stage. Several workers have shown that this is the case in many different organisms, some closely related to the isolate under study. Schünemann (1930) and Cadman (1931) reported amoeboid fusion in *Didymium nigripes*. Schünemann followed these fusion products through the stage of the polynuclear young plasmodium and observed no

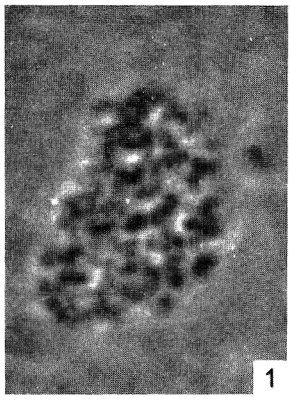
karyogamy. Cadman concluded that karyogamy did occur and that subsequently the zygotes fused to form plasmodia.

Three observations exist for the isolate under study. Von Stosch (personal communication to Dr Norman Kerr) concluded that the ancestral 101 strain was apogamous, i.e. that meiosis and karyogamy are not present during the life-cycle. His conclusions were based on the absence of meiosis at the time it is found in heterothallic isolates of this species and on chromosome counts which showed approximately the same number of chromosomes in amoeboid and plasmodial nuclei. Von Stosch examined nuclei with chromosome numbers approaching 80. Recently N. Kerr (1967) showed by means of time-lapse cinephotomicrography that the S3 strain under study is capable of forming plasmodia without either plasmogamy or karyogamy.

Therrien (1966) measured DNA spectrophotometrically in a strain derived from the ancestral wild-type 101, found two classes of nuclei in his amoeboid cultures, and concluded that the larger cells represented zygotes and that karyogamy preceded plasmodial differentiation. Of 100 amoeboid nuclei he measured, Therrien placed 27 in what he designated as a haploid 2C class and 73 in a diploid 4C class. In this investigation identical culture conditions were not employed and the only nuclei of strain 101 which were studied were 'axenically' grown amoebae; of these cells 8% could be placed in a class which corresponds to Therrien's haploid 2C (class A), 8% in an intermediate class (39–50 chromosomes), 75% in Therrien's diploid 4C (51–76 chromosomes) and 6% in class D. Inspection of Therrien's data shows that he, too, found nuclei with amounts of DNA intermediate to the two ploidy classes and greater than the diploid 4C class. Thus while data from two different techniques agree closely, it is important to note that the 'axenically' grown amoebae used in the present study contain 'diploid' cells which are not plasmodia under liquid culture conditions. When such cells are plated on GPY/5 agar for 'P' clone assay (N. Kerr, 1961) only amoeboid plaques are recovered and no 'P' clones are formed, indicating that none of the cells plated had undergone plasmodial differentiation before plating even though 75% of them were presumably 'diploid'. Furthermore, all of the cells plated continued to divide as amoebae for 2 days before plasmodial differentiation began.

Therrien's analysis showed that all the plasmodial nuclei he surveyed belonged to one size class—the diploid 4C; it is curious that Therrien chose to recognize only one size class of nuclei in his preparations for examination of his graph suggests that several size classes could have been recognized. Plasmodia of all strains examined in this study, including the wild-type 101 which Therrien used, contained nuclei which differed in size. Examination of 500 nuclei of plasmodia grown from spores stored in lyophil tubes showed, on the basis of a simple nuclear sizing technique (visual estimation of nuclear diameter), that plasmodia derived from spores of the strain given to Therrien contained 78% large (15% may be polyploid) and 22% small nuclei.

The present investigation shows that in two strains of the isolate of *Didymium nigripes* under study there is no ploidy level difference between a population of amoebae and plasmodia derived from this population. Although nuclear and cytoplasmic fusion may precede plasmodial initiation, such fusion is not necessary for plasmodial differentiation to occur. The absence of nuclear fusion prior to plasmodium formation is regarded as evidence supporting the hypothesis that the C6 and S3 strains of this isolate may lack a sexual cycle.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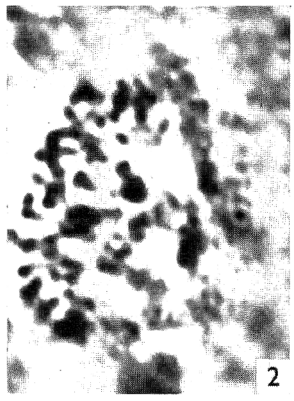
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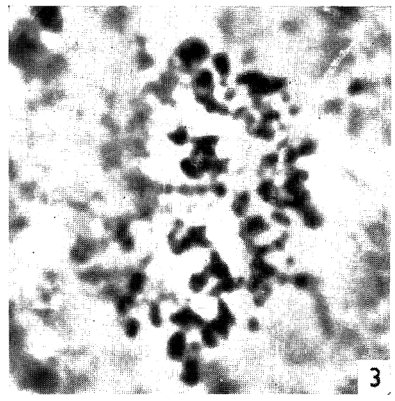
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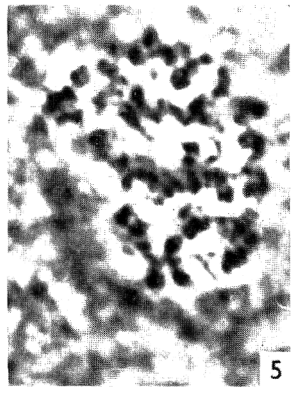
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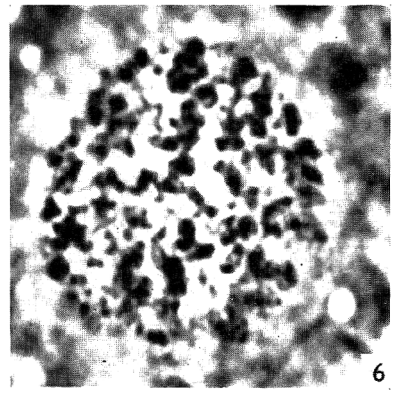
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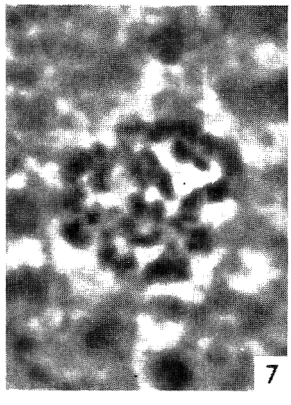
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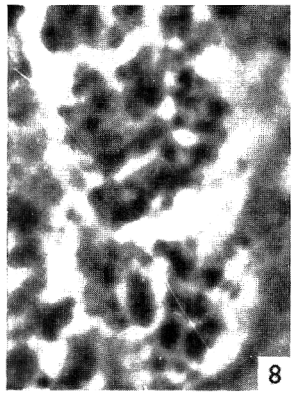
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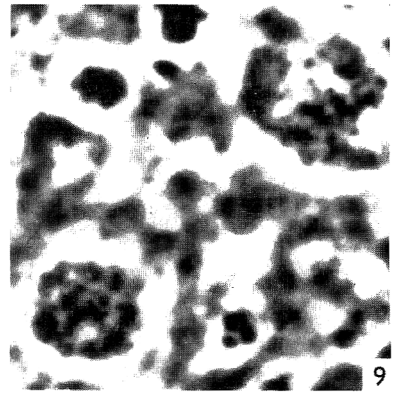
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EXPLANATION OF PLATE

All figures ×4000.

Fig. 1. A20 axenic amoeba; class A.

Fig. 2, 3. Two focal levels through S3 axenic amoeba; Class C.

Fig. 4, 5. Two focal levels through 101 wild-type axenic amoeba; class D.

Fig. 6. C6 plasmodium; prometaphase.

Fig. 7. C6 axenic amoeba; less than 30 chromosomes.

Fig. 8. C6 axenic plasmodium; class A.

Fig. 9. C6 plasmodium; class A.

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Observations on the Differentiation of Plasmodia into Fruiting Bodies by the True Slime Mould, *Didymium nigripes*

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(*Accepted for publication* 4 *March* 1968)

SUMMARY

Under defined experimental conditions plasmodia of *Didymium nigripes* began to differentiate into fruiting bodies 16–18 hr after transfer to bacteria-free agar. The process has been divided into a number of easily recognizable stages and the duration of each stage determined. Plasmodia lost ability to continue vegetative growth at a time before the actual fruiting bodies began to form.

INTRODUCTION

The mature fruiting body of the true slime mould *Didymium nigripes* consists of a rounded black spore-mass supported above the substrate on the apex of an acellular stalk. Under defined conditions (Kerr & Sussman, 1958) the spores germinate and give rise to uninucleate myxamoebae which, when plated with a bacterial food source, feed and multiply by binary fission. After a period of exponential growth, the myxamoebae differentiate into plasmodia which grow to form macroscopic multinucleate networks of rhythmically streaming protoplasm. Plasmodia can be maintained as such by subculturing them every second day to agar plates which have been spread with bacteria. They move across the agar as they grow, engulfing the bacteria. If they are not subcultured before they reach the far side of the plate, they usually sweep back and forth across the plate about one and one-half times before starving sufficiently to differentiate into fruiting bodies. When a plasmodium is transferred to bacteria-free agar so that the loss of its bacterial food supply is more abrupt, the differentiation into fruiting bodies is more uniformly timed. The purpose of the present study was to divide the morphogenesis of plasmodia to fruiting bodies into stages and to determine the timing of each stage. The point at which plasmodia were no longer able to resume vegetative growth even in the presence of a bacterial food source was also determined. Such information is a necessary prerequisite to the study of the differentiation at a molecular level.

METHODS

Didymium nigripes was grown in monoxenic culture with *Aerobacter aerogenes* on dilute glucose peptone yeast-extract (GPY/5) agar as previously described (Kerr & Sussman, 1958). Plasmodia were maintained as such by subculturing a 1 cm.^{2} piece every 2 days to GPY/5 agar which had been spread with *A. aerogenes* 2 days earlier.

To induce fruiting, plasmodia which had advanced one-half to three-quarters of the way across a GPY/5 agar plate were transferred to bacteria-free 1·5% Ionagar no. 2 buffered with 0·01 M-phosphate (pH 6·6). Cylinders (3·5 mm. diam.) which had been

cut from the solid protoplasmic region of the advancing plasmodial fronts with a sterile brass cork-borer were transferred by using a hoe-like nichrome wire transfer tool, which enabled restoration of the original size and orientation of the plasmodial discs on the agar.

At 30 min. intervals the morphology of each transfer was noted. After a period of time each starving plasmodium was tested to see whether, at that time, it was committed to fruiting, by selecting its morphologically most advanced part and transferring it back to GPY/5 agar spread with *Aerobacter aerogenes*. The desired part of the plasmodium, along with the underlying agar, was cut out with a 1 cm.^{2} nichrome cutter and inverted on the bacterial agar, so that the plasmodium was in contact with the lawn of bacteria; 24 hr later each transfer had fruited or grown as a plasmodium. All material was incubated at 23°.

Time-lapse cine films were made, in black and white and in colour with high-speed Ektachrome type B, using a Bolex H 16 Reflex camera, of both the top and side views of fruiting plasmodia (Kerr, 1965). Sequences were taken with the use of extension tubes between the camera and lens, and through a Zeiss microscope using a $\times 2{\cdot}5$ objective and an $\times 8$ ocular.

RESULTS AND DISCUSSION

During the morphogenesis of plasmodia into fruiting bodies on bacteria-free agar, the following stages were distinguished.

Advancing front stage. During the first 16–18 hr after it had been transferred to non-nutrient agar, the starving plasmodia continued to migrate across the agar (Pl. 1, fig. 1). The duration of this stage was the most variable part of fruiting, being dependent on the time since subculture of the parental plasmodia. The use of plasmodia which had migrated three-quarters of the way across the GPY/5 agar plate instead of one-quarter of the way shortened this stage by several hours.

Accumulation stage. The plasmodial fronts stopped their advance and increased in area to form broad accumulations of protoplasm which were as thick as a large plasmodial vein. The veins flowing into such accumulations gradually lost all of their protoplasm to them as they dried and became the hypothallus (Pl. 1, fig. 2).

Mounding stage. Ridges of protoplasm pushed up in the accumulations. Each ridge gradually subdivided into mounds of unequal size which were usually connected to one another by single veins of streaming protoplasm (Pl. 1, fig. 3). The streaming between the mounds gradually slowed and then stopped as all of the protoplasm entered the mounds. The last amounts of protoplasm to enter the mounds became lumpy and flowed in discrete pieces.

When mounds were manipulated with a microneedle, their structural strength was greater than that of a plasmodial vein. This might have been due either to the mounds becoming surrounded by a stronger membrane than that around the plasmodial veins, or to a stiffening of the protoplasm in the mounds.

Rising stage. The protoplasm in the mounds rose above the substrate to form pulsating peg-like structures (Pl. 1, fig. 4). After each forming fruit had reached about two-thirds of its final height, the acellular stalk began to become visible. The protoplasm which was to form the stalk constricted around the more internal protoplasm in a progressive fashion, beginning first at the bottom, so that eventually all of the more internal protoplasm was squeezed out at the top of the forming stalk. In the

time-lapse films the protoplasm, presumably as a continuation of the rhythmic streaming characteristic of plasmodia, continued to pulsate while it rose off the substrate.

White fruit stage. The protoplasm in the head of the fruit continued to pulsate for about an hour after the completion of the stalk. The fruit remained colourless, like the plasmodium, for several hours.

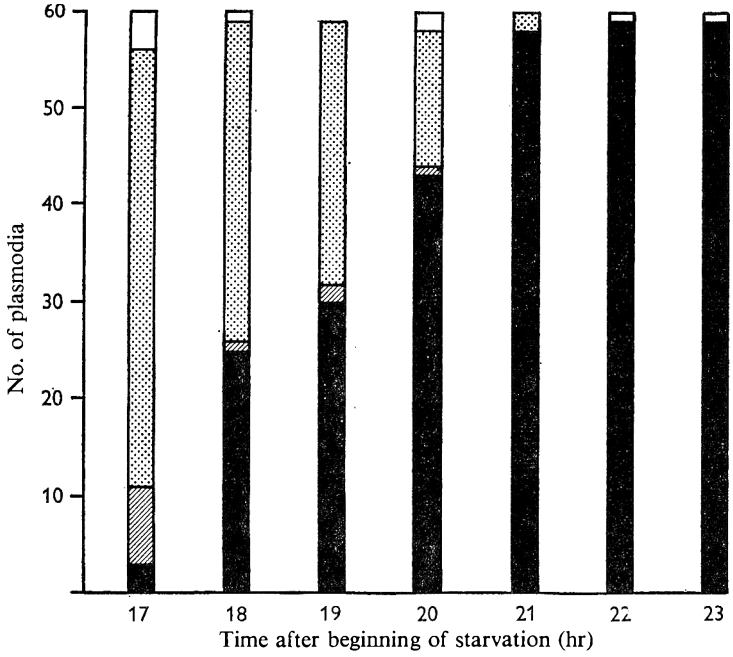

Fig. 1. *Didymium nigripes*: the commitment of plasmodia starved for varying periods of time to complete the fruiting process. □, Plasmodia neither grew vegetatively nor fruited; ▨, the entire plasmodium resumed vegetative growth; ▦, a portion of the plasmodium fruited, the remainder resuming vegetative growth; ■, the entire plasmodium differentiated into fruiting bodies.

Table 1. *Didymium nigripes: timing of the fruiting sequence*

Stage	Duration (hr) ± 1 S.E.
Advancing front stage	16–18
Accumulation	3·4±0·125
Mounding	1·6±0·0163
Rising	2·0±0·0126
White fruit	7·0
Pigment synthesis	2·5

Pigment synthesis stage. The pale-white fruiting body slowly turned pink. The colour gradually deepened in intensity to a brown, and finally to black, the colour of the mature fruiting body. This change in colour is due to the synthesis of a melanin-like material which is a structural element of the spore walls (personal communication from E. Haskins).

Through the use of the standardized transfer technique, all transfers from a single

plasmodium went through the above stages in relative synchrony. The average time spent in each stage by 60 transfers from at least 12 plasmodia, transferred on three separate days, is given in Table 1. To determine the time after which starving plasmodia could no longer revert to the vegetative state when placed in contact with a bacterial food supply, at timed intervals plasmodia were returned to GPY/5 agar spread with bacteria. The results are shown in Fig. 1. Three groups of 20 plasmodia were tested after each hour of starvation. Between 17 and 21 hr of starvation the percentage of plasmodia which were committed to form fruiting bodies increased steadily.

From the data given in Table 1 it can be seen that almost all of the variability in the time of fruiting occurred in the advancing front stage, which lasted 16–18 hr after the transfers to non-nutrient agar. Since the total time from the beginning of starvation to the completion of pigment synthesis was from 32 to 34 hr, this represents a variation of only 6% in the time of fruiting. This variation almost certainly was due to minor differences in the distance the parental plasmodium had grown across the GPY/5 agar plate before portions of plasmodium were transferred to starvation conditions. Pieces of plasmodium (1 cm.^{2}) were subcultured to one side of GPY/5 agar plates which had been spread with bacteria 2 days earlier. The plasmodia spent about 2 days moving across such a plate. Since plasmodia can ingest bacteria only at their leading edge, it is obvious that a recently subcultured plasmodium has substantially greater feeding interface per unit of protoplasmic mass than does a plasmodium which is more than half way across a growth plate. The number of undigested bacteria in food vacuoles must also be greater per unit of protoplasm in a recently subcultured plasmodium. If equal amounts of recently subcultured plasmodium or advanced plasmodia are transferred to plain agar, one would expect the advanced plasmodium to starve more quickly than the recently subcultured plasmodium. Hence the 2 hr variation in the length of the advancing front stage almost certainly represented minor differences in the nutritional state of the parental plasmodia.

A comparison of the data given in Table 1 with Fig. 1 indicates that advancing front stages were not yet irreversibly committed to form fruiting bodies. The mounding stage did not begin until 19·4 to 21·4 hr after the beginning of starvation, by which time over 60% of the plasmodia were already irreversibly committed to differentiate into fruiting bodies. The irreversible commitment to form fruiting bodies must, therefore, have taken place during the accumulation stage, at a time well before the actual site of fruiting body formation had been determined. This represents an example of a case in which differentiation precedes morphogenesis in a clear-cut manner. Although the data presented here were obtained from small, carefully chosen pieces of plasmodium, the various stages can easily be recognized under conditions where larger amounts of protoplasm, sufficient for analysis by techniques such as acrylamide gel electrophoresis, can be obtained.

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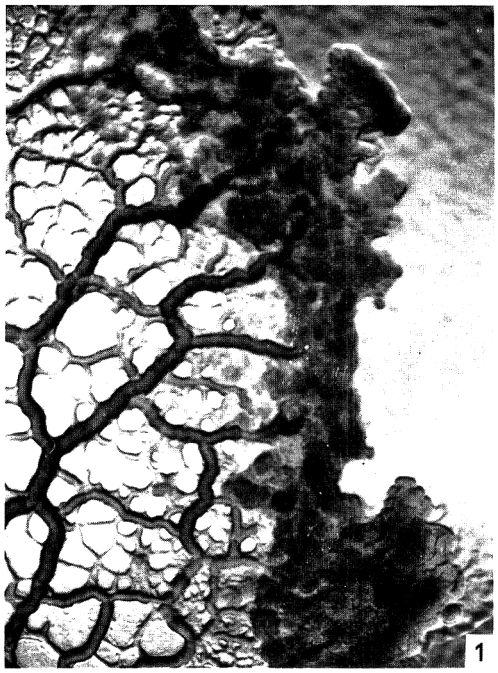
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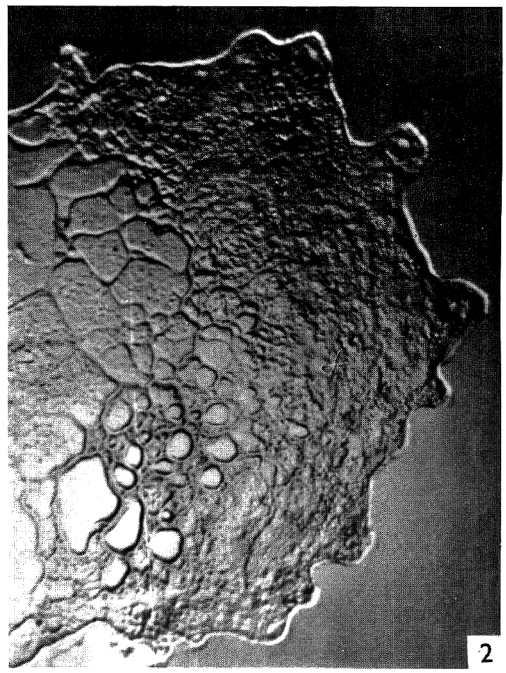
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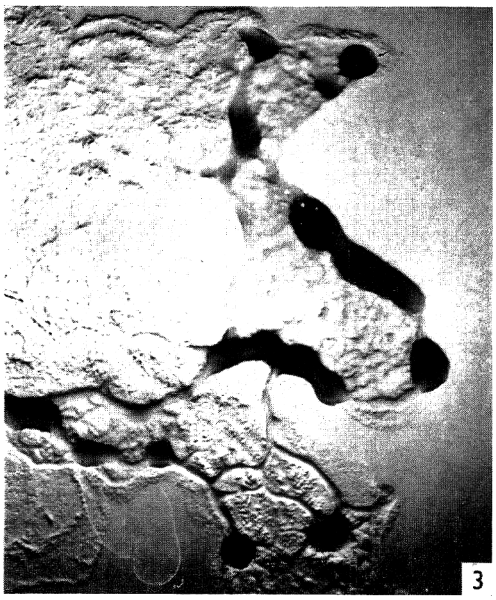
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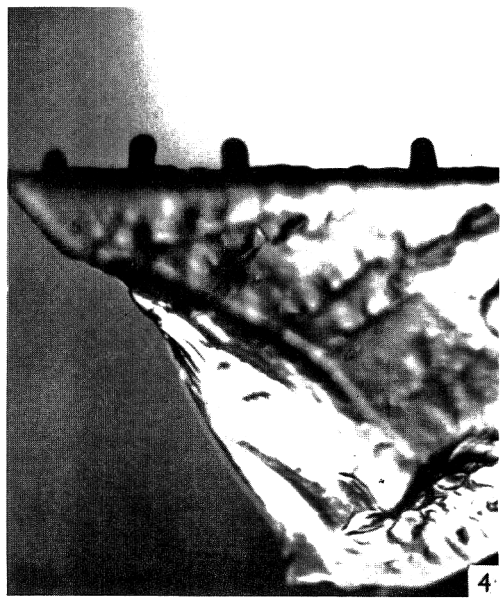
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EXPLANATION OF PLATE

Didymium nigripes: stages in the differentiation of plasmodia into fruiting bodies × 10.

Fig. 1. The advancing front stage. The plasmodium is still advancing across the agar.

Fig. 2. The accumulation stage. The plasmodium has ceased to advance across the agar and an accumulation of protoplasm has formed.

Fig. 3. The mounding stage. Mounds, still connected to one another by veins of streaming protoplasm, appear within the accumulation.

Fig. 4. The rising stage, side view. The presumptive fruiting bodies are shaped as the mounds rise above the agar surface.

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Lysis of the Limiting Membrane of *Mycoplasma gallisepticum* by Chemical Agents

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SUMMARY

Lysis of *Mycoplasma gallisepticum* by various chemical reagents and enzymes was examined. Lysis was measured by determining the release of intracellular constituents, and compared with the results obtained by the turbidimetric method. The ultracentrifuge was used to compare solutions of isolated membrane in 2-chlorethanol, phenol + acetic acid + water mixture, aqueous urea, and aqueous sodium lauryl sulphate, all of which readily dissolved membranes and whole organisms.

INTRODUCTION

Chemical lysis of the limiting membranes of several Mycoplasma species has been studied by Rodwell (1956), Smith & Rothblat (1960), Razin (1963), Razin & Argaman (1962, 1963) and Razin, Morowitz & Terry (1965). These membranes, which are thought to be three-layered lipoprotein membranes (see, for instance, Razin, 1963), were readily lysed by surface-active agents, alcohols and a lipase.

As a preliminary to studies of the macromolecules of *Mycoplasma gallisepticum*, lysis of its enveloping membranes by several chemical reagents was examined. An attempt was made to compare the turbidimetric methods of following lysis used by previous workers with a method which measures the release of intracellular constituents. The ultraviolet spectrum of the cell contents shows an absorption maximum at 260 mμ, due principally to nucleotide material; this provides a convenient method of determining the cell contents released on lysis. A quantitative index of lysis was sought by using extinction measurements at 260 mμ. Some mild reagents which lyse the organisms were used to dissolve isolated membranes, and membrane solutions were examined in the ultracentrifuge.

METHODS

Organism. Mycoplasma gallisepticum strain X95 (PG31) was grown on Difco PPLO broth supplemented with 20% normal horse serum and 10% (w/v) yeast extract; penicillin and thallium acetate were added to inhibit the growth of other organisms. After incubation at 37° for 3 days the cultures were concentrated by using a continuous-flow M.S.E. centrifuge and the concentrates sedimented at 6000 *g* for 30 min. at 2° in a refrigerated centrifuge (M.S.E. Ltd., London). The sediments were resuspended in sterile phosphate-buffered saline (0·15 M-phosphate, 0·85% (w/v) sodium chloride; pH 7·2; autoclaved) and recentrifuged three times. The final washed product was a thick paste of organisms containing 17–18% dry solids.

Lysis. For the turbidity and ultraviolet absorption experiments organisms were taken up in M-sucrose solution containing sodium chloride (0·05 M) and the suspensions shaken vigorously to break up aggregates. In a typical turbidity experiment a suspension of 8 mg. wet organism/ml. was made and 0·3 ml. of solution of lytic agent was added to each 3 ml. sample of Mycoplasma suspension. Extinctions were read at 500 mμ against appropriate controls after 1 hr, the suspensions being shaken before reading. For ultraviolet absorption experiments the concentration of organism was 2–3 mg. wet/ml. After standing with the reagents for 1 hr at 4°, suspensions were sedimented at 9000 ***g*** for 30 min. at 4° and extinctions read at 260 mμ. Proteolysis by pepsin (1 mg./ml.) was done in M-sucrose in 0·2 M-acetate buffer (pH 3·0) for 24 hr at 37° and the suspensions were then treated as above.

Isolation of cell membranes. Organisms were suspended in de-ionized water and disrupted ultrasonically at 20 Kc./sec. (60 W M.S.E.–Mullard ultrasonic oscillator), the disruption being done in an ice water bath. Ultrasonic treatment was followed by centrifugation at 6000 ***g*** for 30 min. at 2° to remove unbroken organisms; the membranes in the supernatant fluid were then collected by ultracentrifugation at 30000 ***g***.

Ultracentrifugation. This was done with schlieren optics in a Beckman Spinco Model E ultracentrifuge. Double sector optical cells were used to obtain a baseline and thus establish whether curvature was due to the solute or the solvent.

RESULTS

Qualitative examination of lytic reagents

Several reagents cleared suspensions of organisms rapidly, requiring no more than visual observation. Thus a suspension of organisms (5 mg. wt/ml.) in phosphate-buffered saline was cleared rapidly at room temperature by 1–1·5 vol. of phenol+acetic acid+water (2+1+0·5, w/v/v; Rottem & Razin, 1967), or by 0·5–1 vol. of 2-chlorethanol or 1 vol. of 16 M-urea in water. Although when these reagents were used occasional clumps of organisms remained unless the suspension had been very thoroughly shaken before adding the reagent, sodium lauryl sulphate caused complete lysis rapidly and consistently.

Reagents which did not cause rapid or complete lysis were examined with the help of the turbidimetric and ultraviolet absorption methods for following the lysis. The results obtained by the two methods were in agreement qualitatively. Sodium deoxycholate, saponin, digitonin, and a toxin from *Staphylococcus aureus* (known to possess lipase activity) all resulted in some degree of lysis. Pepsin in acetate buffer (pH 3·0) caused no more lysis during 24 hr at 37° than a control solution containing buffer but no enzyme. Aggregation and settling limited the reliability of the turbidimetric method of following lysis, but the ultraviolet absorption method did not suffer from these drawbacks, required only one third as much material, and was more sensitive to small degrees of lysis.

Quantitative comparison of lytic agents

The ultraviolet adsorption method was used for quantitative studies. Degree of lysis (%) was expressed in two ways:

$$\text{(A)}\quad (E_x/E_{SLS}) \times 100; \qquad \text{(B)}\quad 100 \times (E_x - E_c)/(E_{SLS} - E_c),$$

where E denotes the extinction at 260 mμ, and the suffix X represents the test solution, C the untreated control and *SLS* the suspension cleared completely by using sodium lauryl sulphate.

Expression (A) is analogous to that used by Razin (1963) in treating turbidimetric results. Expression (B) allows an estimate of the lysis by the reagent after correction for lysis by autolysis or physical handling. The amounts of lysis caused by some chemical reagents are set out in Table 1.

Table 1. *Extent of lysis of Mycoplasma gallisepticum, estimated by extinction at 260 mμ*

The concentrations refer to the reaction mixture. A = $(E_X/E_{SLS}) \times 100$ and B = $100 \times (E_X - E_C)/(E_{SLS} - E_C)$, where E is the extinction at 260 mμ and the suffixes X, C and *SLS* refer to test solution, untreated control, and sodium lauryl sulphate-treated sample respectively.

Reagent	A	B
Sodium lauryl sulphate 0·1 M	100	100
Control	15	0
Sodium dexycholate 0·1 M	80	75
n-Butanol 3 %	30	10
Cetyltrimethylammonium bromide	20	7
Saponin 50 μg./ml.	35	25
Digitonin 25 μg./ml.	35	25
EDTA 0·1 %	15	0
Magnesium chloride 0·2 M	15	0

Preparation of isolated membranes

To determine the optimum period for ultrasonic treatment, equal samples of a suspension of organisms in de-ionized water were treated ultrasonically for periods from 10 sec. to 30 min. An untreated control was used as well as a sample which was completely lysed with sodium lauryl sulphate. The ultraviolet absorptions of the preparations were examined after centrifugation. The results indicated that 20–30 min. ultrasonic treatment was necessary to approach complete lysis. Examination in the electron microscope of a centrifuge pellet (30,000 *g*, 1 hr) obtained after 30 min. ultrasonic treatment indicated that it consisted of membranes which did not appear to be very extensively degraded.

Dissolution of isolated membranes

Membranes dissolved readily in 0·01 M-sodium lauryl sulphate in water on shaking. When a solution of approximately 10 mg. wet wt membrane/ml. was examined in the ultracentrifuge a single peak was obtained, resembling that obtained by Razin (1967), but the sedimentation coefficient of 2·5 S was somewhat lower than the value of 3·0 S obtained by Razin.

Solutions of membranes in 2-chlorethanol or 8 M-urea in water showed no sedimenting components at rotor speeds up to 59,780 rev./min. Strongly curving baselines were however observed. A solution in phenol+acetic acid+water (2+1+0·5, w/v/v) showed several minor fast-moving components, and a strongly curving baseline, at rotor speeds of 40,000 rev./min. Membrane suspensions did not clear on extensive dialysis against de-ionized water.

DISCUSSION

Although results obained by turbidimetry were in qualitative agreement with those obtained by measuring the release of cell contents from the organisms, the latter technique was preferable for the quantitative measurement of lysis. Razin (1964) used an ultraviolet absorption technique in measuring osmotic lysis, which is not accompanied by much membrane dissolution. Lysis by some chemical agents may also occur without dissolution of the membranes; in such cases the turbidimetric method might be misleading.

The bulk of the work done previously on the chemical lysis of Mycoplasma has been concerned with the saprophytic *Mycoplasma laidlawii*. The present results indicate that the membrane of the parasitic *M. gallisepticum* is qualitatively similar to that of *M. laidlawii* in its behaviour towards various chemical reagents. Membranes dissolved in 2-chlorethanol, urea or phenol+acetic acid+water give patterns with base-lines which show strong curvature at the meniscus and cell bottom, indicating the presence of compounds of low molecular weight. These solvents thus do not appear to produce the same subunits as those obtained using sodium lauryl sulphate, suggesting that the situation may be more complex than that envisaged either by Razin *et al.* (1965) or by Engelman, Terry & Morowitz (1967).

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Protease Production by *Bacteroides amylophilus* Strain H 18

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SUMMARY

Bacteroides amylophilus strain H 18 produced protease(s), active at pH 7·0, when grown anaerobically in a simple maltose, ammonium sulphate, cysteine, sodium bicarbonate and salts medium. Protease production was neither induced nor repressed by a wide range of nutrients. Protease was synthesized by exponentially growing organisms and 20% of it was liberated into the growth medium. The cell-bound protease was completely accessible to the protein substrate. Protease production was proportional to ammonium sulphate and maltose concentration in the ranges in which these nutrients were growth limiting.

INTRODUCTION

The breakdown of dietary protein in the rumen is a process which has a significant effect on the retention of nitrogen by ruminants (Blackburn, 1965). Rumen micro-organisms are completely responsible for this proteolysis and bacteria have been shown to be actively implicated (Blackburn & Hobson, 1960*a*). Early attempts to isolate the bacteria effecting this protein breakdown resulted in the growth of facultative anaerobes (Appleby, 1955; Blackburn & Hobson, 1960*b*) but the application of more rigorous anaerobic methods resulted in the isolation of true rumen anaerobes which had considerable proteolytic activity (Blackburn & Hobson, 1962). Most of these bacteria were Gram-negative species, which was considered to be unusual since, with the exception of members of *Pseudomonas* and *Vibrio* genera (Pollock, 1962), most bacteria which produce exo-enzymes are Gram-positive. Protease production by Gram-negative anaerobic bacteria has been little investigated; its study thus seemed to be of general interest and it was also hoped that knowledge of the mode of action of these proteases might enable protein hydrolysis in the rumen to be controlled. *Bacteroides amylophilus* was selected for investigation principally because it rapidly hydrolyses casein in growth media, but it also has other features of interest. Most of the strains examined produced relatively small quantities of free amino acid from protein, indicating the absence of peptidases, and in common with other proteolytic rumen bacteria they appeared to utilize ammonia in preference to preformed amino acids and peptides (Abou Akkada & Blackburn, 1963). The growth requirements of *B. amylophilus* are very simple and rumen fluid is not essential for its growth, but it has an absolute requirement for maltose or for α-1,4-linked glucose polymers (Blackburn & Hobson, 1962). It produces an active amylase and its capacity to hydrolyse casein is not diminished by subculture. In practical terms the object of this work was to define the conditions for the growth of *B. amylophilus* which would give maximum protease production, so that the proteolytic enzyme(s) might be studied in more detail. To define these conditions the following questions had to be answered: is

protease production inducible or repressible? At what stage of the growth cycle is proteolytic activity produced, is it liberated free into the growth medium, is it cell bound or is it located intracellularly? If it is liberated free into the growth medium is its appearance associated with cell lysis?

METHODS

Organism. Bacteroides amylophilus strain H18 was isolated from a dilution of $1/10^8$ in a roll tube of casein + rumen fluid medium (Blackburn & Hobson, 1962), from sheep no. 6 (Blackburn & Hobson, 1960*c*). This strain was identical to previous isolates (Blackburn & Hobson, 1962) and conformed to the description of the type species (Hamlin & Hungate, 1956).

Media. The basal medium, based on that of Hungate (1950), contained (g./l.): K_2HPO_4, 0·45; KH_2PO_4, 0·45; $(NH_4)_2SO_4$, 0·9; NaCl, 0·9; $MgSO_4$, 0·09; $CaCl_2$, 0·09; resazurin, 0·001; L-cysteine hydrochloride, 0·5; $NaHCO_3$, 5·0. The mineral and resazurin solutions plus any other additions to the medium were placed in a screw-capped bottle and the volume made to 900 ml. with distilled water. The medium was autoclaved at 120° for 15 min. and the cap screwed tight immediately on removal from the autoclave; this resulted in a partial vacuum in the bottle when it had cooled. Autoclaved 1·0% (w/v) L-cysteine hydrochloride (50 ml.) and 10% (w/v) sodium bicarbonate solution (50 ml.) which had been sterilized by heating to 100° were added to the medium under a stream of oxygen-free CO_2. The medium could then be dispensed into tubes closed with rubber-bungs; this and subsequent steps were done under an atmosphere of CO_2. Other media constituents were added at 3·0 g./l., except for casein (5·0 g./l.) and agar (25·0 g./l.). Yeast extract (Difco) and Tryptose (Bacto) were from Bacto Laboratories, Detroit, U.S.A.; vitamin-free acid hydrolysed casein (Casamino acids) from Allen and Hanbury Ltd., London, E. 2; soluble starch and maltose were AR grade from B.D.H. Ltd., Poole, Dorset; proteose peptone from Oxoid, London, E.C. 4; cysteine hydrochloride from L. Light Ltd., Colnbrook, Bucks.; corn steep liquor (MX9A) from Brown and Polson, Trafford Park, Manchester 11. Casein was prepared from Glaxo casein C (Glaxo Ltd., Greenford, Middlesex) by the acid precipitation method of Blackburn & Hobson (1962). Throughout this paper media are named by their additional constituents, thus maltose medium refers to basal medium + 0·3% (w/v) maltose.

Maintenance. A large number of lyophilized suspensions of *Bacteroides amylophilus* H18 was prepared, the suspending fluid being 10% (w/v) maltose in sterile horse serum. The bacteria have remained viable for 4 years. The usual drying medium containing 10% (w/v) glucose did not yield viable dried bacteria. Stock cultures were maintained on slopes of casein maltose agar medium. Transfers were made each week to fresh slopes by stab inoculation. All cultures were grown at 38° and inocula generally consisted of 0·01 vol. of log phase bacteria in maltose medium.

Concentration of bacterial cultures. The concentration of bacterial growth was read on an EEL nephelometer (Evans Electroselenium Ltd., Halstree, Essex) set to read 100 against an arbitrary standard of glass fragments suspended in agar. EEL readings were converted into equivalent dry weights of bacteria from a calibration curve, 100 EEL units = equiv. 0·49 mg. dry wt bacteria/ml.

Assay of proteolytic activity. The assay procedure was a modification of the Anson

(1938) method. Enzyme solution (1 ml.) was incubated at 38° with 4·0 ml. of 2·0% (w/v) casein, 0·01% thiomersalate, in 0·1 M-KH_2PO_4 adjusted to pH 7·0 with NaOH. Immediately after adding the enzyme 2·0 ml. of the digest was removed and mixed with 2·0 ml. of 0·72 N-trichloroacetic acid. The remaining digest was incubated for up to 24 hr when a further 2·0 ml. was precipitated by 2·0 ml. of 0·72 N-trichloroacetic acid. At least 30 min. were allowed for the tubes to stand at room temperature and precipitated protein then filtered off on Whatman no. 42 paper. The soluble tyrosine was estimated by adding 1·0 ml. of filtrate to 5·0 ml. of 0·55 M-sodium carbonate and making up to 10·0 ml. with distilled water. One ml. of 1/3 Folin–Ciocalteu reagent (B.D.H. Ltd., Poole, Dorset) was added and mixed in rapidly. The tubes were incubated at 38° for 30 min. and extinctions were read at 650 mμ on an EEL Spectra colorimeter (Evans Electroselenium Ltd., Halstree, Essex). The extinction at zero time was subtracted from the extinction of the test and the value compared with a standard tyrosine curve; 1 μg. tyrosine was equivalent to E_{650} 0·0061. The plot of extinction against enzyme concentration was linear up to E_{650} 0·25 (Fig. 1); extinctions higher than this were corrected to the corresponding value on the extrapolation of the linear portion of the curve. The assay of trypsin ($\times$ 2 crystallized, salt free, from L. Light and Co. Ltd., Colnbrook, Bucks.) at pH 8·0 under similar conditions gave a similar curve. It was calculated that 1·0 ml. of the culture (Fig. 1) contained proteolytic activity equivalent to 0·91 μg. trypsin. It was because of this relatively low activity that long incubations had to be used. When assaying the proteolytic activity of whole cultures or of supernatant fluids of cultures it was necessary to oxidize the cysteine with iodine, with starch as an indicator (Tracey, 1948). This decreased the blank value considerably and gave more reliable results, since cysteine was oxidized to some extent in the enzyme digest but to a lesser extent in the zero time control. A unit of proteolytic activity was defined as the amount of enzyme which would solublize the equivalent of 1·0 μg. tyrosine in 1 min. Different times of incubation were used, up to 24 hr for preparations of low proteolytic activity, but there was a linear relationship between time of incubation and tyrosine solublized.

RESULTS

Protease assay

Proteolytic activity was measured at pH 7·0 but there was evidence (Fig. 2) that two proteolytic enzymes with optima at pH 6·5 and 8·0 were produced. The relative proportions of these two enzymes varied in different cultures and often there appeared to be a broad optimum from pH 6·5 to 8·0.

Ammonia dependence

The nutritional requirements of *Bacteroides amylophilus* H 18 are very simple and neither growth nor protease production was stimulated by adding to the maltose medium any of the following substances (Bryant & Robinson, 1962): thiamine HCl, Ca D-pantothenate, nicotinamide, haemin, riboflavin, pyridoxal (2 mg./l. each); *p*-aminobenzoic acid (1 mg./l.); biotin, folic acid, DL-thioctic acid (50 μg./l. each); cobalamin (20 μg./l.); sodium acetate.$3H_2O$ (3·06 g./l.); sodium isobutyrate (17 mg./l.); sodium valerate, sodium isovalerate, DL-α-methyl-*n*-butyrate (19 mg./l. each). The addition of rumen fluid (20%, v/v) or corn steep liquor (0·01–5·0%, v/v) was also

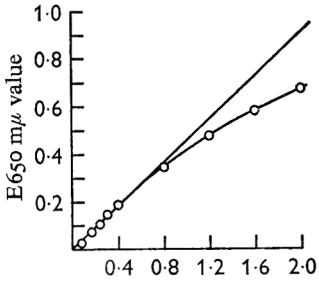

Fig. 1

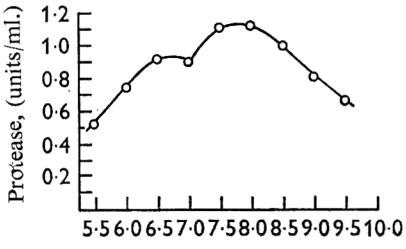

Fig. 2

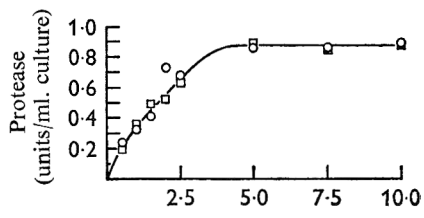

Fig. 3

Fig. 1. Protease concentration curve. The digests contained 80·0 mg. casein, 40·0 μg. thiomersal and 0·4 mmoles KH_2PO_4 adjusted to pH 7·0 with NaOH. An 18 hr culture of *Bacteroides amylophilus* H18 in maltose medium was added in amounts up to 2·0 ml. and the final volumes were adjusted to 5·0 ml. The digests were incubated at 38° for 20 hr and the trichloroacetic acid-soluble tyrosine was assayed as described in Methods.

Fig. 2. Protease activity of *Bacteroides amylophilus* H18 protease as affected by pH. One ml. amounts of an 18 hr culture of *B. amylophilus* H18 in maltose medium were incubated with 80·0 mg. casein, 40·0 μg thiomersal and 0·4 mmoles tris adjusted to the correct pH with maleic acid (pH 5·5–7·0) or HCl (pH 7·5–10·5). The final volume of the digest was 5·0 ml. and the proteolytic activity was assayed as described in Methods.

Fig. 3. Ammonium sulphate concentration and protease production by *Bacteroides amylophilus* H18. Ammonium sulphate was omitted from the basal medium and was added as a sterile solution to the tubed maltose (○—○) and maltose-tryptose medium (□—□) in the concentrations shown. The tubes (20 ml.) were inoculated with a loop from a young culture of *B. amylophilus* H18 in maltose medium. The tubes were incubated for 18 hr. and the protease activity assayed as described in Methods.

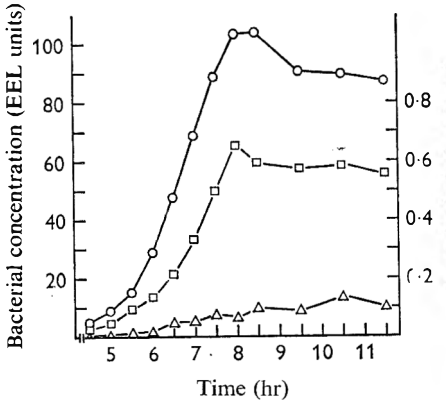

Fig. 4

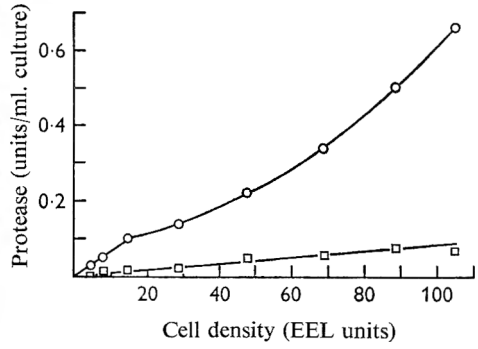

Fig. 5

Fig. 4. Rate of protease production by *Bacteroides amylophilus* H18. 250 ml. maltose medium was inoculated with 2·0 ml. of 18 hr culture of *B. amylophilus* H18 grown in maltose medium. Ten ml. amounts were removed at time intervals and the cell densities were read (○—○). The proteolytic activity of the whole culture (□—□) and of the cell free supernatant (29,000 *g* 10 min.) (△—△) were determined as described in Methods.

Fig. 5. The relationship between cell density and protease production. The data from Fig. 4 are replotted, total protease (○—○) and cell-free protease (□—□).

without effect. Growth and protease production were dependent on the presence of ammonium ions in the growth media. Figure 3 shows that protease production in maltose medium was proportional to ammonium sulphate concentration up to 3·5 μ-moles $(NH_4)_2SO_4$/ml. medium when maltose became limiting; tryptose did not replace ammonium sulphate as a growth factor. The bacterial concentrations showed a similar relationship with ammonium sulphate concentration but were more irregular due to the tendency of *B. amylophilus* to lyse when the bacteria ceased to multiply. The relative rates of production of bacteria and protease are better illustrated in Figs. 4 and 5. Figure 4 shows plots of bacterial concentration, total and free protease, against age of culture in maltose medium. Protease production began early in the logarithmic phase of growth and continued until the bacteria ceased to divide. The cell-free protease constituted at a maximum 20% of the total protease and no immediate increase in free protease was associated with the decrease in bacterial concentration. The data in Fig. 4 are replotted in Fig. 5 to show more clearly the relationship of bacterial concentration and protease production. The cell-free protease was proportional to the bacterial concentration but the total protease was not, especially during the mid-logarithmic growth phase. The proportion of cell-free (29,000 *g*; 10 min.) protease increased in cultures on further incubation to 42, 50 and 87% after 24, 48 and 72 hr, respectively. The total protease, after 72 hr, had fallen to 0·31 protease units/ml. which showed a loss of 54% of the highest activity observed. In the logarithmic phase of growth most of the activity was associated with the intact bacteria. Treatment with toluene or ultrasonic disintegration did not result in an increase of protease, indicating that all the protease was superficially located at the bacterial surface and directly accessible to the casein molecules.

Table 1. *Effect of organic nitrogen sources on growth and protease production of Bacteroides amylophilus H18*

Media compositions and protease assay are described in Methods. The media were incubated for 24 hr and centrifuged 29000 *g* for 10 min. Unhydrolysed casein was measured by a Biuret method (Layne, 1957). Cell densities could not be measured in casein-containing media, due to opacity.

	Medium							
	Maltose	Maltose tryptose	Maltose proteose-peptone	Maltose Casamino-acids	Maltose casein	Maltose Tryptose casein	Maltose Proteose-peptone casein	Maltose Casamino-acids casein
Total protease (units/ml.)	0·62	0·72	0·63	0·27	0·63	0·67	0·68	0·36
Total protease in cell-free supernatant (%)	44	39	50	21	25	28	29	20
Casein hydrolysed (%)	—	—	—	—	80	83	83	87
Cell density (EEL units)	77	60	96	49	—	—	—	—

Effect of organic nitrogen

It was observed (Fig. 3) that the presence of tryptose did not stimulate protease production, nor did mixtures of tryptose, proteose peptone and Casamino acids with casein (Table 1). The bacterial concentrations in 24 hr cultures have little significance

but there was definite inhibition of growth by 0·3 % (w/v) Casamino acids. In experiments not reported this inhibition by 0·3 % (w/v) or more Casamino acids was confirmed, while lower concentrations were without effect in influencing growth and protease production. A considerable proportion of the casein originally present (50 mg./ml. growth medium) was hydrolysed in all the media, irrespective of their composition. That tryptose and proteose peptone were without effect on growth rate and protease production is illustrated in Fig. 6. The mean generation time in all media was 68 min. The addition (5 g./l.) of glutamic acid, aspartic acid, lysine or arginine did not allow *Bacteroides amylophilus* H18 to grow in the basal medium and had no effect on growth or protease production in the maltose medium.

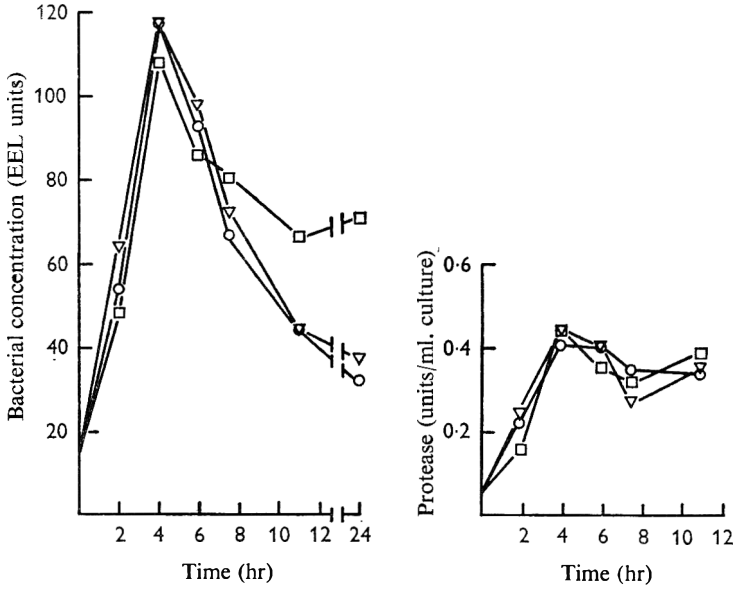

Fig. 6. Protease production by *Bacteroides amylophilus* H18. Three 20 ml. 16 hr cultures of B. *amylophilus* H18 in maltose medium were sedimented at 3000 *g* for 10 min. and the cells inoculated directly into 100 ml. bottles of: maltose medium (□—□), maltose-tryptose medium (○—○) maltose-proteose and peptone medium (▽—▽). The bottles were incubated and samples removed for cell density and protease determinations as described in Methods.

Table 2. *The fermentation products of Bacteroides amylophilus H18*

The four media were inoculated and incubated for 24 hr. The fermentation products were separated and assayed by the method of Wiseman & Irwin (1957).

	Medium			
Products	Maltose	Maltose Tryptose	Maltose casein	Maltose Tryptose casein
Acetic acid (μmoles/ml.)	11·7	15·9	11·7	12·7
Formic acid (μmoles/ml.)	11·5	11·4	8·1	14·4
Succinic acid (μmoles/ml.)	14·2	13·5	12·1	12·8

Maltose utilization

The final products of protein degradation in the rumen include branched- and straight-chain volatile fatty acids (Blackburn, 1965) but the data in Table 2 do not suggest that tryptose or casein were degraded in this way by *Bacteroides amylophilus* H 18, as no increment was found in acetic or formic acid concentrations in media con-

Table 3. *Effect of maltose concentration on growth yields and protease of Bacteroides amylophilus H 18*

Maltose was added as a sterile filtered solution to the basal medium to give the concentrations indicated below. The maltose concentration before and after 16 hr growth of *B. amylophilus* was measured by the anthrone method of Fairbairn (1953). The protease assay is described in Methods. The dry weight of cells was calculated from the cell density.

Initial maltose concentration (mg./ml.)	Final maltose concentration (mg./ml.)	Maltose used (mg./ml.)	Cell density (EEL/ units)	Protease (units/ml.)	Cell yield (g. cells/ mole maltose)	Protease yield (units/mg. maltose)
1·16	0·32	0·84	20	0·01	39·8	0·01
2·30	0·46	1·84	41	0·39	37·4	0·21
3·21	0·48	2·73	51	0·65	31·4	0·24
4·88	1·38	3·50	100	0·74	47·6	0·21
6·50	1·90	4·60	102	0·92	37·2	0·20
7·62	3·60	4·02	98	0·78	40·8	0·19
8·89	4·83	4·06	100	0·82	40·6	0·20
9·45	5·85	3·60	101	0·78	47·0	0·22

taining tryptose or casein, and no other volatile fatty acids were produced. Carbon dioxide was an essential growth factor for *B. amylophilus* H 18 and the fermentation products suggest that: maltose + $2CO_2$ → 2 acetic acid + 2 formic acid + 2 succinic acid. Yields of bacteria and protease were proportional to the amount of maltose fermented and the total yield of both, expressed per ml. culture, increased up to a maltose concentration of 0·6% (w/v) (Table 3). The average yield of bacteria was equivalent to 40 g. dry wt./mole maltose. A high concentration of maltose remained unfermented even where it was growth-limiting.

Utilization of other energy sources

Growth and protease production occurred to the same extent in starch medium as in maltose medium and the addition of casein, tryptose or Casamino acids did not stimulate further protease production. Casein was hydrolysed in starch media and autolysis of the bacteria also occurred. No energy source other than α-1,4-linked glucose polymers was found to support growth, and the presence of α-oxoglutaric acid, pyruvic acid, succinic acid, acetic acid, butyric acid or formic acid (5 g./l.) did not affect growth or protease production.

Bacteroides amylophilus H 18 produced small colonies in roll tubes of casein agar medium containing maltose at 0·2 g./l. These colonies were surrounded by a dense zone of opacity and when transferred to casein maltose slopes they produced a similar dense opacity in the casein. A total of five transfers on slopes of this medium did not produce an inoculum capable of growth in a liquid medium of the same composition.

Effect of pH value of medium

The pH value of the basal medium, which contained 0·5 % (w/v) $NaHCO_3$, was pH 6·7 when equilibrated with a 100 % CO_2 gas-phase. The buffering capacity of this medium was very poor and *Bacteroides amylophilus* H 18 when supplied with a surplus of maltose, lowered the medium to pH 5·5 after 26 hr. As shown in Table 4 the presence of twice the usual $NaHCO_3$ concentration resulted in an increase to only pH 6·9, but after 26 hr the pH value had decreased less than in the normal medium. The protease yields in this experiment were very low and the yield at 26 hr was not greatly increased by the additional buffering of the medium, but on further incubation the yield almost doubled in the well buffered medium while it fell in the normal medium.

Table 4. *Effect of additional buffering on protease production*

2·0 % maltose was used instead of the usual 0·3 % to ensure that maltose was not growth limiting. Twice the normal concentration of $NaHCO_3$ was added to one medium. 100 ml. amounts of the media were inoculated and samples withdrawn at 26 and 52 hr for pH, cell density and protease determinations, as described in Methods.

	Basal medium + 2·0 % (w/v) maltose			Basal medium + 2·0 % (w/v) maltose + additional 0·5 % (w/v) $NaHCO_3$		
Hr	pH	Cell density (EEL units)	Protease (units/ml.)	pH	Cell density (EEL units)	Protease (units/ml.)
0	6·7	0	0	6·9	0	0
26	5·5	94	0·20	6·4	93	0·25
52	5·5	88	0·18	5·3	122	0·47

Effect of E_h value of media

The addition of $Na_2S.9H_2O$ (0·025 %) to the maltose medium (containing cysteine) did not affect growth or protease production. A similar result was obtained when sodium dithionite (0·003 %) was used. The concentration of sodium dithionite used reduced phenosafranine; the measured reduction potential of this medium was −410 to −470 mV (Pt and calomel electrodes).

Bacteroides amylophilus H 18 grew in media in which cysteine was replaced by sodium dithionite or by sodium sulphide + ascorbic acid (0·05 %). L-Methionine (0·05 %) could not replace cysteine in media reduced by ascorbic acid. Protease production was similar in all media in which growth occurred.

Effect of cations in media

A fivefold increase in the normal concentration of Ca^{2+} or of Mg^{2+} did not increase either growth or protease production by *Bacteroides amylophilus* H 18. Further supplementation of the maltose medium with similar concentrations (0·45 g./l.) of $MnCl_2$, $CoCl_2$ or $FeSO_4$ was without effect.

DISCUSSION

The curve for activity against pH of the *Bacteroides amylophilus* protease suggested the presence of two enzymes, although Matsubara *et al.* (1958) showed that the crystalline protease of *Bacillus subtilis* strain N has an irregular plateau of activity

from pH 7·0 to pH 11·0. Production of total protease began with bacterial division and ceased when growth stopped and a small, but constant, proportion (20%) of the protease was released into the medium. This may be truly extracellular (Pollock, 1962) as was the extracellular pH 5·7 micrococcus protease of McDonald (1965). However, on prolonged incubation the total protease content of a culture decreased but the proportion of cell-free protease increased and this and the very sharp decrease in culture density at the end of logarithmic growth, a characteristic of many rumen bacteria (Bryant & Robinson, 1961), might indicate that a proportion of bacteria were lysing during growth and that this became apparent only when growth ceased.

The protease production was not subject to either product repression or induction since protein, peptides or amino acids in the medium did not influence its formation, nor was a general metabolic repression demonstrated. The growth requirements of *Bacteroides amylophilus* are simple and additional nutrients did not affect either growth or protease production. The observations of Hobson, McDougall & Summers (1968) that some 93% of the cell nitrogen could be accounted for by ammonia-^{15}N disappearance in media with and without additional identified sources of nitrogen confirmed the results of Abou Akkada & Blackburn (1963) and explain the fact that additional organic nitrogen sources did not influence growth, except that the presence of 0·1% (w/v) tryptose in the medium did decrease the lag period, probably by stabilizing the E_h value.

The products of maltose fermentation, acetic, formic and succinic acids were those found by Hamlin & Hungate (1956). The absolute dependence of *Bacteroides amylophilus* H18 on maltose or starch as energy source made it impossible to investigate the effect of different energy sources on protease production. Presumably the cell is impermeable to a wide range of energy sources but is permeable to α-1,4-linked polymers of glucose as is the *Micrococcus* sp. ATCC407 (Williams & McDonald, 1966). *Bacteroides amylophilus* H18 lacks the capacity to utilize maltose at concentrations less than 0·32 mg. maltose/ml. Hobson & Summers (1967) also noted this property of *B. amylophilus* H18, and to a lesser extent *Ruminococcus albus* (Hungate, 1963) showed an impaired ability to concentrate, in this case, cellobiose.

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The Protease Liberated from *Bacteroides amylophilus* Strain H18 by Mechanical Disintegration

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SUMMARY

Disintegration of lyophilized *Bacteroides amylophilus* H18 suspended in water, by agitation with glass beads, gave an extract containing 40% of the total cell protease activity. Better yields of an esterase which hydrolysed *p*-toluenesulphonyl-L-arginine methyl ester (TAME) were obtained by disintegration in 0·1 M-phosphate buffer (pH 7·0). Ultrasonic disintegration of fresh suspensions was used to obtain larger quantities of cell extract. The protease had a broad plateau of activity between pH 5·5 and 9·5; the esterase had maximum activity at pH 8·0. Divalent cations had relatively little effect on either activity but 5×10^{-3} M-$CaCl_2$ restored activity to EDTA-inhibited esterase. The protease was not inhibited by EDTA. Both activities were inhibited by di-isopropylphosphofluoridate but the protease was incompletely inhibited (87%). Neither activity was activated nor inhibited by thiol reagents. In addition to TAME, *N*-α-benzoyl-L-arginine methyl ester (BAME), *N*-α-benzoyl-L-arginine ethyl ester (BAEE), *N*-α-benzoyl-DL-arginine-*p*-nitroanilide and lysine ethyl ester were hydrolysed, indicating a trypsin-like specificity. The esterase differed from trypsin in not hydrolysing N-α-benzoyl-L-arginine amide (BAA) nor *N*-α-benzoyl-DL-arginine-naphthylamide (BANA) and in hydrolysing BAME more rapidly than TAME. There was some hydrolysis of *N*-α-benzoyl-L-leucyl-2-naphthylamide and some amino peptidase activity as shown by the hydrolysis of L-analyl-2-naphthylamide, L-leucylglycine and L-leucinamide. TAME competitively inhibited at least 64% of the protease activity. The K_m for casein was 0·17% (w/v). Casein at concentrations greater than 3·0% (w/v) caused substrate inhibition. The rate of 'tyrosine' liberation was proportional to protease concentration provided that less than 0·5 mg. 'tyrosine' was liberated from the 80 mg. casein in the standard assay. Protease concentration to the power 2/3 or the power 1/2 was proportional to the rate of hydrolysis but the straight line did not go to the origin.

Bacteroides amylophilus H18 extracts contained much nucleic acid which could not be separated from the protease activity by ammonium sulphate, protamine sulphate nor manganese chloride precipitation. Continuous electrophoresis was also ineffective. Ion-exchange chromatography separated the nucleic acids, but the protease activity was scattered in so many fractions that the purification was only three-fold and the recoveries poor.

INTRODUCTION

Blackburn (1968) showed that proteolytic activity at pH 7·0 was produced by exponentially growing *Bacteroides amylophilus* strain H18 in a simple maltose, cysteine, sodium bicarbonate/CO_2, ammonia and salts medium. Protease production was apparently not subject to any form of metabolic control and only 20% of the

protease was released into the medium in the exponential growth phase. Since preliminary experiments showed that this small amount of free protease could not satisfactorily be precipitated by ammonium sulphate, the protease attached to the bacteria was selected for examination. At first lyophilized bacteria were used as source of protease, which was liberated by disintegration of a suspension of bacteria in water or in buffer. In later experiments soluble protease was obtained from ultrasonically disintegrated suspensions of fresh bacteria. The proteolytic properties of extracts obtained in this way were examined to determine the optimum conditions for protease assay. The effect of activators and inhibitors, the range of synthetic substrates hydrolysed, the kinetics of casein hydrolysis and some methods of purification were investigated.

METHODS

Strain. Bacteroides amylophilus strain H18 was isolated by Blackburn (1968) and was maintained as described in that paper.

Media. The preparation and composition of the media were described by Blackburn (1968). The basal medium contained (g./l.): K_2HPO_4, 0·45; KH_2PO_4. 0·45; $(NH_4)_2SO_4$, 0·9; NaCl, 0·9; $MgSO_4$, 0·09; $CaCl_2$, 0·09; resazurin, 0·001; L-cysteine hydrochloride, 0·5; $NaHCO_3$, 5·0. Maltose medium contained maltose 3·0 g./l.; maltose tryptose medium contained maltose and tryptose, each 3·0 g./l. Five-litre glass flasks containing 5 l. growth medium were inoculated each with 50 ml. young culture of *Bacteroides amylophilus* H18 and were grown anaerobically under CO_2 for 18 hr at 38°. The bacteria were sedimented in a Sharples (Sharples Centrifuges Ltd., Stroud, Glos.) continuous-flow rotor at 50,000 rev./min. and at a rate of 1 l./min. The bacteria were removed from the rotor, suspended in water, again centrifuged down, and then either stored at −20°, disintegrated ultrasonically or lyophilized.

Disintegration by glass beads. A suspension (8 ml.) of lyophilized bacteria (20·0 mg./ml.) were shaken with 4·0 ml. ballotini beads in a Mickle disintegrator (H. Mickle, Hampton, Middlesex) at maximum amplitude for 15 min. at about 20°. Almost complete disintegration resulted. The disintegrated bacteria were separated from the glass beads which were retained on a coarse sintered-glass filter. The glass beads were washed with the 8·0 ml. suspending solution. The cell debris was sedimented at 20,000 *g* at 4° and the supernatant fluid taken to be a soluble extract.

Ultrasonic disintegration. Suspensions of bacteria were disintegrated ultrasonically either with M.S.E. 60 W. (Measuring and Scientific Equipment Ltd., Spencer Street, London), 20 kcyc. disintegrator in 10 ml. batches for 3 min. at 0°, or with the Dawe Soniprobe Type 1130 A (Dawe Instruments Ltd., Acton, London) in 25 ml. batches in the continuous flow head with the probe cooled to 13°. The disintegrated suspension was centrifuged at 35,000 *g* for 1 hr at 4° and the supernatant fluid removed. Thiomersal (1 mg./100 ml.) was added as a preservative and the extract retained at 4° for 7 days or indefinitely at −20° without loss of activity. Some extracts were lyophilized and retained in vacuum at −20°, without loss in activity.

Protease assay. Protease activity was assayed at pH 7·0 by the method of Anson (1938) as modified by Blackburn (1968), with denatured casein as substrate. One unit of proteolytic activity was defined as the amount of enzyme which would liberate the equivalent of 1·0 μg tyrosine from casein in 1 min. Specific activity was defined as protease units/mg. N (Kjeldahl). A modification of the standard assay procedure was

used to determine protease activity in fractions from electrophoretic and chromatographic separations. A portion of the fraction (0·2 ml.) was removed and incubated with 2·0% casein (1·8 ml.) for 0·5 to 24 hr depending on the activity of the fraction. Trichloroacetic acid solution (0·72 N; 2·0 ml.) was added and the tube centrifuged at 2000 *g* for 10 min.; 1 ml. of the supernatant fluid was then assayed for tyrosine. No enzyme blanks were used in these assays, only a casein-reagent control tube.

Assay of non-trichloroacetic acid-precipitable peptide. Peptide was measured in trichloroacetic acid filtrates by a modification of the method of Lowry, Rosebrough, Farr & Randall (1951) in order to neutralize trichloroacetic acid, as suggested by McDonald & Chen (1965). One ml. 0·5% (w/v) $CuSO_4.5H_2O$ in 1·0% (w/v) sodium potassium tartarate was added to 50 ml. 2·0% Na_2CO_3 in 0·2 N-NaOH; 5·0 ml. of this mixture was added to 1·0 ml. trichloroacetic acid filtrate. This tube was incubated at 38° for 30 min. and 1·0 ml., 1·0 N-Folin & Ciocalteu reagent was added (B.D.H. Ltd., Poole, Dorset) with rapid mixing, the tube was incubated for a further 15 min. and the extinction read at 700 mμ. Crystalline bovine serum albumin (Armour Laboratories) was used as a standard.

Casein-precipitating activity. Casein-precipitating activity was measured by the method of Sandvik (1962). Agar plates were poured, 2 mm. thick, of the following composition: 0·5% casein, 1·5% agar, 10^{-3} M-$CaCl_2$ and thiomersal, 1 mg./100 ml. Holes 7·0 mm. diam. were cut in the agar with a cork borer. Doubling dilutions in water of the enzyme preparation were made, and 0·02 ml. from each dilution placed in a hole in the agar. The plates were incubated for 20 hr at 38° and the zones of precipitation in the casein were measured. One unit of casein-precipitating activity was defined as that amount of enzyme which would give zone of 0·5 mm. around the agar well. Thus when a 1/64 dilution gave a 0·5 mm. zone the preparation would contain 64 units. A plot of $\log_{10}$ concentration in casein-precipitating units against zone diameter gave a straight line and was used to determine the casein-precipitating activity in unknown preparations, from the diameter of the zone of precipitation.

Esterase assay. Esterase activity was measured by the method of Schwert, Neurath, Kaufman & Snoke (1948) by using a Radiometer TTTIC pH-stat (Radiometer Ltd., Copenhagen, Denmark). The digest contained 0·1 M-ester substrate (1·0 ml.), water (8·0 ml.) and enzyme preparation (1·0 ml.) and was incubated at 25° under N_2. Titration was with 0·1 N-NaOH from an 0·5 ml. micrometer syringe.

Peptidase assay. Peptidase activity was measured by the method of Smith (1955) with Mn^{2+} as activator and titration of the free carboxyl by the method of Grassman & Heyde (1929).

Amidase assay. Amidase activity was measured by the method of Schwert *et al.* (1948), ammonia by the method of Conway (1957).

Hydrolysis of N-α-benzoyl-L-arginine-p-nitroamilide (BAPA). The hydrolysis of BAPA was measured by the method of Erlanger, Kokowsky & Cohen (1961).

Hydrolysis of 2-naphthylamides. This was measured by coupling the free 2-naphthylamine with fast garnet GBC (George T. Gurr Ltd., London, S.W. 6) as described by Hopsu & Glenner (1963).

$MnCl_2$ precipitation of nucleic acids. M-$MnCl_2$ (0·05 vol.) was added to the extract of *Bacteroides amylophilus* H18 at 4° and the precipitate removed by centrifugation at 10,000 *g* at 4° (Korkes, del Campillo, Gunsalus & Ochoa, 1951).

Protamine sulphate precipitation of nucleic acids. The addition of protamine sulphate

(Sigma London Chemical Company Ltd., London, S.W. 6) in water (1·5 %, w/v) to dialysed extract of *Bacteroides amylophilus* H 18 at 4° was continued until no further flocculation occurred (Heppel, 1955). The precipitate was removed by centrifugation at 10,000 *g*.

Ammonium sulphate precipitation. Solid ammonium sulphate was added to the extract of *Bacteroides amylophilus* H 18 at 4°, precipitate centrifuged down at 20,000 *g* and dialysed against distilled water. The amount of ammonium sulphate added was expressed as % of saturation.

Continuous electrophoresis. The Elphor continuous electrophoresis apparatus (Bender and Hobein, Munich) was used as described by Hannig (1961). A sample was introduced continuously through an entry point and flowed with buffer between two glass plates, to be collected in tubes at the bottom. A potential was applied across the plates, at right angles to the buffer flow, and charged molecules or particles in the sample were deflected across the plates. The apparatus was cooled to 6°, the plates which were separated by a gap of 0·5 mm. enclosed a volume of 120 ml. A buffer flow of 50 ml./hr was used, so 3 hr was allowed for the sample to reach the collecting rack.

Ion-exchange chromatography. Diethylaminoethyl-Sephadex A-50 (DEAE-Sephadex) was packed into columns after the treatment recommended by the manufacturers (Pharmacia, Uppsala, Sweden). The recommendations of Peterson & Sober (1962) were followed in the treatment of DEAE-cellulose (Whatman DE 50, 100–200 mesh; W. and R. Balston Ltd., Maidstone, Kent) and carboxymethyl-cellulose (Whatman CM 30, 100–200 mesh) and in packing the columns under pressure. In all experiments 1·9 × 30 cm. columns were used. The sample was dialysed against the starting buffer and gradient elution with an increasing molarity of NaCl was used. Either linear gradients or almost linear exponential gradients were used and the molarity of NaCl in the fractions was calculated according to Peterson & Sober (1957). All chromatography was at 4° in a cold laboratory.

Nitrogen by Keldjahl method. Total nitrogen was determined by a modification of the procedure of Weil-Malherbe & Green (1955). To the sample (equiv. 5–200 μg. N), in a 2 × 15 cm. Pyrex test tube, was added 1·5 ml. of 9·0 N-H_2SO_4 containing 6·25 mg. HgO + 200 mg. K_2SO_4. The water was boiled off and digestion was complete after heating for 1 hr. The tubes were then cooled and the contents made to 5·0 ml. Ammonia in 2·0 ml. amounts was determined by the method of Conway (1957).

Ribosomes. Ribosomes were prepared from *Bacteroides amylophilus* by the procedure of Nathans & Lipmann (1961).

Buffers. Phosphate buffers contained KH_2PO_4 adjusted to the desired pH value with NaOH. Tris buffer was adjusted to the desired pH value with HCl and veronal buffers contained sodium 5,5-diethylbarbiturate adjusted with HCl.

Materials. A.R. chemicals were used where available. Trypsin, 2X crystallized, salt free was obtained from L. Light and Co. Ltd. (Colnbrook, Bucks); soy bean trypsin inhibitor from Worthington Biochemicals Sales Co. (New Jersey, U.S.A.) Glyclyglycine, L-leuclyglycine, *N*-α-benzoyl-L-arginine ether ester. HCl, *N*-α-benzoyl-K-arginine methyl ester. HCl, *N*-α-*p*-toluenesulphonyl-L-arginine methyl ester. HCl, N-α-benzoyl-DL-arginine-2-naphthylamide. HCl, L-lysine ethyl ester. di HCl, L-alanyl-2-naphthylamide, L-histidyl-2-naphthylamide, L-arginyl-2-naphthylamide. HCl and α-L-aspartyl-2-naphthylamide were obtained from Koch–Light Ltd. (Colnbrook, Bucks.).

Glyclyglycylglycine, L-leucinamide. HCl, *N*-α-benzoyl-L-arginine amide. HCl. H_2O

and *N*-α-acetyl-L-tyrosine ethyl ester.H_2O were obtained from B.D.H. Ltd., (Poole, Dorset). *N*-α-Benzoyl-L-leucyl-2-naphthylamide.HCl and *N*-α-benzoyl-DL-arginine-*p*-nitroamilide.HCl were obtained from Sigma London Chemical Company (London, S.W. 6).

Denatured casein was prepared from Glaxo casein C (Glaxo Ltd., Greenford, Middlesex) by heating and acid precipitation (Blackburn & Hobson, 1962).

RESULTS

Preliminary experiments on the liberation of protease from *Bacteroides amylophilus* H18 were done with pooled lyophilized bacteria which had been stored in vacuum at −20°. This makes it possible to compare the efficiency of various extractants on a uniform source of protease. This was important as considerable variation in protease content was found between batches of bacteria grown at different times. Not more than 10% of the protease activity was lost on lyophilization. The protease activity of lyophilized bacteria was stable at −20° for at least one year. Only small amounts (5–7%) of the protease was liberated into the suspending medium of water, 2·0% NaCl or 0·1% M-phosphate buffer, unless the bacteria were disintegrated.

Table 1. *Extracts from disintegrated Bacteroides amylophilus H18*

Lyophilized or fresh suspensions of *B. amylophilus* H18 in water or in 0·1 M-phosphate buffer (pH 7·0), were disintegrated by agitation with glass beads (Mickle disintegrator) or by an ultrasonic disintegrator. The properties of representative extracts are described, the extract being the supernatant fluid after centrifugation at 20,000 *g* for 1 hr.

Bacterial source	Lyophilized bacteria	Cell* debris	Lyophilized bacteria	Fresh bacteria	Trypsin
Suspending fluid	Water	Buffer	Buffer	Water	—
Cell concentration (mg. dry wt/ml.):	10·0	—	12·5	5·0	—
Disintegrator	Mickle	—	Mickle	Ultrasonic	—
Total protease in extract (%)	40	6	26	49	—
Protease (units/ml.)	8·85	1·10	5·75	23·1	8·84
Casein precipitating activity (units/ml.)	2	32	16	64	—
TAME-esterase (units/ml.)	290	170	218	1046	1865
Esterase:protease	33	100	38	45	211
mg. N/ml.	0·312	—	0·325	1·58	0·0013
Protease specific activity	28·3	—	17·7	14·6	6830

* Re-extraction with 0·1 M-phosphate buffer (pH 7·0) of the debris from bacteria disintegrated in aqueous suspension by the Mickle disintegrator.

—, No result.

Protease liberation

Disintegration of suspensions of lyophilized *Bacteroides amylophilus* H18 in water gave extracts which contained 37–46% of the total protease in the bacteria. Some of the properties of a typical extract are given in Table 1. When the cell debris, which had been sedimented, was re-extracted with 0·1 M-phosphate buffer (pH 7·0) a further 6% of the total protease was extracted. Very little casein-precipitating activity was extracted into water but on re-extraction with 0·1 M-phosphate buffer this activity came into solution. Similarly, a higher proportion of TAME-esterase activity was extracted into buffer than into water. There was considerable variation in the proportion of total

protease made soluble by disintegration in phosphate buffer; it ranged from 20–43% of the total. Disintegration in 0·1 M-veronal buffer (pH 8·6), or in 0·1 M-tris buffer (pH 8·0) gave extracts similar in properties to those obtained in 0·1 M-phosphate buffer (pH 7·0) and since growth was more reproducible in the latter medium, this was generally used. All the fresh bacteria were harvested from maltose tryptose medium and were ultrasonically disintegrated since larger quantities of bacteria could be processed in this way than by Mickle disintegration. The properties of a representative extract from fresh bacteria are included in Table 1 and some properties of trypsin are given for comparative purposes. The proportion of protease made soluble varied in different extractions from 42–54% of the total and the extract contained all three enzyme activities. The specific activity varied depending on the activity in the bacterial suspension, which fluctuated from batch to batch.

Table 2. *Properties of protease, TAME-esterase and casein-precipitating activities of an extract from Bacteroides amylophilus H18*

The extract was dialysed against three changes of distilled water at 4°. The concentrations of the reagents quoted are those finally attained in the digests, or in the agar medium in the case of casein-precipitating activity. There was no pre-incubation with the reagent except for di-*iso*propylphosphofluoridate. One ml. $5{\cdot}4 \times 10^{-8}$ M-di-isopropylphosphofluoridate in isopropyl alcohol was added to 2·0 ml., 0·1 M-phosphate buffer (pH 7·0), and 5·0 ml. extract (16·5 protease units, 0·74 TAME-esterase units, 1·19 mg. N/ml.). The mixture was kept at 4° for 18 hr and the protease and TAME-esterase activities then assayed.

Treatment	Protease	TAME-esterase	Casein-precipitating activity
Dialysis	No effect	No effect	Variable loss in activity
pH value	Plateau, pH 5·5 to 9·5. Minor peaks at pH 6·0, 7·6 and 8·0	pH 5·5 0; 6·0 29; 6·5 70; 7·0 90; 8·0 100; 8·5 89 (% max. activity)	Activity in range pH 6·0 to 8·0
Ca^{2+}	10^{-2} M, 7; 2×10^{-2} M, 10; 5×10^{-2} M, 14 (% inhibition)	5×10^{-3} M, no effect	10^{-3} M gave clarification of zone
Mg^{2+}	1×10^{-2} M, 34% inhibition	—	10^{-3} M, no effect
Mn^{2+}	10^{-2} M, 22% inhibition	10^{-2} M, no effect	10^{-3} M, no effect
EDTA	10^{-2} M, 8% increase	10^{-3} M, 17% inhibition. 2×10^{-3} M, 42% inhibition; activity restored by 5×10^{-3} M-Ca^{2+}	10^{-3} M, inhibition
Hg^{2+}	2×10^{-3} M, 61% inhibition	10^{-3} M, 60% inhibition	(Precipitation of casein)
Thiomersal	$2{\cdot}5 \times 10^{-3}$ M, no effect	$2{\cdot}5 \times 10^{-3}$ M, no effect	$2{\cdot}5 \times 10^{-3}$ M, no effect
Iodoacetate	4×10^{-3} M, no effect	4×10^{-3} M, no effect	4×10^{-3} M, no effect
Cysteine	10^{-2} M, no effect	10^{-2} M, no effect	10^{-2} M, no effect
2,3 Dimercapto-1-propanol	—	3×10^{-3} M, no effect	—
Soybean trypsin inhibitor	20 μg./ml., no effect	10 μg./ml., no effect	20 μg./ml., no effect
Di-isopropylphosphofluoridate	$1{\cdot}4 \times 10^{-4}$ M, 87% inhibition	$6{\cdot}8 \times 10^{-5}$ M, 100% inhibition	—

Properties of extracts from Bacteroides amylophilus H18

Extracts from fresh bacteria were used throughout. In Table 2 are presented the results of a number of experiments which show the effects of various reagents on protease, TAME-esterase and casein-precipitating activities. The effect of pH on proteolytic activity indicated the presence of more than one protease but the single peak for TAME-esterase activity showed that this activity was probably due to only one enzyme. No change was observed in casein precipitating activity in the range pH 6·0–8·0. The divalent cations inhibited protease activity by precipitating the casein substrate. Calcium (10^{-3} M) was necessary in the casein agar plates to give clearly visible zones of casein precipitation.

Table 3. *Hydrolysis of rates various substrates, at pH 8·0, by Bacteroides amylophilus H18 extract*

Substrate	Incubation time (min.)	Substrate concentration (mM)	Hydrolysis rate (mμ moles/mg. N/min) *B. amylophilus* H18 extract
N-α-Benzoyl-L-arginine methyl ester	30	10·0	2870
N-α-Benzoyl-L-arginine ethyl ester	30	10·0	2890
N-α-Benzoyl-L-arginine amide	1440	33·3	0
N-α-Benzoyl-DL-arginine-2-naphthylamide	180	0·25	0
N-α-Benzoyl-L-leucyl-2-naphthylamide	60	0·25	9·4
N-α-Benzoyl-DL-arginine-p-nitroanilide	20	0·94	11·1
Lysine ethyl ester	30	10·0	54·3
N-α-p-Toluenesulphonyl-L-arginine methyl ester	30	10·0	671
N-α-Acetyl-L-tyrosine ethyl ester	120	10·0	0
L-Alanyl-2-naphthylamide	60	0·25	23·0
α-L-Aspartyl-2-naphthylamide	180	0·25	0
L-Arginyl-2-naphthylamide	180	0·25	0
L-Histidyl-2-naphthylamide	180	0·25	0
α-Naphthyl acetate	180	0·25	0
Glycyl ethyl ester	120	10·0	0
Glycylglycylglycine	960	50·0	11
Glycylglycine	960	50·0	0
L-Leucylglycine	1320	50·0	11
L-Leucinamide	120	33·3	37·1

Substrate specificity. The range of specificities of extracts from *Bacteroides amylophilus* H 18 was examined against a range of synthetic substrates. The results (Table 3) confirmed that a trypsin-like endopeptidase activity was present as *N*-α-benzoyl-L-argine methyl ester, *N*-α-benzoyl-L-arginine ethyl ester, *N*-α-benzoyl-DL-nitroanilide, *N*-α-*p*-toluene-sulphonyl-L-arginine methyl ester and L-lysine ethyl ester were hydrolysed, but unlike trypsin, the preparation did not hydrolyse *N*-α-benzoyl-L-arginine amide or *N*-α-benzoyl-DL-arginine-2-naphthylamide. There was no chymotrypsin-like activity as *N*-α-acetyl-L-tyrosine ethyl ester was not hydrolysed. L-Alanyl-2-naphthylamide and N-α-benzoyl-L-leucyl-2-naphthylamide were hydrolysed and there was some peptidase activity as glycylglycylglycine, leycylglycine, leucinamide and L-alanyl-2-naphthylamide were also hydrolysed; none of the other substrates tested was hydrolysed. All the hydrolyses were measured at pH 8·0, although the rate of hydrolysis of *N*-α-benzoyl-L-leucyl-2-naphthylamide was 72% higher at pH 9·0 (Table 4). The

hydrolysis of *N*-α-benzoyl-L-leucyl-2-naphthylamide and of L-alanyl-2-naphthylamide was inhibited by Mn^{2+} (Table 4), but the hydrolysis of L-leucinamide was increased while that of *N*-α-benzoyl-L-arginine methyl ester was unaffected.

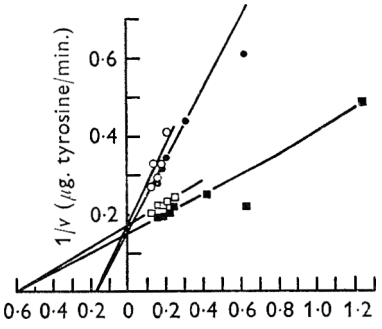

Fig. 1

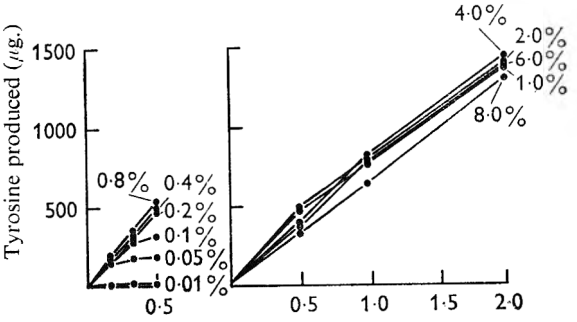

Fig. 2

Fig. 1. The determination of the K_m for casein from a Lineweaver & Burk plot. Digests at pH 7·0 (0·4 m-moles phosphate buffer) contained TAME (100 μmoles) and different concentrations of casein. *Bacteroides amylophilus* H18 extract (6·9 protease units) was added to tubes with TAME (●—●) and without TAME (■—■). To a second series was added *Bacteroides amylophilus* H18 extract (5·9 protease units) with TAME (○—○) and without TAME (□—□). Total volume, 5 ml. Incubation time, 20 min., rate of 'tyrosine' liberation measured.

Fig. 2. Effect of substrate concentration on protease activity. Digests at pH 7·0 (0·8 m-mole phosphate buffer) contained *Bacteroides amylophilus* extract (16·3 protease units) and a range of casein concentrations as illustrated. Total volume, 10·0 ml., 'tyrosine' liberation measured.

Table 4. *The hydrolysis of some synthetic substrates by an extract of Bacteroides amylophilus H18*

The extract was incubated for 1 hr with the manganese chloride, before the addition of substrate.

Substrate	Optimum pH value	$MnCl_2$ (2×10^{-3} M)
N-α-Benzoyl-L-leucyl-2-naphthylamide	9·0	63% inhibition
N-α-Benzoyl-L-arginine methyl ester	8·0	No effect
L-Leucinamide	8·0	73% increase
L-alanyl-2-naphthylamide	8·0	66% inhibition

Effect of casein concentration. The K_m for casein was 1·7 mg./ml. as determined for two concentrations of *Bacteroides amylophilus* H18 extract (Fig. 1). There was a considerable inhibition of caseolytic activity at pH 7·0 by TAME (2×10^{-2} M); this was as high as 64% inhibition at a casein concentration of 1·6 mg./ml. Figure 1 shows that the inhibition was competitive as the plots of $1/S$ against $1/V$, with and without TAME, intercepted at $1/V$. *N*-α-Benzoyl-L-arginine amide and L-leucinamide (2×10^{-2} M) did not inhibit the protease.

In the presence of 1·63 protease units/ml. digest, an increase in casein concentration up to 2·0% (w/v) resulted in an increased rate of 'tyrosine' liberation for 0·5 hr (Fig. 2), but there was a progressive decrease in the rate of 'tyrosine' liberation with an

increase in casein concentration from 2·0–8·0 %, w/v. After incubation for 2 hr there was a decrease in the rate of tyrosine production from all concentrations of casein except from the 8·0 % (w/v) casein digest. Figure 3 shows that the decrease in rate of 'tyrosine' production was due to substrate exhaustion and not to protease inactivation, as the addition of casein after incubation for 2 hr increased the rate of proteolysis in digests containing: casein (%) 0, 1, 2, 3.

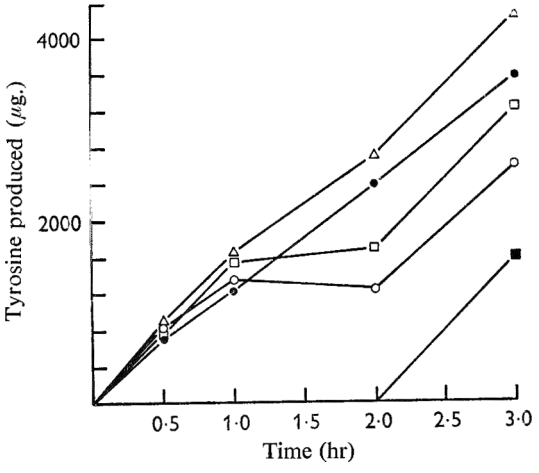

Fig. 3. Effect of substrate concentration on protease activity Digests at pH 7·0 (0·8 m-mole phosphate buffer) contained *Bacteroides amylophilus* H18 extract (30·8 protease units) and a range of casein concentrations (% w/v): 0·0 (■—■), 1·0 (○—○), 2·0 (□—□), 3·0 (△—△), 4·0 (●—●). Total volume 10·0 ml. After 2 hr the casein concentration in each digest was increased by 2·0 % (w/v). 'Tyrosine' liberation measured and expressed as the total μg. 'tyrosine' that the initial amount of protease would have produced.

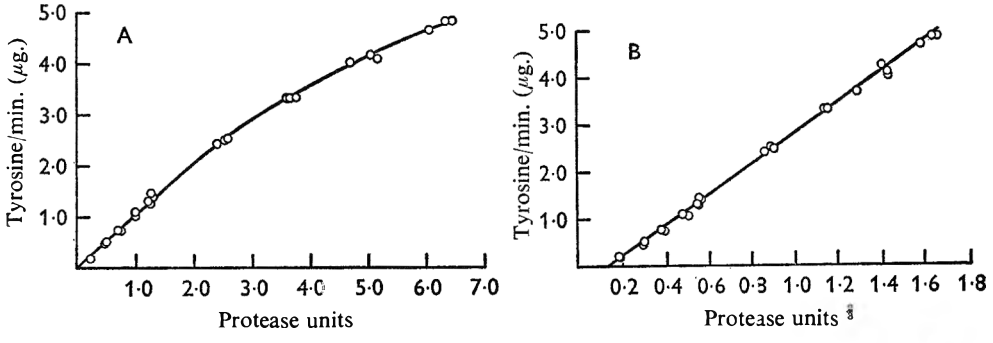

Fig. 4. Effect of protease concentration on the rate of 'tyrosine' liberation. Digests at pH 7·0 (0·4 m-mole phosphate buffer) contained casein (80 mg.) and various amounts of *Bacteroides amylophilus* H18 extract in a total volume of 5·0 ml. The digests were incubated for 4 hr and the rate of 'tyrosine' liberation measured. The number of protease units (maximum rate of liberation of 'tyrosine'/min.) was plotted against the actual rate of 'tyrosine' liberation in Fig. 4A. In Fig. 4B, protease units $^{\frac{1}{3}}$ were plotted against the actual rate of 'tyrosine' liberation.

Effect of enzyme concentration. The rate of proteolysis was not directly proportional to protease concentration except at low protease concentration (Fig. 4A). The rate was proportional to protease concentration up to the point where 0·5 mg. of 'tyrosine'

had been liberated from 80 mg. casein substrate. When protease (concentration$^{\frac{1}{2}}$) was plotted against the rate of 'tyrosine' liberation (Miller & Johnson, 1951), a linear relationship was observed (Fig. 4B) but the straight line did not meet the origin. Substituting protease (concentration) $^{\frac{1}{2}}$ (Schütz, 1885) gave a poorer fit. A plot of log protease concentration against log rate of 'tyrosine' liberation showed a linear relationship and from the slope it was deduced that protease (concentration) $^{0.94}$ should be proportional to the rate. A plot of these data showed that this was correct for protease concentrations liberating less than 820 μg. 'tyrosine' from 80 mg. casein. The maximum rate of production of non-trichloroacetic acid-precipitible peptide by an extract containing 23·1 protease units, was 372 μg. peptide/min. In other words, 1·0 μg. 'tyrosine' was contained in every 16·1 μg. peptide liberated from casein.

Purification of protease. The absorption spectrum of *Bacteroides amylophilus* H 18 extracts showed (Fig. 5) that a considerable amount of nucleic acid was present as there was an absorption peak at 260 mμ. The ratio $E_{280}:E_{260}$ indicated that 6·0% nucleic acid was present in the dialysed extract (Layne, 1957).

Table 5. *Ammonium sulphate fractionation of an extract from Bacteroides amylophilus H 18*

An extract from ultrasonically disintegrated *B. amylophilus* H18 (20 ml.; pH 7·0) was treated with increasing amounts of solid $(NH_4)_2SO_4$ at 4° and the resulting precipitates were sedimented at 20,000 *g*, dialysed against water and made up to 20 ml.

	$E_{280}:E_{260}$	Nitrogen (mg./ml.)	Protease (units/ml.)	Specific activity	Protease recovery	Purification
Original extract	0·81	1·58	23·1	14·6	—	—
40% to 50% $(NH_4)_2SO_4$ fraction	0·89	0·70	12·1	17·4	52	× 1·2

Ammonium sulphate precipitation. The major portion of the protease activity was precipitated between 40 and 50% saturation with ammonium sulphate; the yield varied in different experiments from 52 to 83% of the original activity. Very little purification of the protease activity was achieved (Table 5) in relation to total nitrogen or to nucleic acid and recovery was poor. The fractional solution of an ammonium sulphate precipitate, by a decreasing linear concentration gradient of ammonium sulphate (Zahn & Stahl, 1953) did not result in a discrete peak of protease activity and little purification was achieved.

Manganese chloride precipitation. Manganese chloride consistently precipitated a large portion of the nucleic acid (44–50%, 260 mμ-absorbing material) but it also precipitated much of the protease; in various experiments 131, 117 and 70% was precipitated leaving in solution 84, 60 and 62% respectively. There was an increase in total activity of the fractions but Mn^{2+} itself did not activate protease activity. The dialysed $MnCl_2$ supernatant fluid did not respond to ammonium sulphate fractional precipitation in a more satisfactory manner than did the untreated extract.

Protamine sulphate precipitation. When protamine sulphate was added to a dialysed extract of *Bacteroides amylophilus* H 18 until no further precipitate was formed, the clear supernatant fluid contained no nucleic acid and no protease activity.

Elphor continuous electrophoresis. Protease activity was found in a sharp peak (tubes 5–7) in which 82% of the original protease was recovered, where also was found 56% of the 280 mμ-absorbing material. (Fig. 6). On re-running this fraction

(pooled tubes 5–7), under the same conditions, there was some broadening of the peak (tubes 4–16) but there was no further separation of the protease activity from the nucleic acid (E_{260} peak).

Ion exchange chromatography. Protease activity was eluted from DEAE-Sephadex by a sodium chloride gradient in 0·005 M-phosphate buffer (pH 5·8) in a complex manner (Fig. 7). There was one major peak in which 65 % of the protease activity was recovered (Expt. no. 1, Table 6) but there were at least three other peaks and the protease activity was scattered over 180 fractions. The protease peaks did not coincide with E_{280} absorbing material which was eluted in nine or more peaks. A steeper NaCl gradient (Expt. no. 2, Table 6) gave essentially the same elution pattern but was more

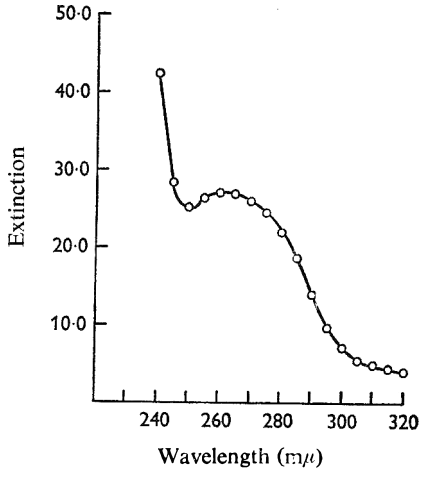

Fig. 5

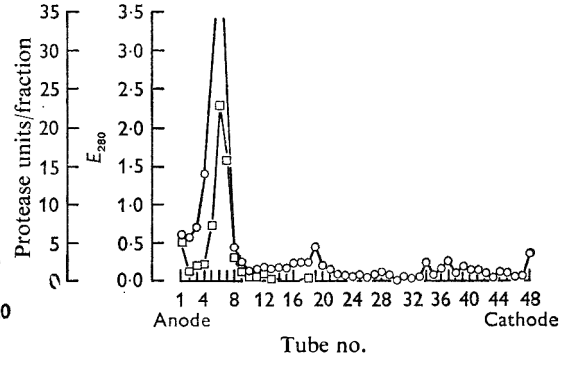

Fig. 6

Fig. 5. The absorption spectrum of an extract from *Bacteroides amylophilus* H18.

Fig. 6. Elphor electrophoresis of *Bacteroides amylophilus* H18 extract. An ultrasonic extract of *B. amylophilus* H18 (3·0 ml.) containing 56·4 units protease activity was run in 0·1 M-tris + 0·2 M-acetic acid (pH 8·3) at 1680 V. and 230 mA. and was collected in 3·4 ml. fractions. The sample was introduced above tube 39. The protease activity (□—□) and E_{280} (○—○) was measured in each fraction.

compressed. The main peak of activity was contained in eighteen fractions from which 45 % of the protease activity was recovered. The pooled fractions of this peak contained only 3·7 % of the original E_{280}-absorbing material and the ratio $E_{280}:E_{260}$ 1·13 indicated that a considerable portion of nucleic acid had been removed. In this experiment a further 20 and 7 % protease activity was recovered in two minor peaks in tubes 97–119.

In Expts. 3–7 in Table 6 a lyophilized extract was used, redissolved and dialysed against the starting buffer. The product of $N \times 6{\cdot}25$ indicated that only 66 % of the dry weight was protein + nucleic acid. Similar elution patterns were found using a NaCl gradient in 0·005 M-phosphate (Expts. 3 and 4) and in 0·05 M-phosphate (Expts. 5 and 6) at pH 7·0. In Expts. 4 and 5 steeper gradients of NaCl were used to compress the peaks, but this did not greatly affect the degree of purification, which at best (Expt. no. 3) was only three-fold. There was a low ratio $E_{280}:E_{260}$ in all the protease peaks obtained by ion-exchange chromatography at pH 7·0 on DEAE-cellulose

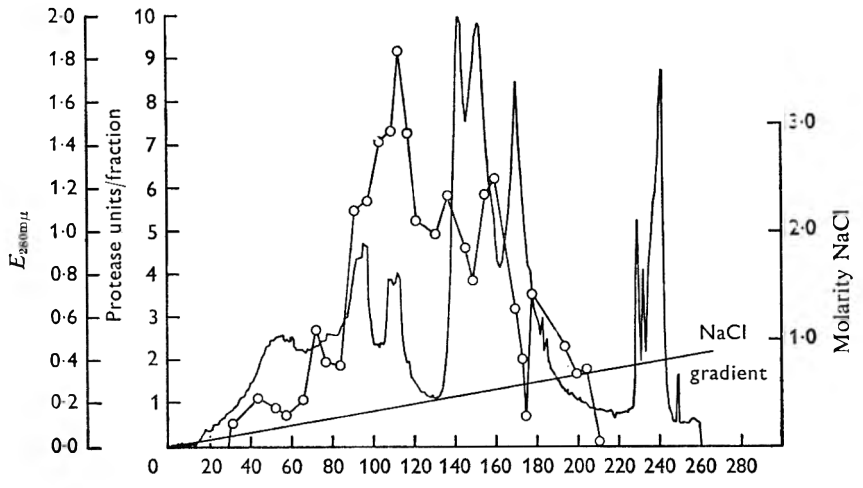

Fig. 7. DEAE-Sephadex chromatography of *Bacteroides amylophilus* H18 extract. The extract (30 ml.) containing 381 protease units after dialysis against 0·005 M-phosphate buffer (pH 5·8) was introduced into a 1·9 × 30 cm. column of DEAE-Sephadex. Elution was by a linear gradient of NaCl and fractions were collected in 3·5 ml. volumes. The E_{280} (—) and protease content (○—○) of the fractions was measured.

Table 6. *Ion-exchange chromatography of Bacteroides amylophilus H18 extracts*

NaCl gradients were used to elute the extract from 1·9 × 30 cm. columns of ion-exchanges.

Expt. no.	Extract	No. of peaks	Fraction vol.	Phosphate buffer (M)	pH	Exchanger	Main peak between gradient	Protease recovery in main peak (%)	Purification
1	381 units	4	3·5	0·005	5·8	DEAE-Sephadex	0·2 to 0·3 M (50–120)	65	—
2	381 units	4	3·5	0·005	5·8	DEAE-Sephadex	0·2 to 0·3 M (79–96)	45	—
3	18·9 mg. N, 405 units	4	3·25	0·005	7·0	DEAE-Cellulose	0·1 to 0·3 M (91–131)	59	3·12
4	18·9 mg. N, 405 units	4	3·25	0·005	7·0	DEAE-Cellulose	0·1 to 0·3 M (101–120)	37	2·50
5	21·0 mg. N, 450 units	4	4·5	0·05	7·0	DEAE-Cellulose	0·1 to 0·3 M (102–135)	39	2·76
6	21·2 mg. N, 450 units	4	4·5	0·05	7·0	DEAE-Cellulose	0·2 to 0·4 M (137–159)	38	2·71
7	21·2 mg. N, 450 units	—	4·5	0·05	6·4	CM-Cellulose	—	67	0·6

—, No result.

which showed that there was little purification with regard to nucleic acid. There was a loss of esterase activity as the ratio esterase activity:protease activity in the main protease peak of experiment 5 was 21 compared to 45 in the original extract. Protease activity was not retained by CM-cellulose at pH 6·4, there was a 33 % loss in activity and a decrease in specific activity.

Gel filtration. Protease activity was completely excluded from Sephadex-G200 and emerged in the void volume of 0·05 M-phosphate buffer (pH 7·0).

Centrifugal fractionation. Further centrifugation of *Bacteroides amylophilus* H 18 extract yielded two sediments (Table 7) which contained 49 % of the original protease activity and 27 % of the original nitrogen. The light sediment fraction in particular showed an increase in specific activity and also an increased ratio $E_{280}:E_{260}$. There was an increase in the total activity in the fractions.

Ribosome-bound protease. A preparation of *Bacteroides amylophilus* H 18 ribosomes had a specific activity of 7·0 while that of the soluble (75,000 *g*) portion of the disintegrated bacteria was 12·7.

Table 7. *Centrifugal fractionation of Bacteroides amylophilus H 18 extract*

Ten ml. of an aqueous solution (0·2 % w/v) of a lyophilized extract from *B. amylophilus* H 18 was centrifuged at 105,000 *g* for 4 hr. The solid sediment and the light sediment were washed once with water and the washings added to the soluble fraction.

Fraction	Ratio $E_{280}:E_{260}$	Total protease (units)	Fraction of original activity (%)	Total nitrogen (mg.)	Specific activity	Purification
Soluble	0·70	330	73	14·0	23·5	1·1
Light sediment	0·85	50	11	0·7	71·4	3·4
Solid sediment	0·72	174	38	4·9	35·5	1·7
Complete extract	0·71	450	—	21·0	21·2	—

— No result.

DISCUSSION

Only 20 % of the total protease of *Bacteroides amylophilus* H 18 was released into the medium during experimental growth of the bacteria and preliminary experiments showed that ammonium sulphate precipitation gave poor yields of protease. Unlike the cell-bound protease of a *Micrococcus* sp. (McDonald, 1962) only 5–7 % of the protease of *B. amylophilus* was liberated into 2·0 % NaCl, but Mickle disintegration of lyophilized cells suspended in water gave good yields of protease with low TAME-esterase and casein-precipitating activities. These latter activities were extracted more efficiently into a buffered suspending medium, suggesting that at least two proteases were present in the cells. Both protease and TAME-esterase activities were extracted by ultrasonic disintegration of fresh cells suspended in water. The TAME-esterase had an optimum pH at 8·0, but the protease had a broad optimum with minor peaks similar to the two broad peaks of protease activity at pH 6·5 and 8·0 for whole cultures of *B. amylophilus* (Blackburn, 1968).

Neither protease nor esterase activities were inhibited by soybean trypsin inhibitor, but both activities were similar to trypsin in that they were inhibited (the esterase completely and the protease 87 % by di-*iso*-propylphosphofluoridate, although both the protease and esterase activities of trypsin are inhibited to the same extent (Jansen & Balls, 1952). The TAME-esterase also differed from trypsin in that it failed to hydrolyse *N*-α-benzoyl-L-arginine amide (BAA) and *N*-α-benzoyl-DL-arginine-2-naphthylamide (BANA). In failure to hydrolyse the latter substrate the enzyme is

similar to Kallikrein, which in other respects resembles trypsin (Werle & Kaufmann-Boetsch, 1960). The fact that the rate of hydrolysis of TAME by trypsin is 50 times that of the rate of BAA hydrolysis (Schwert, Neurath, Kaufmann & Snoke, 1948) could not explain the lack of detectable hydrolysis of BAA by extracts of *B. amylophilus* as the digests were incubated for 132 times the time required to give a 10% hydrolysis of an equivalent amount of TAME. Although the esterase resembled trypsin in the hydrolysis of *N*-α-benzoyl-L-arginine methyl ester (BAME) and -ethyl ester (BAEE), *N*-α-benzoyl-DL-arginine-*p*-nitroamilide and lysine ethyl ester, it differed from trypsin in the ratio of rate of hydrolysis of TAME: rate of hydrolysis of BAME (1:0·23, compared to 1:5·2 for trypsin (Schwert *et al.* 1948). There was no hydrolysis of *N*-α-acetyl-L-tyrosine ethyl ester, the substrate for chymotrypsin, but there was some hydrolysis of *N*-α-benzoyl-L-leucyl-2-naphthylamide (BLNA). The pH optimum for hydrolysis of BLNA was higher than that for the other substrates examined, but the enzyme responsible for this hydrolysis was inhibited by 2×10^{-3} M-$MnCl_2$, as was the enzyme hydrolysing L-alanyl-2-naphthylamide. The enzyme(s) responsible for these hydrolyses is thus almost certainly distinct from the enzyme hydrolysing L-leucinamide and L-leucylglycine as this was activated by Mn^{2+}.

The extent of inhibition of casein hydrolysis by TAME indicated that a considerable proportion of the total protease activity was due to an enzyme with a trypsin-like specificity. *N*-α-Benzoyl-L-arginine amide, which was not hydrolysed, did not act as an inhibitor. On the assumption that the hydrolysis of *N*-α-benzoyl-L-leucyl-2-naphthylamide was by an endopeptidase different from that responsible for the TAME hydrolysis, L-leucinamide was tested as a possible inhibitor. It did not inhibit any of the protease activity. Sufficient quantities of *N*-α-benzoyl-L-leucyl-2-naphthylamide were not available to test its effect as a competitive inhibitor. There was a complex relationship between the rate of casein hydrolysis and the substrate concentration, but the casein concentration of 1·6% (w/v) which was routinely used in the assay of protease activity was within the optimum range. In every 16·1 μg. of peptide released from casein there was 1·0 μg. of 'tyrosine'. Ellinger & Boyne (1965) found 1·0 μg. of tyrosine in 15·1 μg. casein, so it is probable that the protease of *Bacteroides amylophilus* did not preferentially hydrolyse peptide bonds involving tyrosine.

A number of bacterial proteases with a trypsin-like activity have been described. A streptococcal protease hydrolysed peptide bonds involving the carboxyl groups of arginine, lysine, histidine, glutamic acid and aspartic acid (Mycek, Elliott & Fruton, 1952). Hall, Kunkel & Prescott (1966) described a semi-purified protease from *Bacillus licheniformis* which hydrolysed TAME and was inhibited by di-*iso*-propylphosphofluoridate.

Extracts of *Bacteroides amylophilus* contained much nucleic acid, which may have accounted for the difficulties encountered in the purification of the protease. Some 49% of the protease activity in the extracts was sedimentable by centrifuging at 105,000 *g* for 4 hr. Presumably the protease was bound to small particles of cell membrane or wall which were fragmented by the ultrasonic disintegration. As all the protease was excluded from Sephadex G-200 it may have been all particle-bound. The trypsin-like protease prepared by Hall *et al.* (1966) also emerged in the void volume of Sephadex G-200.

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Deoxyribonucleic Acid Base Composition and Taxonomy of Thiobacilli and some Nitrifying Bacteria

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SUMMARY

The DNA base composition of 14 authentic strains of the genus *Thiobacillus*, determined by caesium chloride density gradient centrifugation, was found to vary from 51 to 68 mole % guanine + cytosine (G + C). The mole % G + C values for the various species are as follows: *T. thiooxidans* and *T. concretivorus* 51–52, *T. neopolitanus* and *T. ferrooxidans* 56–57, and *T. thioparus*, *T. thiocyanoxidans*, *T. denitrificans*, *T. novellus* and *T. trautweinii* 62–68. Another group of chemoautotrophic bacteria, the nitrifiers *Nitrosomonas europaea* and *Nitrobacter agilis*, were found to have a G + C content of 52 and 65 %, respectively. The results are compared with other types of taxonomic studies made with these and related bacteria.

INTRODUCTION

Thiobacilli are Gram-negative, non-sporulating, rod-shaped bacteria, usually found in soils and in marine and fresh water (Vishniac & Santer, 1957; Trudinger, 1967*a*). It might be assumed from their common function in oxidizing certain inorganic sulphur-containing compounds and in utilizing carbon dioxide as sole carbon source, that species within the genus *Thiobacillus* are closely related genetically. These physiological criteria may not however be sufficient in themselves to support this conclusion, since many different types of micro-organisms fix carbon dioxide, e.g. the nitrifying bacteria (Alexander, 1961), and some heterotrophic organisms readily oxidize sulphur compounds (Trudinger, 1967*b*).

The taxonomy of thiobacilli has recently been studied by Hutchinson, Johnstone & White (1965, 1966, 1967). They used a multivariate analysis (Beers & Lockhart, 1962; Sneath, 1957) to demonstrate that the genus consists of well-defined species and an unusual absence of intermediate forms. This method, however, has the disadvantage that it does not analyse directly the relationships between all the species of the genus *Thiobacillus*. This is because some of these species are aerobic, others anaerobic, some acidophilic, whereas others prefer more neutral pH conditions. Moreover, although this numerical analysis was based upon 100 or so phenotypic properties, it accounts for only a small proportion of the bacterial genome. Another way of examining the interrelationships between a group of micro-organisms is to compare the over-all base composition of their DNA complement. It has been applied with success to the pseudomonads (Mandel, 1966) and other bacteria. A list of the DNA base compositions of some bacteria was given by Hill (1966). For bacterial species to be closely related, it is necessary that their DNA should have a similar over-all base composition.

This method has the advantage over multivariate analysis in that a comparison of the thiobacilli can be made directly on the basis of the whole genome. We have extracted DNA from various species of thiobacilli and from some nitrifying bacteria, and examined the over-all base composition of each by CsCl density gradient centrifugation.

METHODS

Organisms. Strains of *Thiobacillus thiooxidans*, *T. concretivorus*, *T. novellus*, *T. trautweinii* and *T. ferrooxidans* were supplied by the National Collection of Industrial Bacteria (NCIB), Torrey Research Station, Aberdeen, Scotland (see Table 1). Strains 1P and 2P, originally from C. P. Parker, Melbourne, Australia, were procured from Dr R. Swaby, C.S.I.R.O., Division of Soils, Adelaide, South Australia. Samples of *T. neopolitanus* and *T. denitrificans* were obtained from Dr P. A. Trudinger (Baas-Becking Laboratories, Canberra). Dr I. M. H. Aleem (Research Institute for Advanced Studies, Baltimore, Maryland, U.S.A.), supplied strains of *Ferrobacillus ferrooxidans* (hereafter referred to as *T. ferrooxidans*; for discussion of the nomenclature see Hutchinson *et al.* 1966) and *Nitrobacter agilis*. The *Nitrosomonas europaea* strain used was obtained from Dr Jane Meiklejohn (Rothamsted Experimental Station, England).

Growth conditions. All the bacteria used here were grown in media listed below, either in flasks on a gyrotary shaker where the total volume was 1 l. or less, or in carboys up to 40 l. capacity. In the latter case sterile air was forced through the medium by using sintered glass aerators attached to a compressed air line. The culture media were either sterilized at 121° for 45 min. or, when more than 10 l. were used, the medium was filtered through a Millipore filter assembly (diam. 142 mm., pore size 0·22 μ, with prefilter). The larger carboys were sterilized by placing a specially constructed ultraviolet lamp inside them for several hours. The energy output of this lamp was 95 microwatts/cm.^{2} at 1 m. distance, measured at 253·7 mμ.

A minimum of 10% (v/v) inoculum was used to seed the cultures in all these experiments. All cultures were grown at 30° and the growth rate followed by counting the bacteria periodically in a haemacytometer. A generation time of not less than 14 hr was recorded for these chemoautotrophic bacteria.

Media. Thiobacillus thioparus, *T. novellus* and *T. trautweinii* were grown in the culture medium containing thiosulphate as described by Vishniac & Santer (1957) for *T. thioparus*; this medium was initially at pH 7. Growth of *T. thioparus* and *T. novellus* decreased the pH value of the medium, while that of *T. trautweinii* increased it. *Thiobacillus thiooxidans* and *T. concretivorus* were grown in a medium similar to that for *T. thioparus*, except that the initial pH value was made to 5·6 by substituting the following amounts of phosphate buffer (g./l. medium): K_2HPO_4, 0·5 and KH_2PO_4, 7·5.

The two strains of *Thiobacillus ferrooxidans* were grown in a medium containing (g.): $(NH_4)_2SO_4$, 3; KCl, 0·1; $MgSO_4.7H_2O$, 0·5; $Ca(NO_3)_2$, 0·01; KH_2PO_4, 0·2; in glass-distilled water, 700 ml. To this solution, which was autoclaved separately, was added 45 g. $FeSO_4.7H_2O$ and 1 ml. 10 N-H_2SO_4 dispensed in 300 ml. distilled water. The medium was then at pH 2·7.

The medium for *Nitrosomonas europaea* contained (g./l. distilled water): $(NH_4)_2SO_4$, 4; KH_2PO_4, 0·5; and (mg./l.) $MgSO_4.7H_2O$, 50; $CaCl_2.2H_2O$, 4; chelated iron, 0·1

(Nicholas & Rao, 1964). Phenol red was added as internal indicator and the medium adjusted with 20% (w/v) K_2CO_3 solution to pH 8.

Nitrobacter agilis was grown in the following medium (g./l.): KNO_2, 0·3; K_2HPO_4, 0·175; $MgSO_4.7H_2O$, 0·175; NaCl, 0·1; $KHCO_3$, 0·5; and chelated iron, 0·1. A sterile 10% (w/v) solution of KNO_2 was added automatically to maintain a value of 200 mg./l. during growth.

Isolation of DNA. About 1–4 g. wet weight of bacteria was collected from 10 to 40 l. of culture by continuous flow centrifugation in a Sorvall RC-2 refrigerated unit. The bacteria were stored at $-17°$. The procedure of Marmur (1961) for Gram-negative bacteria was used to prepare the DNA, except where otherwise stated in the results. The DNA preparations were characterized by u.v. absorption, and deoxypentose, pentose and organic phosphate determinations. The preparations were stored in 0·05 M-tris HCl (pH 8) at $-17°$.

Determination of DNA base composition. The base composition of the various samples was determined by CsCl density gradient centrifugation as follows: CsCl (1·128 g.) was dissolved in 0·86 ml. 0·01 M-tris HCl buffer (pH 8) containing approximately 5 mμmoles of the DNA to be tested and 5 mμmoles of a DNA from either calf thymus or *Escherichia coli* as a reference. *E. coli* DNA was prepared from *E. coli* B cells according to the method of Marmur (1961), and the DNA from calf thymus purified following the procedure of Kay, Simmons & Downce (1952). Optical grade CsCl was obtained from Stanley H. Cohen (Yonkers, New York). The refractive index of the solution containing the DNA samples was measured to check the final CsCl concentration. This solution was then used for density gradient centrifugation in a Spinco Model E analytical ultracentrifuge fitted with a titanium rotor. The u.v. photographs taken after 17 hr. at 44,770 rev./min. and 25°, were used to determine the density of the unknown DNA. For this purpose, tracings were made on a Beckman Analytical recording densitometer equipped with a film attachment. The buoyant densities of the reference DNA samples were taken as 1·710 g. cm.$^{-3}$ for *E. coli* DNA and 1·699 g. cm.$^{-3}$ for calf thymus DNA, and the base composition of the unknown sample calculated as described by Schildkraut, Marmur & Doty (1962), using the equation $\rho = 1{\cdot}660 + 0{\cdot}098$ (GC), where ρ = buoyant density.

RESULTS AND DISCUSSION

DNA isolation

Since most of the chemoautotrophic bacteria investigated are Gram-negative, they were easily lysed by detergent as described by Marmur (1961). There were, however, two exceptions, *Thiobacillus concretivorus* and *T. ferrooxidans*.

Thiobacillus concretivorus, strain 1P. Unlike strains 2P and 9514, strain 1P did not lyse with sodium lauryl sulphate. To prepare a DNA sample from this strain, it was necessary to break the bacteria with aqueous phenol mixtures. Bacteria (2 g. wet wt) were suspended in 20 ml. 0·05 M-tris HCl (pH 8) containing 2% (w/v) sodium lauryl sulphate, then shaken with 20 ml. phenol saturated with aqueous M-tris HCl (pH 8). After centrifugation at 6000 *g* for 10 min., the upper aqueous layer was collected and the DNA precipitated from it with 2 vol. of ethanol. The method of Marmur (1961) was then followed to achieve further purification. Despite the different extraction technique used, the base composition of the DNA prepared from strain *T. concretivorus* 1P was the same as that for strains 2P and 9514 (see Table 1).

Thiobacillus ferrooxidans. In these experiments, *T. ferrooxidans* organisms were not lysed by detergent alone, lysozyme followed by detergent, grinding with glass powder (500-mesh), or even by shaking with aqueous phenol mixtures at 20° or 60°. A DNA preparation was obtained by suspending 0·6 g. wet wt bacteria in a mixture containing 2·5 ml. of 0·1 M-tris HCl (pH 8) and 2·5 ml. phenol previously saturated with aqueous M-tris HCl (pH 8). This suspension was given ultrasonic treatment for 1 min. at 0° with an M.S.E. model 60 W. ultrasonic disintegrator (20 kcyc./sec.). After centrifugation at 6000 *g* for 10 min. the upper aqueous layer was removed and the DNA precipitated with 2 vol. of ethanol. The purification was then continued as described by Marmur (1961). This method, however, did not always yield DNA. It is possible that small amounts of ferric oxide and perhaps other compounds of iron in the bacteria

Table 1. *DNA base compositions of Thiobacillus species*

Species	Strain	Buoyant density (g. cm.$^{-3}$)	Base composition (mole % G+C)
T. trautweinii	NCIB 9549	1·725	66
T. novellus	NCIB 9113	1·727	68
	NCIB 8093	1·725	66
T. denitrificans	Baas-Becking (Trudinger)	1·723	64
T. thioparus	NCIB 8349	1·725	66
	NCIB 8370	1·721	62
T. thiocyanoxidans	NCIB 5177	1·722	63
T. neopolitanus	Baas-Becking (Trudinger)	1·715	56
T. ferrooxidans	(Aleem)	1·716	57
T. thiooxidans	NCIB 9112	1·711	52
	NCIB 8085	1·711	52
T. concretivorus	NCIB 9514	1·710	51
	1P	1·710	51
	2P	1·711	52

complexed with some or all of the DNA and RNA, rendering them insoluble during cell breakage. Ferric ions are known to precipitate DNA (Hammerstein, 1924). Although differential centrifugation removed some of the free ferric compounds from the whole bacteria, this did not improve the extraction of the nucleic acids, presumably because of the iron compounds within the cells (Dugan & Lundgren, 1965). Addition of Na-EDTA during lysis was without effect. After several attempts, DNA was isolated from Aleem's strain of *T. ferrooxidans* but as yet none has been obtained from strain NCIB9490. The DNA preparation from Aleem's strain gave a band much broader than usual in the CsCl density gradient, indicating a relatively low molecular weight. This was probably a result of shearing forces operating during the ultrasonic treatment.

DNA base composition of Thiobacillus species

The buoyant densities of CsCl solutions and the corresponding base compositions of DNA preparations from the Thiobacillus species are listed in Table 1. In general, the value for buoyant density is reliable to $\pm 0{\cdot}001$ g. cm.$^{-3}$, and so the probable error in base composition as determined by this method is $\pm 1\%$ G+C (Schildkraut, Marmur & Doty, 1962).

The DNA base composition for all the members of the genus *Thiobacillus* tested were in the range 51–68 mole % G+C. Further subdivision into three groups can be made, based on this parameter. Thus one group with 62–68 % G+C included *T. trautweinii* (the only facultative autotroph in the genus), *T. novellus*, *T. denitrificans*, *T. thioparus* and *T. thiocyanoxidans*. These organisms thus have G+C content similar to some of the pseudomonads (Hill, 1966), in particular *Pseudomonas aeruginosa* (64–68 %) and *Pseudomonas fluorescens* (60–64·5 %). Since these heterotrophs also oxidize certain inorganic sulphur compounds (Trudinger, 1967*b*), it would be of interest to study further their relationship to the genus *Thiobacillus*.

Another group which included *Thiobacillus thiooxidans* and *T. concretivorus* showed a base composition of 51–52 % G+C, and was therefore clearly separated from the rest of the genus. *Thiobacillus neopolitanus* and *T. ferrooxidans* were distinct from both these groups since the base composition was intermediate (56–57 % G+C). This does not necessarily mean that these two bacteria are closely related to each other, but it does suggest that they are not closely allied to the other members of the genus.

The DNA preparation from *Thiobacillus thiocyanoxidans* NCIB 5177, by CsCl density gradient centrifugation, gave a second minor band. This band made up to 10–20 % of the total DNA present, and had a buoyant density of 1·718 g. cm.$^{-3}$, and a G+C content of 59 %. This observation illustrates an advantage of the buoyant density method over other techniques for base composition determination, since it shows that all the preparations investigated in the present work with the exception of *T. thiocyanoxidans* NCIB 5177, had only one species of DNA present with respect to over-all base composition. We have no proof at present that this minor band is not derived from a second or contaminating organism in the *T. thiocyanoxidans* culture. This minor band, like the major component, is shifted by 0·013 g./cm.$^{-3}$ to a higher buoyant density in CsCl solutions following heat denaturation, and so is double stranded. It may be of interest that *Pseudomonas stutzeri*, another organism capable of oxidising inorganic sulphur compounds, was found by Mandel (1966) to have a minor 'satellite' DNA component.

Brief reports of the DNA base composition of two Thiobacillus species have previously been recorded. Marmur, Falkow & Mandel (1963) quote a value of 68–70 mole % G+C for *T. thiocrus* (method unspecified); this is reasonably close to the figure reported here. Bohacek, Kocur & Martinec (1965) give a value of 58–59 % G+C for *T. novellus* which is different from our measurements made on two authentic strains.

Comparison with a multivariate analysis of Thiobacillus species

Hutchinson *et al.* (1965, 1966, 1967) were able to recognize six groups within the genus *Thiobacillus* by means of a multivariate analysis. The order of similarity (S values) of these groups, as indicated by this numerical analysis, is compared in Table 2 with data from the over-all DNA base composition. Where S values can be compared directly (groups 0–4), the order tends to follow that of the base composition.

To make this comparison, *T. concretivorus* is included in group 5 with *T. thiooxidans*, whereas *T. thiocyanoxidans* is placed in group 3 with *T. thioparus*. Hutchinson *et al.* (1965) stated that there was not sufficient difference in S values between *T. thiocyanoxidans* and *T. thioparus*, or between *T. concretivorus* and *T. thiooxidans*, to justify subdividing either group 3 or group 5. The results for DNA base composition

reported here tend to support this view (see Table 1). Another point of agreement between the two investigations is the unequivocal demonstration of the difference between *T. thioparus* and *T. neopolitanus* (Table 2). Some doubt had been expressed earlier about this (see discussion by Hutchinson *et al.* 1965).

Table 2. *Comparison of multivariate analysis and over-all DNA base composition*

Species	Group no. (multivariate analysis)	Range of S values*	Mean S value*	DNA base composition (mole % G+C)
T. trautweinii	0	94–63	71	66
T. novellus	1	49–46	47	66–68
T. denitrificans	2	—	—	64
T. thioparus†	3	45–24	35	62–66
T. neopolitanus	4	37–18	27	56
T. ferrooxidans	6	—	—	57
T. thiooxidans‡	5	—	—	51–52

* The S values listed are taken from Table 4 of Hutchinson *et al.* (1965). They refer to S values of the various strains, all with respect to the 3F strain of *T. trautweinii*. This particular strain was chosen since it represents an extreme case, and so enables all the species to be arranged in an order of similarity. *T. denitrificans* is listed between *T. novellus* and *T. thioparus* by reason of the results of aerobic tests made by Hutchinson *et al.* (1967).

† Includes *T. thiocyanoxidans*.

‡ Includes *T. concretivorus*.

Table 3. *DNA base composition of Nitrobacter agilis and Nitrosomonas europaea*

Bacterium	Energy yielding reaction	Buoyant density (g. cm.$^{-3}$)	DNA base composition (mole % G+C)
N. agilis	$NO_2^- \rightarrow NO_3^-$	1·724	65
N. europaea	$NH_4^+ \rightarrow NO_2^-$	1·711	52

Starkey (1935) and Baalsrud (1954) suggested that *Thiobacillus trautweinii* be excluded from the genus *Thiobacillus*. The results of Hutchinson *et al.* (1965) support this view, although they state that their investigations were not designed to test this point specifically. Although it has a G+C content within the range found for other species classified in the genus, the present DNA base composition studies carried out on this organism do not allow a positive decision to be made on this point.

As mentioned in the Introduction, a DNA base composition study enables a comparison to be made between all members of the genus, regardless of cultural conditions. Thus, as well as substantiating the suggestion of Hutchinson *et al.* (1965) that *Thiobacillus thiooxidans* and *T. ferrooxidans* are clearly separable, the present work goes further and establishes that *T. thiooxidans* (including *T. concretivorus*) are not closely related to the rest of the genus.

DNA base composition of the nitrifying bacteria

Although *Nitrosomonas europaea* and *Nitrobacter agilis* are both nitrifying bacteria (Alexander, 1961), they are quite distinct from each other since they utilize different inorganic nitrogen compounds, and have a distinctive cell ultrastructure (Murray,

1963). This is also confirmed by their widely different DNA base composition (Table 3). The value obtained here for *N. europaea* is similar to that quoted by Marmur *et al.* (1963) for a '*Nitrosomonas* sp.' (54–56% G+C), and by Anderson, Pramer & Davis (1965) who found 50–51% G+C for *N. europaea*. The DNA samples from the nitrifying bacteria have a G+C content within the range found for the other group of chemoautotrophs, *Thiobacillus* spp. Because it is possible to have the same over-all base composition with entirely different base sequences (and thus with different genetic 'messages'), this method does not permit us to decide whether or not these chemoautotrophs are closely related genetically.

CONCLUSIONS

DNA base composition studies of species of the genus *Thiobacillus* have confirmed some of the similarities and differences previously indicated between the species in a numerical analysis (Hutchinson *et al.* 1965, 1966, 1967). The present work suggests a possible relationship between some members of the genus (*T. trautweinii*, *T. novellus*, *T. denitrificans* and *T. thioparus*) and certain pseudomonads, e.g. *Pseudomonas aeruginosa* and *P. fluorescens*, which oxidize sulphur compounds. Although the base compositions of the chemoautotrophic nitrifying bacteria examined are in the range obtained with Thiobacillus species the method does not permit any conclusions about the relationship between them.

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Active Resistance to Apple Scab

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SUMMARY

Two partially characterized groups of compounds were isolated from an apple variety (Miller's Seedling) which has polygenic resistance to apple scab. These compounds inhibited germination of conidia of *Venturia inaequalis* and interfered with pigment production in log-phase cultures of the organism. They were more abundant in varieties of apple resistant to scab than in susceptible varieties, and increased in the leaves of resistant varieties after inoculation with *V. inaequalis* conidia. When applied to the foliage of a variety of apple normally susceptible to scab these compounds conferred a measure of protection against the disease. The subject of physiological resistance of plants to microbial attack is discussed.

INTRODUCTION

Previous work at East Malling has shown the importance of the part played in the plant/disease syndrome *Malus* sp./*Venturia inaequalis* by certain of the simple apple-leaf phenolic substances and their analogues (Kirkham, 1957; Kirkham & Hunter, 1965). More recently the specific effects on the transport of solutes in the host by extracellular melanoproteins produced by the pathogen has been studied (Hignett & Kirkham, 1967). The present paper discusses some further host factors which have been found in a resistant apple variety. Comparison of extracts of apple leaves by chromatography or high voltage electrophoresis showed that varieties having a high degree of field resistance to the apple scab organism contained a number of compounds not observed in varieties which are susceptible to scab. The most notable compounds were present in two bands that fluoresced bright light-blue, in the presence of ammonia vapour, under ultraviolet radiation (3650 Å). These, for convenience, have been called Blue I and Blue II. Methods have been evolved for their isolation from apple leaves, particularly those of the variety Miller's Seedling.

METHODS

Experiments were made with plants of Miller's Seedling worked on MM. 104 root-stocks, or with rooted shoots of the MM. 109 clone. The host material and fungus were handled and trials recorded as before (Kirkham & Hunter, 1965). Pigment production in deep culture was followed by the methods already reported (Hignett & Kirkham, 1967). Young leaves were obtained from plants grown either in greenhouses or in orchards.

An extract of 'phenolics' was made by macerating 100 g. leaves in a blender with 200 ml. ice-cold disodium hydrogen phosphate (0·2 M, pH 8) containing cysteine

hydrochloride (0·005 M) to reduce polyphenoloxidase activity (Walker, 1964). The extract was filtered through two thicknesses of fine muslin and centrifuged at 7000 *g* and 5° for 15 min. The clear, pale yellow-green supernatant fluid was decanted, the precipitate being discarded. The supernatant fluid was extracted several times with ethyl acetate; the ethyl acetate layer (containing chlorophylls, phloridzin, phloretin, chlorogenic acid) was discarded, and the aqueous residue concentrated to a syrup on a rotary evaporator under water-pump vacuum at 35°. The syrup was dialysed against distilled water for 12–18 hr in a cold room at 5°. The diffusate, which consisted mainly of compounds Blue I and Blue II, together with the lower molecular weight sugar, phenolic, amino acid and peptide components of apple, was evaporated to small volume.

In an alternative method leaves were macerated in 50% (v/v) aqueous methanol. After filtration the extract was acidified (pH 5) with acetic acid and concentrated under vacuum on a rotary evaporator. The water-soluble components were decanted from the precipitated chlorophylls and, after extraction with ethyl acetate, were worked up in the same way as the buffer extracts.

Apple phenolics including Blue I and Blue II could also be obtained from young shoots of Miller's Seedling. The dormant shoots were powdered in a hammer mill and the powder extracted with 50% (v/v) aqueous methanol.

Several methods were used for the separation of pure Blue I and II from the crude concentrate.

Chromatography. Whatman no. 1, no. 20 and 3 MM papers were used. Solvents were B.A.W.: *n*-butanol + acetic acid + water (63 + 10 + 27, by volume); P.E.W.: *n*-propanol + ethyl acetate + water (7 + 2 + 1, by volume); and 2% acetic acid. Formic acid (2%) was used to develop thin-layer chromatograms, with Schleicher & Schull cellulose powder no. 145 (0·5 mm.) as the adsorbent.

Electrophoresis. Phosphate buffer (pH 7; equal volumes 0·02 M-NaH_2PO_4 + 0·02 M-Na_2HPO_4) or morpholine + acetic acid buffer (pH 8; 1·1% + 0·6%, by vol.) was used with Whatman 3 MM paper at 26·7 V./cm.

Electrophoresis or chromatography gave distinct spots or bands which could be detected under ultraviolet radiation (3650 Å) in the presence of ammonia vapour. The spots or bands which fluoresced bright light-blue were cut from the paper, eluted with distilled water, the eluates concentrated, and stored at 0°.

Gel filtration on columns of either Sephadex G 10 or Bio-Gel P2 was of limited use for the separation. Sephadex tended to absorb materials irreversibly from the plant extract and thus decreased the efficiency and loading capacity of the column. Bio-Gel did not give a clean separation between the components of the mixture. Changing the solvent from chloroform-saturated distilled water to 2% acetic acid or weak salt solutions did not improve the separation.

RESULTS

Biological activity of compounds Blue I and Blue II

In vitro. The effects of Blue I and Blue II on the germination of conidia of *Venturia inaequalis* were studied by using a modification of the method of Montgomery & Moore (1937). Solutions (0·1 ml. E_{255} 4·8 in a 1 cm. cell) of Blue I and Blue II were dried on glass slides and suspensions (0·1 ml.) of conidia applied to the dry deposits.

In the controls the conidial suspension was put on to an untreated slide. The slides were incubated at 18° and observed at intervals.

The fungistatic effect (Table 1) was not observed in the presence of 2 % Oxoid malt extract (Batch 114) nor when the Blue I or Blue II were present in lower concentrations. Solutions of Blue I and Blue II were added to 5-day cultures of *Venturia inaequalis* growing in early log phase. After 12 days the mycelium was removed by centrifugation (20,000 *g* for 10 min.). The extracellular pigment was then fractionated by precipitation from the culture fluid with increasing concentrations of ethanol (Hignett & Kirkham, 1967). The extinctions of the N 50 fraction (50 %, v/v, ethanol in water) and N 75 fraction (75 %, v/v, ethanol in water) were compared with those of control cultures. The N 50 pigment produced in the presence of Blue I and Blue II was not only present in larger amounts than in the control culture but was also more opaque to ultraviolet radiation when compared on a dry weight basis (Table 2). The extinction of the N 75 pigment was unaltered but less was produced when Blue I or II were present.

Table 1. *Effects of Blue I and Blue II on germination of conidia of Venturia inaequalis*

	Time (hr)					
	20	24	48	72	120	
	Estimated germination (%)					Observations
Control	90	90+	90+	90+	90+	Germination and growth normal
Blue I*	10	10	10	10	10	Germination poor; no growth
Blue II*	10	10	10–20	20–30	30+	Germination patchy; slight growth

* 0·1 ml. E_{255} 4·8 in a 1 cm. cell.

Table 2. *Effects of Blue I and Blue II (Final E_{255} in medium 0·72 (1 cm. cell)) on pigment production by log phase cultures of Venturia inaequalis*

	N 50			N 75		
Culture	Weight (mg.)	Total extinction*	Total extinction/mg.	Weight (mg.)	Total extinction*	Total extinction/mg.
Control	4·9	8·1	1·65	125	76	0·605
Blue I added	8·2	26	3·34	103	63	0·61
Blue II added	6·25	20·4	3·07	72	57	0·79

* Total extinction, equivalent to E_{275} × volume (ml.).

In vivo. Tests were made in a temperature-controlled greenhouse on plants of MM. 109 clonal rootstock. 1·5 ml. solutions of Blue I (E_{255} 8·0) or Blue II (E_{255} 4·8) were added to an equal volume of the inoculum which was then applied to leaves by means of an atomiser. The incidence of disease on leaves treated with inoculum to which Blue I or Blue II had been added was less than 3 % lesion cover. Lesion cover on the control leaves amounted to 48 %.

As Blue I and Blue II had been shown to interfere with conidial germination *in vitro*,

an experiment was made in which Blue I and Blue II were sprayed separately on to MM. 109 leaves at different times after the inoculum had been applied.

Figure 1 shows that inhibitory action of both Blue I and Blue II was restricted to a 48 hr period after inoculation. When applied after 6 days there was no difference in degrees of infection between a Blue II treatment and the control.

Leaves of the apple cultivar Miller's Seedling were inoculated with *Venturia inaequalis* conidia and removed from the plants either at once or 24 hr later. Epidermal scrapes were made on a freezing microtome stage (Kirkham & Hunter, 1965) and

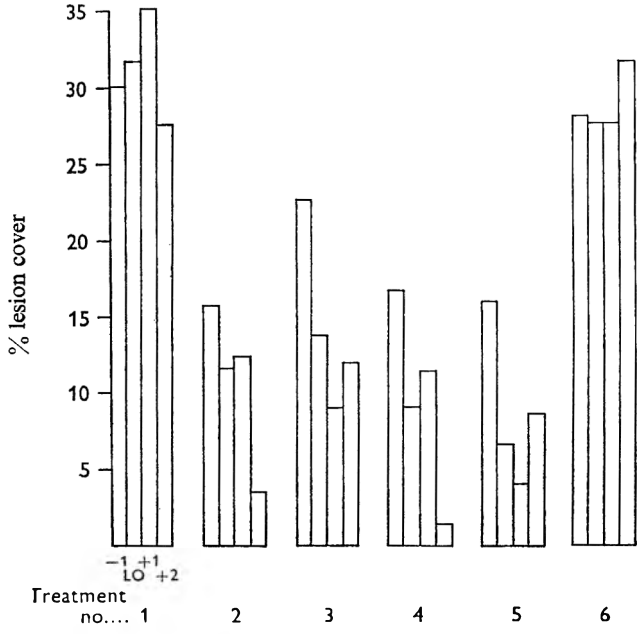

Fig. 1. Effects of Blue I and Blue II [1·5 ml. E_{255} 4·8 (1 cm. cell)] on the incidence of *Venturia inaequalis* on MM 109 rootstocks (four recorded leaves: −1, LO, +1, +2). (1) Control. (2) Blue I applied 24 hr after inoculation. (3) Blue II applied 24 hr after inoculation. (4) Blue I applied 48 hr after inoculation. (5) Blue II applied 48 hr after inoculation. (6) Blue II applied 6 days after inoculation.

dried in a vacuum desiccator over phosphorus pentoxide. Leaf areas sampled were in each case equivalent to 65·8 cm.^{2} and yielded a dry sample weighing 25 mg. The dried epidermal material was shaken with 0·5 ml. 80% (v/v) methanol in water, and samples of the supernatant fluid were used for thin-layer chromatography. The amounts of fluorescent components present were compared in a recording densitometer (Chromoscan) in the presence of ammonia vapour (Hignett, 1967). Although the intensity of fluorescence due to the first spot had increased twofold 24 hr after inoculation, that due to the second spot had increased by a factor of seven and that due to the third spot by nearly four times (Table 3).

Characterization of Blue I and Blue II compounds

Comparison was made on thin-layer chromatograms of Blue I and Blue II, eluted after electrophoresis and used in slide tests and greenhouse trials, with the blue fluorescent material in Miller's Seedling epidermal scrapes.

Blue I gave one rather indistinct spot, R_F 0·75–0·9, with 2% formic acid and proved to be homogeneous in a variety of other chromatographic solvents.

Blue II, on the other hand, gave three spots, R_F values 0·8, 0·7 and 0·56, with 2% formic acid. Rechromatography of the spot with R_F 0·7 (using the same solvent) gave two spots R_F values 0·8 and 0·7. Rechromatography of the spot with R_F 0·56 gave two spots R_F values 0·8 and 0·56. That there were two substances present, each of which existed as an equilibrium mixture of two isomers, was confirmed by electrophoresis.

Table 3. *Increase in intensity of fluorescence of TLC spots obtained from Miller's Seedling leaves 24 hr after inoculation with Venturia inaequalis*

	Spot	R_F	Control*	24 hr*	Increase†
Blue I+II	1	0·8	2·7	4·8	×2·4
	2	0·7	1·6	4·0	×7·25
Blue II	3	0·56	1·6	2·8	×3·75

* Area under densitometer curve in cm.²

† Numerical results compared by method of Hignett (1967).

Comparison of the R_F values obtained with those of the spots measured in the Miller's Seedling epidermis (Table 3) showed that the increase in materials contained in spot 1 of the latter was an increase in Blue I and the faster running Blue II isomers, while increases in spots 2 and 3 were due to increased amounts of Blue II only. Thus Miller's Seedling leaves responded to invasion by *Venturia inaequalis* by producing large quantities of Blue I and Blue II.

Neither Blue I nor Blue II reacted with diazotized *p*-nitroaniline, ferric chloride, ammoniacal silver nitrate or Fehling solution. Both appeared faintly blue under ultraviolet radiation after chromatography or electrophoresis, and fluoresced bright light-blue in the presence of ammonia vapour. Determination of pK values was done by electrophoresis at various hydrogen ion concentrations; pK_a values of 3·5 and 8·5 were found, indicating carboxyl and phenolic hydroxyl ionisations. The ultraviolet spectra of both compounds had $\lambda_{max.}$ 300 mμ and $\lambda_{min.}$ 250 mμ. Sealed tube hydrolysis at 100° with 5 N-hydrochloric acid or 5 N-sodium hydroxide gave tars and no identifiable product.

DISCUSSION

The attack of *Venturia inaequalis* on apple has been found to induce a complex of inter-related reactions. The pathogen, by producing melanoprotein pigments, influences the solute transport in the leaf of a susceptible variety, making the leaf a more favourable environment for growth. The response to infection which we have observed in the resistant variety Miller's Seedling is an increase in the quantities of some of the components of the phenolic fraction present in the leaves. The amounts of the substances referred to for convenience as Blue I and Blue II, for example, increased by factors from two to seven. Blue I and Blue II have been found to be detrimental to the growth of the fungus, not only in their prevention of germination of conidia, but also in their interference with normal pigment production in culture and normal growth of the fungus on leaves. This type of response to infection we propose to call 'active resistance'. This concept in the field of natural resistance of plants to microbial attack needs some clarification. Although some excellent reviews of this subject have

been published (Walker & Stahmann, 1955; Farkas & Kiraly, 1962) some confusion has been evident of late arising mainly from the misuse of the term 'phytoalexin' and a stretching of the original concepts behind the term. In a recent paper Cruickshank (1966) quoting the hypothesis of Müller & Börger clearly stated that: '...phytoalexin...is formed or activated only when the host cells come into contact with the parasite'. In further discussion he stated: 'Phytoalexins have not been detected in uninfected fresh plant tissues nor do they normally occur as wound responses.' This clearly defines the position and occurrence of phytoalexins. The picture is immediately clouded, however, by the section in the same paper on non-microbiological induction of phytoalexins. Under the influence of the heavy metal ions and metabolic inhibitors mentioned in that section, the production of pisatin, ipomeamarone and isocoumarin must be due to a non-specific reaction of the host. It follows, therefore, from the original definition of the term, that pisatin is not a phytoalexin, nor are ipomeamarone or isocoumarin. We believe that such a reaction to physical or chemical damage, occurring in the absence of an invading organism, should not be confused with a true host/parasite interaction and that the original definition of a phytoalexin should be strictly observed.

It may be that this effect of a parasite on the host can be closely imitated by physical or chemical means, but, considering the specificity of the host/parasite interaction and the generalized effects of the inhibitors mentioned, this appears to be unlikely. To dispel this confusion we propose the adoption of the following five classes of natural resistance of plants to attack by parasites. Here we would stress that we are considering only biochemical factors involved with fungal infections and neither morphological differences between plants nor reactions between plants and other parasites.

The first three classes are characterized by little or no biochemical interaction between host and parasite.

Passive resistance. This is shown by plants which have in their tissues a preformed fungicidal or fungistatic agent in sufficient concentration to prevent the germination of the fungus or to stop its establishment if germination and infection have taken place. The onion/*Colletotrichum circinans* complex is an example of this passive resistance (Walker & Stahmann, 1955).

Wound reaction. This is the general non-specific reaction of the plant to disruptive damage of any sort which gives rise to a protective layer.

Hypersensitivity. This type of resistance is characterized by the death of the host cells at the site of invasion by the pathogen. This isolates the invading organism, which dies either from the effects of having its supply of nutrients cut off or from the action of toxins produced by the death of the host cells (Shay & Williams, 1956, Class I reaction).

In the remaining classes of resistance there is a direct biochemical interaction between host and parasite typified by the production of antifungal compounds.

Phytoalexin production. This should be restricted to mean the production of a specialized metabolite not present in the healthy host. This metabolite (the phytoalexin) is formed by interaction between host and invading organism and is inhibitory to the latter.

Active resistance. This is the resistance of the type shown by the apple variety Miller's Seedling to *Venturia inaequalis* discussed in the present paper. Active resistance implies the stimulated production of fungistatic agents by the host, under the influence of the

parasite. The agents produced differ from phytoalexins in that they are present before inoculation, and are increased in response to the pathogen. It is not yet clear whether this is a *de novo* synthesis or a mobilization of reserves.

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Laboratory Cultivation of Some Human Parasitic Amoebae

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SUMMARY

Cultural conditions and supplementary substances necessary for the laboratory cultivation of some human parasitic amoebae were investigated by using a basal solution containing inorganic salts, citrate and lactate. Three supplementary components were found necessary: starch grains, animal protein (soluble or insoluble) and living bacteria. Restraint of bacterial growth by antibiotics, and of the development of an alkaline reaction (due to pellicle-forming aerobes) by carbon dioxide, improved the amoebic growth. Under these conditions, all the common parasitic amoebae examined, except *Iodamoeba butschlii*, were grown and maintained for long periods in laboratory culture.

INTRODUCTION

This work began with the observation of *Entamoeba histolytica* trophozoites in a culture after incubation for 24 hr at 37° of cyst-containing faeces suspended in a simple defined medium suitable for growing *Escherichia coli* (Robinson, 1951). A method for cultivating the parasitic amoebae was then developed in which a liquid culture of *E. coli* in this defined medium + starch + erythromycin was overlaid on a slope of saline agar in a quarter-ounce screw-capped bottle (gas-phase air) and inoculated with cyst-containing faeces (of normal bacterial content). By this process *E. histolytica* was grown in primary culture and in the first subculture, while other amoebic species occasionally appeared in recognizable numbers. But even when normal (i.e. amoeba-free) faeces was added, further amoebic subcultures did not grow. It was decided to examine the nature of the supplements which were needed to obtain continuous subculture of amoebae in *E. coli* cultures in the defined medium + starch + erythromycin overlaid on saline agar.

METHODS

Saline agar slopes. Agar 1·5% (w/v) + NaCl 0·7% (w/v) in water was distributed in 2·5 ml. volumes in quarter-ounce screw-capped glass bottles and sloped after autoclaving.

Antibiotics. Solutions of erythromycin, ristocetin and streptomycin (0·5%, w/v), chloramphenical (0·2%, w/v) and polymyxin (5000 units/ml.) were used.

Rice starch. Rice powder (British Drug Houses Ltd.) after drying was dry-sterilized in partly filled quarter-ounce bottles with caps screwed tight.

Phthalate (0·05 M) diluent. Potassium hydrogen phthalate 10·02 g. and sodium hydroxide 2 g. were dissolved in 1 l. water, adjusted to pH 6·5 and the solution autoclaved.

Defined medium R for growing Escherichia coli. Concentrated stock solution consisted of 125 g. sodium chloride, 50 g. citric acid monohydrate, 12·5 g. potassium

dihydrogen phosphate, 25 g. ammonium sulphate, 1·25 g. magnesium sulphate heptahydrate and 100 ml. lactic acid (British Drug Houses Ltd., 90·08 %) in 2·5 l. water. For R medium, one volume of concentrated stock solution was diluted with nine volumes of 0·33 % (w/v) sodium hydroxide, adjusted to pH 7 and autoclaved. Stock more than 4 weeks old was used to avoid pH change on autoclaving.

Basal amoebic medium BR. Escherichia coli strain B was incubated for 48 hr at 37° in shallow layers of medium R in sealed flat bottles; this living culture was the basal medium BR.

Supplemented media for amoebic growth. The general method of preparing supplemented media was to boil a proposed supplement in medium R, filter through Whatman no. 1 paper, autoclave the filtrate at pH 7, inoculate with *Escherichia coli* B, incubate at 37° in shallow layers for 48 hr and store the living culture at room temperature until required. Media with 70 different complex supplements were thus prepared; the main supplements used are indicated in Table 1; the preparation of four are described below:

Medium BRS (*serum added*). Equal volumes of medium BR and Seitz-filtered heated (56°) serum (sheep) were mixed and incubated for 48 hr at 37°. Sheep serum was better than any other.

Medium BRMt (*serum metaprotein added*). Metaprotein (Cole, 1919) was prepared to exclude soluble (at pH 7) material, by sulphosalicylic acid precipitation of human serum. The precipitate was washed with water, the suspension boiled, dissolved in NaOH, filtered, re-precipitated by acetic acid and the precipitate washed for 4 days, and autoclaved as an aqueous cream. For use, 5 ml. was added to 100 ml. medium R, (adjusted to pH 7) steamed, inoculated with *Escherichia coli* B and incubated.

Medium BRT (*tripe extract added*). Tripe (500 g.) was sliced into 1 cm. cubes, boiled with 1 l. medium R for 4 hr on 3 successive days, filtered through lint, the extract autoclaved, inoculated with *Escherichia coli* B and incubated.

Medium BRG (*gastric mucin added*). Gastric mucin (Armour) was triturated in medium R to give 1 or 2 % (w/v), autoclaved, inoculated with *Escherichia coli* B and incubated.

The inocula of amoebae used and the maintenance of the amoebic cultures. Except for two strains of *Entamoeba histolytica*, all the amoebae used were derived from the faeces of patients and were subcultured two or three times weekly in phthalate dilutions (1/2 to 1/10) of medium BRS and medium BRT, containing antibiotic (usually erythromycin) and starch, as a liquid overlay on the saline agar slopes. Bactopeptone was often added to about 0·2 %. All cultures were cleared of Blastocystis and in later work *Pseudomonas aeruginosa*.

Method for removal of Blastocystis. Culture sediment (0·4 ml.) was allowed to settle for 2 min. in 4 ml. of 0·1 N-HCl, the fluid removed and the remaining settled fragments taken up in a few ml. of liquid overlay from the culture to be inoculated (Smedley, 1956).

Methods for antagonizing bacteria in amoebic cultures. These methods were required for two purposes: (*a*) the elimination of bacterial species, (*b*) the slowing of multiplication of a mixed flora on which the present medium is based. The interactions of the bacterial species in these amoebic cultures were too complex for their control to be anything but empirical; no transfer of trophozoites (as opposed to cysts) was possible in the absence of living bacteria in the inoculum (apart from the presence of *Escherichia coli* in the culture medium). For purpose (*a*) four or five successive amoebic sub-

cultures were made in medium diluted to the maximum degree (by medium R or phthalate solution) which permitted the amoebae to grow at all, and containing the appropriate antibiotic. For total elimination of bacteria a prolonged series of tests on successive amoebic subcultures was needed to be sure that an apparently eliminated bacterial species had not become latent. For purpose (*b*) continuous dosage with antibiotic (usually erythromycin 10–200 μg./ml.) was added to the medium. When bacterial aerobes increased, to the detriment of amoebic growth (see later), a stream of carbon dioxide was used to displace most of the air above the culture for a few seconds before screwing up the cap of a bottle (this process was also used with polymyxin for elimination of *Pseudomonas aeruginosa*). Escape from antibiotic control, i.e. development of resistance in the bacterial flora of amoebic cultures, was the commonest cause of the dying out of amoebae kept for long periods as stock strains. This occurred more commonly when chloramphenical or penicillin was used than with erythromycin. It was corrected by dispersion of whole sediment from an amoebic culture in saline medium containing several antibiotics in high dosage. After settling for 30 min. the saline solution was replaced by amoebic culture medium + starch, the whole process (growth + treatment) being done, as often as necessary, in 9 ml. volume in a screw-capped one-ounce bottle without a saline agar slope.

Testing for amoebae in supplemented media. The various supplemented media (after *Escherichia coli* B had been grown in them) were diluted 1/2 to 1/10, usually with phthalate diluent, occasionally with medium R or medium BR, + starch + antibiotic solution, and overlaid on saline agar slopes at the time of inoculation with amoebae. Sediment from the first bottle was then transferred, after 1–4 days at 37°, to a second similar bottle, and so on, until a sufficient number of subcultures showed that the amoebae grew continuously in the given medium. Microscopic examination of wet preparations in iodine solution was the means of assessment; this gave information about the state of the starch, the bacteria, the degree of protein coagulation and the carbohydrate metabolism (colour in iodine) of the trophozoites, as well as their number, size and disposition (clumped or diffuse). Generally, number and size of trophozoites, indicating satisfactory growth, ran parallel (in small as well as large species) to iodine-staining of the cytoplasm, which itself constituted a criterion of good growth. From this information, variations and replications were made as the amoebic subculture series proceeded, to prolong it as far as possible.

CONCLUSIONS

Growth maxima from the main groups of supplemented media are shown in Table 1. Because different strains of the same amoebic species did not always give the same results on the same medium it was concluded that the effect of the supplement on the amoebic growth was because of the accompanying bacteria. This was supported by the observed lag in amoebic growth on first transferring a species from one supplemented medium to another, which suggested that adaptation of the bacterial flora was involved. It also accorded with the fact that continual adjustment of concentration and type of antibiotic was one of the principal means by which prolonged growth of the protozoal series was obtained with the less easily grown amoebic species and less effective ented supplemmedia. Another means of affecting the bacterial flora, and so prolonging a series of amoebic subcultures, was by addition of some of the substances (particularly

Bacto-peptone and Lab-Lemco) shown at the end of Table 1 as incapable of supporting amoebic growth by themselves alone.

For *Entamoeba coli*, *Entamoeba hartmanni* and *Endolimax nana*, a single strongly growing strain of each was selected for the experiments. For *Dientamoeba fragilis*, two strains were used which, though mainly similar in behaviour on the various supplements, were different on medium BRG ('Newman' died after 53; 'Raja' after 17 subcultures) and with a mucoid material prepared from human colon (Newman 2, Raja 21 subcultures). For *Entamoeba histolytica*, 8 strains were used and discrepancies

Table 1. *Maximum numbers of positive serial subcultures obtained with different species of human parasitic amoebae with various supplements*

Figures with + indicate that amoebae were still growing when discarded; figures without + indicate that amoebae died at this stage.

Source	Media and supplements, etc.	Protozoa				
		Entamoeba histolytica	*Ent. coli*	*Ent. hartmanni*	*Endolimax nana*	*Dientamoeba fragilis*
Serum	BRS; basal medium + serum (see Methods)	100+	100+	100+	100+	100+
	BRMt; basal medium + metaprotein (see Methods)	57+	40+	20+	63+	11+
	Extract of heat-coagulated human serum	30+	14+	16	14+	14+
	Same, Seitz-filtered	15+	3	13	2	0
	Inspissated serum slope + phthalate extract overlay after BR	18+	20+	8+	28+	8+
	Seromucoid (Winzler *et al.* 1948)	16+	9+	16+	8+	2
Colon	Human colonic mucoid purified (Hawk, 1923)	12+	27	8	2	21
Tendon	Ox tendo-mucoid (Hawk, 1923)	10+	5+	2	5	0
Stomach	BRT (see Methods)	64+	78+	16+	112+	63+
	Tripe mucoid (Hawk, 1923)	13+	4	16	22+	10
	BRG (see Methods)	50+	49	40	41	53
Muscle	Extract of horse muscle	22+	2	0	0	2
	NaOH extract of ox heart, pptd. by HCl, washed, used solid	15+	1	22+	4	13
	Filtrate from previous	2	0	1	1	1
Liver	Extract of human liver	14	1	2	2	4
Fish	Extract of tinned salmon	25+	2	0	5	8
	Extract of fresh plaice	10	1	0	0	0
Hen eggs	Extract of hard-boiled eggs	16+	12+	12+	4	17+
Gelatin	Steamed at 2 % (w/v) in R	3	3	2	2	2
	As previous, but incubated with *Proteus mirabilis* (instead of *Escherichia coli*)	5	11	3	2	1
Yeastrel, Marmite, Lab-Lemco, Bacto-peptone, Proteose peptone, Bacto-casitone, Bacto-casamino-acids, asparagine, glucosamine, cholesterol, fish solubles, distiller's solubles, vegetable mucoid from *Plantago coronopus*, faeces and controls of BR without supplement.		< 3	< 3	< 3	< 3	< 3

occurred from the maxima recorded in Table 1: one died after 8 subcultures on inspissated serum, two after 3 with fibrinogen extract, one after 4 with horse-meat extract and two after 3 with plaice extract. Differences observed between species (e.g. *E. hartmanni* required more concentrated supplement and more varied antibiotic control of bacteria, than *E. histolytica*) were like those found between more and less fastidious strains of one amoebic species. This suggested that the nutritional requirements of the various parasitic amoebae differed in degree but not in kind. If it be assumed that the maximum amoebic survival (owing to traces of growth factor in the inoculum and in some of the cultures themselves) is three subcultures, then the results of Table 1 indicate that many bacterial nutrients (final group in Table 1) were incapable of supporting amoebic growth, but that protein supplements were able to do so. When protein was completely removed from any of the supplements amoebic growth did not occur. For example, fibrinogen was removed from solution as coagulum on autoclaving; the resulting clear solution did not support amoebic growth. But a cloudy suspension of fibrinogen, prepared by repeated heating, trituration and lint filtration, gave the amoebic growth maxima shown in Table 1. The clearest extracts shown in Table 1 were those from human liver, plaice and horse meat, which give poor amoebic growth maxima. The point was confirmed by showing that the removal of 'cloud' by the Seitz filter removed also the property of supporting amoebic growth, e.g. from medium BRG in Table 1, except in the case of seromucoid, which (unlike the tissue mucoids) passed the Seitz filter and was not coagulated by heat.

Table 2. *Insufficiency of either phosphotungstic precipitate (X: 1 ml.) or filtrate (Y:1 ml.) of deproteinized mucoid-containing serum filtrate to support two successive cultures (a) and (b) of Entamoeba histolytica and Entamoeba coli, unless reinforced by extract (Z: 1 ml.) of heat-coagulated whole serum in R medium.*

Defined medium = R. Culture of *Escherichia coli* B in R = BR.

Bottle	Composition of 3 ml. overlay, with starch grains + erythromycin 20 μg./ml. on saline agar slope			Trophozoites/ml. after 3 days incubation			
				Entamoeba histolytica		*Entamoeba coli*	
				(*a*)	(*b*)	(*a*)	(*b*)
1	X	BR	R	10,000	0	0	0
2	Y	BR	R	0	0	0	0
3	X	Y	R	3,000	0	1,000	0
4	X	BR	Z	50,000	26,000	35,000	31,000
5	Y	BR	Z	75,000	16,000	30,000	13,000

An experiment with one strain of *Entamoeba histolytica* and one of *Entamoeba coli* showed that the efficiency of serum as growth factor was not simply due to its seromucoid content: when deproteinized serum filtrate containing seromucoid was separated by phosphotungstic acid into mucoid-containing precipitate incorporated in medium BR at about 0·1 % (w/v), as in serum, and into mucoid-free filtrate similarly incorporated (after dialysis) in medium BR, both supplements needed addition of cloudy filtrate of heat-coagulated whole serum boiled in medium R to restore amoebic growth in subculture (Table 2).

The suitability of mucoid supplement as amoebic growth supplement led to a test of

gastric mucin (Armour and Co.), with which (alone of the manufactured products tested) long runs of subcultures sometimes resulted. Continuously viable cultures of *Entamoeba histolytica* with centrifuged 0·05 % (w/v) commercial gastric mucin in saline were reported by Dolkart & Halpern (1958) who stated that their results were improved by adding small amounts of egg medium.

Once the need for protein for amoebic growth became clear, the question was: were proteolytic bacteria necessary to split it? Accordingly, the bacteria accompanying some of the *Entamoeba histolytica* strains were examined after the amoebae had grown. Anaerobic plates showed predominance of *Escherichia coli* but aerobically heavy growths of Aerobacter, Achromobacter, Proteus and Pseudomonas species occurred; *Streptococcus faecalis* was always present and Clostridium species absent. After elimination of the proteolytic Proteus, Pseudomonas and Bacillus species by the technique given above, serial amoebic subcultures were made on medium BRMt with parallel inoculations into nutrient gelatin at each transfer. Of 36 such cultures, most showed gelatin liquefaction after 3 days and all but two after 14 days. The organism responsible was found to be a proteolytic variety of *S. faecalis* which was impossible to eliminate; although it could be decreased apparently to zero for a time, it always reappeared in the cultures. Two *Entamoeba histolytica* strains were isolated monoxenically from faecal cysts by the method of Singh, Das & Saxena (1963) by using 24 hr instead of the recommended 48 hr in dilute HCl suspension and inoculation into overlays of BRS and BRT media with starch and erythromycin (10 μg./ml). Blastocystis (one strain) grew but was removed by sedimentation in HCl solution (above). Both these strains were successfully transferred to medium BRMt and gave runs of more than 8 serial subcultures, tested by parallel inoculations of aerobic and anaerobic plates at 37° and 25°, as well as by inoculation on nutrient gelatin. The plates showed *Escherichia coli* B to be the only organism accompanying the amoebae; 30 gelatin controls did not liquefy in 14 days.

The finding that proteolytic bacteria were not essential for *Entamoeba histolytica* and, by inference, for the other strains used, supported the idea that no liquid amoebic growth factor was liberated by bacteria from protein. Attempts to grow amoebae by the diffusion of growth factor through agar layers or viscose sacs always failed.

At this point the microscopical appearance of the cultures became relevant, for it had been noticed that the growth of all amoebic species tended to be in foci, the individuals being clustered upon bacterial colonies. Plate 1, fig. 1, 2 give a cross-section of the trophozoites in such clusters. Over 100 *Endolimax nana* individuals were calculated to be present in the bunch shown in Pl. 1, fig. 2. The failure to find a soluble metabolite suggested that the trophozoites might be ingesting protein fragments directly, hence they appeared to be clumped on the bacterial colonies, but were really on the protein beneath. Microscopic study of the sediments from BRMt cultures left no doubt that this was not so: the trophozoites could be seen in contact with bacterial networks which had often formed around protein fragments; but to these fragments themselves no trophozoites were ever seen to be attached. Plate 1, fig 3 shows such an arrangement in the case of *Entamoeba coli*. Because the outline of the protein fragment was sacrificed in focusing the amoebae, a diagram of the positions in the microphotograph is given beside it. The conclusion of this part of the work was therefore that 'amoebic growth factor' was ingested by trophozoites in the form of protein-nourished bacterial bodies.

The gas phase in amoebic cultures

Experiment 1. For growth of my amoebae in the manner described above, it was not necessary to tighten the cap of the bottle; but growth did not occur in a shallow layer of supplemented BR medium + starch + antibiotic in an open container without a saline agar slope. This observation was investigated by serial subcultures of *Entamoeba histolytica* under varied physical conditions in 0·8 ml. volumes of medium BRS and medium BRG in 70 × 11 mm. tubes in a moist incubator. Figure 1 gives as a diagram the conditions and amoebic counts (at peak) with medium BRG. The results showed that continuous serial subcultures were possible under these conditions: (*a*) with 1·5 ml. saline agar slope; (*b*) with slope replaced by a glass rod of equal volume; (*c*) with bung in tube, but not under conditions (*d*) with open tube in standard bacteriological anaerobic jar (pure hydrogen); (*e*) with open tube in air. When the height of the liquid

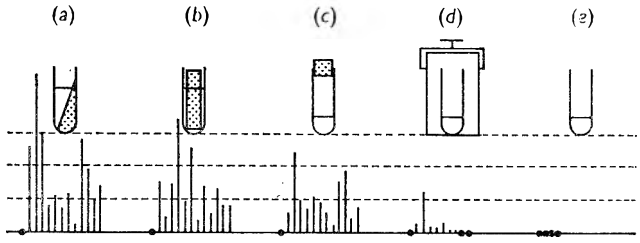

Fig. 1. Growth of *Entamoeba histolytica* under five physical conditions in 70 × 11 mm. tubes, containing starch and 0·8 ml. volumes of medium BRG diluted 1/5 in R with erythromycin 20 μg./ml. The base line represents zero, the three dotted lines 100, 200 and 300 thousand trophozoites/ml. The vertical lines represent the trophozoite counts in successive cultures. Conditions: (*a*) with 1·5 ml. saline agar slope; (*b*) with 1·5 ml. glass rod; (*c*) with bung in tube; (*d*) with tube in hydrogen atmosphere of anaerobic jar; (*e*) with tube open to air.

column was increased in (*d*) and (*e*) to that of (*a*) and (*b*) by increasing the volume, growth still failed after a few subcultures in hydrogen (*d*), but succeeded in the tube open to air (*e*). Therefore, when open to air, the cultures showed a critical surface: height ratio, although they were not inhibited by air in the bunged tube (*c*). This finding was confirmed many times with various media in various vessels, and with various species of amoebae.

Experiment 2. Drops of amoebic cultures mixed on a tile with pH indicators showed very rapid alkaline increase suggestive of loss of CO_2. On releasing the bottle caps, bubbles rose from below the base of the slope, showing that gas had been retained under pressure. Abundance of CO_2 was shown in the cultures by passing a stream of hydrogen into lime water. For rough estimations, overlays from cultures in medium BRS were pooled, and 10 ml. titrated with 0·05 N-Na_2CO_3 from pH 7 to maximum colour with phenolphthalein (corresponding to excess Na_2CO_3); this took 23 ml.; a blank titration with medium BRS from pH 7 took 6 ml. Since 1 ml. of 0·05 N-Na_2CO_3 absorbs 0·0011 g. CO_2, this gives 1·87 g. CO_2/l. culture, if all the acidity in this range were due to CO_2.

Experiment 3. Other gas-phase experiments were done with liquid BRS + starch cultures in 8 × 2·5 cm. one-ounce glass bottles closed by screw caps when required (incubation in a water bath at 37°). Bubbling gases through these cultures was un-

successful, and gas was therefore passed through a reservoir to lessen pressure changes, to emerge from a nozzle 2 cm. above the level of medium in an uncapped bottle. Each run of 4 days comprised four bottles containing equal amounts of medium, starch, antibiotic when needed, and inoculum. Two bottles received gases; the third was capped and the fourth uncapped as control. Amoebae were counted daily in a haemocytometer. The gas flow was kept at about 1 l./hr. Culture volumes were restored daily by adding water. These experiments were to compare the effects of carbon dioxide and of oxygen as shown in Fig. 2. The following results were obtained with three runs.

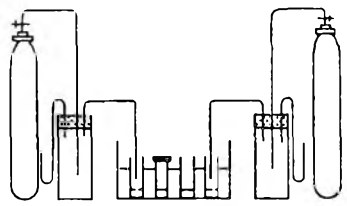

Fig. 2. Diagram of Expt. 3. Passage of gas from cylinder through jar with manometer tube to issue above liquid culture in bottle placed in water bath at 37°. Each run comprised two such arrangements (for comparison of two gases), accompanied by control cultures in capped and uncapped universals.

(1) Four 9 ml. volumes of medium BRS 1/5 in medium R + erythromycin 7 μg./ml. + starch were inoculated with *Entamoeba histolytica*. Amoebic counts (48 hr) were 27,500/ml. with 5% (v/v) CO_2 in oxygen, nil with oxygen, nil in uncapped control and 250,000/ml. in the capped control. (2) With the same amoebic strain and the gases as above, with 9 ml. volumes of undiluted medium BRS containing erythromycin 70 μg./ml. + starch, the amoebae/ml. (maximum at 48 hr) were: nil for the uncapped control; 20,000 for O_2; 360,000 for the $O_2 + CO_2$; 570,000 for the capped control. (3) With *Endolimax nana*, 3·5 ml. volumes of undiluted medium BRS with erythromycin 33 μg./ml. + starch, and gases O_2 and CO_2, the amoebae/ml. (maximum at 72 hr) were: 20,000 in uncapped control, 20,000 in O_2; 110,000 in CO_2; 170,000 in the capped control.

Nelson & Jones (1964) recorded improvement in amoebic growth by the use of CO_2 + bicarbonate buffer. From the present experiments it was at first concluded that CO_2 was necessary for amoebic growth; but it became clear as the work proceeded that inhibition of amoebic growth under these experimental conditions was due to the culture becoming alkaline because of the growth of a pellicle of aerobic bacteria. The pellicles on the cultures of Expt. 3 incubated in a mixture of CO_2 and O_2 were usually just detectable as very thin transparent skins, while the thick white rings of those without CO_2 were striking, and when bromthymol blue was added, the downward spread of an alkaline reaction was seen. The phenomenon was like that on which Simmons (1926) based his citrate + salts + bromthymol blue agar test for distinguishing *Aerobacter aerogenes* by the development of a blue colour, which did not occur when the culture was sealed; that is when CO_2 was retained above the surface of the medium; the development of carbonate from organic acids (Ayers & Rupp, 1918) in presence of abundant oxygen was inhibited by relatively small amounts of CO_2.

Experiment 4. Serial cultivation of amoebae on BR-based medium in the anaerobic jar gave variable numbers of positive subcultures on different occasions, but always

failed eventually. This was apparently due to progressive diminution of the bacterial flora because of oxygen lack. It also occurred when the jar was filled with CO_2 instead of H_2, and when the hydrogen-filled jar contained a CO_2 absorbent. Thus absence of O_2 from the gas phase prevented the growth of aerobic bacteria which promoted amoebic growth, provided that excessive alkali production did not occur. This was corroborated by the parallel effect on amoebic growth observed throughout this work of anaerobiosis or (*a*) partial CO_2 atmosphere, and (*b*) chemical antibiotics; both factors increased amoebic growth by restraining bacterial growth to a point beyond which they lessened it. Also, because of this effect, optimal concentrations of antibiotic were lower when CO_2 was added to the gas phase above the culture.

Experiment 5. Since the above experiments made it likely that the effect of the agar slope in the amoebic cultures was to establish a pH gradient by retaining CO_2, thus preventing alkalinization of the culture sediment from above while permitting aerobic bacterial growth at the surface, it seemed that the slope should be replaceable for long-term maintenance, by the alternate stimulation and repression of the growth of the aerobic bacteria. An experiment was made in which a strain of *Entamoeba histolytica* was maintained for 3 months without recourse to a saline agar slope by changing from shallow to deep conditions and vice versa. The rubber-capped 120 × 14 mm. tubes from (Capon & Heaton) transfusion sets were used with 9 ml. volumes of liquid medium for the deep conditions, which proved necessary once for every three shallow sub-cultures of 3 ml. volumes in one-ounce bottles.

The necessity for starch grains. Media were developed in which other components, e.g. living *Trypanosoma cruzi* in the medium of Phillips (1962), replaced living bacteria; in such media, starch particles appear to be unnecessary. But in the medium described above, no amoebic growth was ever obtained without particulate starch. This was tested for all the parasitic amoebae: the substitution of sugars, dextrin or soluble starch was ineffective in promoting the growth of any species in the absence of starch grains. In confirmation, an experiment with *Entamoeba histolytica* may be quoted. Trophozoites in a faecal specimen were observed to ingest a yeast. These trophozoites were isolated, grown separately and added to cultures of the amoeba in presence and in absence of starch. Without starch, the yeast was not ingested and the amoebae died; with starch, the yeast was ingested *in vitro* as it had been *in vivo*.

DISCUSSION

I have found only one report of previous work aimed to find an 'amoebic growth factor': Nakayama (1958) used a method, like that described here, of adding organic supplements to citrate + Ringer solution overlaying an agar slope. His conclusion is opposed to mine, since he found that protein preparations could be replaced by a variety of soluble protein breakdown products (e.g. proteose, glucosamine). It could be argued from the present results that particulate as opposed to soluble protein is required for amoebic nutrition. Even in 'clear liquid serum' medium a little coagulum was always produced.

Haematoxylin stains commonly show bacteria to be adherent to trophozoites from any source, and the probable ingestion of bacteria by *Entamoeba histolytica* has been confirmed by electron micrographs of bacteria in food vacuoles (Fletcher, Maegraith & Jarumilinta, 1962). 'Nests' (Balamuth & Howard, 1946), and 'crowding' of tropho-

zoites (Everitt, 1950), were terms used by these workers, without explanation. In the medium described in the present paper amoebic bunches (such as those shown in Pl. 1, figs. 1–3) were seen to be satellites upon bacterial colonies, parts of which often persisted as wisps, binding two or three trophozoites together after separation from the bunch. The siting of the amoebae thus followed that of the accompanying bacteria; the prominence of bunching seen in the present work was possibly due to the favouring of bacterial growth in coherent networks (see Pl. 1, fig. 3). Thus a scheme for the processes of growth in the type of amoebic culture described here might be formulated as follows. (1) At some points in the sediment there is focal decrease of pH value (perhaps due to liberated CO_2, held under the butt of the agar slope) sufficient to allow bacterial attack on starch grains. (2) Enough acid is formed from the starch to permit the coating of growing bacteria by protein (analogous to the coating of red blood cells for phagocytosis), from which trophozoites, by ingestion of such bacteria, obtain the ability ('growth factor') to ingest and metabolize grains of starch, which iodine staining shows to be important in their metabolism on the present medium. (3) The amoebae congregate and reproduce on the bacterial colonies, ingesting starch rapidly and possibly stimulating further bacterial growth in their neighbourhood by dextrin formation. (4) The break up of the amoebic clusters follows that of the bacterial colonies on which the amoebae are sited; this seems to be associated with further acid production. This scheme suggests that 'protein-conditioning' of bacteria for ingestion constitutes a difference between parasitic amoebae which require it, and free-living amoebae, which do not.

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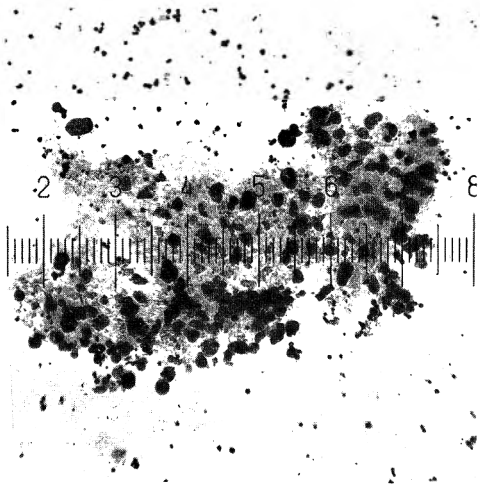

Fig. 1

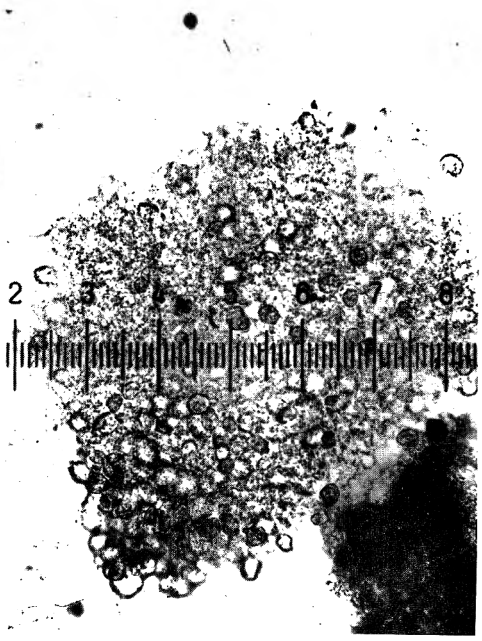

Fig. 2

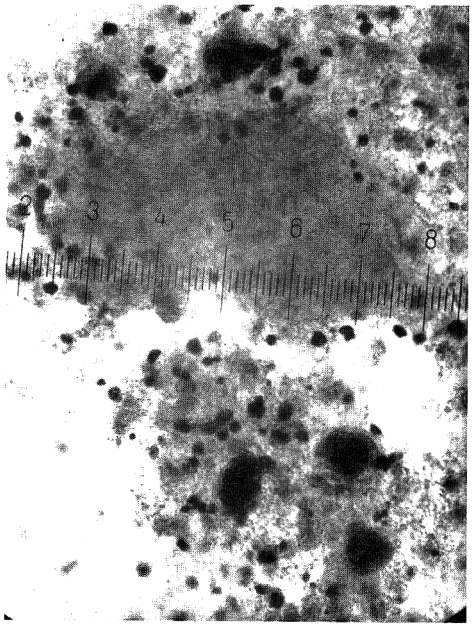

Fig. 3

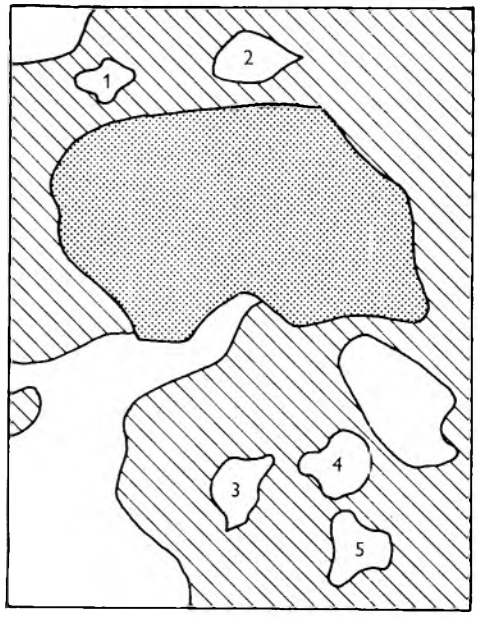

Fig. 4

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EXPLANATION OF PLATE

Fig. 1. *Entamoeba histolytica* (12353/60) culture. Trophozoites clustered on bacterial colony. Wet preparation in iodine. One scale division = 12·2 μ.

Fig. 2. *Endolimax nana* (2687/62) culture. Trophozoites clustered on bacterial colony. Wet preparation in iodine. One scale division = 3·4 μ.

Fig. 3. *Entamoeba coli* (10390/61) culture. Five trophozoites in bacterial network formed around (denser) fragment of metaprotein. Wet preparation in iodine. One scale division = 3·4 μ.

Fig. 4. Diagram of Fig. 3. Bacterial network striated, protein fragment shaded and *Entamoeba coli* numbered.

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Ascus Cytology of *Podospora anserina*

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SUMMARY

The ascus cytology of *Podospora anserina* (Ces.) Rehm, a secondarily homothallic Pyrenomycete fungus, has been followed under the light microscope from the crozier, through karyogamy and the three nuclear divisions in the ascus (the first two constituting a meiosis and the third a mitosis) to the first spore mitosis and spore maturation. The spindles remain widely separated at division II during which segregation for mating type is said to occur. After division III, in which the spindles are transverse, the ascus contains four pairs of sister nuclei. The realignment of the nuclei so that two non-sister nuclei are included in each of the four spores is brought about by the movements of the centrosomes or, more likely, of their outer portions. Spore delimitation, which starts from these organelles, appears as a thin cleavage line separating the spore plasm from the epiplasm of the ascus. A mitosis in the spore initial followed by degeneration of one of the four daughter-nuclei in the primary appendage leads to an imbalance in the mating-type factors in the spore. The wall of the mature spore is separable into three layers, the middle one of which is pigmented. The mucilaginous secondary appendage formed at each end of the spore is already present before the primary appendage is cut off.

INTRODUCTION

Podospora anserina (Ces.) Rehm is a Pyrenomycete fungus which has been the subject of many genetic studies. Its life-cycle was described by Ames (1932, 1934) and Dodge (1936) and some observations on its cytology were published by Moreau & Moreau (1951), Franke (1957, 1962) and Heslot (1958). An attempt is made here to give a more complete account of the cytology.

METHODS

Cultures of *Podospora anserina* were grown in Petri dishes on a medium of sheep dung agar, at room temperature in continuous light. Small pieces of sterile filter paper were mixed with the medium to enhance perithecial production.

Blocks of agar, approximately 3 mm^3 and containing numerous young perithecia, were fixed for 15 min. under vacuum in either 1:3 (v/v) acetic acid:ethanol solution, or a 1:3 (v/v) propionic acid ethanol solution, after which they were left in fixative at room temperature for periods of 24 hr to 6 weeks. The blocks were then washed for 2–24 hr under running water before treatment with a saturated solution of cytase (S/401 G.T. Gurr Ltd) for 10 hr. They were again washed in running water for 1 hr and then placed in a 400 μg./ml. solution of ribonuclease and incubated for 12–24 hr at

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35°. The blocks were then washed for a further hour, mordanted at room temperature in a 4% (w/v) iron alum solution and given a final wash for 30 min. prior to staining.

Perithecia were removed from the blocks and the asci dissected out into a drop of propiono-carmine on a glass slide. After adding more stain, the slide was heated to 60° and the asci squashed flat under a coverslip, excess stain being blotted up. Slides were then ringed with D.P.X. Mountant and allowed to cool down to room temperature.

Satisfactory preparations were obtained by this method, showing all stages from synapsis in young asci to division IV in the ascospores. However, in studying the croziers, the Feulgen-propiono-carmine technique of McIntosh (1954) gave better results in that hydrolysis for 8 min. in N-HCl caused the young asci to separate clearly on squashing and enabled the relative positions of the cross-walls and nuclei to be easily seen. Observations of maturing spores were made difficult by their increasing pigmentation.

All preparations except Pl. 2, fig. 20 were examined and photographed on a Zeiss Standard Universal Microscope fitted with a Zeiss Icon 35 mm camera. Photographs were taken on Kodak Panatomic-X film with the aid of phase contrast and a medium green filter, and developed in Kodak Microdol-X developer. All prints were made on Ilfobrom paper. Plate 2, fig. 20 was photographed on a Baker phase-contrast microscope fitted with a Trilux condenser and a Watson fixed-plate camera, using a Kodak B. 4 half-tone plate.

RESULTS

Croziers, karyogamy and division I

A typical crozier with a binucleate, penultimate cell and unicucleate terminal and stalk cell is shown in Pl. 1, fig. 1. The two nuclei in the penultimate cell fuse soon after the penultimate cell begins to enlarge to form the ascus (Pl. 1, fig. 2–4). Meanwhile, the stalk and terminal cell of the crozier unite (Pl. 1, fig. 3, 4) and the binucleate cell so formed is capable of growing out to form another crozier. This conforms with the normal development in Eu-ascomycetes and does not support the early observations from sections by Faull (1905).

The diploid nucleus resulting from karyogamy contains a single very large nucleolus. Contraction of the chromosomes and synapsis occur while the ascus is still only 15–20 μ in length (Pl. 1, fig. 2 (right), 4). When the ascus reaches 40–50 μ in length, the chromosomes are somewhat longer and are fully paired (Pl. 1, fig. 5, 6). When the ascus is 100–120 μ long, the chromosomes are fully extended at full pachytene (Pl. 1, fig. 7–9). At this stage chromomeres are readily seen on the chromosomes and the nucleolus has a diameter of about 7 μ. Synapsis, while the chromosomes are in a contracted condition, followed by pachytene elongation have been described at meiosis in other Pyrenomycetes including *Neurospora crassa* (Singleton, 1953) and *Sordaria fimicola* (Carr & Olive, 1958).

At early diplotene (Pl. 1, fig. 10, 11) and diakinesis (Pl. 2, fig. 12) the chiasmata are apparent. At diakinesis seven contracted bivalents can be counted in well spread out preparations (Pl. 2, fig. 12). By metaphase I (Pl. 2, fig. 13, 14) the seven highly condensed bivalents can be seen near the centre, but at slightly different levels, on the well developed intranuclear spindle. There are crescent-shaped centrosomes, at each pole and astral rays can be seen in Pl. 2, fig. 14, (top). The spindle is always arranged

parallel to the long axis of the ascus. It remains clearly visible throughout anaphase (Pl. 2, fig. 15–18). The nucleolus, pushed out to one side of the spindle at metaphase, diminishes in size and gradually disappears during anaphase. New nucleoli are produced in each of the two daughter nuclei (Pl. 2, fig. 19).

Division II

In the two daughter-nuclei, now well separated from each other, the chromosomes show the relic anaphase arrangement (Pl. 2, fig. 19), and remain considerably contracted throughout division II, although the reformed nucleoli increase in size. In Pl. 2, fig. 20, the upper nucleus is at metaphase and the lower at prometaphase, illustrating a slight basipetal succession in the nuclear divisions in the ascus. The spindles at division II have well marked rod-shaped centrosomes at their poles and are orientated either vertically or obliquely in the ascus, but they are so far apart that it is unlikely that they slip past each other at anaphase (Pl. 2, fig. 20). This division completes meiosis.

Interphase II and division III

Following division II there is a well marked interphase (Pl. 2, fig. 21) during which the nuclei lie in two widely separated pairs, each pair presumably consisting of sister nuclei. Almost invariably, the centrosomes of the upper pair face the base of the ascus while those of the lower pair face the apex, as noted by Heslot (1958). The chromosomes become greatly elongated during this interphase.

In all the preparations seen of division III, spindle orientation was transverse to the long axis of the ascus (Pl. 2, fig. 22, 23) and not vertical as suggested by Franke (1957). Spindle elongation at late anaphase III brings the centrosomes and associated chromosome groups close to the wall of the ascus (Pl. 2, fig. 22, 23, lower right). This division is a normal mitosis.

Interphase III to ascospore delimitation

Interphase III nuclei are characterized by a distinct nuclear 'beak' apparently formed by the drawing out of the nuclear membrane at its point of attachment to the centrosome (Pl. 3, fig. 24–27). From many observations of asci at this stage it is clear that there is a regular arrangement of the nuclei in pairs resulting from division III (Pl. 3, fig. 27). Starting from the apex of the ascus, the centrosomes (or part of them) then move in a downward direction, away from their related nuclei, and come to lie close to the ascus wall (Pl. 3, fig. 28). In the next stage, which is less clear owing to an increase in granularity of the ascus cytoplasm, the centrosome bodies can be seen as densely staining plates, in pairs, close to the wall of the ascus (Pl. 3, fig. 29). On close examination a light-coloured cleavage line can be seen, starting from each of the paired centrosome bodies, extending back around two nuclei and enclosing them within long, cigar-shaped spore initials. It is apparently by the movement of the centrosome bodies away from their own nuclei and their association in pairs below non-sister nuclei that two such nuclei become included within each of the four binucleate spore initials (Pl. 3, fig. 30). Occasionally, uninucleate spores are cut out (Pl. 3, fig. 31) and are of the normal form. Asci with the uninucleate spores may contain three binucleate and two uninucleate spores or one trinucleate, one uninucleate and two binucleate spores.

Division IV, maturation of the spore and development of spore appendages

A fourth division within the spore initial, previously described by Moreau & Moreau (1951) and Heslot (1958) but denied by Franke (1957), is confirmed (Pl. 3, fig. 32). The division occurs in the spore head in such a way that four nuclei are produced, three remain in the head and one migrates into the narrower tail, and the latter now becomes cut off by a wall. This nucleus aborts and the cytoplasmic contents disappear to form a vacuolated, hyaline primary appendage. The wider, distal part of the spore initial containing three nuclei enlarges to form the spore itself. It becomes darkly coloured during development. When the spore is crushed the wall separates into three layers, the middle one of which is pigmented. There is a germ pore at the apical end of the spore. Two mucilaginous, secondary appendages, one almost apical at the side of the germ pore and one below the primary appendage, can be discerned at a very early stage of spore development, before the primary appendage has been cut off, but observations made with the optical microscope did not reveal how these appendages arise.

DISCUSSION

The haploid chromosome number of seven agrees with a metaphase I count by Franke (1962) and is in conformity with the seven linkage groups recorded by Kuenen (1962) from a genetic analysis of gene mutants.

The spindle orientation is more or less vertical at I and II (with the products of division I widely separated from each other) and transverse in III. This differs from the account of Franke (1957), but agrees with that of Heslot (1958). It is through the movements of the centrosome bodies that the four pairs of sister-nuclei resulting from division III are realigned to form four pairs of non-sister nuclei which are then incorporated in the four binucleate spore initials. This process is important since *Podospora anserina* is secondarily homothallic and the two nuclei in each spore are normally of different mating type, segregation for this factor having occurred, according to the genetic evidence, in division II (Rizet & Engelmann, 1949). These cytological observations agree with the theoretical scheme put forward by Esser & Kuenen (1965) which shows that on the spindle orientation described here, and post-reduction, each spore will contain a (+) and a (−) nucleus.

The mitosis in the binucleate spore and the degeneration of one of the four resulting nuclei in the primary appendage leads to the formation of a spore containing either one (+) and two (−) nuclei or one (−) and two (+) nuclei. No consideration appears to have been given by geneticists to possible consequences of this imbalance.

The term 'centrosome' or 'centriole' has been used rather loosely for the same body in the literature on the cytology of the Ascomycetes. In *Podospora anserina* the centrosome has been depicted as a disc in division I, as a rod in divisions II and III and as a plate at the nuclear 'beak' and spore delimitation stages. Similar appearances have been described in other Pyrenomycetes such as *Neurospora tetrasperma* (Colson, 1934), *N. crassa* (Singleton, 1953), *Podospora* spp. and *Sordaria macrospora* (Heslot, 1958) and *S. fimicola* (Carr & Olive, 1958; Doguet, 1960), and it has been noted that the centrosome appears to enlarge at the later divisions. Singleton (1953) suggested that this growth might be in some way related to the functioning of the centrosome in spore delimitation and perhaps did not involve that part of the centrosome which has

genetic continuity in relation to the spindle. Recent work by Schrantz (1967) on the fine structure of the Discomycete *Pustularia cupularis* has shown that the so-called centrosome is bipartite; the inner disc is associated with the spindle fibres and the outer disc carries the astral rays, which do not themselves fuse to form the cell wall, but do, in some way, appear to control the delimitation of the spore by the endoplasmic reticulum, outside the rays themselves. The centrosome in yeast also consists of two discs (Robinow & Marak, 1966).

In the light of these observations it seems reasonable to suggest that in *Podospora anserina* also, it is only the outer part of the centrosome which moves away from the nucleus with which it was associated and functions in spore delimitation while the inner part remains closely associated with the nuclear membrane, ready to function at the next mitosis. This hypothesis needs to be tested by electron-microscope studies.

Heslot (1958) suggested three functions for the centrosome—the control of nuclear division, the delimitation of the ascospores and the orientation of the spores. It now appears that the first function belongs to the inner disc of the centrosome and the two latter functions to the outer part. To these an additional function, the association of nuclei of different mating type prior to spore formation in the secondarily homothallic *P. anserina*, can be added. Doguet (1960) has argued that the spindle controlling body probably divides during interphase and that during the subsequent nuclear division the spindle is formed between the two separating halves. The close association of the centromeres of the chromosomes and this body throughout each interphase may be significant.

Although the techniques employed in this investigation were not the most suitable for showing the astral rays and their possible role in spore delimitation, it was clearly demonstrated that cleavage of the spore plasm is initiated from the centrosome bodies and gradually extends up to include two non-sister nuclei.

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. Crozier on ascogenous hypha, with penultimate, binucleate cell beginning to enlarge. × 2240.

Fig. 2. Ascogenous hyphae and young asci. × 1400.

Fig. 3. Two young asci, uninucleate after karyogamy, and fusion of the apical and stalk cell of the croziers. × 1400.

Fig. 4. Group of ascogenous hyphae, croziers and asci. × 1400.

Fig. 5. Young ascus with fusion nucleus showing chromosomes synapsed. × 1400.

Fig. 6. Young ascus. Paired chromosomes have begun to elongate. × 1400.

Fig. 7. Ascus with chromosomes at early pachytene. Note enlarged nucleolus and chromomeres visible on some of the chromosomes. × 1400.

Fig. 8, 9. Two focal planes of ascus at full pachytene. × 1400.

Fig. 10, 11. Two focal planes of ascus at early diplotene. × 1400.

PLATE 2

Fig. 12. Early diakinesis. Chiasmata visible. × 1400.

Fig. 13. Metaphase I. Contracted bivalents on vertical spindle. Crescent-shaped centrosomes at poles. Nucleolus pushed to one side. × 2500.

Fig. 14. Metaphase I. Seven bivalents can be counted. Indications of astral rays at upper pole of spindle. × 1400.

Fig. 15, 16, 17. Anaphase I. Three focal planes showing chromosomes grouping at poles of the vertical spindle. × 1400.

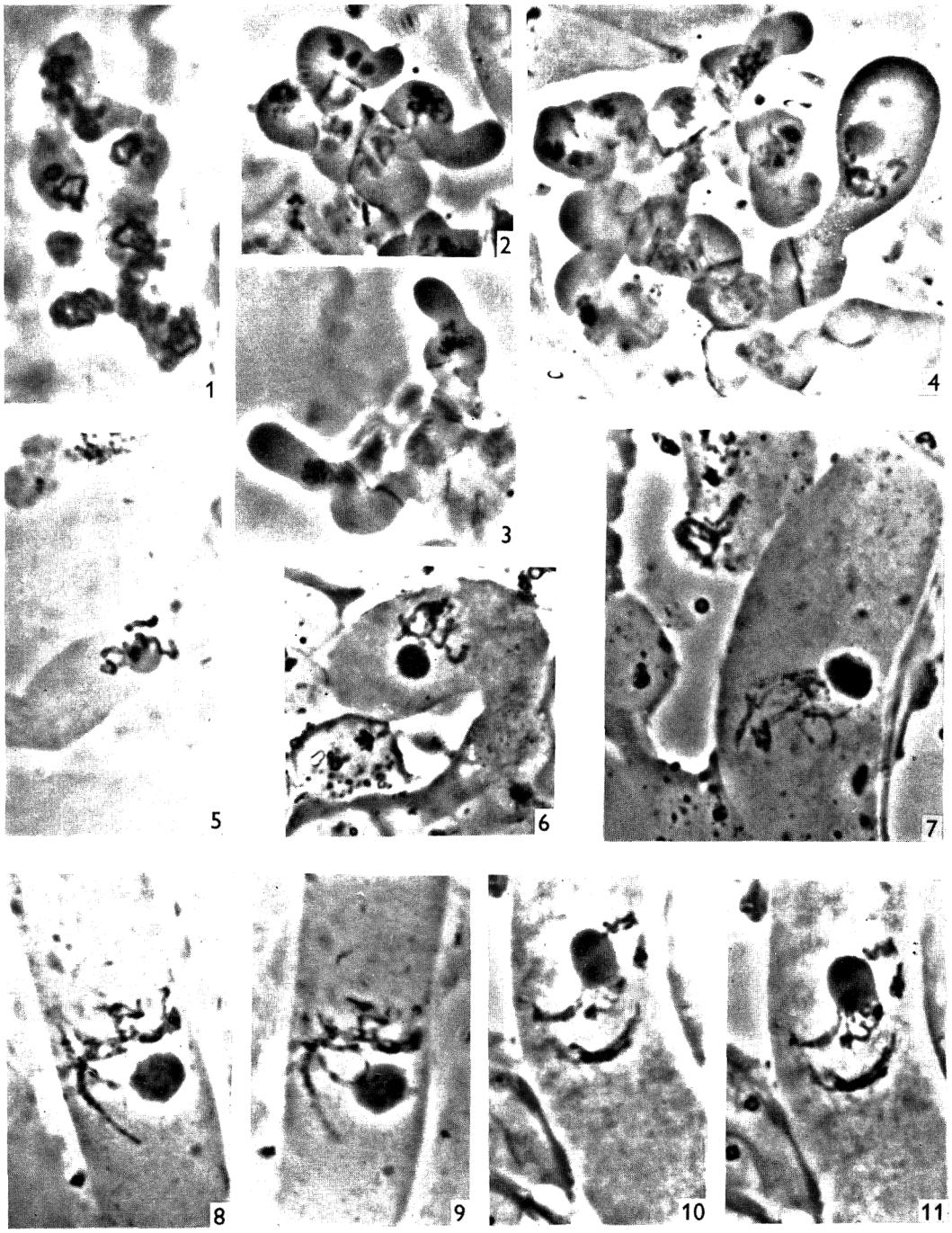
Fig. 18. Late anaphase I. Chromosomes at each pole. Spindle fibres seen as broad band. Nucleolus degenerating. × 1400.

Fig. 19. Telophase I. The spindle has disappeared. A nucleolus has appeared related to each of the two groups of chromosomes. × 1400.

Fig. 20. Metaphase and prometaphase II. Spindles vertical and oblique. Centrosomes visible as rods. × 1400.

Fig. 21. Interphase II–Prophase III. Two pairs of sister-nuclei widely separated in the ascus. × 1400.

Fig. 22, 23. Anaphase III in two focal planes. Spindles horizontal. Rod shaped centrosomes at poles pushed towards ascus wall. × 1400.



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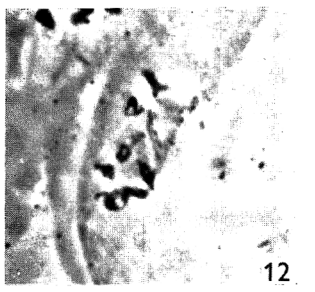
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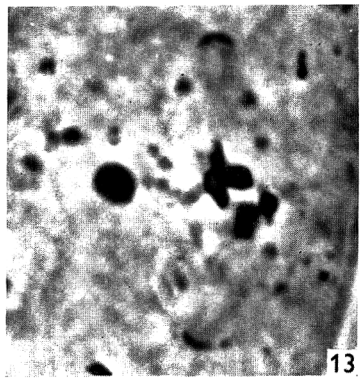
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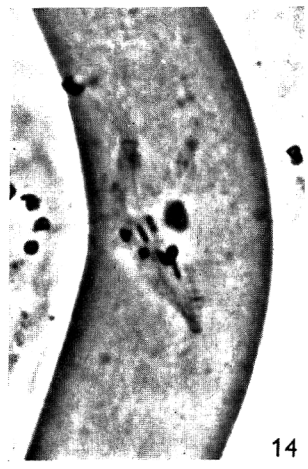
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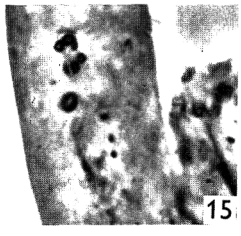
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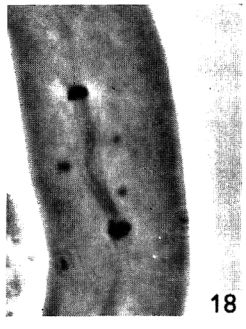
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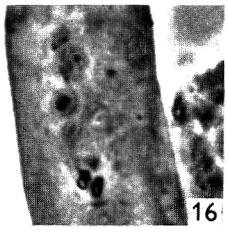
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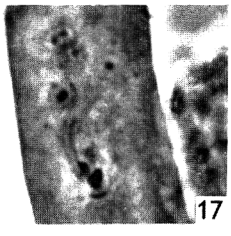
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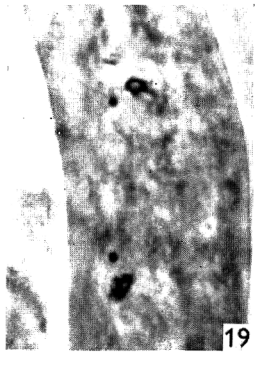
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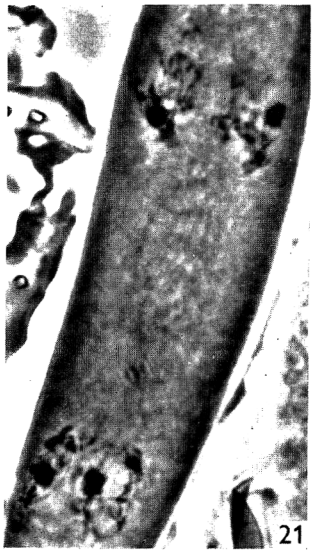
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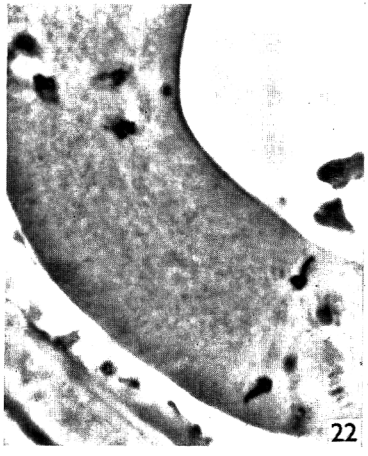
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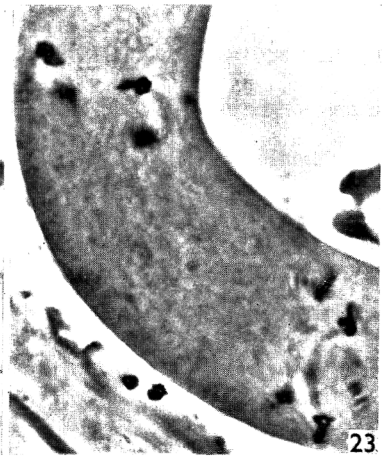
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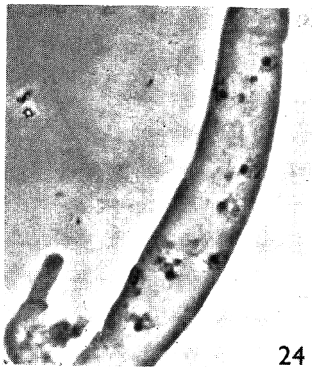
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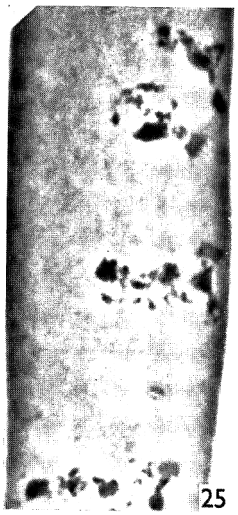
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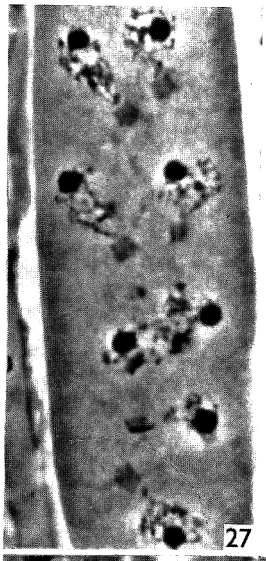
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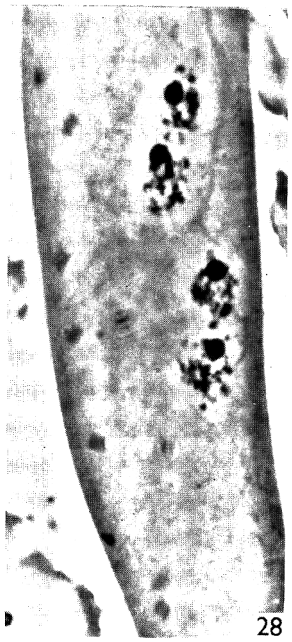
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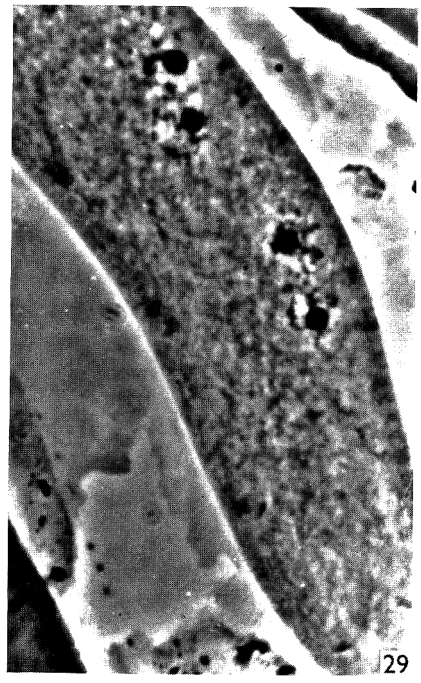
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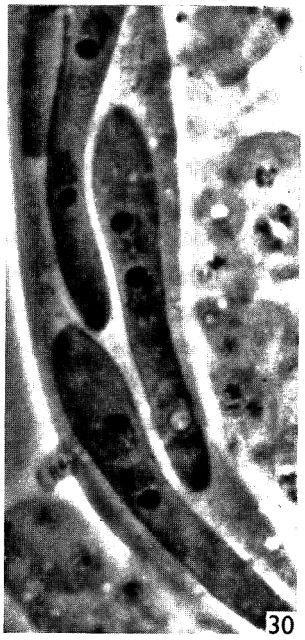
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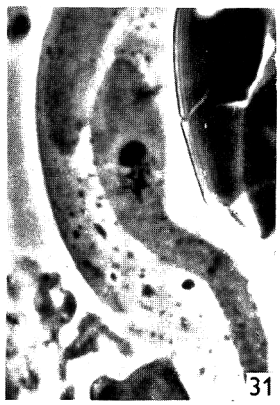
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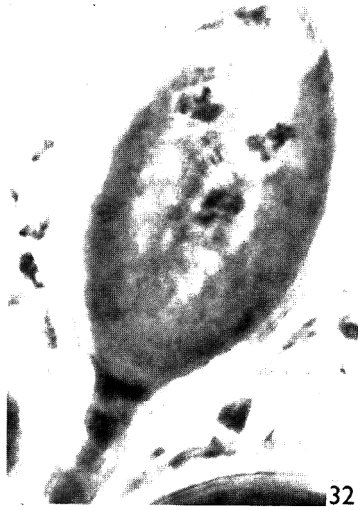
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PLATE 3

Fig. 24. Ascus showing eight nuclei at interphase III. Centrosomes visible in association with nuclei. × 560.

Fig. 25. Part of ascus with six of the nuclei at interphase III. Prominent nuclear 'beaks' formed by association of nuclear membrane with centrosome. × 1400.

Fig. 26. Part of an unsquashed ascus stained with Feulgen-propionocarmine technique. Centrosomes seen as deeply stained plate-like structures in pairs near to ascus wall. × 1400.

Fig. 27. Pairs of sister nuclei and centrosomes undergoing a basipetal orientation. × 1400.

Fig. 28. Part of ascus, showing later stage in orientation. Centrosomes aligned in pairs adjacent to the ascus wall (left), following a downward migration away from their respective nuclei. × 1400.

Fig. 29. Part of ascus at early stage of spore delimitation. Two pairs of nuclei have been cut out by a thin cleavage line in the ascus cytoplasm which is initiated from the centrosomes seen on the left of the ascus. × 1400.

Fig. 30. Three of the four binucleate spore initials shown as discrete bodies after completion of cleavage. Enucleate epiplasm remains in the ascus. Spore initial beginning to show wider spore head and narrower tail portion, the latter towards base of ascus. × 1400.

Fig. 31. Uninucleate spore of normal shape. × 1400.

Fig. 32. Maturing ascospore with three nuclei in the main spore head and one in the tail, now cut off by a septum as the primary appendage. × 1400.

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Fine Structure of the Wall and Appendage Formation in Ascospores of *Podospora anserina*

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(*Accepted for publication* 15 *March* 1968)

SUMMARY

The ascospores of *Podospora anserina* (Ces.) Rehm are delimited by a double membrane system. The primary spore wall develops within this, the outer part of the double membrane being pushed out to form the spore membrane and the inner part forming the plasmalemma of the spore. Starting in the middle of the matrix of the expanding primary wall, a secondary wall is laid down and gradually extends to the outer periphery of the spore wall. Later, a thick tertiary wall is formed at the inner side of the secondary wall by blocks of electron-dense material between which channels of the primary wall matrix remain. This is the pigmented layer of the spore wall. On the innermost side of the spore wall, a part of the original primary wall remains.

The primary appendage at the base of the spore arises as part of the spore initial, but, after it has been cut off by a septum, its contents degenerate and it is bounded only by the primary and secondary wall layers. The secondary appendages, formed at the apex of the spore and at the bottom of the primary appendage, are considered to be actively growing processes bounded by the spore membrane.

INTRODUCTION

In this study the electron microscope has been used to extend the optical microscope observations by Beckett & Wilson (1968) on the development of the spore wall and the primary and secondary appendages (Fig. 1 F) of *Podospora anserina*.

METHODS

Perithecia of *Podospora anserina* were removed from cultures (Beckett & Wilson, 1968) and the contents dissected out into a drop of phosphate buffer on a glass slide. Asci so obtained were then transferred to a 2% (w/v) potassium permanganate solution maintained at pH 7·2 with 0·1 M-phosphate buffer and fixed for 45 min. to 1 hr at room temperature. The material was then washed in distilled water, dehydrated in a graded ethanol series, soaked in propylene oxide and finally embedded in either Araldite or Epon. Various staining procedures were employed involving both saturated aqueous uranyl acetate solution and lead citrate (Reynolds, 1963). Details of these are given in the explanation of the plates.

Sections were cut with glass knives on either a Huxley ultramicrotome or an LKB Ultratome II. Observations and photographs were made using AEI EM 6 and AEI EM 6 B electron microscopes. Correlative observations of fresh material were made on a Baker phase-contrast light microscope.

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RESULTS

Spore wall development

Delimitation of the spore initial in the ascus of *Podospora anserina* is by a double membrane system as in *Pustularia cupularis* (Schrantz, 1967). Soon after the completion of this process the primary spore wall may be detected as a conspicuous electron-

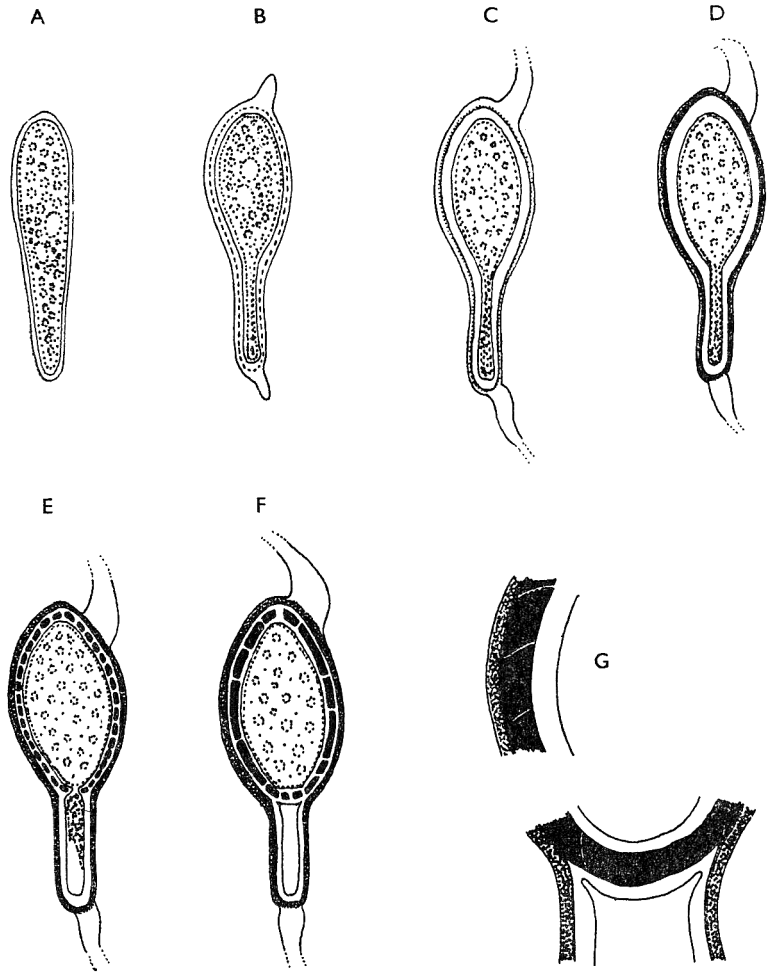

Fig. 1. Semi-diagrammatic representation of spore wall development and appendage formation in *Podospora anserina*. Nuclei omitted. A, spore initial with primary wall. B–D, Stages in formation of secondary appendages and secondary-wall layer and early stages in differentiation of spore head and primary appendage. E–G, Stages in formation of tertiary-wall layer in spore head and septum, not in primary appendage.

transparent layer between the two unit membranes, the outer of which becomes the spore membrane and the inner the plasmalemma of the spore. With the gradual widening of the primary wall new electron-dense material is deposited within it. This material appears as discrete blocks measuring about 120 Å × 250 Å (Pl. 1, fig. 2)

which later merge to form a continuous layer (Pl. 1, fig. 3, 4; Pl. 2 fig. 9–11). Prominent at this stage are numerous membrane-bound vesicles, often grouped together, resembling the lomasome structures described by Moore & McAlear (1961). The electron-transparent primary wall then widens considerably and more of the electron-dense secondary material is deposited towards the outer side of the wall until it fills the whole space extending to the spore membrane (Pl. 1, fig. 3, 4; Fig. 1 D). These primary and secondary wall layers are laid down round the whole spore initial, the expanding spore head and the narrower tail portion.

During maturation the ascospores undergo a gradual pigmentation, the main spore head changing from hyaline to green to dark brown. Observations on sections of spores at the green stage show, in the wall around the spore head, the deposition of a tertiary wall layer to the inside of the secondary layer, consisting of blocks of electron-dense material separated by regions in which the primary wall material remains continuous (Pl. 1, fig. 6; Fig. 1 E–G). The channels may be homologous with the ectodesmata described by Kirk (1966) in *Ceriosporopsis halima*. The tertiary wall represents the pigmented layer of the main spore head and in the completely mature spore is seen in section as a wide band of electron-dense material running between the primary wall layer on the inside and the secondary wall layer on the outside (Pl. 1, fig. 7; Fig. 1 G). The dense granules seen at the outer edge of the primary wall of the mature spore closely resemble the pigment granules observed by Delay (1966) in the spore wall of *Ascobolus immersus*.

At the point at which the spore head joins the cylindrical primary appendage a septum is formed by the inward growth of the primary wall and the intercalary tertiary wall (Pl. 1, fig. 8; Fig. 1 E, F). This septum limits the growth of the pigmented tertiary wall of the spore head, while the wall of the primary appendage remains hyaline and two-layered (Pl. 1, fig. 5, 6, 8; Fig. 1 G). No median sections were obtained of this septum and it was not determined whether a pore was present in it. A single germ pore does exist at the extreme apical tip of the main spore head and passes through all the wall layers (Pl. 1, fig. 8).

Appendage formation

Two types of spore appendages are formed on the ascospores of *Podospora anserina* (Pl. 1, fig. 1; Fig. 1 B–F). The primary appendage is initially continuous with the main spore head and contains all the organelles common to the latter except the nuclei (Pl. 1, fig. 1; Fig. 1 A–D). After nuclear division in the spore initial (Moreau & Moreau, 1951; Beckett & Wilson, 1968) and the formation of the septum at the base of the spore head, the cytoplasm and the one daughter-nucleus which migrated into the appendage degenerate (Pl. 1, fig. 8; Fig. 1 E, F).

The second type of appendage present on the ascospore is the secondary appendage. Normally one of these is formed near the apex of the spore, to one side of the germ pore, and another at the basal tip of the primary appendage (Pl. 1, fig. 1; Fig. 1 B–F). These secondary appendages are long, hyaline structures as seen with the light microscope and often become folded within the ascus.

The first indications of the formation of secondary appendages are seen with the electron microscope as out-pushings of the spore membrane, normally at each end of the spore initial, but sometimes along the sides of the spore also (Pl. 2, fig. 9). Later the lateral out-pushings are usually lost and a concentration of dense material can be seen

accumulating at the spore apices between the rudimentary secondary wall and the spore membrane (Pl. 2, fig. 10). Further development produces a distinct cylindrical appendage, completely bounded by the spore membrane (Pl. 2, fig. 11) and situated at each end of the spore.

Very rarely, small lateral appendages persist during development of the spore and may be seen enclosed by the spore membrane, on both sides of the spore where the spore head joins the primary appendages (Pl. 3, fig. 12). Similar appendages were first reported by Moreau (1953) as a result of optical-microscope observations.

DISCUSSION

Earlier investigators of the spore wall have used such terms as epispore, mesospore and endospore, terms which indicate the relative positions of the various layers making up the wall. Recently Kirk (1966) has attempted to establish homologies between the epispore, mesospore and endospore of different species within a family by cytochemical methods. In this study it has seemed more logical to designate the wall layers according to the order in which they develop, especially since both secondary and tertiary layers are laid down within the expanding matrix of the primary wall and are not completely separate entities.

Delay (1966) considered that the pigment granules which colour the spore coat of *Ascobolus immersus* originate in the epiplasmic vacuoles outside the spore. The position of similar, electron-dense granules seen in the primary wall layer of *Podospora anserina* suggests an origin within the spore. Carroll (1966) has demonstrated the presence of small electron-dense vesicles within the spore which pass out through the primary wall and deposit material on the outside of the spore in *Ascodesmis sphaerospora*.

Comparing the spore appendages of *Podospora anserina* with those of some marine Pyrenomycetes recently investigated with the light microscope (Kirk, 1966), the primary appendage of *P. anserina* and the apical appendages of *Corollospora maritima* begin their development in the same way as parts of the spore initial, but the later stages in their development diverge. The terminal mucilaginous appendages of *Ceriosporopsis halima* and the secondary appendages of *P. anserina* have some superficial resemblances. In the former, Wilson (1965) suggested that the appendages grow as a result of the extrusion of material from spore vacuoles through apical pores into an out-pushed epispore, while Kirk (1966) considered that the appendages develop from the epispore material itself owing to a change-over from chitin to mucilage production at these sites. In *P. anserina* there is no extrusion of material through a pore in the formation of the secondary appendages; they appear to grow *in situ*, but are contained within the spore membrane.

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. Photomontage showing L.S. of young ascospore with two nuclei (*N*) in spore head (*SH*), primary appendage (*PA*), and apical and basal secondary appendages (*ASA*, *BSA*). $KMnO_4$ fixation, sections stained with lead citrate and embedded in Araldite. × 2250.

Fig. 2. L.S. of parts of walls of two adjacent young ascospores *AS* 1, *AS* 2, showing electron-dense blocks of secondary-wall material (*SW*) forming towards outer edge of primary wall (*PW*) which lies between the spore membrane (*SM*) and the plasma membrane (*PM*). Technique as for fig. 1. × 100000.

Fig. 3. L.S. of part of ascospore wall showing widening of primary wall (*PW*) and a continuous layer of electron-dense secondary-wall material (*SW*) between the primary wall and spore membrane (*SM*). $KMnO_4$ fixation, sections stained with uranyl acetate and lead citrate, embedded in Epon. × 20,000.

Fig. 4. L.S. of spore wall at later stage. Continued deposition of secondary-wall material (*SW*) has now filled space between spore membrane (*SM*) and remaining primary wall (*PW*). $KMnO_4$ fixation, uranyl acetate after fixation, sections stained with lead citrate, embedded in Epon. × 20,000.

Fig. 5. L.S. of part of spore at point where spore head (*SH*) joins primary appendage (*PA*). Part of septum (*S*) is shown and early stages of tertiary-wall development (*TW*). $KMnO_4$ fixation, sections stained with uranyl acetate and lead citrate, embedded in Epon. × 22,500.

Fig. 6. L.S. of similar part of spore to fig. 5. Tertiary-wall material (*TW*) in electron-dense blocks in septum (*S*) and spore head (*SH*), but not in primary appendage wall (*PAW*). Pore-like regions (*E*) can be seen between blocks of primary-wall matrix. $KMnO_4$ fixation, sections stained with uranyl acetate and lead citrate, embedded in Epon. × 45,000.

Fig. 7. L.S. of part of wall of nearly mature spore head. Three layers seen: a wide primary-wall layer (*PW*) with numerous electron-dense granules (*G*) along outer edge, a wide, electron-dense tertiary-wall layer (*TW*) with persistent ectodesmata or pores (*E*), and a narrower electron-dense secondary-wall layer (*SW*) on the outside. $KMnO_4$ fixation, sections stained with lead citrate, embedded in Araldite. × 30,000.

Fig. 8. Light micrograph of ascospore at green pigmented stage showing restriction of pigment to spore head. Germ pore (*GP*) at apex. × 900.

PLATE 2

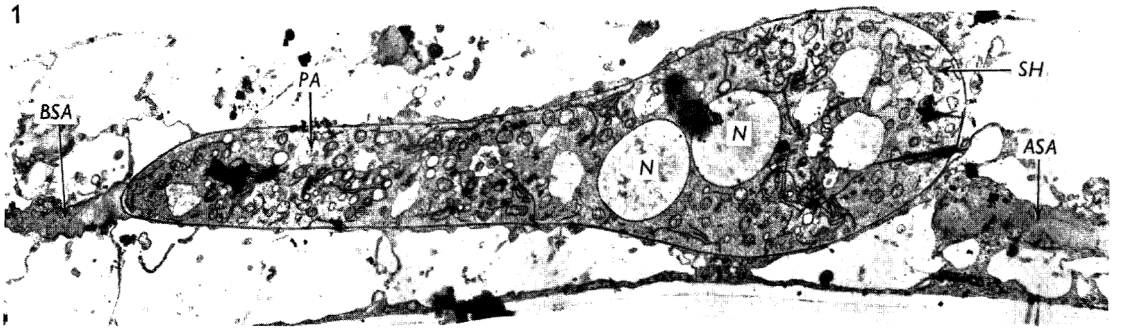
Fig. 9. L.S. of spore initial showing the pushing out of the spore membrane (*SM*) during early stage of secondary appendage formation. Lomasomes (*LO*) can be seen associated with developing spore wall. $KMnO_4$ fixation, uranyl acetate staining after fixation, embedded in Epon. × 11,500.

Fig. 10. L.S. of part of spore head apex showing concentration of secondary appendage material (*SA*) beneath spore membrane (*SM*). An almost continuous line of secondary-wall material (*SW*) can be seen outside the electron-transparent primary wall (*PW*). Fixation, staining and embedding as for fig. 9. × 23,000.

Fig. 11. L.S. of part of spore head apex showing further development of apical secondary appendage (*ASA*). Fixation staining and embedding as for fig. 9. × 23,000.

PLATE 3

Fig. 12. L.S. of part of spore showing abnormal lateral secondary appendages (*LSA*). $KMnO_4$ fixation, uranyl acetate staining after fixation, embedded in Epon. × 15,000.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N
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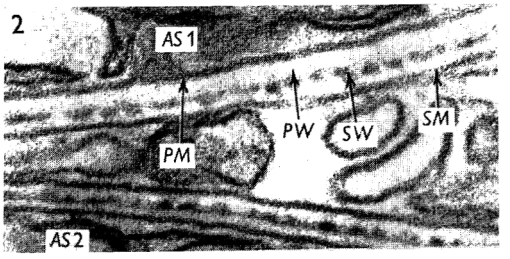
SH
ASA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AS1
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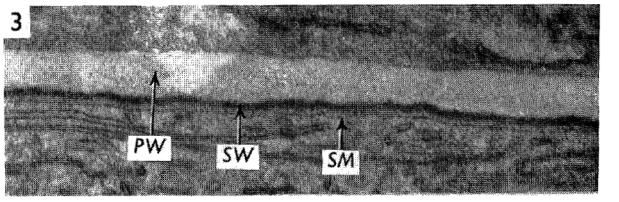
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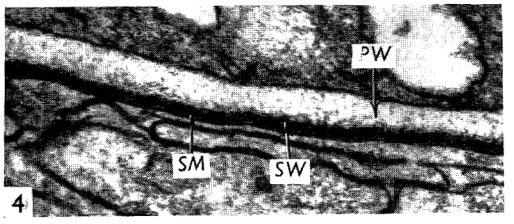
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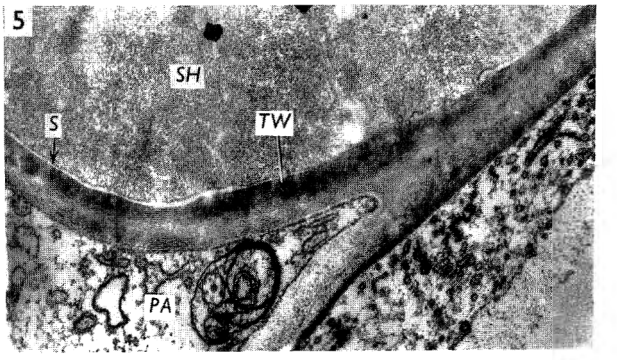
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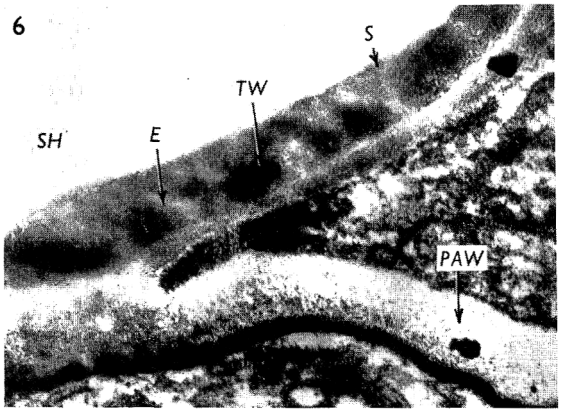
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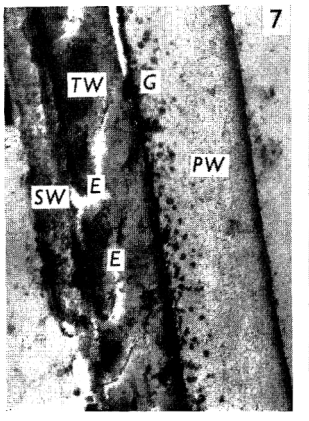
PAW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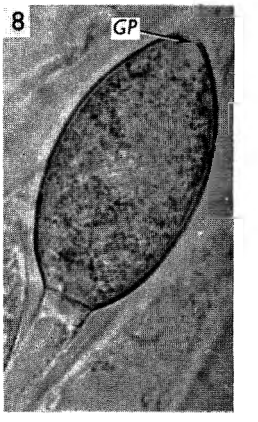
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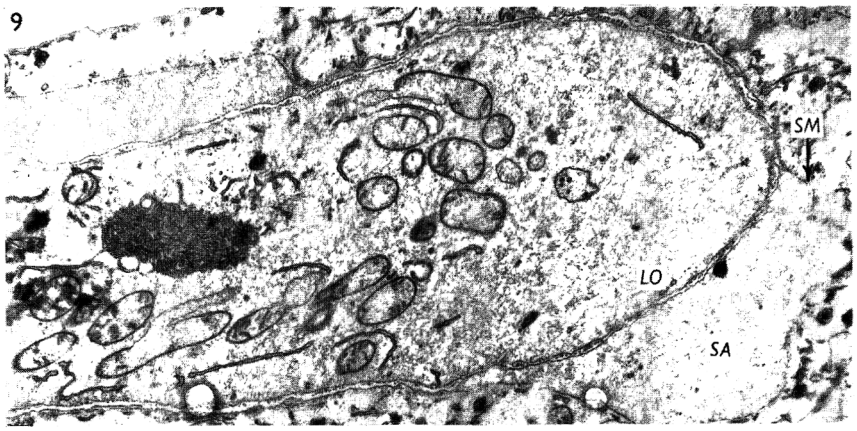
GP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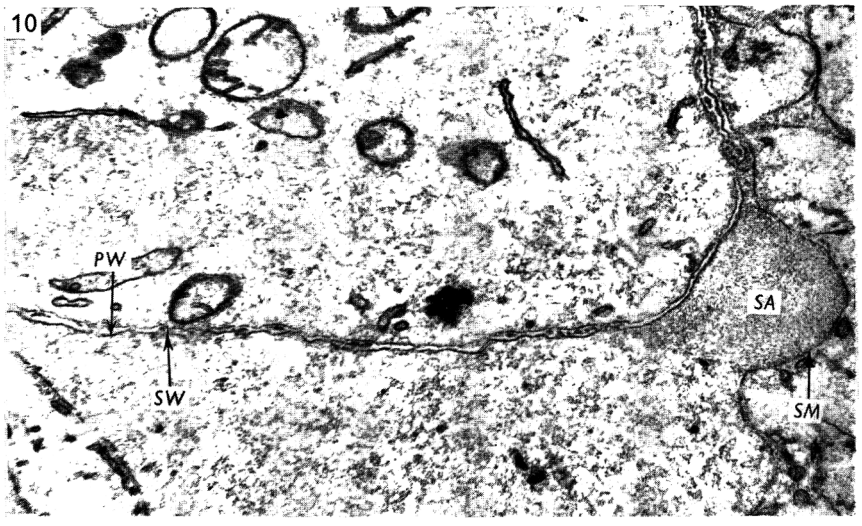
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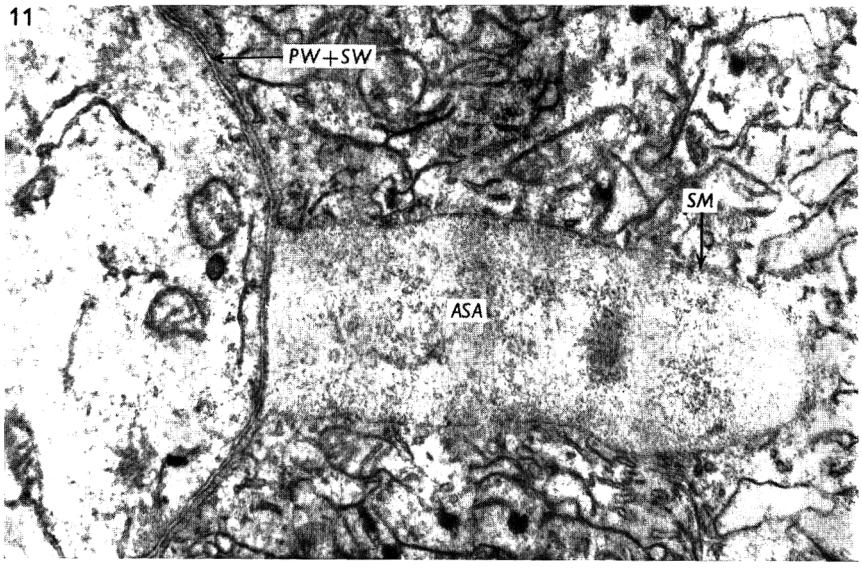
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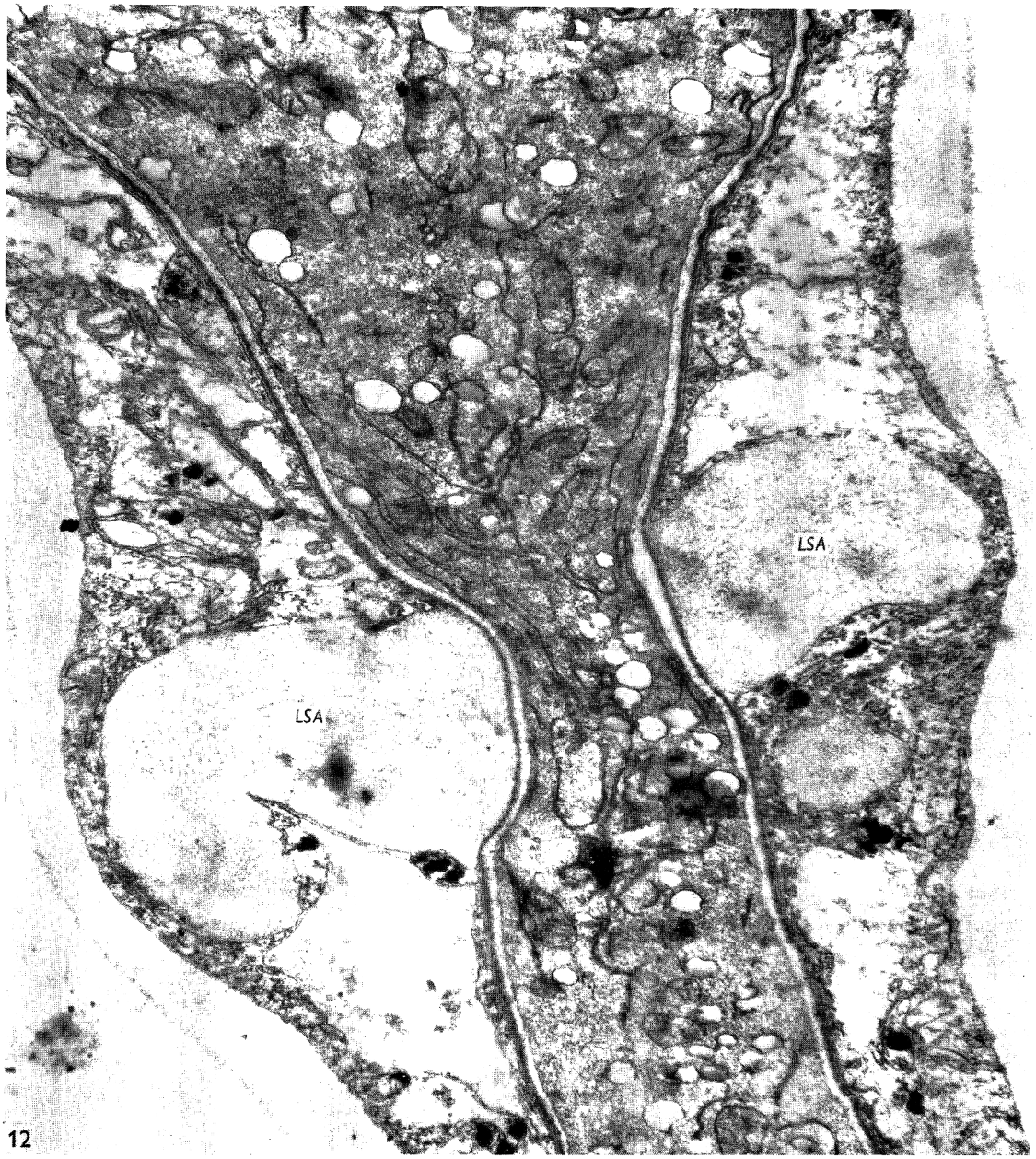
SM
ASA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Observations on the Chromatinic Bodies of Two Species of the Actinoplanaceae

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(*Accepted for publication* 18 *March* 1968)

SUMMARY

The structure and distribution of the chromatinic bodies of two species of the genus *Actinoplanes* were studied with light microscopy. Results obtained from observation of material stained by the technique of DeLamater (1951), Feulgen (Rafalko, 1946), or Piéchaud (1954), were supplemented by observation of living material. A technique for observing living and stained preparations with phase-contrast or dark-field microscopy gave results far superior to those obtained with bright-field microscopy. Both strains studied had a nearly identical structure and distribution of chromatinic bodies. Colonies consisted of two hyphal types; substrate hyphae and palisade hyphae (Couch, 1950). The chromatinic bodies of the substrate hyphae appeared variously as short rods or as small bands of material that extended across the width of the hyphae. The palisade hyphae contained bodies ranging from dumb-bell shaped structures to multiple entities composed of 3–4 interconnected subunits. The distal portions contained dense rods of chromatinic material. The chromatinic rods of the palisade hyphae progressed into the sporangium along with the ingrowth of the sporogenic hyphae into the sporangial envelope. Within the sporangium these rods appeared to contract and undergo repeated divisions into smaller subunits; a single subunit was incorporated into each spore. A striking similarity in structure and distribution of the chromatinic bodies was noted between members of the Actinoplanaceae and members of the Streptomycetaceae.

INTRODUCTION

The structure and distribution of the chromatinic bodies in the genus *Streptomyces* (*sensu* Waksman & Henrici, 1943) were studied by Badian (1936), Klieneberger-Nobel (1947), McGregor (1954), Saito & Ikeda (1958), Hopwood & Glauert (1960) and Shamina (1964). Couch (1954), who used the nuclear staining techniques of Robinow (1941) and DeLamater (1951), reported chromatinic bodies in Actinoplanes (strain no. 20) to be similar to those found by Klieneberger-Nobel (1947) is Streptomyces, however, no pictures were presented. The purpose of the present paper is to give an illustrated account of the structure and distribution of the chromatinic bodies in two species of the Actinoplanaceae and to compare these findings with those reported by various authors for Streptomyces.

METHODS

Two species of the genus *Actinoplanes* were selected for study. One of these, strain 20, was selected by Couch (1954) because it formed abundant vertical palisade hyphae and produced abundant sporangia on a variety of culture media. This strain, no. 20,

forms its spores in vertical rows within the sporangia (Pl. 2, fig. 11; Pl. 4, fig. 27). The other strain, 1668, forms its spores in branched coils within the sporangia. Strain 1668 was isolated from soil collected in Hyderabad, South India, whereas strain 20 was isolated from soil collected near Tuscaloosa, Alabama, U.S.A. Both strains will be described in a later publication.

Stock cultures of the two strains were maintained in pure culture on Czapek agar (Fisher Scientific, Fairlawn, New Jersey, U.S.A.). For enhancement of sporulation, cultures were at times grown on the surface of a grass–water medium prepared by autoclaving 30 g. Paspalum grass in 1 l. water.

Living material was prepared for study by squashing a small portion of a sporulating colony from either agar or liquid culture in water under a coverslip. To increase the contrast of the chromatinic bodies, living material was mounted in 5–30% (w/v) concentrations of gelatin and studied with phase-contrast microscopy (Mason & Powelson, 1956). Similar observations were made in Zeiss W-15 non-hardening mounting medium for phase-contrast microscopy.

For staining, a small block (1 × 1 × 10 mm. long) from a sporulating culture was crushed under a coverslip, the coverslip removed and the crushed material was fixed for 3–5 min. in the vapour of 2% (w/v) osmic acid. This material was fixed to the slide with Haupt's adhesive and allowed to dry. After drying, the slides were placed in distilled water for 5 min. The chromatinic bodies were stained by the technique of DeLamater (1951), Feulgen (Rafalko, 1946), or Piéchaud (1954). For the Feulgen and DeLamater methods, the preparations were hydrolysed with N-HCl for 10 min., then rinsed in distilled water. Feulgen preparation: the preparations were rinsed in sulphurous acid for 2 min., stained in Grübler's leuco-basic fuchsin for 2 hr, rinsed in sulphurous acid for 2 min., differentiated in tap water for 15 min., dehydrated in a graded ethanol/water series, and mounted in Zeiss hardening mounting medium for phase-contrast microscopy. DeLamater preparation: the preparations were stained for 2·5 hr in a solution prepared by adding 1 drop thionyl chloride to 10 ml. 0·5% (w/v) thionin, rinsed in distilled water, dehydrated in a graded ethanol/water series, and mounted in Zeiss hardening mounting medium for phase-contrast microscopy. The Piéchaud stain was prepared by adding 2 drops 0·5% (w/v) eosin-Y to 20 drops of G. T. Gurr's Giemsa stain just before use. The fixed material was stained for 30–60 min. After staining the preparations were mounted in Zeiss W-15 non-hardening mounting medium for phase-contrast microscopy.

Observations were made with a Zeiss Photomicroscope equipped with a 12 V., 60 W. lamp. Photographs were taken on Kodak Panatomic-X film at magnifications ranging from × 160 to × 500. A Zeiss no. 467806 interference wide-band green filter was used for the phase-contrast and bright-field photographs.

RESULTS

The mounting of living material in gelatin greatly increased the contrast of the chromatinic bodies; however, better results were obtained by mounting the living material in Zeiss W-15 non-hardening medium for phase-contrast microscopy. This mounting medium so greatly increased the contrast of the chromatinic bodies when viewed with phase-contrast microscopy that the bodies appeared as if they were stained. When compared with living hyphae mounted in water, no noticeable distortions which

could be attributed to the Zeiss medium were observed in the structure of the cells or cellular contents of the hyphae.

After staining by the Piéchaud, DeLamater, or Feulgen techniques, the nuclear material was at first very distinct and deeply stained, but all the stains faded very rapidly and within 24 hr the chromatinic bodies were barely visible with bright-field microscopy. These same faded preparations, when observed with either phase-contrast or dark-field microscopy, exhibited chromatinic bodies that were more distinct than when originally observed with bright-field mirocopy. These methods of observations were somewhat better for the Feulgen preparations than for the DeLamater or Piéchaud preparations.

The structure and distribution of the chromatinic bodies of both strains studied were so nearly identical that a detailed description of the findings for one strain makes unnecessary separate descriptions of the two strains. Strain 20 usually gave slightly better staining results and was most often used to make illustrations.

As in *Streptomyces* sp. (Klieneberger-Nobel, 1947), the colonies of the Actinoplanaceae are composed of two distinct hyphal types (Couch, 1950): substrate and palisade hyphae (Pl. 1, fig. 1). The substrate and palisade hyphae seemed to be homologous respectively with the primary and secondary hyphae of *Streptomyces* sp., as described by Klieneberger-Nobel (1947). The substrate hyphae were branched and thinner than the palisade hyphae (Pl. 1, fig. 1–3). The palisade hyphae arose vertically as simple branches from the substrate hyphae, as reported for *Streptomyces* sp. by Hopwood (1960) and Shamina (1964), and grew to the agar surface where they were characteristically terminated by sporangia. The formation of an 'initial cell' as reported by Klieneberger-Nobel (1947) for *Streptomyces* sp. was not observed.

The chromatinic bodies of the substrate hyphae appeared essentially the same whether mounted in the Zeiss non-hardening mounting medium while living or stained by any of the three methods used (Pl. 1, fig. 2, 3). The chromatinic masses existed in a wide variety of sizes and shapes. Variation ranged from short rods to small bands of material that extended across the width of the hyphae (Pl. 1, fig. 2). Septa divided the hyphae into cells which included from 1 to 4 of the chromatinic bodies. Although there was considerable diversity in size and shape of the chromatinic bodies of the substrate hyphae, they were usually small dense bodies which were markedly similar to the chromatinic bodies described for the substrate hyphae of *Streptomyces coelicolor* (Hopwood & Glauert, 1960).

As with the substrate hyphae, all the methods of study yielded a similar structure and distribution of the chromatinic bodies in the palisade hyphae. The palisade hyphae were characterized by having two distinct types of chromatinic bodies (Pl. 2, fig. 4–9; Pl. 4, fig. 20–27). The lower half to two-thirds of a palisade hypha contained chromatinic bodies that were very complex and considerably larger than those in the substrate hyphae. These bodies varied in shape from dumb-bell shaped structures to multiple entities composed of 3 to 4 interconnected subunits (Pl. 2, fig. 4–6; Pl. 4, fig. 20–27). Individual cells of the hyphae contained 1–6 such bodies.

The upper portions of the palisade hyphae contained dense rods of chromatinic material that appeared of such a size as to be the equivalent of several of the chromatinic bodies seen in the lower portions of the palisade hyphae (Pl. 2, figs. 8, 9; Pl. 4, fig. 20–27). Similar rod-shaped structures were reported by Hopwood & Glauert (1960) for *Streptomyces coelicolor*. Chromatinic bodies that resembled elongate rods

were observed in the upper portion of young palisade hyphae prior to (Pl. 2, fig. 8; Pl. 4, fig. 20), during (Pl. 2, fig. 9; Pl. 4, fig. 21–26), and after sporangial formation (Pl. 4, fig. 27). Whether the rods are a result of fusion and/or elongation of the chromatinic bodies found in the lower portion of the palisade hyphae was not determined.

The squashing of young and mature sporangia (Pl. 2, fig. 10, 11) under coverslips frequently resulted in the disruption of the sporangium (Pl. 2, fig. 12). With the rupture of the sporangial wall, the sporogenic hyphae contained therein were spread out (Pl. 2, fig. 12) in a manner that greatly facilitated the study of the chromatinic bodies present in the sporangia.

With the formation of the sporangia, the chromatinic rods of the palisade hyphae were found to progress into the sporangium along with the growth of sporogenic hyphae into the sporangial envelope (Pl. 2, fig. 8, 9; Pl. 3, fig. 13, 14; Pl. 4, fig. 21–24). In strain 20 this process continued until the sporangium was filled with parallel rows of sporogenic hyphae, each of which contained a dense rod of chromatinic material (Pl. 3, fig. 13; Pl. 4, fig. 23, 24). In strain 1668 the sporangium was filled with a coil of branched sporogenic hyphae with each hypha containing a dense rod of chromatinic material. Within the sporogenic hyphae of both strains, the rods of chromatinic material appeared to contract and undergo repeated divisions into smaller subunits (Pl. 3, fig. 15, 16; Pl. 4, fig. 25, 26). These subunits were observed as paired dense bodies within the threads of sporogenic hyphae (Pl. 3, fig. 17, 18; Pl. 4, fig. 26). Crosswalls were formed at wide intervals between these bodies (Pl. 3, fig. 17). Further septation of the sporogenic hyphae resulted in only one chromatinic body being included between any two septa. Constrictions occurring simultaneously at the septa produced chains of spores within the sporangium (Pl. 2, fig. 11; Pl. 4, fig. 27).

Each liberated, mature spore contained a single, round chromatinic body which, as is generally the case with bacterial spores that have been hydrolysed, appeared to have collapsed and formed a crescent along one side of the spore wall (Pl. 3, fig. 19).

DISCUSSION

The technique for observing stained preparations with phase-contrast or dark-field microscopy was recently discovered while examining Feulgen preparations made by Couch in 1953. These preparations were completely faded when observed with bright-field microscopy. However, when viewed with phase-contrast or dark-field microscopy they exhibited chromatinic bodies that were considerably more distinct than when observed as unfaded preparations in 1953 with bright-field microscopy. It appeared that the different refractive index of the stained but faded chromatinic bodies, although not detectable with bright-field microscopy, was greatly enhanced by either phase-contrast or dark-field miroscopy. Even though this technique for observing stained, but faded, preparations was slightly better for the Feulgen stain than for the other stains used, this technique might be found useful by other investigators who at times may need to study stained preparations that have faded.

The structure and distribution of the chromatinic bodies in the living and stained preparations of the Actinoplanes strains studied were almost identical. The Zeiss W-15 non-hardening mounting medium proved to be a useful mountant for viewing material with phase-contrast or dark-field microscopy, and did not create any distortions in

the cells of the hyphae. Hopwood & Glauert (1960) reported that chromatinic bodies of *Streptomyces coelicolor* when stained with the Piéchaud technique, which obviates the necessity of preliminary acid hydrolysis, were sharper and hence had more definite outlines than when stained with either the DeLamater or Feulgen techniques. However, the Piéchaud method as used in the present work yielded results that were not noticeably different from those obtained with either the DeLamater or Feulgen technique.

The two strains considered here, as well as other members of the Actinoplanaceae that have undergone preliminary observation, all exhibited striking similarity in structure and distribution of the chromatinic bodies to that reported for members of the Streptomycetaceae (Badian, 1936; Klieneberger-Nobel, 1947; Saito & Ikeda, 1958; Hopwood & Glauert, 1960; Shamina, 1964). This similarity between the chromatinic bodies of members of the Actinoplanaceae and those of the Streptomycetaceae corresponds with results reported by Couch (1954). Chromatinic bodies similar to those reported to occur at each major stage in the life-cycle of streptomycetes were found to occur at apparently homologous stages in the life-cycle of strains of the Actinoplanaceae. Strains included in both of these families exhibit two distinct hyphal types: the substrate hyphae, and the palisade or secondary hyphae. The substrate hyphae of the Actinoplanaceae are similar to those of the Streptomycetaceae in frequency of branching, in diameter of hyphae, and in variability of chromatinic bodies. The palisade hyphae of the Actinoplanaceae are similar to the secondary hyphae of streptomycetes in that in both they arise as simple branches from the substrate hyphae, lack numerous branches, are larger than the substrate hyphae, and contain large and complex chromatinic bodies. The sporogenic hyphae of the two families go through very similar stages in the process of sporogenesis. Indeed, if one were to remove the sporangial wall from the sporogenic hyphae of some members of the Actinoplanaceae, it would be difficult to distinguish these organisms from members of the Streptomycetaceae. This similarity in structure and distribution of the chromatinic bodies in these two groups indicates a close relationship between them.

The results presented in this paper seem to be corroborated in the electron micrographs of thin sections of representatives of the Actinoplanaceae by Lechevalier, Lechevalier & Holbert (1966). Although these authors did not mention chromatinic material, electron-transparent regions within the sporogenic hyphae shown in their micrographs probably represent the chromatinic material. These electron transparent regions appeared to undergo processes similar to those described in this paper.

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. Strain 1668 from Czapek agar culture. Feulgen stain, phase-contrast. Palisade hyphae (*P*), substrate hyphae (*S*), origins of plaisade hyphae (arrows), sporangia (*SP*). × 2000.

Fig. 2, 3. Substrate hyphae of strain 20. Fig. 2, Feulgen stain, phase-contrast, × 1,000. Fig. 3, Zeiss W-15 non-hardening mounting medium, × 1330. Chromatinic bodies (*C*).

PLATE 2

Fig. 4–6. Chromatinic bodies in lower portions of palisade hyphae. Feulgen stain, phase-contrast. Fig. 4, × 1200. Fig. 5, 6, × 4000.

Fig. 7. Young palisade hypha. Feulgen stain, phase-contrast, Proximal chromatinic bodies (*X*), distal chromatinic bodies (*Y*), origin of palisade hypha (arrow). × 1500.

Fig. 8, 9. Distal portions of young palisade hyphae. Feulgen stain, phase-contrast. Distal thread of chromatin (*Y*), young sporangia (*S*), × 2200.

Fig. 10–12. Sporangia of strain 20. Fig. 10, Young sporangium with sporogenic hyphae, × 1400.

Fig. 11, Mature sporangia filled with spores, × 1000. Fig. 12, Open sporangium exposing sporogenic hyphae, × 1000. Phase-contrast. Sporangial wall (*W*).

PLATE 3

Fig. 13–17. Sporogenic hyphae from sporangia of strain 20. Feulgen stain, phase-contrast. Chromatinic material (*C*), septa (*S*).

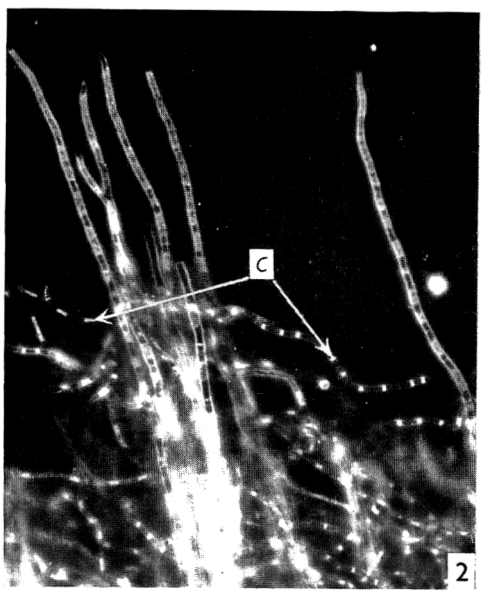
Fig. 13, Dense rods of chromatin inside sporogenic hyphae. × 4000. Fig. 14, Sporogenic hyphae spread out to show distribution of chromatinic material, × 2040. Fig. 15, 16, Division of chromatinic bodies into smaller subunits, × 3600. Fig. 17, Paired chromatinic bodies inside sporogenic hyphae. × 2600.

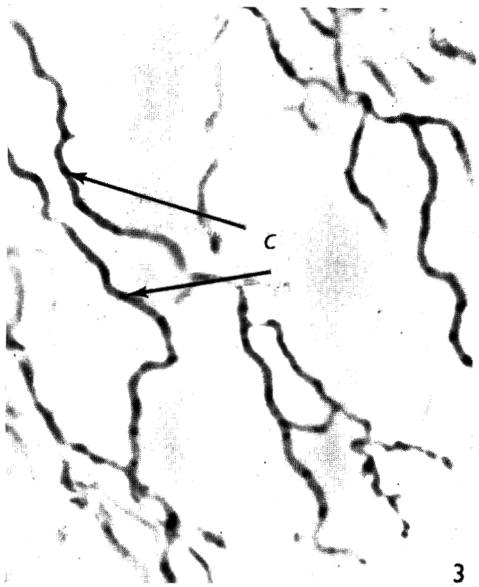
Fig. 18. Sporogenic hyphae broken from a sporangium at the tip of a palisade hyphae. Feulgen stain, dark-field. Chromatinic bodies (*C*). × 1680.

Fig. 19. Spores of strain 20. Feulgen stain, phase-contrast. Chromatinic bodies (*C*). × 3250.

PLATE 4

Fig. 20–27. Semi-diagrammatic drawings of palisade hyphae of strain 20 to show chromatinic bodies at various stages during sporangial formation. Chromatinic material is stippled. Hyphae are nearly straight but shown curved to conserve space. × 1400.





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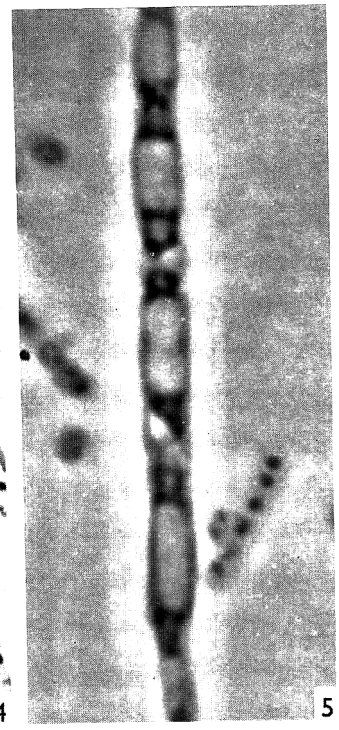
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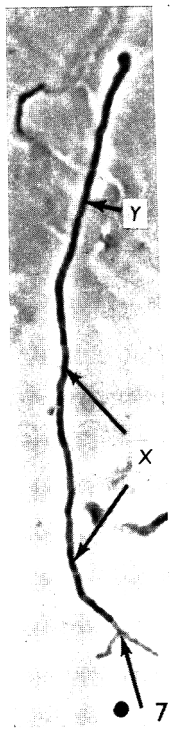

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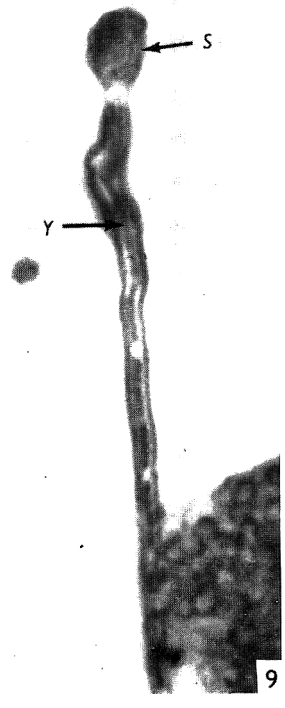

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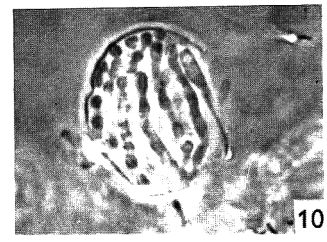
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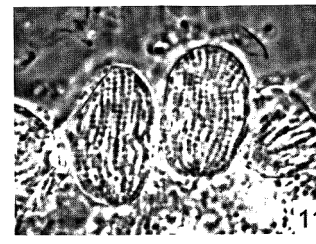
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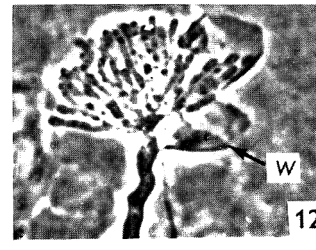

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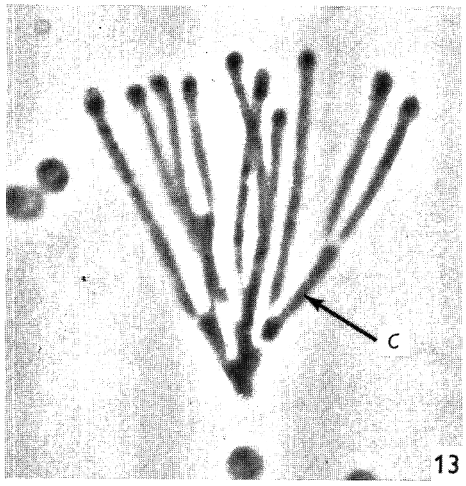
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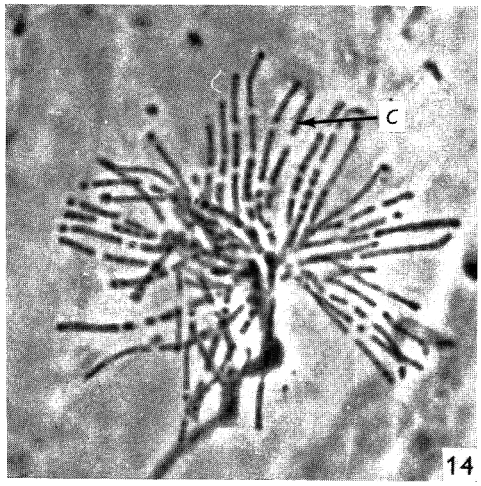
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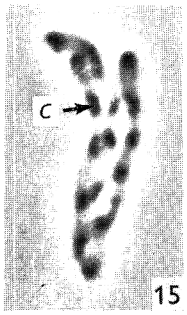
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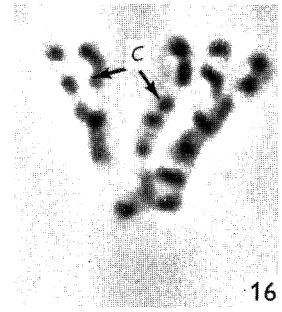
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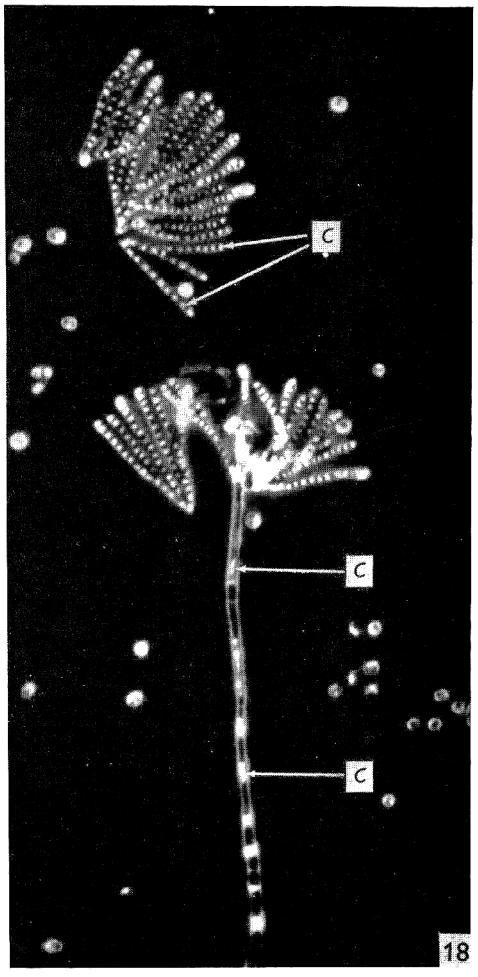
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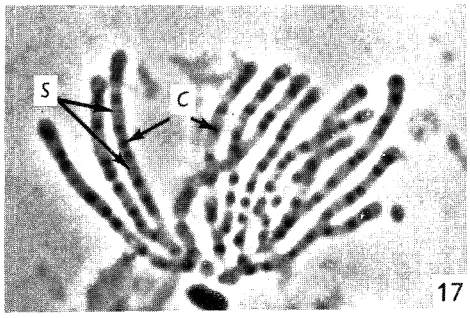
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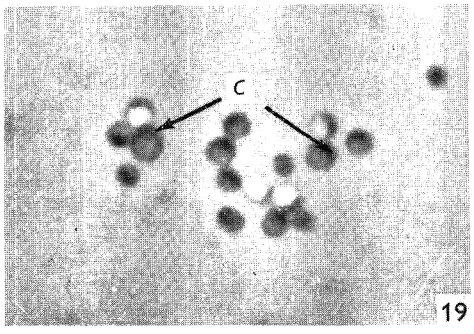
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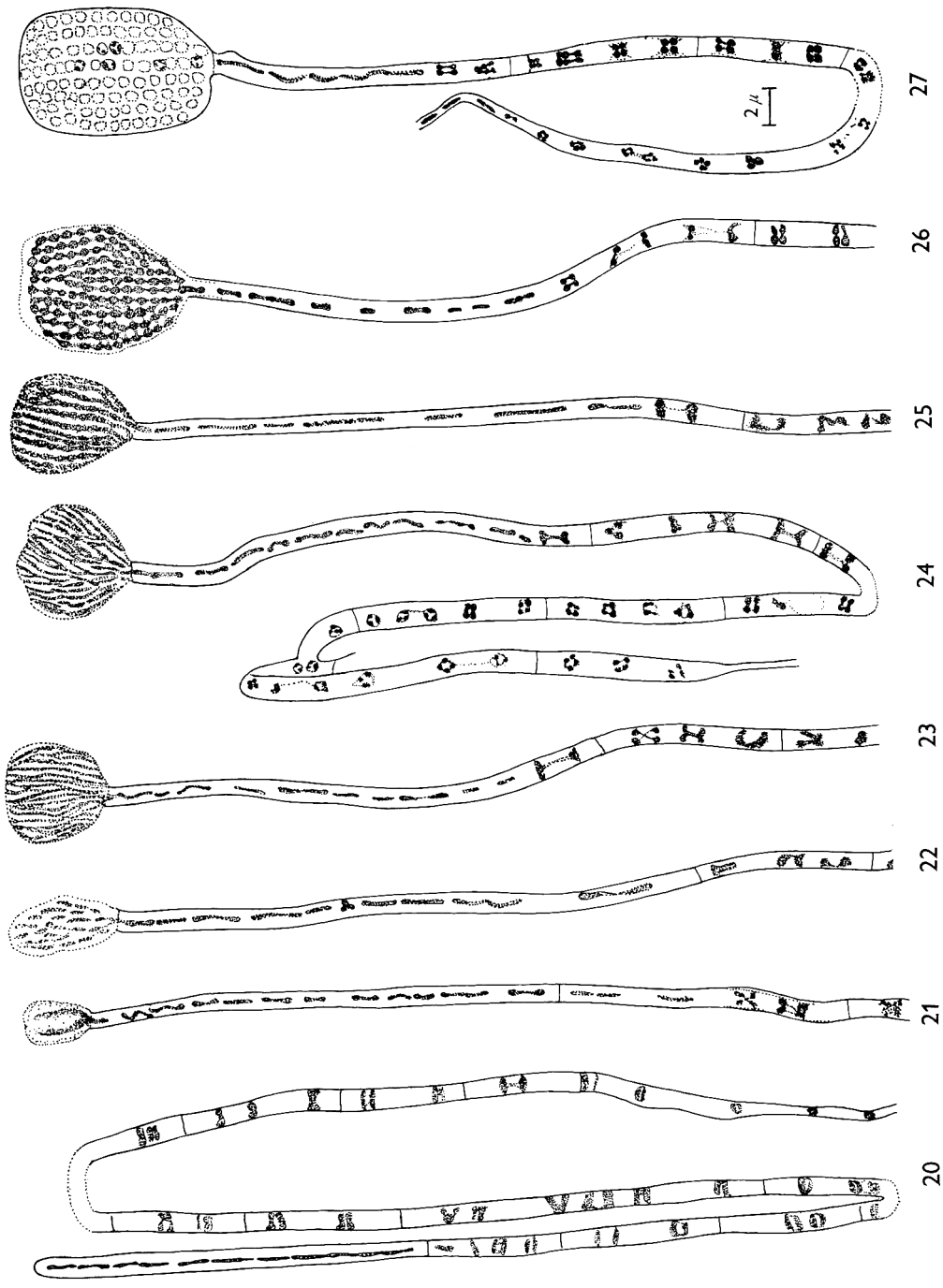

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The Autolysis of *Aspergillus flavus* in an Alkaline Medium

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SUMMARY

The autolysis of *Aspergillus flavus* subjected to a combined action of a continuous flow of air and mechanical agitation has been studied. We have obtained a degree of autolysis amounting to 85%. The total loss of nitrogen in autolysing mycelium of *A. flavus* reached 82%, whereas the loss of phosphorus was 90% from the beginning of autolysis. Total carbohydrates were reduced from 524 mg./sample, at 0 day, to 119 mg./sample by the 16th day of autolysis, which represents a loss of 77%. Glucose was the only sugar found in significant amount in autolysing mycelium of *A. flavus*, being 96% the total loss. The concentration of mannitol decreased to nearly a third at the end of the log phase. The disappearance of this substance during pre-autolysis and autolysis amounted to 97·7% with respect to the maximum weight present at the 8th day of incubation. Fourteen different free amino acids were identified in the mycelium of *Aspergillus flavus* during the pre-autolytic and autolytic stages of growth. These amino acids increased their content between the 4th and the 6th days of incubation, to the highest concentration observed. Seventy-nine per cent of this maximum amount disappeared by the end of the log phase, whereas 78% of the remainder disappeared from mycelium during autolysis (16 days). From the total amount of bound amino acids in mycelium of *A. flavus* it seemed that the make-up of the mycelial protein does not undergo great changes in the interval between pre-autolysis and the initiation of the autolytic phase of growth.

INTRODUCTION

During our investigations of the chemistry of autolysis in cultures of filamentous fungi (Lahoz, Reyes & Beltrá, 1966; Lahoz, 1967; Lahoz, Reyes, Beltrá & Garcia-Tapia, 1967) we have often suspected that the effect of a continuous flow of air on the process of autolysis might be to increase the amount of material released by the mycelium and consequently increase the degree of autolysis.

In exploratory experiments with *Aspergillus flavus* grown in a stirred alkaline culture medium and with a continuous supply of air (4 l./min.) we obtained a degree of autolysis amounting to 85%, which is to our knowledge the highest value recorded for filamentous fungi. It was decided to examine autolysis under these conditions.

METHODS

Chemicals. The chemicals used in the preparation of the culture medium were of analytical purity and they were obtained from Probus S.A. (Barcelona, Spain) and from the British Drug Houses Ltd. (Poole, Dorset, England). Standards used in chromatographic work were from the Sigma Chemical Co. (St Louis, Mo., U.S.A.).

Organism. Aspergillus flavus Link (our collection no. 2427) came from the Centraalbureau voor Schimmelcultures, Baarn, Holland.

Culture medium and conditions of culture. The organism was grown in the Czapek–Dox medium of the following composition (g./l. distilled water): glucose, (anhydrous) 50; $NaNO_3$, 2·0; KCl, 0·5; $MgSO_4.7H_2O$, 0·5; KH_2PO_4, 1·0; $FeSO_4.7H_2O$, 0·01; pH 4·2. The fungus was grown in submerged culture with continuous aeration and mechanical stirring in a 14 l. fermentor (NBS Continuous Culture Apparatus, model CF 500, New Brunswick Scientific Co., Inc., New Brunswick, New Jersey, U.S.A.). Incubation was at 24–25°, aeration at 4·0 l./min. and the culture stirred at 150 rev./min. throughout. The empty fermentor was sterilized at 120° for 2 hr and afterwards the fermentor containing the basal medium was sterilized at 120° for 20 min. A concentrated solution of glucose autoclaved separately at 110° for 20 min., was added aseptically to the basal medium in the fermentor; the final volume of medium was 10 l.

Inoculum. A spore suspension (10 ml.) was obtained by gently rubbing the surface of a slope of *Aspergillus flavus* grown on malt agar incubated for 15 days at 24–25° in the dark. With this spore suspension two conical flasks of 300 ml. capacity each, containing 100 ml. of Czapek–Dox medium, were inoculated and incubated for 24 hr on a reciprocating shaker (60 × 10 cm. strokes/min.) at 25°. One of these flasks was used as inoculum for the fermentor.

The time of incubation was usually 30–40 days; the water lost by evaporation was daily replaced. At convenient intervals samples (250 ml.) of culture were taken, and mycelium separated from culture fluid by filtration. Culture filtrate was kept frozen until analysed. Samples of mycelium were washed on the filter with distilled water and dried at 60–70° to constant weight.

Analytical methods. Total carbohydrates were determined by the anthrone method of Morris (1948) with glucose as standard.

The intensity of the colour of the culture filtrate was measured with a Klett-Summerson photocolorimeter with a blue filter.

Total N, P, crude fat, glucose, mannitol, amino acids and kojic acid were determined as previously described (Lahoz *et al.* 1966; Lahoz, 1967; Lahoz *et al.* 1967).

Fractionation of mycelium. Weighed samples of dry mycelium, harvested at different times, were exhaustively extracted with light petroleum (b.p. 50–70°) in a beaker (100 ml./g. dry mycelium) with mechanical stirring for 1 hr; the solvent was removed by distillation and the residue left in the flask constituted fraction I. This defatted mycelium was carefully removed from the beaker, dried at 80°, weighed and extracted twice by cold (22°) distilled water (100 ml./g. defatted mycelium) with mechanical stirring for 1 hr; the cold-water extracts obtained were combined (fraction II) and stored at −10°. To test for completeness of extraction the mycelial residue was extracted a third time and the resulting extract concentrated under reduced pressure and a sample of it chromatographed for sugars and amino acids. Free sugars and free amino acids contained in fraction II were separated by paper chromatography and

individually estimated as described previously (Lahoz *et al.* 1966; Lahoz, 1967; Lahoz *et al.* 1967). The water-extracted mycelium was dried at 80° to constant weight (fraction III). A portion (40 mg.) of fraction III was hydrolysed with 3 N-HCl (1 ml./10 mg. dry mycelium) in sealed ampoules at 100° for 12 hr. The hydrolysate was dried at 60–70°, the residue dissolved in the initial volume (4 ml.) of distilled water, again evaporated to dryness at 70°, again dissolved in 4 ml. distilled water and finally dried in a desiccator over $NaOH + P_2O_5$. This residue was dissolved in water (4 ml.), samples (0·10 ml.) chromatographed on Whatman no. 1 filter paper for carbohydrates (Lahoz *et al.* 1966).

A portion (40 mg.) of fraction III was heated with 6 N-HCl (1 ml./10 mg. dry mycelium) in sealed ampoules at 110° for 18 hr, the hydrolysate processed as for carbohydrates, and samples (0·07 ml.) of the final hydrolysate chromatographed for amino acids.

RESULTS

Aspergillus flavus incubated in medium containing nitrate as N source with aeration gave cultures showing pH values generally in the range pH 4·5–8·5, in some experiments rising to pH 8·6 and even 9·0. In a typical experiment (Table 1) the culture changed from pH 4·8 at day 11, when autolysis began, to pH 8·5 at the 9th day of

Table 1. *The autolysis of Aspergillus flavus during incubation in Czapek–Dox medium in submerged culture at 25° with aeration (4 l./min.) and mechanical stirring (150 rev./min.) in fermentors. Autolysis* began at 11–12 days of incubation*

Time of incubation (days)	Time of autolysis* (days)	pH	Residual glucose (g./l.)	Colour intensity (Klett values)	Kojic acid (mg./100 ml.)
4	—	5·0	33·0	n.t.†	0
6	—	4·5	23·5	n.t.	6
8	—	4·5	12·6	39	20
9	—	4·5	7·4	n.t.	60
11	0	4·8	0·7	65	125
12	1	5·3	0·6	80	162
13	2	6·5	0·6	175	195
14	3	7·1	0·5	260	158
16	5	7·6	0·3	355	61
18	7	8·0	0·2	395	21
20	9	8·5	0·1	390	0
24	13	8·5	0·1	490	0

* Autolysis was measured as decrease in mycelial dry weight.
† n.t. = not tested.

autolysis (day 20 of incubation), being constant thereafter. The criterion of autolysis adopted throughout this work was the loss in mycelial dry weight. According to that, autolysis begun on the 11th day of incubation. Residual glucose (Table 1) was present at about 0·5 g./l. at the beginning of autolysis. The colour of the culture fluid (Table 1) continuously increased in intensity and was equivalent to about 400 Klett scale units between 20 and 25 days. When kojic acid was produced its content in the culture fluid increased to a maximum value and thereafter decreased as the culture aged. At the end of the autolysis no kojic acid remained. In a typical experiment the maximum content of kojic acid in the culture fluid coincided with the beginning of autolysis.

In a representative experiment the highest amount of mycelial weight (Table 2), 1·81 g./250 ml. broth, was attained at day 11 of incubation, being of 0·28 g./250 ml. broth by the 16th day of autolysis—that is, an 85% decrease of mycelial dry wt. The total-N content of the mycelium (Table 2) continuously decreased from the end of the

Table 2. *The autolysis of Aspergillus flavus mycelium during incubation in Czapek–Dox medium in submerged culture at 25° with aeration (4 l./min.) and mechanical agitation (150 rev./min.) in fermentors*

(Values for yield of mycelium, total N, P, carbohydrates and crude lipids from mycelium. The term 'sample' indicates the dry weight of mycelium contained in 250 ml. of broth.)

Time of incubation (days)	Time of autolysis (days)	Mycelium (g. dry wt/ sample)	Total N (g./ sample)	Total P (g./ sample)	Total carbohydrates (g./ sample)	Petroleum ether extract (g./ sample)
6	—	1·214	0·050	n.t.*	0·327	0·174
8	—	1·623	0·057	n.t.	n.t.	0·290
11	0	1·807	0·046	0·010	0·524	0·386
12	1	1·599	n.t.	n.t.	n.t.	n.t.
13	2	1·550	n.t.	n.t.	n.t.	n.t.
16	5	1·214	0·031	0·007	0·461	0·162
18	7	0·625	0·017	0·003	n.t.	0·076
20	9	0·517	0·015	0·003	0·196	0·040
24	13	0·262	0·008	0·001	n.t.	0·018
27	16	0·278	0·008	n.t.	0·119	0·015

* n.t. = not tested.

Table 3. *Fraction II. variation in the content of water-extractable materials, total carbohydrates, mannitol and free gluose in mycelium of Aspergillus flavus during pre- and autolytic phase of growth*

Results are expressed as mg. substance(s)/sample of original dry mycelium. Other details are given in the text.

Time of incubation (days)	Time of autolysis (days)	Water extractable materials (mg./ sample*)	Total carbohydrates in aqueous extracts (mg./sample*)	Mannitol (mg./ sample*)	Glucose (mg./ sample*)
6	—	476	50	70	42
8	—	514	57	90	43
11	0	361	43	37	25
16	5	221	24	21	13
18	7	104	11	9	5
20	9	75	7	7	4
24	13	30	4	2	2
27	16	—	3	2	1

* The term 'sample' indicates grams of dry mycelium contained in 250 ml. of brew.

log phase to the 24th day of incubation, being constant thereafter, the total loss of N during the period of autolysis amounting to 82% with respect to the 11th day of incubation. Phosphorus lost during autolysis 90% of its maximum initial content (Table 2). Total carbohydrates present in autolysing mycelium of *Aspergillus flavus*,

524 mg./sample, at the 11th day of incubation (Table 2) fell to 119 mg./sample by the 27th day of incubation, which represents a loss of 77 % with respect to the content at the 11th day of incubation, whereas the content of lipids, measured as the total light petroleum extractable material, continually increased at the end of the log phase. During autolysis the loss of these materials was 96 % with respect to the 11th day of incubation.

Behaviour of free glucose and mannitol. Free glucose was the only sugar present in significant amounts in the autolysing mycelium of *Aspergillus flavus* under these conditions. The decreasing concentrations of total carbohydrate, glucose and mannitol in fraction II are shown in Table 3. The behaviour of bound glucose in fraction III is shown in Table 4, where the amount of free glucose released on hydrolysis of fraction III is recorded.

Behaviour of free amino acids. Free amino acids increased in content between the 4th and the 6th days of incubation, being at the highest (Table 5) concentration on the 6th day of incubation. During pre-autolysis and autolysis the amount continuously decreased till the 27th day of incubation. Seventy-nine per cent of the maximum amount of the free amino acids contained in mycelium at the 6th day of incubation disappeared at the end of the log phase, whereas 78 % of the remainder disappeared during autolysis. The bound amino acids released on hydrolysis of fraction III are shown in Table 6.

Table 4. *Total reducing substances and glucose released in the hydrolysis of the defatted, ex-water mycelium (fraction III) by 3 N-HCl during 12 hr at 100° in sealed ampoules (other details are given in the text)*

Time of autolysis (days)	Total reducing substances (mg./ sample* of original dry mycelium)	Glucose (mg / sample* of original dry mycelium)
0	148	40
5	128	29
7	77	15
9	51	10
13	35	6
16	34	5

* The term 'sample' indicates the weight of dry mycelium contained in 250 ml. of brew.

DISCUSSION

These experiments seem to indicate that comparatively strong aerobic conditions during autolysis increase the extent to which the processes of breakdown of mycelium took place, which partially explains the high value for the degree of autolysis encountered in these conditions. Excepting actinomycetes, as a group of filamentous organisms, this is to our knowledge the highest loss of mycelial weight reported in the literature of filamentous fungi during autolysis.

In static cultures of *Aspergillus flavus* the maximum content of free amino acids (4·92 g./100 g. dry mycelium) occurred at the 5th day of incubation, with a subsequent decrease (Pillai & Srinivasan, 1956), the 15th day of incubation being the 0 day of autolysis. Similarly, in our experiments nearly 80 % of the total free amino acids

Table 5. *Intracellular free amino acids present in the mycelium of Aspergillus flavus during pre-autolytic and autolytic phase of growth in a physiologicaly alkaline medium (Czapek–Dox) with aeration (4 l./min.) and mechanical agitation (150 rev./min.) at 25°*

Autolysis began at 11–12 days of incubation. Results are expressed as mg. amino acid/sample* (column A) and as g. amino acid/100 g. dry wt of initial mycelium (column B).

Time of incubation (days)	4		6		11		16		18		27	
Time of autolysis (days)	—		—		0		5		7		16	
Amino acids	A	B	A	B	A	B	A	B	A	B	A	B
Leucine	11·4	1·22	—	—	—	—	—	—	—	—	—	—
Phenylalanine	T	T	T	T	T	T	T	T	T	T	T	T
Valine	12·1	1·30	14·3	1·18	8·7	0·48	3·2	0·26	1·3	0·21	0·2	0·09
Tryptophan + tyrosine	3·4	0·37	24·0	1·98	6·1	0·34	4·6	0·38	1·8	0·30	—	—
Proline	T	T	T	T	T	T	T	T	T	T	T	T
Alanine	11·0	1·18	14·9	1·23	3·8	0·21	2·5	0·21	0·6	0·10	0·3	0·12
Glutamic acid	13·5	1·45	—	0·78	4·0	0·22	1·8	0·15	1·1	0·17	0·1	0·05
Serine + glycine + aspartic acid	11·0	1·18	13·4	1·10	4·7	0·26	2·5	0·21	0·9	0·14	0·2	0·06
Arginine	5·7	0·61	5·1	0·42	—	—	1·2	0·10	0·5	0·08	T	T
Histidine	6·2	0·67	8·3	0·68	—	—	—	—	0·4	0·07	T	T
Lysine	T	T	T	T	T	T	T	T	T	T	T	T
Total amount %		6·76		7·37		1·51		1·31		1·07		0·32

* The term 'sample' indicates g. dry mycelium contained in 250 ml. of brew.
T = Present in traces only.
— = No estimation was carried out.

Table 6. *Bound amino acids in the mycelium of Aspergillus flavus, during the pre-autolytic and autolytic phase of growth, released in the acid hydrolysis of fraction III*

Results are expressed as g. amino acid/100 g. fraction III. Other details are given in the text.

Time of incubation (days)	6	8	11	16	18	20	24	27
Time of autolysis (days)	—	—	0	5	7	9	13	16
Amino acid								
Leucine	1·14	1·98	1·48	2·00	0·92	0·80	0·88	0·86
Phenylalanine	T	T	T	T	T	T	T	T
Valine	1·28	2·16	1·52	1·84	1·20	0·75	1·08	0·96
Tyrosine	T	T	T	T	T	T	T	T
Proline	T	T	T	T	T	T	T	T
Alanine	0·80	1·20	1·48	1·32	0·72	0·74	0·66	0·76
Glutamic acid	1·16	1·18	1·40	1·44	1·04	1·10	0·94	0·84
Aspartic acid + glycine	1·30	1·40	2·04	2·00	1·00	1·16	1·14	0·92
Glucosamine	2·08	2·00	2·00	1·88	2·24	1·36	2·32	1·44
Arginine	0·52	0·88	0·68	0·74	0·36	0·46	0·46	0·28
Asparagine	T	T	T	T	T	T	T	T
Total amount	8·28	10·80	10·60	11·22	7·50	6·40	7·40	6·06

T = present in traces only.

present on the 6th day of incubation had (7·37 %) disappeared before the initiation of the autolytic phase of growth (1·51 % at the 0 day of autolysis). *Aspergillus terreus* behaved in a very similar fashion when grown in a physiologically acid medium as static cultures. In these cultures the maximum concentration (2·45 g./100 g. dry mycelium) of the free amino acids in mycelium at the 9th day of incubation diminished by 86 % during these 9 days prior to autolysis (Lahoz *et al.* 1967). The diminution of these compounds in the mycelium during the end of the log phase well before autolysis sets in (as measured by the loss of mycelial dry weight) seems to be a common pattern for these fungi irrespective of the medium and of the conditions of culture. Chattaway, Toothill & Barlow (1962) noted that the maximum concentration of the pool amino acid from growing mycelium of *Microsporum canis* was observed towards the end of the log phase of growth. In Pillai & Srinivasan's experiments, referred to above, with *Aspergillus flavus* grown as static cultures in Czapek–Dox medium the disappearance of free amino acids from mycelium during the first 15 days of autolysis amounted to 42 %, whereas in the present results this disappearance reached 78 %, thus indicating that in the presence of a continuous flow of air and mechanical stirring autolysis affected free amino acids to a greater extent than in stationary cultures.

The constancy in the content of glucosamine (values in the neighbourhood of 2 g./100 g. fraction III, almost throughout the whole period of incubation, Table 6) released upon acid hydrolysis of fraction III seemed to indicate that chitin is very little affected by autolysis in these conditions. This is in agreement with Emiliani & Ucha de Davie's (1962) observations working with *Aspergillus phoenicis*. These workers stated that chitin is not attacked, or very little attacked, during the induced autolysis of mycelium of *A. phoenicis* by 0·2 M-acetic acid.

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R (Transmissible Drug-resistance) Factors in *Salmonella typhimurium*: Pattern of Transduction by Phage P22 and Ultraviolet-protection Effect

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SUMMARY

R factors from Enterobacter strains isolated outside Japan were transferred to *Salmonella typhimurium* LT 2 and their resistance traits transduced by phage P 22. Of five *fi*$^+$ factors conferring resistance to tetracycline (Tc), streptomycin (Sm), sulphonamides (Su) and chloramphenicol (Cm), three, one of which conferred also resistance to kanamycin (Km), behaved like previously reported factors in that: Tc was transduced by itself but never with any other trait; all other traits were usually co-transduced; no transductants could transmit resistance by conjugation, even after infection with F′-13 *lac*. The other two *fi*$^+$ factors, which conferred also resistance to benzylpenicillin (Pn), behaved similarly, except that: Pn was transduced only at a low rate, and was never co-transduced with any other trait; and a few transductants when given F′-13 *lac* became able to transmit. Four *fi*$^-$ factors, two of type Tc, Sm, Su, Pn and two of type Tc, Su, Pn differed in that: nearly all Tc transductants acquired also all the other traits; Pn was usually co-transduced with the other traits; and some transductants could transmit by conjugation, even without F′-13 *lac*. The various transductant classes obtained by treatment with P 22 grown on LT 2 carrying both an *fi*$^+$ (Tc, Sm, Su, Cm, Km) and *fi*$^-$ (Tc, Sm, Su, Pn) factor could be accounted for by transduction of fragments of either one or the other of these factors. The sorts of transductants produced by lysates of a strain possessing Tc, Sm, Su, Cm, Km determinants obtained by growing a strain carrying an *fi*$^+$ (Tc, Sm, Su, Cm) factor with one carrying an *fi*$^+$ (Km) factor suggested that the strain carried two *fi*$^+$ factors, not a recombinant factor. Both *fi*$^-$ and *fi*$^+$ factors conferring resistance to benzylpenicillin conferred also resistance to cephalothin and cephaloridine and caused the constitutive production of a β-lactamase, active on both benzylpenicillin and on cephalothin. All four *fi*$^-$ and one of the five *fi*$^+$ factors resembled *col*I factors in that they protected strain LT 2 against the bactericidal effect of ultraviolet irradiation.

INTRODUCTION

Transmissible resistance to several antibiotics and to sulphonamides was encountered in Shigella strains in Japan in 1955, and soon became very common in that country (Watanabe, 1963; Mitsuhashi, 1965). Such resistance results from the presence in the bacteria of a non-chromosomal genetic determinant or plasmid, termed an R factor, composed of DNA of a characteristic base composition (Falkow, Citarella, Wohlhieter & Watanabe, 1966). R factors reported from Japan commonly confer

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four resistance traits, namely, resistance to tetracycline (Tc), streptomycin (Sm), sulphonamides (Su) and chloramphenicol (Cm); less often, only one, two or three of these traits. Similar R factors are now prevalent in many parts of the world and factors determining resistance to the neomycin-kanamycin (Km) group (Lebek, 1963; Carpenter & Drabble, 1965) or to the penicillins (Anderson & Datta, 1965; Carpenter & Drabble, 1965) have been encountered. Some R factors, termed *fi*$^+$, when introduced into Hfr or F$^+$ lines of *Escherichia coli* decrease or abolish their fertility and susceptibility to the male-specific phages, probably by repressing formation of F-pili (Watanabe *et al.* 1964*a*; Meynell & Datta, 1965; Datta, Lawn & Meynell, 1966). The *fi* property divides R factors into two groups. The presence in a host of an R factor of one group interferes with its acquisition and maintenance of another factor of the same group, but not with that of a factor of the other group (Watanabe *et al.* 1964*a*). R factors or their component genes may be transduced by general transducing phages. The *Escherichia coli* phage P 1 commonly transduces an entire R factor, i.e. most transductants acquire all the drug-resistance traits conferred by the factor, and also the ability to transmit these traits by conjugation. In Salmonella, by contrast, the general transducing phages, P 22 for group B and ϵ_{15} and ϵ_{34} for group E, transduce only fragments of the R factor. The Tc trait when present is transduced separately from the other traits, which usually travel together; and the transductants cannot transmit their resistance traits by conjugation—presumably because the transduced fragments of the R factor lack its genes which confer ability to conjugate (Watanabe & Fukusawa, 1961; Harada, Kameda, Suzuki & Mitsuhashi, 1963). Most isolates of another sort of plasmid, namely, colicine factors of the *colIa* and *colIb* classes, decrease the sensitivity of their bacterial host to the bactericidal action of ultraviolet irradiation (Howarth, 1965; Howarth-Thompson, 1966) and we have found (Drabble & Stocker, 1966) that some R factors have a similar effect in *Salmonella typhimurium* strain LT 2. We here report a comparative study of the transduction and u.v.-protecting properties of various R factors. They include factors conferring penicillin-resistance, discovered in *S. typhimurium* strains isolated in England (Anderson & Datta, 1965); the R factor conferring resistance to kanamycin (Lebek 1963); and several R factors found amongst Shigella strains from various parts of the world examined at the Shigella Reference Laboratory, Central Public Health Laboratory, Colindale, London, N.W. 9. (Carpenter & Drabble, 1965).

METHODS

Bacterial strains and phages. The strains from which the R factors were obtained are listed in Table 1. Strains with SL numbers were from the collection maintained in this laboratory. The *Escherichia coli* strains W 1895 and 200 U were provided by Dr P. Gemski and came originally from J. Lederberg and F. Jacob respectively. Phage P 22 was used for transduction. The male-specific phage R 17 (Loeb, 1960) was used to test the *fi* character of R factors.

Media. Nutrient broth was Oxoid no. 2, code CM 67. Nutrient agar was Oxoid blood agar base (code CM 55). For some purposes peptone agar (Evans's bacteriological peptone, 1 %, w/v) was used instead. The defined medium was that of Davis & Mingioli (1950) with glucose, 1 mg./ml., solidified with Oxoid no. 3 agar at 15 g./l. Appropriate amino acids and purines were added at 10 μg./ml.; vitamins at 1 μg./ml.

Antibacterial drugs. Bacterial sensitivity was tested by inoculation on nutrient agar

Table 1. *R factors and strains from which they were isolated*

R Factor		Strain from which isolated			
Name*	Characters	Species	Strain no.	Characters†	References
Brighton	Tc, Sm, Su, Pn; *fi*⁻	*Salmonella typhimurium*	2 M 1818	Phage type 1 a	Anderson & Datta (1965)
Bradford	Tc, Sm, Su, Pn; *fi*⁻		2 M 1872	Phage type 1 a	Datta & Kontomichalou (1965)
Enfield	Tc, Su, Pn; *fi*⁻		2 M 1736	Phage type 1 a	
Utrecht	Tc, Su, Pn; *fi*⁻		3735	Phage type 1 a	—
Munich	Tc, Sm, Su, Cm, Km; *fi*⁺	*Escherichia coli*	—	Colicinogenic	Lebek, (1963); Watanabe, Ogata & Sato (1964*b*)
S. Africa	Tc, Sm, Su, Cm; *fi*⁺	*Shigella sonnei*	C 63/1174	Colicinogenic, type 12	
Peru	Tc, Sm, Su, Cm; *fi*⁺	*Shigella flexneri*	571/64	Serotype 2 a	
Zambia	Tc, Sm, Su, Cm, Pn; *fi*⁺	*Shigella flexneri*	747/64	Serotype 6 (var. Manchester)	Carpenter & Drabble (1965)
Singapore	Tc, Sm, Su, Cm, Pn; *fi*⁻	*Shigella sonnei*	C 62/381	Colicinogenic, type 6	
Barnet	Km; *fi*⁺	*Shigella sonnei*	C 64/258	Colicinogenic, type 14 (?)	

The Shigella strains were supplied by Dr P. Carpenter (Shigella Reference Laboratory, Central Public Health Laboratory, Colindale, London N.W. 9) and the other strains by Dr N. Datta (Bacteriology Department, Postgraduate Medical School, London W. 12).

* Name indicates place of origin of the source strain. The *Shigella sonnei* strain carrying R-Barnet was isolated in England from a patient thought to have been infected in Continental Europe.

† Information supplied by the Laboratories from which strains were obtained.

or peptone agar containing the following: tetracycline, 25 μg./ml.; streptomycin sulphate, either 25 μg./ml., to test the low-level resistance conferred by some of the R factors, or 1 mg./ml., to test the high-level (mutational) resistance of some strains; chloramphenicol, 25 μg./ml.; benzylpenicillin, 60 μg./ml.; kanamycin, 25 μg./ml. Sulphonamide-sensitivity was determined by inoculation to defined medium containing sulphathiazole, 1 mg./ml. The drug-resistance pattern of sets of 25 transductants, etc., and of two control strains was determined by replication with a 27-prong replicator to test plates of the above media. Nutritional characters and sugar fermentation characters were similarly determined by replication to appropriate media.

Conjugational transfer of plasmids. R factors and the F′-13 *lac* episome were transferred from appropriate donor strains to recipients by growing them together for about 18 hr in broth at 37° without shaking or aeration, then plating on medium selective for bacteria of the recipient strain which had received one of the characters of the plamid. The R factors originally harboured in *Salmonella typhimurium* strains, all of which conferred penicillin resistance, were first transferred in this way to SL 675 (LT 2 *pur pro str*), selection being for penicillin resistance and for the high-level streptomycin resistance of the recipient. The R-Munich factor was transferred directly from its original *Escherichia coli* host to SL 726 (LT 2 *met trp ara gal*), using medium containing kanamycin with citrate as sole energy source to select the Salmonella recipient. Attempts to transfer R factors directly from their original Shigella hosts to SL 726 were unsuccessful. They were therefore first transferred to an *E. coli* K 12 *met* F^- strain, selecting for nicotinic-acid independence and for one of the drug-resistance traits conferred by the R factor. Each of these R factors was then easily transferred from the *E. coli* to SL 675, selection being made for the high-level streptomycin resistance of the acceptor as well as an R-factor-determined resistance trait. The R factors thus introduced into the streptomycin-resistant LT 2 line, SL 675, were next transferred to a streptomycin-sensitive LT 2 line, SL 726 (LT 2 *met trp ara gal*) with selection for the pur^+ pro^+ of the recipient and for one of the resistance traits of the R factor.

The *fi* character of the R factors was determined after transfer from LT 2 to an *Escherichia coli* K 12 *met* Hfr line, W 1895. To test for inhibition of fertility the R derivatives of W 1895 were scored for ability to produce lac^+ *str-r* recombinants when crossed to a K 12 *lac str-r* F^- line, 200 U. The W 1895 lines given R factors were also tested for sensitivity to the male-specific phage R 17 (on tryptone yeast-extract agar containing 0·002 M-$CaCl_2$).

Transduction. Phage P 22 was propagated by the soft-agar layer method on the SL 726 (LT 2 *met trp ara gal*) derivatives carrying the various R factors. The lysates after centrifugation were Seitz-filtered or held at 60° for 1 hr. The strain used as transductional recipient was SL 862 (LT 2 *trp his*). A young broth culture was mixed with phage at a multiplicity of about 10. After 10 min. the mixture was centrifuged and the bacteria washed twice, then resuspended at ×20 the original concentration in 1/4 strength Ringer solution. Drug-resistant transductants were selected by spreading 0·1 ml. volumes of the concentrated suspension on duplicate plates of peptone agar containing a single antibiotic, or of supplemented defined medium with sulphathiazole. As controls, bacteria to which no phage had been added were similarly washed and plated. Suitable dilutions of the transduction mixtures were also plated on defined medium supplemented with tryptophan, to detect the rate of transduction of his^+.

The drug-resistance pattern of transductants was determined, after purification, as described above. The ability of representative drug-resistant transductants to transmit their resistance traits by conjugation was tested by overnight growth in broth with strain RS 75, a streptomycin-resistant LT 2 line resistant to P 22 (and in consequence unsusceptible to transduction) (Gemski & Stocker, 1967). Drops from overnight mixed cultures of each transductant with RS 75 were spotted on drug-supplemented peptone agar selective for the high-level streptomycin-resistance of the acceptor and one of the resistance traits of the transductant. Transductants found unable to transmit were infected with the F'-13 *lac* episome by growth with *Salmonella typhimurium* strain SL 2110 (*leu cys mal rou* F'-13 *lac*), followed by selection on appropriate medium. The *lac*$^+$ derivatives were then tested for ability to transmit drug-resistance, as before.

Test for protection against u.v. radiation. The effect of R factors on susceptibility to killing by ultraviolet irradiation was tested in *Salmonella typhimurium* SL 675 (*pur pro str*). Overnight 37° broth cultures were diluted 1/10 in broth; the diluted cultures were incubated at 37°, without aeration or shaking, for 4–5 hr, by which time they contained $3–5 \times 10^8$ viable bacteria/ml. Serial decimal dilutions were made in quarter-strength Ringer solution containing 10% (v/v) nutrient broth. Drops (volume 0·02 ml.) from appropriate dilutions were delivered to the surface of peptone agar plates. When the drops had dried, sets of plates were exposed for various times, usually 30 sec. and 60 sec., at 69·5 cm. from a 15 W Phillips TUV Germicidal Lamp (low-pressure Hg arc). Colonies on the irradiated, and unirradiated control, plates were counted after overnight incubation at 30°. The medium and temperature of incubation were chosen to give conveniently small colonies.

Table 2. *Maximum antibiotic concentrations (μg./ml.) allowing growth of strain SL 726 and of its derivatives carrying R-Munich or R-Brighton*

	SL 726	SL 726 (R-Munich)	SL 726 (R-Brighton)
Tetracycline	0·5	60	50
Streptomycin	1	20	60
Sulphathiazole	40	2500	2500
Chloramphenicol	2·5	1000	2·5
Kanamycin	0·5	1000	0·5
Neomycin	0·5	100/150	0·5
Benzylpenicillin	1·5	1·5	360
Cephalothin	1·5	1·5	100
Cephaloridine	1·25	1·25	35

Inocula of about 10^4 log-phase cells were added to 3 ml. tubes of nutrient broth containing graded concentrations of drug. Growth was inferred from turbidity after 20 hr at 37°.

RESULTS

Pattern of resistance conferred by R factors

Table 2 records the minimum concentration of various drugs required to inhibit the growth of a small inoculum of a *Salmonella typhimurium* LT 2 line carrying no R factor, and of its derivatives carrying R-Munich (Tc, Sm, Su, Cm, Km) or R-Brighton (Tc, Sm, Su, Pn). When the R factor conferred resistance, the minimal inhibitory concentration was increased at least 20-fold. Several R factors conferring resistance to benzylpenicillin, derived either from *S. typhimurium* strains or from Shigella strains,

conferred also substantial resistance to 6-amino penicillanic acid, cloxacillin, phenethicillin, methicillin, cephalothin and cephaloridine. *Salmonella typhimurium* and *Escherichia coli* lines given the complete R-Brighton factor by conjugation or transduction and an LT 2 line given only its benzylpenicillin-resistance trait by transduction gave positive reactions in the test of Foley & Perret (1962), indicating production of a β-lactamase, active on both benzylpenicillin and also, though less strongly, on cephalothin. A strain of *Bacillus cereus* with an inducible penicillinase, included as control, gave as expected a positive reaction only with benzylpenicillin. Prior exposure of the colonies to chloroform vapour did not diminish the size of the zones produced in the penicillinase test, except in the case of the inducible *B. cereus* strain. That is, the R factors caused constitutive production of penicillinase. However, the chloroform-exposed colonies produced zones more slowly than the untreated colonies in the test for cephalothin destruction.

Conjugal transfer of R factors

All the R factors were transferred from their original hosts to the *Salmonella typhimurium* LT 2 line SL 726, as described under Methods. In each instance it was easy to isolate an LT 2 derivative with all the resistance traits of the original strain. This suggests that the complete R factor was being transferred. The *Escherichia coli* strain and several of the Shigella strains used as sources of R factors were colicinogenic, but the LT 2 derivatives chosen for investigation had not acquired colicinogeny with the R factor. Some R factors are very unstable in *S. typhimurium* LT 2 (Watanabe, 1963). In our experiments spontaneous loss of resistance traits was not so frequent as to interfere with experimental design. To investigate its stability, the LT 2 (R-Munich) line, resistant to tetracycline, streptomycin, sulphonamide, chloramphenicol and kanamycin, was subcultured in broth daily. After five subcultures 50 %, and after 13 subcultures 99 %, of the population had lost resistance to all drugs except tetracycline. After 16 subcultures 40 % of the population was no longer tetracycline-resistant, and 99 % no longer resistant to the other drugs.

Strains carrying two R factors

Strains presumably carrying both an fi^- and an fi^+ R factor were isolated from mixed cultures of derivatives of the *Salmonella typhimurium* strains SL 699 (*met trp*) and SL 726 (*met trp ara*). One strain carried R-Munich (Tc, Sm, Su, Cm, Km) and the other R-Brighton (Tc, Sm, Su, Pn). Doubly resistant colonies were selected on peptone arabinose indicator agar containing Pn and Km. The medium distinguished the arabinose-fermenting SL 699 from the arabinose-negative SL 726 line. Whichever strain acted as donor the fraction of bacteria carrying R-Munich which acquired R-Brighton was about 10^{-3}, and the fraction of bacteria carrying R-Brighton which acquired R-Munich was about 2×10^{-5}. The colonies selected carried all the resistance traits of both donor and recipient, and were presumed to carry both factors. Strains apparently carrying two fi^+ R factors were prepared by growing *S. typhimurium* SL 726 (*met trp gal*) carrying either R-S. Africa (Tc, Sm, Su, Cm) or R-Peru (Tc, Sm, Su, Cm) with the Shigella strain C 64/258, carrying R-Barnet (Km). On plating the mixtures on peptone galactose indicator medium containing Cm and Km, double-resistant galactose-fermenting Shigella colonies were distinguishable from doubly resistant galactose-non-fermenting Salmonella colonies. The fraction of Shigella organisms carrying R-Barnet

which acquired the episome from Salmonella was about 5×10^{-7} for both R-S. Africa and R-Peru. For the reverse transfer, of R-Barnet from Shigella to Salmonella, the fraction was about 5×10^{-7} when the recipient carried R-S. Africa, but 20-fold higher when the recipient carried R-Peru. All the colonies isolated in this way carried all the unselected resistance traits of the original strains. The Salmonella strains presumably carrying two *fi*$^+$ R-factors appeared quite stable and could be used in transductional studies with phage P 22.

Transduction of R factor resistance traits by phage P 22

Yield of transductants. No colonies, or negligibly few, appeared on the control plates, except when selection was made for low-level streptomycin-resistance, when they were usually so numerous as to obscure possible transductant colonies on the experimental plates. In some experiments, however, transductants recognizable because of having unselected donor resistance traits were obtained, together with mutants, by selection for low-level streptomycin-resistance. The numbers of transductant colonies obtained by selection for various resistance traits are recorded in

Table 3. *Yield of drug-resistant and of his*$^+$ *transductants after treatment with phage P 22 grown on donor strain carrying various R factors*

(Donor strain was SL 726 (= LT 2 *metA trpB*) carrying the indicated R factor. Recipient strain was SL 862 (= LT 2 *hisD trpA*). Phage was added at multiplicity about 10.

R factor(s) carried by donor	No. of colonies/plate* on medium selective for						
	his$^+$	Tc	Sm	Su	Cm	Pn	Km
R-Munich	336	14	b.g.†	n.d.‡	25	—§	13
R-S. Africa	87	11	b.g.	n.d.	70	—	—
R-Peru	94	16	b.g.	n.d.	85	—	—
R-Brighton	211	132	> 1000	7	—	> 1000	—
R-Bradford	280	98	b.g.	19	—	42	—
R-Enfield	310	189	—	300	—	244	—
R-Singapore	117	7	b.g.	1	70	13	—
R-Zambia	128	4	b.g.	0	94	1	—

* Plate inoculum about 10^3 bacteria for drug-resistance selection, and about 10^7 bacteria for selection of *his*$^+$.

† b.g. means that any transductant colonies were obscured by numerous resistant-mutant colonies, present also on control plate of bacteria without phage.

‡ n.d. means this selection was not done.

§ — means not relevant, since R factor of donor does not confer resistance to this agent.

Table 3, together with the frequency of *his*$^+$ transduction, tested as a control. These figures, however, in some instances certainly underestimate the true number of transductants, for on occasion selection of resistance to one drug gave more transductants resistant to a second drug than were obtained by direct selection with the second drug. Also repeat experiments sometimes gave quite different numbers of colonies. For instance, in the experiment recorded in Table 3 the numbers of transductants obtained by selection for streptomycin-resistance or penicillin-resistance after treatment with phage grown on the donor carrying R-Brighton were exceptionally high (of the same order of magnitude as the number of *his*$^+$ transductants)—but the frequency of unselected donor resistance traits confirmed that the clones were indeed transductants, not mutants.

Table 4. *Resistance patterns of transductants and their ability to transmit resistance traits by conjugation*

R factor in donor (name, resistance pattern and *fi* character)	Transductants: Resistance pattern*						No.† amongst those selected by						Proportion‡ infective	
							Tc	Sm	Su	Cm	Pn	Km	As isolated	With F′-13 *lac*
R-Brighton (Tc, Sm, Su, Pn; *fi*⁻)	Tc	Sm	Su		Pn		46	7	43		3		9/38	2/29
	Tc	—	Su		Pn		2	/	4		0		1/3	0/1
	—	Sm	Su		Pn		/	39	0		44		1/44	0/43
	—	Sm	—		Pn		/	2	/		0			
	—	—	Su		Pn		/	/	0		1			
					No. tested		48	48	47		48		11/85	2/73
R-Bradford (Tc, Sm, Su, Pn; *fi*⁻)	Tc	Sm	Su		Pn		45	5	13		44		4/50	6/46
	Tc	—	Su		Pn		2	/	4		0		0/2	0/2
	—	Sm	Su		Pn		/	0	0		4		0/4	2/4
	Tc	—	—		—		1	/	/		/			
					No. tested		48	5	17		48		4/56	8/52
R-Enfield (Tc, Su, Pn; *fi*⁻)	Tc		Su		Pn		48		20		16		3/33	1/30
	—		Su		Pn		/		4		32		3/15	0/13
					No. tested		48		24		48		6/48	1/43
R-Munich (Tc, Sm, Su, Cm, Km; *fi*⁺)	Tc	—	—	—		—	98			/		/	0/48	0/41
	—	Sm	Su	Cm		Km	/			94		76	0/81	0/81
	—	Sm	Su	—		Km	/			/		8	0/6	0/1
	—	—	Su	Cm		Km	/			0		2		
	—	Sm	Su	Cm		—	/			1		/	0/1	0/1
	—	—	—	Cm		Km	/			1		1	0/1	0/1
	—	—	—	—		Km	/			/		11	0/6	0/3
	—	—	—	Cm		—	/			2		/	0/1	0/1
					No. tested		98			98		98	0/144	0/129
R-S. Africa (Tc, Sm, Su, Cm; *fi*⁺)	Tc	—	—	—			10			/			0/10	0/8
	—	Sm	Su	Cm			/			39			0/22	0/19
					No. tested		10			39			0/32	0/27

Table 4 *cont.*

R factor in donor (name, resistance pattern and *fi* character)	Transductants: Resistance pattern*					No.† amongst those selected by						Proportion‡ infective	
						Tc	Sm	Su	Cm	Pn	Km	As isolated	With F′-13 *lac*
R-Peru (Tc, Sm, Su, Cm; *fi*$^+$)	Tc	—	—	—		16			/			0/16	0/10
	—	Sm	Su	Cm		/			44			0/20	0/18
	—	Sm	—	Cm		/			1			0/1	
	—	—	—	Cm		/			2			0/2	
					No. tested	16			47			0/39	0/28
R-Zambia, (Tc, Sm, Su, Cm, Pn; *fi*$^+$)	Tc	—	—	—	—	4			/	/		0/4	0/4
	—	Sm	Su	Cm	—	/			42	/		0/22	2/22
	—	—	—	—	Pn	/			/	1		0/1	
					No. tested	4			42	1		0/27	2/26
R-Singapore (Tc, Sm, Su, Cm, Pn; *fi*$^+$)	Tc	—	—	—	—	8			/	/		0/8	0/7
	—	Sm	Su	Cm	—	/			45	/		0/21	1/19
	—	Sm	—	Cm	—	/			1	/		0/1	
	—	—	—	Cm	—	/			1	/			
	—	—	—	—	Pn	/			/	12		0/12	4/11
					No. tested.	8			47	12		0/42	5/37

* Resistance traits of R factor of donor not present in transductant class are indicated by —.
† Classes necessarily absent because sensitive to the drug used for selection are indicated by /.
‡ No. of clones able to transmit resistance by conjugation/no. of clones tested.

Resistance pattern of transductants. The resistance patterns of transductants selected by various drugs are recorded in Table 4. In the case of the five *fi*+ factors conferring multiple resistance, no transductant selected as tetracycline-resistant had any other resistance trait from the R factor present in the donor strain; and no transductant selected as chloramphenicol-resistant had the Tc trait of the R factor concerned, though most had its Sm and Su traits, and also its Km trait in the case of R-Munich. Most of the 98 transductants selected for acquisition of the Km trait of R-Munich had acquired also its Sm, Su and Cm traits, but none had its Tc trait. Thus the Km determinant of this *fi*+ factor is transduced by phage P 22 as a member of the Sm, Su, Cm group—as reported by Watanabe *et al.* 1964*b*. Of the five *fi*+ factors tested two, R-Singapore and R-Zambia, conferred the Pn trait, in addition to the Tc, Sm, Su and Cm traits. The Pn trait was not present amongst 12 transductants selected by tetracycline (none of which showed any other resistance trait) nor amongst 89 selected by chloramphenicol (though nearly all of these had the unselected Sm and Su traits). Few colonies were obtained on selection for benzylpenicillin resistance (Table 3); no other resistance trait was present in the twelve transductants tested. It thus appears that in the *fi*+ factors R-Singapore and R-Zambia the determinant of benzylpenicillin-resistance is transduced by phage P 22 separately from the Tc determinant and from the Sm, Su, Cm group.

Table 5. *Resistance patterns of transductants derived from donors carrying two R factors*

R factors in donor (name, resistance pattern and *fi* character).	Transductants: Resistance pattern*						No.† amongst those selected by Tc	Sm	Su	Cm	Pn	Km
R-Munich (Tc, Sm, Su, Cm, Km; *fi*+) and R-Brighton (Tc, Sm, Su, Pn; *fi*−)	Tc	—	—	—	—	—	26‡	/	/	/	/	/
	—	Sm	Su	Cm	—	Km	/	0	0	31	/	23
	—	Sm	Su	—	—	Km	/	0	0	/	/	1
	—	—	Su	Cm	—	Km	/	/	0	18	/	2
	—	—	—	Cm	—	Km	/	/	/	0	/	2
	—	—	—	—	—	Km	/	/	/	/	/	6
	Tc	Sm	Su	—	Pn	—	21§	15	41	/	20	/
	Tc	—	Su	—	Pn	—	2§	/	4	/	2	/
	—	Sm	Su	—	Pn	—	/	4	5	/	25	/
	—	Sm	—	—	Pn	—	/	0	/	/	1	/
	—	—	Su	—	Pn	—	/	/	0	/	2	/
	Tc	Sm	—	—	Pn	—	0	1	/	/	0	/
				No. tested			49	20	50	49	50	34
R-S. Africa (Tc, Sm, Su, Cm, *fi*+) and R-Barnet (Km; *fi*+)	Tc	—	—	—	—	—	50			/		/
	—	Sm	Su	Cm	—	—	/			50		/
	—	—	—	—	—	Km	/			/		12
				No. tested			50			50		12
R-Peru (Tc, Sm, Su, Cm; *fi*+), and R-Barnet (Km; *fi*+)	Tc	—	—	—	—	—	45			/		/
	—	Sm	Su	Cm	—	—	/			50		/
	—	—	—	—	—	Km	/			/		12
				No. tested			45			50		12

* Resistance traits of R factor of donor not present in transductant class are indicated by —.
† Classes necessarily absent because sensitive to the drug used for selection are indicated by /.
‡ All 26 transductants of this class grew as large colonies on the tetracycline selection plates.
§ These 23 transductants grew as small colonies on the tetracycline selection plates.

Three *fi*⁻ factors, two of type Tc, Sm, Su, Pn and one of type Tc, Su, Pn and all derived from *Salmonella typhimurium*, were tested. In contrast with the results with *fi*⁺ factors nearly all transductants selected by tetracycline had all the other resistance traits of the factor concerned, and many of those selected by streptomycin, sulphathiazole or benzylpenicillin had acquired tetracycline resistance (and other unselected resistance traits). Furthermore all of 144 transductants selected as benzylpenicillin-resistant had acquired some or all of the other traits of the factor concerned. Most R-Utrecht transductants selected by tetracycline or by benzylpenicillin had all the three resistance traits of R-Utrecht. It thus appears that both the Tc and Pn determinants of these *fi*⁻ R factors, unlike those of the *fi*⁺ factors tested, can be transduced by phage P 22 as part of a group comprising also the Sm and Su determinants.

Transduction from donors carrying two R factors. When drug-resistant transductants were selected after treatment with phage P 22 grown on donor strains carrying two R factors, R-Munich (*fi*⁺, Tc, Sm, Su, Cm, Km) and R-Brighton (*fi*⁻, Tc, Sm, Su, Pn), the classes obtained indicated independent transduction of the two R factors—or rather parts of them. No transductant had two resistance traits, one derived from one factor and one from the other factor, and the different resistance patterns observed corresponded to transductant classes observed when the donor carried only one or the other of the two factors (Table 5). Two sizes of colony were observed when tetracycline-resistance was selected. All of 26 large colonies proved to be resistant to tetracycline only, and thus resembled the only class of tetracycline-resistant transductant obtained when the donor carried only the *fi*⁺ R-Munich; all of 23 small colonies were resistant to several agents, including benzylpenicillin, and corresponded to the common classes of tetracycline-resistant transductant observed when the donor carried only the *fi*⁻ R-Brighton. Lysates of two strains each with the Km trait derived from the *fi*⁺ R-Barnet and the Tc, Sm, Su, Cm traits from another *fi*⁺ factor produced both Km and Sm, Su, Cm transductants—but unlike the lysate of the strain carrying the *fi*⁺ R-Munich factor (Tc, Sm, Su, Cm, Km) produced no transductants with both the Km trait and the Sm, Su, Cm traits together.

Ability of transductants to transmit resistance by conjugation. In general, Salmonella clones given R-factor drug-resistance traits by transduction are unable to transmit their resistance properties by conjugation. In testing for conjugation it is necessary to exclude the possible transfer by transduction due to phage liberated from lysogenic R⁺ bacteria, thus a strain unable to absorb P 22 was used as recipient. None of the 300 transductants of various classes derived from the five *fi*⁺ R factors transmit by conjugation. By contrast a minority of the transductant clones with resistance traits derived from each of the three *fi*⁻ R factors gave a positive result. The transductants able to transmit were of several different resistance patterns (Table 4). Several of them were found to transmit all their resistance traits simultaneously. It was reported by Harada, Kameda, Suzuki & Mitsuhashi (1964) that some Salmonella transductants acquired the ability to transmit their resistance by conjugation after infection with F′-13 *lac*. We transferred this factor to many of our non-transmitting transductants, and of 247 *fi*⁺ R-factor transductants, seven acquired the ability to transmit drug resistance. All these were derived from R factors conferring resistance to benzylpenicillin. Of 168 *fi*⁻ R-factor transductants negative in the first test, 11 transmitted resistance by conjugation when they also carried F′-13 *lac* (Table 4). In the few tests made, the majority of acceptor colonies selected for acquisition of any one drug-

resistance trait were found to have acquired all the resistance traits of the donor, and usually also its *lac*$^+$ property.

Effect of R factors on ultraviolet susceptibility

Each R factor except R-Barnet was tested for ability to protect *Salmonella typhimurium* against killing by ultraviolet irradiation. The clones tested had acquired by conjugation all the drug-resistance traits of the R factor concerned, except possibly

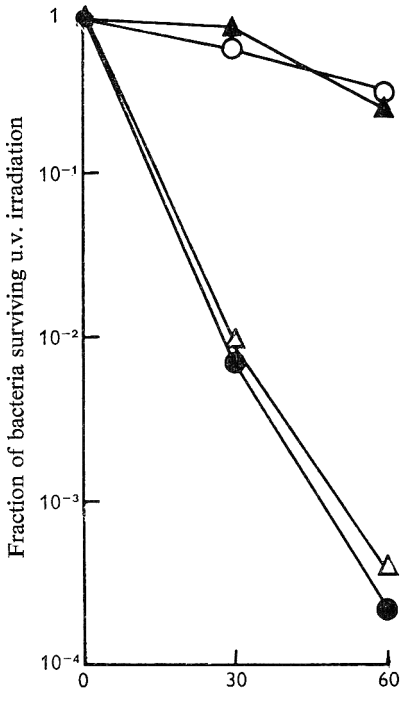

Fig. 1

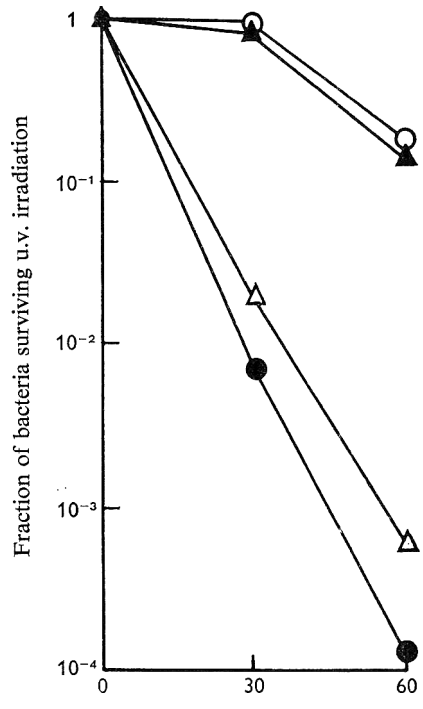

Fig. 2

Fig. 1. Dose/log-survival curves for ultraviolet-irradiated *Salmonella typhimurium* LT 2_{SL675} (*pur pro str*) and its derivatives carrying R factors. ●—●, R$^-$; ○—○, R-Brighton; ▲—▲, R-Munich; △—△, R-Singapore. Three other *fi*$^-$ factors gave curves similar to that for R-Brighton. Four other *fi*$^+$ factors gave curves similar to that shown for R-Singapore.

Fig. 2. Dose/log-survival curves for u.v.-irradiated *Salmonella typhimurium* LT 2_{SL748} (*met trp str rfb*) and its derivatives given resistance traits by contact with transductants carrying either some or all of the resistance traits of R-Brighton. ●—●, R$^-$; ○—○, R$^+$ (Tc, Sm, Su, Pn); ▲—▲, R$^+$ (Sm, Su, Pn); △—△, R$^+$ (Pn).

streptomycin-resistance, undetermined because the LT 2 strain used (SL 674) was already streptomycin-resistant. At least two clones were tested for each R factor, always with consistent results (Fig. 1). All the four *fi*$^-$ factors greatly decreased the susceptibility of strain SL 675 (LT 2 *pur pro str*) to killing by u.v. radiation. Of the *fi*$^+$ factors tested, R-Munich protected but the other four did not. The five factors which conferred u.v. resistance gave similar protection, about equal to that given by a *colI* factor; for instance, the survival was between 0·15 and 0·45 after 60 sec.

exposure, a dose which permitted survival of only about 3×10^{-4} of the R^- strain. The data though scanty show that all the protecting factors caused the appearance of an initial shoulder in the dose/log-survival curves. Two clones which had acquired only Tc and Tc, Su from R-Munich (*fi*+, Tc, Sm, Su, Cm, Km) by conjugation were tested. Both showed the protecting effect. Segregation of the u.v.-protecting character was seen amongst three LT 2 lines given some or all of the resistance traits of R-Brighton (*fi*−, Tc, Sm, Su, Pn) by contact with transductants which had also acquired the ability to conjugate. A clone with only Pn was not protected, whereas one with all four traits and another with all except Tc had the same u.v. resistance as clones given the complete R factor by conjugation (Fig. 2).

DISCUSSION

The four *fi*− R factors examined differed from previously described R factors in two important respects. First, nearly all transductants which acquired tetracycline resistance also acquired all or most of the other resistance traits of the factor concerned, instead of acquiring only tetracycline resistance. Secondly, about 10% of the transductant clones tested were able to transmit their transduced resistance traits during growth in mixed culture, instead of none or very few having this ability (Watanabe & Fukusawa, 1961; Harada *et al.* 1963). A minority of the transductants which were unable to transmit their traits by conjugation acquired this ability after they were given the plasmid F′-13 *lac* (see Harada *et al.* 1964). We cannot explain these different kinds of behaviour during transduction by phage P 22. Perhaps in these factors, though not in others, the gene for tetracycline resistance is so closely linked to the other drug-resistance genes that a DNA molecule of the length which can be contained within a P 22 phage coat is likely to encompass all the drug-resistance genes of the factor. Those transductants able to transmit all the resistance traits of the factor by conjugation presumably receive the whole of the DNA comprising the R factor, and this therefore must also be capable of enclosure within a single P 22 phage coat. It is therefore perhaps surprising that only a minority of transductants receive the ability to conjugate instead of all or nearly all, as when R factors are transduced by the larger phage P 1 in *E. coli*. The four R factors with which this occurred were also the only *fi*− factors amongst those we examined; but it is possible that they may have a recent common origin since the strains from which they were obtained were all *Salmonella typhimurium* of phage type 1 a, isolated in Great Britain or Holland at about the same time.

The *fi*+ factors which conferred more than one resistance trait fell into two groups. With three factors the pattern of the transduction was as previously reported for other factors in Salmonella; namely tetracycline resistance was never co-transduced with the other traits, which usually travelled together; and none of the transductants could transmit by conjugation, even after acquisition of F′-13 *lac*. The determinant for the kanamycin (and neomycin) trait of the R factor isolated by Lebek (1963) behaved as though closely linked with the Sm, Su and Cm determinants, in confirmation of the report of Watanabe *et al.* (1964*b*). The other group comprised two *fi*+ factors which conferred resistance to benzylpenicillin (and cephalosporins) in addition to the Tc Sm, Su, Cm combination. The pattern of transduction of these four determinants was the same as that in other *fi*+ factors—except that a few transductants after acquisition

of F′-13 *lac* could transmit by conjugation. The benzylpenicillin resistance trait of these two factors, unlike that of the *fi*⁻ factors and unlike the kanamycin-resistance trait of the *fi*⁺ R-Munich, was never co-transduced with any other trait, though it was transduced by itself at low frequency. Thus, transduction of these factors by phage P 22 produces, not two, but three common transductant classes, none of them able to transmit by conjugation. This may indicate that their DNA is longer than that of previously reported *fi*⁺ factors—or it may be that their benzylpenicillin-resistance gene is located in a region of the R-factor linkage group which in other *fi*⁺ factors does not cause any obvious alteration of host phenotype, so that its acquisition by transductants would not have been detected.

We easily obtained a strain carrying both R-Munich, an *fi*⁺ factor, and R-Brighton, an *fi*⁻ factor—as was to be expected since *fi*⁺ and *fi*⁻ factors do not exclude each other (Watanabe *et al.* 1964*a*). The strain carrying both factors was resistant to six different agents, but each of the numerous classes of transductant obtained could be accounted for by transduction of a fragment either of R-Munich or of R-Brighton. Transductants selected as tetracycline-resistant were of two sorts: large colonies, resistant to no other agent, attributable to transduction of the Tc determinant of R-Munich; and smaller colonies, with several other resistance traits, including the Pn of R-Brighton. This observation confirms the inference of Watanabe *et al.* (1964*a*) that a bacterial host can support the continued presence of both an *fi*⁺ and an *fi*⁻ factor, without these factors undergoing recombination. The Tc, Sm, Su, Cm, Km traits of the LT 2 lines obtained by growing a strain carrying an *fi*⁺ factor of type Tc, Sm, Su, Cm, with a strain carrying the *fi*⁺ factor, R-Barnet, of character Km might have resulted in recombination of the two *fi*⁺ factors (Watanabe *et al.* 1964*a*). However, though the Km trait of the *fi*⁺ R-Munich factor was usually co-transduced with its Sm, Su and Cm traits, the Km trait and the Sm, Su, Cm group of the strains obtained by mixed growth were never co-transduced (Table 5). This suggests that the strains carried both the parental *fi*⁺ factors, rather than a single recombinant factor.

Some of the R factors protected *Salmonella typhimurium* strain LT 2 against the bactericidal effect of ultraviolet irradiation to about the same effect as do most *colIa* and most *colIb* factors (Howarth, 1965; Howarth-Thompson, 1966), whereas other factors had little or no effect on the dose/log-survival curves (Fig. 1). The mechanism of this protection is unknown. Since such plasmids replicate without being incorporated into the chromosome of their host they presumably carry genes which specify enzymes involved in DNA synthesis and perhaps also in DNA repair, and such enzymes might assist in the restoration of u.v.-damaged DNA. All the *fi*⁻ factors we tested protected, as also did one of the five *fi*⁺ factors; but some *fi*⁻ factors do not protect (A. Siccardi, personal communication). The u.v.-protection effect of R-Brighton appeared to segregate during transduction (Fig .2), so this property may prove a useful new marker for the linkage map of some R factors.

The *fi*⁻ R-Brighton and the *fi*⁺ R-Singapore and R-Zambia factors all caused production of β-lactamase for both benzylpenicillin and cephalothin; Datta & Kontomichalou (1965) reported such activity for three R factors, including R 1818, which is the factor we term R-Brighton. In their experiments the presence of R-Brighton in either of two strains of *Escherichia coli*, though it conferred considerable ability to destroy both cephaloridine and 6-aminopenicillanic acid, caused only a twofold increase in the minimal inhibitory concentrations of these drugs; whereas we found

the same factor in *Salmonella typhimurium* increased the minimal inhibitory concentration of cephaloridine from 1·25 to 35 μg./ml. (Table 2) and caused a considerable increase in resistance to 6-aminopenicillanic acid. This difference may result from the different hosts or from the different methods of measuring bacterial sensitivity. The determinants of benzylpenicillin resistance (presumably the structural genes for penicillinases) in our *fi*⁻ factors differ genetically from those in our *fi*⁺ factors in that they are co-transducible with the Sm and Su determinants. It will be interesting to see whether the Pn genes of *fi*⁻ and *fi*⁺ factors, which presumably arose independently, specify chemically different penicillinases. Datta & Kontomichalou (1965) found that the substrate profile of the enzyme determined by R 1818 (= R-Brighton) was very different from that of the enzymes determined by two other R factors.

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New Host-strains for the Lysogenic *Corynebacterium diphtheriae* PARK WILLIAMS No. 8 Strain

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SUMMARY

Investigation of the lysogenicity of seven *Corynebacterium diphtheriae* PW 8 variants revealed the spontaneous liberation, by these strains, of phage particles active against two *C. ulcerans* strains (9304 and 298 G) which are sensitive to *C. diphtheriae gravis* and *mitis* phages. By this means, phage particles with different host-range activity and morphology of plaques were obtained from the PW 8 strains. Further studies showed that some *C. diphtheriae* strains which possess the same sensitivity towards *gravis* and *mitis* phages showed the same spontaneous liberation of PW 8-carried phages. The newly obtained phages were able to convert to toxinogenesis both *C. ulcerans* and *C. diphtheriae* strains.

INTRODUCTION

During the course of lysotyping certain strains of *Corynebacterium diphtheriae*, received from Dr D. T. Simmons of the Commonwealth Serum Laboratories, Melbourne, Australia, we observed that some of the strains spontaneously liberated phage particles active against two *C. ulcerans* strains, 9304 and 298 G. The *C. ulcerans* strains were recently isolated in Rumania and were used as indicator strains for diphtheria phages. Since one of the lysogenic strains from Australia was labelled *intermedius* C.S.L. TORONTO (referred to as TORONTO), we assumed it to be the Toronto variant of the *C. diphtheriae* PW 8 strain and consequently tested it for toxinogenicity in a static broth culture. The identity of the strain TORONTO was established by the production of diphtheria toxin of high potency (100 Lf per ml.) and demonstrating similar lysogenic capacity in all PW 8 collection variants existing in the Diphtheria Department of the 'Dr I. Cantacuzino' Institute. The strain isolated by Park & Williams (1896) and used for large-scale diphtheria toxin production in all countries is known to be different from most lysogenic toxinogenic *C. diphtheriae* strains since it carries a defective phage (Barksdale, Garmise & Horibata, 1960; Barksdale, Garmise & Rivera, 1961; Matsuda & Barksdale, 1966, 1967). Since our observation was in disagreement with the findings of others which stated the defective inducible nature of the PW 8-carried phage, a detailed study was begun to demonstrate more conclusively the spontaneous liberation of phage particles by the PW 8 strains. At the same time, we tried to analyse the relationship existing between PW 8 and *C. ulcerans* strains and to find, if possible, other receptive strains to confirm the spontaneous liberation of PW 8 phages.

METHODS

Corynebacteria strains. The following *Corynebacterium diphtheriae* PW 8 variants from the collection of the Diphtheria Department of the 'Dr I. Cantacuzino' Institute: the WEISSENSEE and DESSAU strains, received from the Weissensee Institute at Berlin; CN 2000, received from the Rijks Instituut vor de Volksgezondheid at Utrecht; MASSACHUSETTS and CN 2000 from the Human Institute at Budapest (the cultures labelled CN 2000 came originally from the Wellcome Research Laboratories, Beckenham, Kent, England, CN 2000 being their number for their original PW 8 strain); and the 474 strain received from the Pasteur Institute, Paris; the Australian strain TORONTO from the Commonwealth Serum Laboratories, Melbourne; the *C. ulcerans* strains were isolated in Rumania, 9304 (−) tox− from a healthy human carrier, and 298 G (−) tox − from a horse.

Other strains used were Freeman's *Corynebacterium diphtheriae mitis* strains nos. 770, 411, 444, 1174, 1180; the *C. diphtheriae mitis*-like strains nos. A 003, A 028, C 43, S 038 supplied by Dr N. B. Groman of the Department of Microbiology, University of Washington, Seattle; and the C. *belfanti* 1030 strain received from Dr H. Oehring of the Hygiene Institut, Friedrich Schiller Universität, Jena. The 9304 (W) tox+ strain was derived from the 9304 (−) tox− strain lysogenized with PW 8 phage.

All strains were investigated for their morphology, biochemical behaviour and *in vitro* toxin production according to the minimal requirements of the scheme for bacteriological diagnosis of *Corynebacterium diphtheriae* (*Bulletin*, 1965).

Detection of lysogeny. The method used for demonstrating liberation of phages was previously described by Saragea & Maximescu (1964). This method demonstrates phage particles, spontaneously liberated from the lysogenic strain in shake culture, by the spot method on agar plates of the sensitive indicator strain.

Toxinogenicity. Tests were performed on filtrates of Pope-Linggood broth cultures of the lysogenized strains: (1) in rabbit skin by the method of Fraser (1931); (2) by the subcutaneous inoculation of guinea-pigs; and (3) by *in vitro* inoculation of monkey kidney tissue cultures. Quantitative determination of large amounts of diphtheria toxin was done by the flocculation test of Ramon & Richou (1950).

The phage types of all strains were established according to the lysotyping scheme for *Corynebacterium diphtheriae* (Saragea & Maximescu, 1964, 1966).

RESULTS

Lysogenicity of PW 8 variants

The lysogenicity testing of the PW 8 variants (WEISSENSEE, DESSAU, CN 2000 from Utrecht, MASSACHUSETTS and CN 2000 from Budapest, 474, and TORONTO), carried out with stock cultures of each strain, showed spotaneous liberation of phages of *Corynebacterium ulcerans* strains 9304 (−) tox− and 298 G (−) tox−. Previous tests performed with *C. ulcerans* strains had established that they were non-lysogenic and non-diphtheria toxin producers. Appearances of lysis with punctiform plaques were noted. By multiplication of the WEISSENSEE, CN 2000 Utrecht, and TORONTO lysates on the 9304 (−) tox− *C. ulcerans* strain, the three phage preparations, 'W', 'U', 'T', with confluent lysis were obtained. The new phage preparations lysed mainly the *C. ulcerans* strains, but had also a weaker activity (a few plaques) on Freeman's strain *C. diphtheriae* 770 (Table 1).

Considering that all PW 8 variants used in this study were stock cultures and that in general among populations of the high toxinogenic PW 8 strain mutants occur displaying different lysogenic qualities (Barksdale *et al.* 1961), we tried to repeat the lysogenicity test, starting from isolated colonies of the WEISSENSEE and CN 2000 variants. The tests performed show similar results (Table 2).

Table 1. *Activity of phage particles spontaneously released by WEISSENSEE, CN 2000 and TORONTO strains*

	PW 8, WEISSENSEE		PW 8, CN 2000 Utrecht		TORONTO	
Sensitive strains	Original lysate*	Phage 'W'†	Original lysate*	Phage 'U'†	Original lysate*	Phage 'T'†
9304	Scanty punctiform plaques	OL	1+s	OL	1+s	OL
298 G	Scanty punctiform plaques	OL	1+s	OL	1+s	OL
770	—	Isolated, normal-sized plaques	.	Isolated, normal-sized plaques	.	Isolated, normal-sized plaques

* An overnight shake culture of the stock PW 8 strains. 1+s = > 50 small, punctiform plaques.
† Phages 'W', 'U' and 'T' originated from the multiplication of the spontaneously released PW 8 phage particles on the 9304 (−) tox− *C. ulcerans* strain. OL = opaque lysis.

Table 2. *The lysogenic state of some isolated colonies from the highly toxinogenic WEISSENSEE and CN 2000 strains*

Strain	Colony no.	Diphtheria toxin, produced in static culture (Lf/ml.)	Type of lysis obtained on *C. ulcerans* strains 9304 and 298 G
PW 8, WEISSENSEE	112	100	Scanty punctiform plaques
	113	90	Scanty punctiform plaques
	114	70	Scanty punctiform plaques
PW 8, CN 2000	115	95	1+s*
	116	95	1+s*
	117	100	1+s*

* 1+s = > 50 small punctiform plaques.

Starting from isolated plaques of the 'W' phage on plates with different host strains (9304, 770 and 411), three kinds of phage preparations with different spectra of activity were obtained (Table 3).

The similarity of the host-range activity of the W/411 and Freeman's B phage is remarkable. Attention is also drawn to (1) the minute plaques of the W/9304 phage obtained on *Corynebacterium ulcerans* strains (Pl. 1, fig. 1*a*, *b*); (2) the normal size plaques of the W/770 and W/411 phages; and (3) the different behaviour towards PW 8 phages of the 770 strain among Freeman's other strains (Table 3).

Table 3. *Host range of PW 8 phages as compared with Freeman's B phage*

Indicator strains	Bacteriophage			
	W/9304*	W/770*	W/411*	Freeman's phage B
411	.	.	CL	CL
444	.	.	CL	CL
1174	.	.	CL	CL
1180	.	.	CL	CL
770	l+n	OL	CL	CL
9304	OL	l+n	l+n	l+n
298 G	OL	l+n	l+n	l+n

* Each of the PW 8 phages was obtained by multiplication of a single titre plaque from the original lysate of the WEISSENSEE strain seeded on the growth of the 9304, 770 and 411 strains.

CL, Clear confluent lysis; OL, opaque confluent lysis; l+n, > 50 normal-sized plaques.

Lysogenic conversion by PW 8 phages

The lysogenization by PW 8 phages of the 9304 (−) tox− *Corynebacterium ulcerans* and 770 (−) tox− *C. diphtheriae mitis* strains demonstrates their converting capacity. Single colonies isolated from the lysis area on solid medium (for each phage, 20 colonies from the respective strain) were tested in parallel with an equal number of colonies from the original strain. The results showed different rates of conversion (20/20) for the *C. diphtheriae* 770 (−) tox− and (5/20) *C. ulcerans* 9304 (−) tox− strains. The conversion of the *C. ulcerans* 9304 (−) tox− strain caused us to analyse the change produced in the lysogenized population in greater detail. For demonstration of the newly formed diphtheria toxin by the lysogenized *C. ulcerans* (9304 (W) tox+) cultures, the *in vitro* Elek precipitation test, the *in vivo* test on rabbit skin (Pl. 1, fig. 2*a*, *b*), the *in vivo* guinea-pig inoculation test and *in vitro* tissue culture inoculation (Pl. 2 fig. 3*a*, *b*) were set up. The results, which are presented in Table 4, show the identity of the newly produced toxin with diphtheria toxin.

The lysogenized diphtheria-toxinogenic 9304 (W) tox+ strain did not change the morphological, biochemical and biological behaviour of a *C. ulcerans* strain, but became resistant to lysis by PW 8 phages.

The phage liberated by the 9304 (W) tox+ strain had the same capacity to convert the original 9304 (−) tox − strain to toxinogenicity as the original phages released by PW 8 strains.

Other strains receptive for the spontaneously released PW 8 phage particles

In view of the fact that *Corynebacterium ulcerans* and *C. diphtheriae* strains are closely related (Petrie & McClean, 1934; Jebb, 1948; Jebb & Martin, 1965), we considered that the receptivity of the two *C. ulcerans* strains for PW 8 phages is not due to their specificity as *C. ulcerans* strains, but to their wide receptivity for diphtheria *gravis* and *mitis* phages. For this reason, we selected from the Diphtheria Department's collection some strains which presented this feature (Table 5): *C. diphtheriae mitis*-like strains nos. A 003, A 028, C 43, S 038, and *C. belfanti* strain no. 1030. All these strains were receptive for spontaneously released PW 8 phages (Pl. 2, fig. 4).

Table 4. *Tests to demonstrate the identity of the toxin produced by the lysogenized Corynebacterium ulcerans strain with diphtheria toxin*

Strain and colony no.	Sensitivity towards PW 8 phages	Lysogenicity against 9304 (−) tox− original strain		*In vivo* tests: Skin test on rabbit (Fraser's method) Test*	*In vivo* tests: Skin test on rabbit (Fraser's method) Control with 5000 i.u. diphtheria antitoxin	*In vivo* tests: Guinea-pig subcutaneous inoculation Test†	*In vivo* tests: Guinea-pig subcutaneous inoculation Control with 2000 i.u. diphtheria antitoxin	*In vitro* tests: Monkey kidney tissue culture inoculation Test	*In vitro* tests: Monkey kidney tissue culture inoculation Control with 5 i.u. diphtheria antitoxin	Elek–Ouchterlony test‡
9304 (W) tox + lysogenized strain 1	−	+	Filtrate	i.N.	i.	+	−	++	−	+
			Culture	i.n.N.	i.n.	++	+++	±		
9304 (W) tox + lysogenized strain 2	−	+	Filtrate	i.N.	i.	+	−	++	−	+
9304 (W) tox + lysogenized strain 3	−	+	Filtrate	i.N.	i.	+	−	++	−	+
			Culture	i.n.N.	i.n.	++	+++	±	−	
9304 (W) tox + lysogenized strain 19	−	+	Filtrate	i.N.	i.	+	−	++	−	+
9304 (W) tox + lysogenized strain 20	−	+	Filtrate	i.N.	i.	+	−	++	−	+
9304 (−) tox− control strain	+	−	Filtrate	i.	i.	+	−	−	−	−
			Culture	i.n.	i.n.	+++	+++	−	−	
PW 8 WEISSENSEE strain	−	+	Filtrate	N.	−	+	−	++	−	+
			Culture	N.	−	+	−	++	−	

* i = infiltration (activity of *C. ulcerans* strains positive for filtrates or cultures); n = haemorrhagic necrosis (activity of *C. ulcerans* cultures); N = necrosis produced by diphtheria toxin (in filtrates and cultures).

† + = guinea-pigs die within 24–48 hr with gelatinous oedema at the site of inoculation and haemorrhagic adrenal gland (symptoms typical of diphtheria toxin neutralized with diphtheria antitoxin); ++ = guinea-pigs die within 24–48 hr with haemorrhagic gelatinous oedema at the site of inoculation (typical of *C. ulcerans* not neutralized by diphtheria antitoxin) and also haemorrhagic adrenal glands (typical of diphtheria toxin); +++ = guinea-pigs die within 48–96 hr with haemorrhagic gelatinous oedema at the site of inoculation (typical of *C. ulcerans*).

‡ Done on a Loeffler culture of each strain.

Table 5. *Lysosensitivity of the indicator Corynebacterium diphtheriae and C. ulcerans strains, and of the PW 8 strain for diphtheria phages according to the lysotyping scheme for C. diphtheriae*

Sensitive strains	Biotype	Bacteriophages: *mitis* 1	2	3	4	5	6	*intermedius* 7	8	9	*gravis* 10	11	12	13	14	15	16	17	18	19	20	21	22	Not specific for type 23	24	Lysogenicity	Toxinogenicity
770	*mitis*	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−
9304	*C. ulcerans*	+	+	+	+	+	−	−	−	−	−	+	−	−	−	−	−	−	−	+	+	+	+	+	+	−	−
298 G	*C. ulcerans*	+	+	+	+	+	−	−	−	−	−	+	−	−	−	−	−	−	−	+	+	+	+	+	+	−	−
A 003	*mitis*-like	+	+	+	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−	+	+	−	+	+	+	+	+
A 028	*mitis*-like	+	+	+	+	+	−	+	−	−	+	−	−	+	+	+	−	−	−	+	+	+	+	+	+	+	+
C 43	*mitis*-like	+	+	+	−	+	−	−	−	−	−	−	+	+	+	+	−	−	−	+	+	−	+	+	+	+	+
S 038	*mitis*-like	+	+	+	+	+	−	−	−	−	−	−	+	+	+	+	−	+	+	+	+	+	−	−	−	+	+
1030	*C. belfanti*	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−
PW 8	*gravis*-like	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+

DISCUSSION

The synthesis of diphtheria toxin occurs through a mechanism controlled by the gene of a bacteriophage which converts the receptive host to lysogeny and at the same time confers upon it the tox+ character (Freeman, 1951; Barksdale, 1959). In general, toxinogenesis and lysogenicity are correlated and the presence of the tox+ character implies carriage of a bacteriophage, the detection of which is dependent on the availability of a host strain. This fact became obvious to us on numerous occasions when apparently non-lysogenic, toxinogenic *Corynebacterium diphtheriae* strains liberated a bacteriophage which could be detected only when tested on a larger set of sensitive strains. By this means the prophage is not difficult to demonstrate. Numerous studies on the mechanism of diphtheria toxin production have focused attention on the behaviour of the highly toxinogenic PW 8 strain. Such studies demonstrated that this strain differs from most lysogenic *C. diphtheriae* strains by the defective phage it carries, which harbours a genetic block that prevents the formation of mature phage (Barksdale, 1959); for this reason, although generally phage liberation and toxin synthesis are closely correlated, this correlation could not be established with the PW 8 strain (Matsuda & Barksdale, 1966). In the present work, using two *C. ulcerans* and some *C. diphtheriae* strains which present the same wide receptivity to diphtheria *gravis* and *mitis* phages, we were able to demonstrate the spontaneous liberation of phage particles by PW 8 strains, with different host range activity, amongst which one was similar to Freeman's phage B. Thus our findings agree with those of Miller, Pappenheimer & Doolittle (1966), who demonstrated in the PW 8 lysate a blocking antigen for anti-B phage sera which caused them to state: 'It is at least as likely that the PW 8 strain carries a normal phage genome...', and with those of Mathews, Miller & Pappenheimer (1966), who showed by electron-microscope photographs a phage resembling phage B in PW 8 lysates. In the present investigation, using suitable host strains, the presence of prophage was easily demonstrated.

Thus the results of this investigation open some new aspects of the PW 8 strain: PW 8 strains are not defective lysogenically, but spontaneously liberate phage particles with different host-range activity; the host strains which display spontaneous liberation of PW 8 phages (*Corynebacterium ulcerans*, *C. diphtheriae mitis*-like, and *C. belfanti*) are strains sensitive for a large number of diphtheria *gravis* and *mitis* phages; phages released by PW 8 strains convert to diphtheria toxinogenesis sensitive strains of both *C. ulcerans* and *C. diphtheriae*; all PW 8 variants are resistant to a large number of *gravis*, *intermedius* and *mitis* phages.

The possibility of demonstrating by means of the new host strains the spontaneous release of PW 8-carried phages, and the classification of the PW 8 strain, according to our conception, as an atypical *gravis* type (McLeod, 1943) opens new prospects for the study of phage-host relationships and bacteriophage-directed synthesis of diphtheria toxin—as well as for the 'iron effect' (Matsuda & Barksdale, 1967) in this highly toxinogenic strain.

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EXPLANATION OF PLATES

PLATE I

Fig. 1. Plaques produced by PW 8 phages on Corynebacterium strains. *a*, Minute plaques produced on 9304 (−) tox− *Corynebacterium ulcerans* strain. *b*, Normal size plaques produced on *C. diphtheriae* strains. Magnification of both *a* and *b* × 20.

Fig. 2. Skin tests in rabbits of toxic filtrates with and without diphtheria antitoxin. *a*: 1, *Corynebacterium ulcerans* filtrate from 9304 (−) tox−; 2 and 3, filtrate from 9304 (W) tox+ lysogenized strain; 4, PW 8 diphtheria toxin. *b*, The filtrates, in the same order, with diphtheria antitoxin. It will be seen that the PW 8 toxin is neutralized in 4, and also the necrosis in 2 and 3.

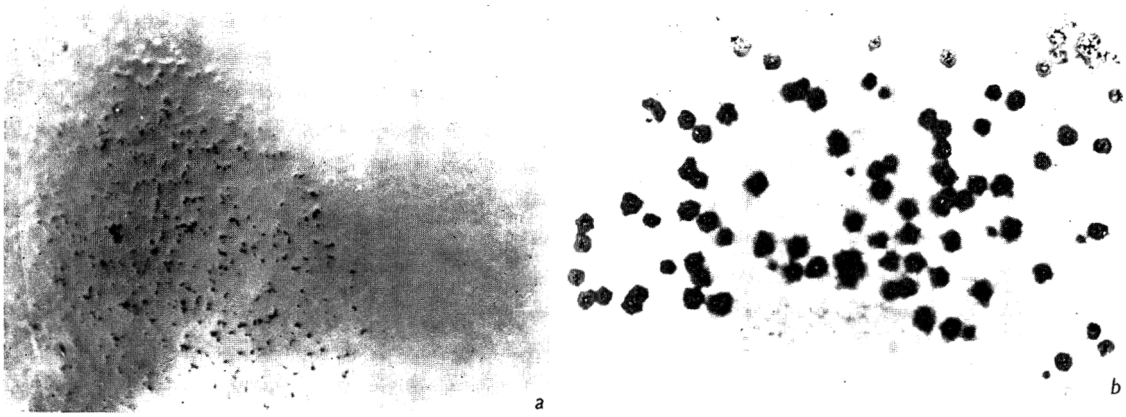

Fig. 1

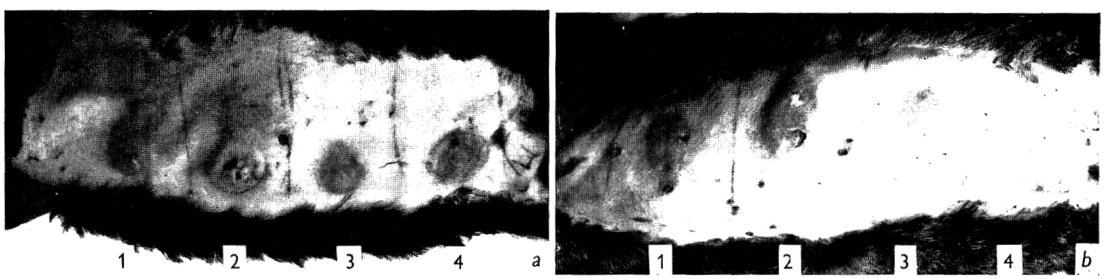

Fig. 2

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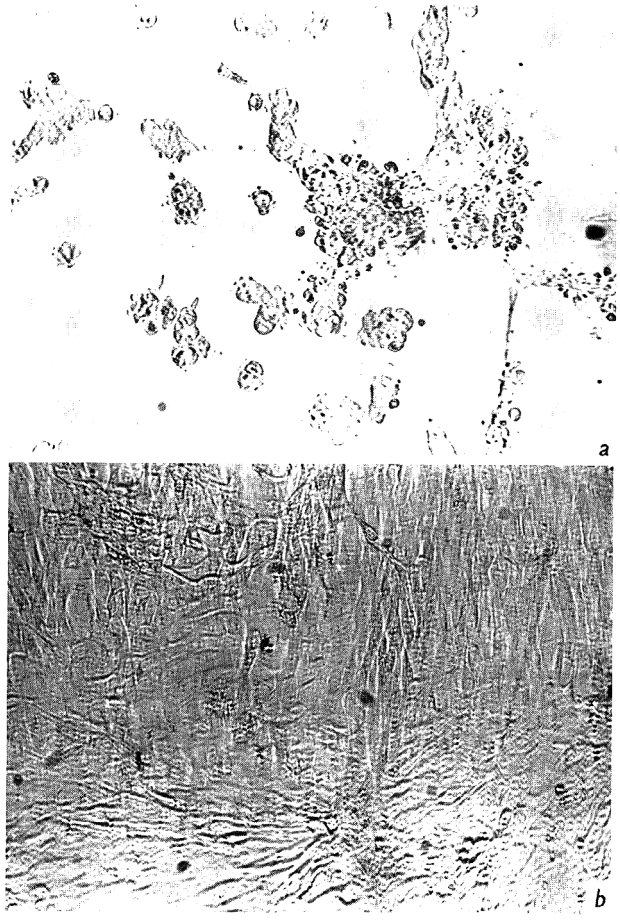

Fig. 3

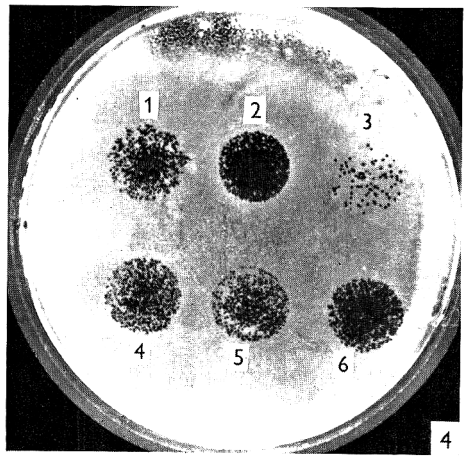

PLATE 2

Fig. 3. Cytopathogenic effect of filtrate on monkey tissue. *a*, Cytopathogenic effect of 9304 (W) tox+ filtrate on monkey tissue culture. *b*, Similar tissue culture in which toxic effect of 9304 (W) tox+ filtrate has been neutralized with diphtheria antitoxin.

Fig. 4. Lysis produced by spontaneously released PW 8 phages by (1) WEISSENSEE, (2) CN 2000 Budapest, (3) MASSACHUSETTS, (4) WEISSENSEE, (5) CN 2000 Utrecht, (6) TORONTO strains seeded on a growth of *Corynebacterium belfanti* strain 1030.

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Production of Plant Growth Substances by *Azotobacter chroococcum*

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SUMMARY

Cultures of *Azotobacter chroococcum* strain A 6 were grown for 14 days in a nitrogen-deficient mineral medium, the supernatant fluid and bacteria extracted and examined by paper partition chromatography with two solvent systems which separate authentic gibberellin (GA 3) and indolyl-3-acetic acid (IAA). Gibberellin-like substances were not detected on the chromatograms examined under ultraviolet (u.v.) radiation, but were detected when chromatograms were cut into ten equal strips representing a sequence of R_F values and the eluates tested in dwarf pea and lettuce hypocotyl bioassays. Certain eluates applied to the roots of tomato seedlings also altered the later growth of stems, leaves and flowers. The Azotobacter cultures contained three gibberellin-like substances, of which probably the dominant was one with an R_F value similar to that of GA 3; the other two were not identified. The average concentration of gibberellin/ml. culture was 0·03 μg. GA 3 equivalent. The gibberellins in Azotobacter cultures probably cause the reported effects on plant development and yield when seeds or roots are inoculated with Azotobacter. Plant growth may also be affected by synthesis of further gibberellins in the root zone when the Azotobacter inoculum colonizes developing roots.

INTRODUCTION

Azotobacter chroococcum has long been used in the Soviet Union to inoculate seeds or roots of crop plants, and increases in yields from this practice have been reported (Mishustin & Naumova, 1962). Recent pot trials and field trials outside the Soviet Union have also shown that frequently plant growth was altered and sometimes yield increased. Jackson, Brown & Burlingham (1964) found that inoculation with Azotobacter accelerated the stem and leaf growth of tomato and shortened the time between bud appearance and petal fall. Rovira (1965) found that the onset of flowering of wheat was hastened, and Denarié & Blachère (1966) that growth of potato haulms and stems of tomato was accelerated by some, but not all, strains of Azotobacter. These responses suggest that Azotobacter probably influences the development of plants by producing growth-regulating substances.

Burger & Bukatsch (1958) and Brakel & Hilger (1965) showed that Azotobacter produced indolyl-3-acetic acid (IAA) when tryptophan was added to the medium; Vancura & Macura (1960), Burlingham (1964) and Hennequin & Blachère (1966) found small amounts of IAA in old cultures grown without added tryptophan. Vancura

(1961) and Burlingham (1964) also detected gibberellin-like substances in old cultures, but Hennequin & Blachère (1966) did not detect these in cultures of six strains of Azotobacter, including three supplied by Vancura and one by Burlingham.

Brown, Jackson & Burlingham (1968) have found that after treating tomato seeds or seedling roots with small amounts (0·5–0·01 μg.) of commercially produced gibberellin GA 3, the plants responded in the same way as after treatments with 14-day cultures of *Azotobacter chroococcum* strain A 6. Treatment of seedling roots with 0·5 μg. IAA, the concentration per ml. in Azotobacter cultures (Burlingham, 1964), had no effect on plant development, and adding 0·5 μg. IAA with GA 3 had no greater effect on growth than GA 3 alone. These results indicated that the active substance in Azotobacter culture was a gibberellin.

The amounts of growth substances in cultures of Azotobacter were determined in the present work by using paper partition chromatography and bioassay techniques. Particular attention was paid to gibberellins; their activity was tested on dwarf peas, lettuce hypocotyls and tomato seedling roots. The first two bioassays are specific for the gibberellins and there is a linear relationship between log dose and log plant response (Brian & Hemming, 1955; Frankland & Wareing, 1960). Indolyl-3-acetic acid had no effect in any of these bioassays.

METHODS

Cultures. Azotobacter chroococcum, strain A6, was grown for 14 days in 100 ml. medium containing (g./l.): sucrose, 5·0; K_2HPO_4, 0·8; $MgSO_4.7H_2O$, 0·2; $FeSO_4.7H_2O$, 0·04; Na_2MoO_4, 0·005; $CaCO_3$, 2·0; pH 7·0; in 500 ml. flasks on a rotary shaker incubated at 26°.

Extraction procedures. Cultures were centrifuged at 3000 rev./min. for 20 min. and supernatant fluid and sedimented bacteria extracted as follows. Analar chemicals were used unless otherwise stated.

Supernatant fluid. After acidifying with N-HCl to pH 2·8–3·0, 50 ml. of supernatant liquid was shaken with 0·5 g. activated charcoal (British Drug Houses Ltd.) for 2 hr. The charcoal was separated by centrifugation, extracted with aqueous acetone (16 ml. 95% (v/v) acetone in water), the acetone evaporated in a current of air at room temperature and the moist residue shaken twice with 1·5 times its volume of ethyl acetate. The combined ethyl acetate extracts were evaporated to dryness in cold air; the residue was dissolved in methanol (0·5 ml.) and examined by paper partition chromatography.

Sedimented bacteria. The deposit was suspended for 24 hr in aqueous acetone (10 ml. 70% (v/v) acetone in water), which was then evaporated in cold air and the extract made to known volume, acidified to pH 2·8–3·0 with N-HCl and shaken with activated charcoal. The charcoal was extracted as described above.

The bacterial fraction was also treated to release 'bound' gibberellin (McComb, 1961). After washing with distilled water, the centrifuged deposit was frozen and the bacteria disrupted in a Hughes press at −20°. The disrupted material was thawed, the volume made to 10 ml., 0·08 g. crude ficin (Koch–Light Laboratories Ltd.) and 0·12 mg. L-cysteine added, and the mixture incubated at 35° for 18 hr. The hydrolysate was then acidified to pH 3·0, activated charcoal added and after adsorption extracted as described.

Uninoculated culture medium without added sucrose was also extracted as described for the supernatant fluid fraction.

Paper partition chromatography. The different extracts were examined on Whatman no. 1 chromatography paper with two solvent systems which separate GA 3 and IAA. Control additions of 25 μg. authentic GA 3 and IAA were developed at the same time.

With solvent system A (freshly mixed isopropanol + ammonia solution sp.gr. 0·880 + water; 10 + 1 + 1 by vol.) chromatograms were equilibrated in solvent-saturated air for 1 to 2 hr and then developed with solvent descending for 16 hr until the front was about 33 cm from the origin. The chromatograms were dried in air. Strips with authentic GA 3 and IAA and one spot of extract were cut and dipped in 5 % (v/v) conc. sulphuric acid in methanol, dried in hot air and exposed for 15 min. to u.v. radiation (wavelength 350 mμ). GA 3 was identified by green fluorescence at R_F 0·53 and IAA by yellow fluorescence at R_F 0·36. IAA was also identified as a pink spot when chromatogram strips were dipped in 0·05 M-$FeCl_3$ in 3 % (v/v) conc. sulphuric acid in methanol mixture.

With solvent system B (benzene + acetic acid + water; 4 + 2 + 1 by vol.) chromatograms were equilibrated overnight with the lower phase of the solvent mixture in the bottom of the tank and then developed with the upper phase of the mixture as descending solvent for 2 hr until the front was about 25 cm. from the origin. Strips from dried chromatograms were dipped in chromogenic reagents and examined under u.v. radiation. GA 3 fluoresced at R_F 0·05 and IAA at R_F 0·55.

With solvent systems A and B the R_F values differed slightly for each development, but GA 3 and IAA were always in the same position relative to each other and never overlapped.

Chromatogram portions not treated with chromogenic reagents were dried for at least 7 days to remove solvents, cut into 10 equal strips representing the sequence of R_F values 0·1–1·0 and the strips eluted separately for bioassays.

Bioassays

Elongation of dwarf pea internodes. Chromatogram strips representing the sequence of 10 R_F values were eluted with acetone, the acetone evaporated in cold air and the residues dissolved in methanol (0·5 ml.).

Seeds of *Pisum sativum* cultivar Meteor, were germinated for 2 days on wet filter paper in Petri dishes incubated at 25°, before planting, 5 per pot, in Eff Soil-Less Compost (Eff Products Ltd., Bracknell, Berks). After 16 days in the glasshouse, plants were selected for uniformity and the distances between third and fifth nodes (interval A) measured. A minimum of ten replicates were each treated with 10 μl. of methanol extract, which was placed on the leaf subtending the third node. Distances between third and sixth nodes (interval B) were measured after 5 days. Control plants were treated with 10 μl. methanol extract of a portion of chromatogram over which only solvents had run. The increment in growth due to treatment was expressed as a percentage calculated from the equation

$$\frac{100\ \{(B-A)\ \text{treated} - (B-A)\ \text{control}\}}{(B-A)\ \text{control}}.$$

A series of plants was also treated with 1·0, 0·1, 0·01 and 0·001 μg. GA 3 in methanol, as above.

Elongation of lettuce hypocotyls. Seeds of lettuce, *Lactuca sativa* cultivar Tom Thumb, were germinated for 2 days in continuous light on wet filter paper in Petri dishes incubated at room temperature (about 18°). Chromatogram strips representing the sequence of R_F values were placed on filter paper moistened with 6 ml. distilled water in 9 cm. diameter Petri dishes. Ten seedlings with roots about 5 mm. long were placed on each chromatogram strip. The seedling hypocotyls were measured after 3 days at room temperature (about 18°) in continuous light and percentage increases in growth over controls calculated. Control seedlings were placed on a portion of chromatogram over which only solvents had run. Seedlings were also placed on filter paper impregnated with 0·1, 0·25, 1·0 or 2·5 μg. GA3.

Tomato bioassay. Tomato seeds, *Lycopersicum esculentum* cultivar Money Maker, were germinated in potting compost. When the cotyledons had expanded, seedlings were transplanted and their roots treated with 0·1 ml. of aqueous extract of test substance. Plants were graded at weekly intervals by measuring stem height and leaf length, and for flower and fruit development, as described by Brown, Jackson & Burlingham (1968). The following extracts were tested by this method. (1) Supernatant fluid fraction; the residue from ethyl acetate extract taken up in 1·0 ml. distilled water. (2) Sterile culture medium; the residue from ethyl acetate extract taken up in 1·0 ml. distilled water. (3) Acetone eluates from chromatogram strips representing a sequence of 10 R_F values; the residues were taken up in 1·0 ml. distilled water. (4) Untreated whole Azotobacter culture and sterile culture medium free from sucrose were also tested.

RESULTS

Detection of growth substances on chromatograms

The supernatant fluid and bacterial fractions from 50 ml. Azotobacter culture contained substances separated by paper chromatography with solvent system A which fluoresced under u.v. radiation at R_F 0 and R_F values 0·02 to 0·06 and 0·1 to 0·16. With solvent system B the extracts fluoresced only at R_F 0. There was no fluorescence corresponding to the positions of IAA or GA 3. Extracted culture medium did not fluoresce after separation in either solvent system.

Detection of gibberellin-like substances by pea bioassay

Supernatant fluid fraction. The supernatant fluid fractions of ten different batches of Azotobacter culture were extracted and separated by paper chromatography with solvent A. Figure 1 is a histogram of the growth effects on peas produced in one such experiment by the sequence of ten eluted strips corresponding to R_F values 0·1 to 1·0. In the different tests growth responses were registered by substances with R_F values 0 to 0·2, 0·4 to 0·7 and 0·7 to 1·0, showing peaks of activity at R_F values 0·15, 0·55 and 0·85. The magnitudes of the peaks ranged from (%): 12 to 68, 16 to 74 and 14 to 42, respectively. Substances with R_F values between 0·5 and 0·7 corresponded in position to authentic GA 3. Calculated from the response curve of peas to standard amounts of GA 3, the original supernatant fluid fraction contained from 0·009 to 0·1 μg. GA 3 equivalent per ml.; the amount differed between batches of cultures, but most contained 0·03 μg. GA 3 equivalent/ml. In three of the ten experiments there was also a peak of activity of 30 to 50 % at R_F 0·3 to 0·4. This substance did not correspond in position on chromatograms to authentic IAA.

Eluates from chromatograms of the supernatant fluid fraction developed with

solvent system B were also tested on peas. Figure 2 shows that significant growth responses were registered by substances with R_F values 0 to 0·4, 0·5 to 0·6 and 0·7 to 0·9. Substances with R_F 0 to 0·1 corresponded in position to authentic GA 3 and in different tests gave growth increases from 10% to 30%. Calculated from the response curve of peas to standard amounts of GA 3, the original supernatant fluid fraction contained on average 0·02 μg. GA 3 equivalent/ml.

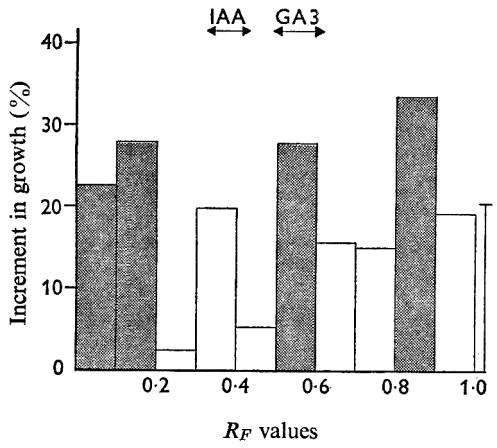

Fig. 1

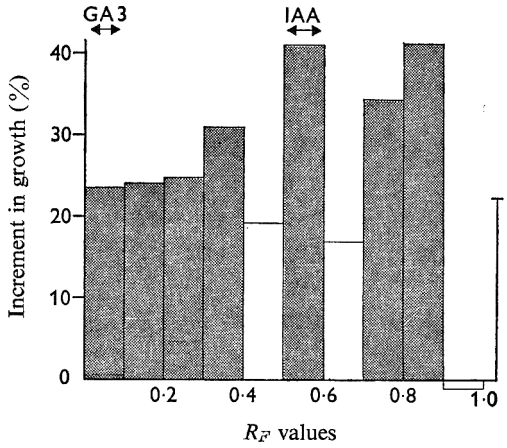

Fig. 2

Fig. 1. Effects on growth of dwarf pea (*Pisum sativum* cultivar Meteor) internodes by components of the supernatant fluid from *Azotobacter chroococcum* cultures separated by chromatography in solvent A (isopropanol + ammonia + water). Vertical line represents least significant difference, $P = 0{\cdot}05$. Shaded portion represents activity significant at 5% level. Horizontal lines at top of figure represent positions of authentic gibberellin GA 3 and indolyl-3-acetic acid (IAA).

Fig. 2. Effects on growth of dwarf pea (*Pisum sativum* cultivar Meteor) internodes by components of the supernatant fluid from *Azotobacter chroococcum* cultures separated by chromatography in solvent B (benzene + acetic acid + water). Conventions as in Fig. 1.

Bacterial fraction. Figure 3 shows the effects on peas of eluates from chromatograms of acetone-extracted bacteria developed with solvent system A. Substances with growth-promoting activity occurred at R_F values 0 to 0·7 and 0·8 to 0·9; that at R_F 0·4 to 0·6 corresponded in position to authentic GA 3. Substances with R_F values 0 to 0·3 and 0·8 to 0·9 were probably the same as those in the supernatant fluid fraction which developed in the same positions. Calculated from the response curve of peas to standard amounts of GA 3, the bacteria from 1 ml. of original culture contained on average 0·01 μg. GA 3 equivalent.

Eluates from crushed and hydrolysed bacteria also produced growth responses of peas at R_F values 0·1 to 0·2, 0·5 to 0·6 (corresponding to authentic GA 3) and 0·8 to 0·9, but the crushed bacteria yielded no more gibberellin than was contained in acetone extracts. The Azotobacter organisms did not contain 'bound' gibberellins.

Uninoculated culture medium gave no growth response.

Lettuce bioassay

Supernatant fluid fraction. Eluted material from the supernatant fluid fraction separated by paper chromatography with solvent system A was tested by lettuce bioassay. Figure 4 shows that substances with R_F values 0 to 0·2 and 0·3 to 0·6 (corresponding in position to authentic GA 3), significantly increased growth of lettuce hypocotyls by 14% and 12%, respectively. Substances with R_F values 0·8 to 0·9 that promoted extension of dwarf peas stems were inactive on lettuce hypocotyl. Eluates from chromatograms run in solvent system B gave a growth response at R_F 0 to 0·2 with a peak of activity of 27% at R_F 0·15. Substances with R_F 0 to 0·1 corresponded in position to authentic GA 3 and gave a growth increase of 10%. Calculated from the response curve of lettuce hypocotyls to standard amounts of GA 3, the supernatant fluid fraction contained on average 0·01 μg. GA 3 equivalent/ml.

Extracted uninoculated culture medium produced no growth responses of lettuce hypocotyls.

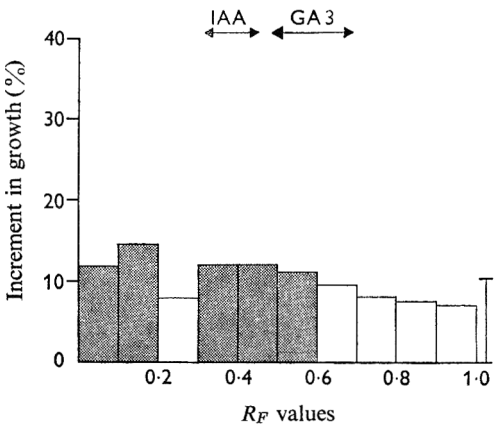

Fig. 3

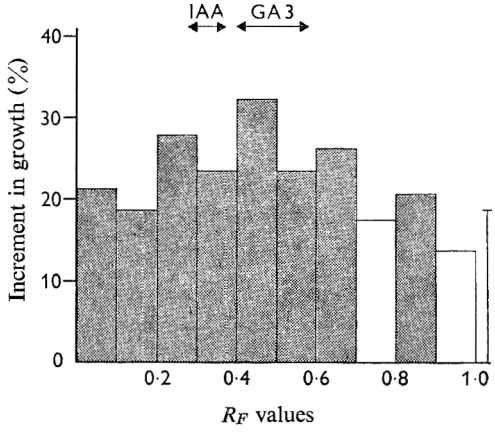

Fig. 4

Fig. 3. Effects on growth of dwarf pea (*Pisum sativum* cultivar Meteor) internodes by components of acetone-extracted *Azotobacter chroococcum*, and separated by chromatography in solvent A (isopropanol + ammonia + water). Conventions as in Fig. 1.

Fig. 4. Effects on growth of lettuce (*Lactuca sativa* cultivar Tom Thumb) hypocotyls by components of the supernatant fluid of *Azotobacter chroococcum* cultures separated by chromatography in solvent A (isopropanol + ammonia + water). Conventions as in Fig. 1.

Tomato bioassay

Whole Azotobacter culture and medium. Tomato seedling roots were treated with whole Azotobacter culture or with uninoculated medium. The Azotobacter culture significantly increased stem length and leaf size until five true leaves were formed; it also shortened the time between appearance of flower buds and petal fall, on the first truss by 4 days, and on the second by 6 days.

Supernatant fluid fraction. The supernatant fluid fraction from the Azotobacter culture behaved like the whole culture in increasing growth of tomato stems and leaves and shortening the time of development of the first truss by 1 day and of the second truss by 5 days.

Extracted chromatogram strips representing a sequence of ten R_F values. After eluting chromatogram strips of the supernatant fluid fraction with acetone, the dry residues were dissolved in distilled water and applied to tomato seedling roots. Material with R_F values 0·4 to 0·6 and 0·7 to 0·8 (solvent system A) significantly accelerated growth of stems and leaves until six true leaves had formed. From then until flower buds were showing, significant responses were caused only by substances with R_F values 0·4 to 0·6. Table 1 shows that substances with R_F values 0 to 0·1, 0·4 to 0·6 and 0·8 to 1·0 shortened, and those with R_F 0·1 to 0·4 and 0·6 to 0·8 lengthened, the time between flower bud appearance and petal fall of the first truss. Only substances with R_F values 0·4 to 0·6 shortened the time of development of the second truss. It was calculated that eluted substances with R_F values 0·4 to 0·6 contained 0·02 μg. GA 3 equivalent/ml.

Table 1. *Effect on truss development in Lycopersicum esculentum cultivar Money Maker of growth substances in Azotobacter culture supernatant fluid separated on chromatograms*

	Position (R_F) of supernatant fluid fraction on chromatogram									
	0–0·1	0·1–0·2	0·2–0·3	0·3–0·4	0·4–0·5	0·5–0·6	0·6–0·7	0·7–0·8	0·8–0·9	0·9–1·0
	Truss development shortened (−) or lengthened (+) (days)									
1st truss	−3	−1	+5	+1	−3	−3	+2	+1	−4	−4
2nd truss	0	0	0	0	−4	−3	0	0	0	0

Table 2. *Effects of substances in Azotobacter supernatant fluid on stem and leaf growth of Lycopersicum esculentum cultivar Money Maker at different stages of development*

R_F value of substance (solvent system B)	No. of leaves formed	Accelerated growth	
		Height	Leaf
0–0·1	2	+	−
0·1–0·2		+	−
0·9–1·0		+	+
0·1–0·2	4	+	+
0·3–0·4		+	−
0·4–0·5		+	−
0·9–1·0		+	−
0·1–0·2	6	+	−
0·3–0·4		+	−

Several substances in eluates from chromatograms developed with solvent system B significantly affected stem and leaf growth of tomato at different stages of plant development. Table 2 shows that substances with R_F values 0·1 to 0·2 accelerated stem growth until six true leaves had formed. The substance corresponding in position to authentic GA 3 (R_F 0·05) accelerated internode growth of young plants only. When developed with solvent system A, substances in the same position as GA 3 were active to truss development. This difference might be caused by phenolic acids interfering in the tomato bioassay because these acids also developed R_F values 0 to 0·1 in solvent system B.

Identification of gibberellin-like substances in the supernatant fluid fraction of Azotobacter cultures

Substances with R_F values 0·1 to 0·2, 0·5 to 0·6 and 0·8 to 0·9 separated with solvent system A were eluted and then developed on paper chromatograms with solvent system B. After development the chromatograms were cut into ten equal strips and each eluted and tested on dwarf peas. Table 3 shows the position of substances that significantly increased growth.

Table 3. *Substances in Azotobacter supernatant fluid which affected the extension of dwarf pea (Pisum sativum cultivar Meteor) internodes (R_F position)*

R_F in solvent A	R_F in solvent B
0·1 to 0·2	0 to 0·1
0·5 to 0·6	0 to 0·1
0·8 to 0·9	0 to 0·1, 0·5 to 0·6, 0·7 to 0·8

Table 4. *R_F values of different gibberellins in two solvent systems*

	Solvent system	
	A Isopropanol + ammonia + water	B Benzene + acetic acid + water
Gibberellin	R_F value	
A 1	0·55	0·05
A 4	0·67	0·67
A 5	0·61	0·51
A 7	0·70	0·65
A 8	0·42	0·0
A 9	0·72	0·90

Material eluted at R_F 0·5 to 0·6 in solvent system A developed at R_F 0 to 0·1 in solvent system B, that is, in the position of GA 3 in both solvent systems and so was probably GA 3 or a very closely related substance such as GA 1 which also developed in the same positions in both solvent systems (Brian, Grove & MacMillan, 1960). Material eluted at R_F 0·1 to 0·2 in solvent system A developed in the position of GA 3 in solvent system B, and again was probably closely related to this compound. The material at R_F 0·8 to 0·9 in solvent system A separated into three components when developed in solvent system B, one with the R_F value of GA 1 or GA 3, and two unidentified gibberellin-like substances. Cavell, MacMillan, Pryce & Sheppard (1967) identified 17 gibberellins but only six were available for tests in the present work. These latter were separated by paper chromatography with the solvent systems A and B and compared with the unknown gibberellin-like substances from Azotobacter. Table 4 shows the R_F values of the authentic gibberellins. The unidentified active components in Azotobacter supernatant fluid fraction could not be related to the authentic compounds. Thus Azotobacter produced three growth-promoting substances which were gibberellin-like in character in that they induced shoot elongation of dwarf mutant plants whose growth responses are thought to be specific to the gibberellins (Phinney & West, 1960). One substance resembled GA 1 or GA 3; the other two were not identified.

DISCUSSION

Of the three gibberellin-like substances detected in the present work in cultures of *Azotobacter chroococcum* strain A 6, the one with the same R_F value as GA 1 or GA 3 was probably the most important. Although the amount was too small to detect by fluorescence on paper chromatograms, bioassays readily detected it and suggested that the concentration in 14-day cultures ranged between 0·01 and 0·1 μg. GA 3 equivalent/ml. This amount of gibberellin-like substance was seemingly enough, when an inoculum of Azotobacter was added to seeds or roots, to alter the later development of tomato plants, possibly because it was taken up by the seedlings at a critical stage of development, when vegetative and reproductive primordia were differentiating. However, not all the gibberellin taken up by the seedlings may have come from the initial inoculum, for gibberellins may have continued to be synthesized for a short period when the roots were being colonized by the Azotobacter inoculum which moved from the seed to the germinating root and multiplied (Jackson & Brown, 1966). Only 14-day Azotobacter cultures grown in a nitrogen-deficient mineral medium have so far been studied; it has yet to be determined whether the conditions of cultivation affect the production of gibberellins by Azotobacter.

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THE SOCIETY FOR GENERAL MICROBIOLOGY

The Society for General Microbiology held its fifty-second General Meeting at Imperial College, London, on Monday, Tuesday and Wednesday, 1, 2 *and* 3 *April* 1968. *The following communications were made:*

ORIGINAL PAPERS

Cell Division following Recovery from 'Thymineless Death' in *Escherichia coli* 15. By W. DONACHIE (*M.R.C. Microbial Genetics Unit, Hammersmith Hospital, London, W.* 12)

Thymine-requiring strains of *E. coli* 15 become increasingly sensitive to plating during growth in the absence of thymine. The progressive decrease in numbers of cells able to give rise to colonies is the classical 'thymineless death' described by Barner and Cohen. We have recently reported that cells can recover completely from their inability to form colonies on agar, if they are allowed to grow again in the presence of thymine in liquid medium. The recovery of the ability of the cells to divide in liquid medium has been followed after various periods of thymine starvation. For this strain, the time of onset of cell division is delayed following re-addition of thymine. The exact time of cell division is delayed in proportion to the period of thymineless growth. Observations on the resumption of DNA synthesis suggests that cell division is delayed until the DNA/mass ratio in the cells is restored to its normal value.

Host Specificity in F′ Heterogenotes of *Escherichia coli*. By S. W. GLOVER. (*M.R.C. Microbial Genetics Research Unit, Hammersmith Hospital, London, W.* 12)

Host-controlled modification of DNA in strains of *Escherichia coli* has two clearly defined characteristics. First, modification, a process which acts directly on DNA and involves the specific alteration of certain base sequences by methylation. As a result, the DNA synthesized in a particular strain may carry a characteristic, strain-specific pattern. Secondly, restriction, a process which may lead to the rapid degradation of DNA introduced into a cell of different host strain specificity.

The host specificities of *E. coli* strain K12 and *E. coli* strain B are different such that DNA from K12 may be degraded in B and vice versa. In both strains mutants have been isolated which either have lost the ability to restrict but are still able to modify DNA, or have lost both the ability to restrict and to modify DNA. The genetic location of both these mutations has been determined in *E. coli* K and *E. coli* B. Both mutations map in the *thr-leu* region.

An F′ factor from *E. coli* K12 carrying *thr leu* and the genes determining host specificity has been used to examine the host-specificity properties of diploids. Restriction was assayed simply by measuring the efficiency of plating of bacteriophage λ grown on either *E. coli* K or *E. coli* B and designated λ.K and λ.B respectively. Modification was assayed again indirectly by growing phage λ on the diploid strains and measuring its efficiency of plating on standard indicator strains.

The results obtained so far indicate that:

(1) In strain K the wild-type alleles are dominant to both of the mutants described above.
(2) The diploid K/K may plate λ.B less efficiently than the haploid strain K.

(3) The diploid K/B restricts both λ.K and λ.B.
(4) The diploid K/B produces λ particles which are able to plate on both strains K and B.
(5) The diploid constructed between K and a mutant of B deficient in both restriction and modification is indistinguishable from the haploid K strain;
(6) The diploid constructed between K and a mutant of B deficient in restriction only, restricts both λ.K and λ.B and produces λ particles which are able to plate on both strains K and B.

These results will be discussed in relation to the several models which have been proposed to explain the genetic basis of host-controlled modification of DNA.

Preliminary Observations on the Antigens of *Mycoplasma hominis*. By RUTH M. LEMCKE and M. R. HOLLINGDALE (*Lister Institute of Preventive Medicine, London, S.W.* 1)

Mycoplasma hominis was fractionated by differential centrifugation after disruption of the cells by decompression under 80–100 atmospheres of nitrogen or by sonic treatment. A 'membrane' fraction, which sedimented at 37,500 *g* and a 'soluble' fraction, which did not sediment at 100,000 *g* both had complement-fixing activity, but gave different precipitin patterns in gel-diffusion and immunoelectrophoresis tests. The membrane fraction also had a capacity for blocking growth-inhibiting antibody at least 32 times greater, on a protein content basis, than that of the soluble fraction. Subfractionation of the soluble fraction on Sephadex G-150 or DEAE-cellulose achieved separation of some of the precipitating components.

Antigens were also extracted from whole cells of *Mycoplasma hominis* by chemical methods. Lipid extracted with chloroform-methanol had some, apparently specific, complement-fixing activity, but did not contain the major serologically reactive components of the organism. In this respect, *M. hominis* differs from *M. pneumoniae*.

Extraction of *Mycoplasma hominis* with cold or warm aqueous phenol yielded no serologically reactive material in the aqueous phase, indicating the absence of lipopolysaccharide antigens. Instead, serologically reactive components of *M. hominis* were extracted in the phenolic phase, and are therefore probably protein in nature. Comparison with the soluble and protein fractions in gel diffusion tests showed that material from the phenolic phase was derived mainly from the cell membrane. Extraction of cells at pH 11·7 also yielded a serologically reactive extract which contained components characteristic of both the membrane and soluble fractions.

The results suggest that distinct antigenic components can be recognized in the cell membrane and in the 'soluble' cell contents, and that antigens capable of blocking growth-inhibiting antibody are located in the cell membrane. The major antigens of *Mycoplasma hominis* appear to be protein in nature, not lipid as in *M. pneumoniae*, or polysaccharide as in *M. mycoides* var. *mycoides*.

Mycoplasma Host Specificity in the Light of Strains Isolated from Pigs. By D. TAYLOR-ROBINSON and Z. DINTER (*Clinical Research Centre, Harvard Hospital, Salisbury, England, and Institute of Virology, Royal Veterinary College, Stockholm, Sweden*)

Mycoplasma hyorhinis and *M. granularum* were isolated some years ago from pigs in the U.S.A. and these observations have often been confirmed (Switzer, W. P. (1967) *Ann. N.Y. Acad. Sci.* **143**, 281). Dinter, Danielsson & Bakos ((1965) *J. gen. Microbiol.* **41**, 77) isolated a number of mycoplasma strains from pigs in Sweden, some of which (SEP strains) were identified as *M. hyorhinis*. In addition, some strains (B strains), which were neither *M. hyorhinis* nor *M. granularum*, comprised four distinct serotypes. Three of these serotypes (B4, B1 and B6) have now been identified by metabolic-inhibition and agar growth-inhibition techniques as *M. laidlawii*, *M. gallinarum* and *M. iners* respectively. The latter two mycoplasmas are usually isolated from birds. One serotype represented by mycoplasma B3 could not be identified. The isolation of avian mycoplasma serotypes from pigs is discussed in relation to mycoplasma tissue specificity and specificity in experimental and natural infections *in vivo*.

The Cell Wall of *Bacillus subtilis* During Sychronous Growth. By A. H. DADD and R. J. L. PAULTON (*Bacteriological Laboratories, Imperial College, London, S.W. 7*)

A method for synchronizing a strain of *B. subtilis* has been devised and utilized to study the incorporation of cell-wall material. The cell wall of this strain consists of two polymers; a mucopeptide comprising glucosamine, muramic acid, glutamic acid, alanine and diaminopimelic acid (DAP), and teichoic acid (Salton M. R. J. & Marshall B. (1959), *J. gen. Microbiol.* **21**, 415).

Despite a step-wise multiplication in cell numbers, dry weight increased logarithmically as did the DAP content of the cell population. However, after extraction of the cells with 10 % trichloro-acetic acid, the acid insoluble DAP showed a discontinuous incorporation. Between divisions there was no increase so that only during the period of cell division did doubling occur. Since DAP is not found in cellular proteins but in walls or wall precursors, the measurement of acid insoluble DAP is an estimate of total cell-wall material, assuming DAP to be a fixed proportion of the cell wall throughout the synchronous cycle. This was found to be true.

Quantitative isolation of the cell wall and the measurement of dry weight, teichoic acid, amino sugar and DAP content gave the same information. The incorporation of cell-wall material therefore accompanied cell division. Consequently, between divisions a fixed amount of cell wall had to contain an enlarging cytoplasm and during this period there was an increase in the reactive groups of the wall. For example, the number of DAP molecules having an NH_2 group free increased by approximately 30 %, suggesting that some stretching of the wall may have occurred.

The Proteolytic Enzymes of *Microsporum fulvum*. By G. E. MATHISON and M. G. SARGENT (*Department of Microbiology, Queen Elizabeth College, University of London*)

The basic features of the proteolytic system of *Microsporum fulvum* have been investigated with the ultimate object of elucidating the role of proteinases in dermatophyte infections. *M. fulvum* synthesizes several proteinases, about 20 % of the total activity being extracellular in growing cultures. Using pH-dependence curves and inhibition data it has been demonstrated that the intracellular and extracellular enzymes are qualitatively quite distinct. Both the intracellular and extracellular preparations have been resolved into two components by cellulose phosphate adsorption. At pH 8 the adsorbed fractions are an intracellular component with a broad pH optimum in the range 6·7–7·7, and an extracellular component with a narrow optimum at pH 9·3. The unadsorbed fractions are intra- and extracellular components with optima at pH 6·7 and 7·7 respectively. Data from inhibition experiments using *p*-chloromercuribenzoate, ethylene diamine tetra-acetate, mercaptoethanol and di-isopropyl phosphofluoridate indicate that most of the components are not homogeneous.

Homogenization of the mycelium, followed by centrifugation at $9{\cdot}55 \times 10^5$ g.-min. partitions the intracellular activity into soluble and particulate fractions. In isotonic sucrose only 30 % of the activity is solubilized. The insoluble material is not readily solubilized by a variety of treatments with buffers or detergents and, when assayed in the presence of isotonic sucrose, 40 % of the total particulate activity is unavailable to the substrate. This fraction may be located in lysosome-like secretory organelles.

When *M. fulvum* is grown on a casein hydrolysate medium no extracellular proteinases are detectable and the intracellular activity is about 30 % of that found in casein-grown cells. On transferring amino acid-grown mycelium to a medium containing casein, extracellular proteinase is formed after a lag and the intracellular specific activity rises to that of fully induced cells. The proteinase liberated during active growth is a true exoenzyme, its liberation being clearly separable in time from that of an intracellular marker (alkaline phosphatase).

Synthesis of Cell-wall Mucopeptide by Particulate Preparations from *Bacillus megaterium* and *Bacillus stearothermophilus*. By P. E. REYNOLDS (*Subdepartment of Chemical Microbiology, Department of Biochemistry, University of Cambridge*)

This paper reports the results of investigations of the effect of temperature on two particulate enzyme preparations that are extremely active in synthesizing mucopeptide.

Bacillus megaterium KM and *B. stearothermophilus* (NCTC 10339) were grown in nutrient media at 37° and 55° respectively. Bacteria were harvested when in the exponential phase of growth, washed in a mineral salts solution and broken by blending with polystyrene beads in an MSE micro-homogenizer. After removal of unbroken cells, cell walls and beads by centrifugation at 5000 *g*, the membrane fraction was obtained by centrifugation at 40,000 *g*. The membrane pellet was washed and resuspended in 0·05 M-tris/HCl buffer, pH 7·8 containing 10^{-2}M-Mg^{2+}. The incubation system contained UDP-*N*-acetyl-glucosamine, UDP-*N*-acetyl-muramyl-ala-glu-[^{3}H]-diaminopimelic acid -ala-ala, 0·05 M-tris/HCl buffer, pH 7·8 containing 10^{-2}M-Mg^{2+} and the particulate enzyme. The system was assayed by filtration on glass fibre discs or by chromatography in isobutyric acid/0·5 N-ammonia (5:3, v/v).

The mucopeptide synthesizing system from *B. megaterium* was active maximally at 22° and incorporated the substrates linearly during a 2 hr incubation period. At higher temperatures (30°, 37°) the system was gradually inactivated and no further incorporation occurred after 1 hr. In addition, some of the material that had been synthesized in the first hr was rendered soluble during the subsequent 60–90 min.

The particulate enzyme from *B. stearothermophilus* incorporated radioactivity into mucopeptide material linearly during 60 min. incubations at 37°, 55° or 65°. This suggests that the protein/lipid complex concerned in mucopeptide synthesis in this organism is very resistant to high temperature since the mean generation time is only 20 min. at 55°.

The maximum enzymic activities observed were 30 and 33 mμmoles radioactive substrate incorporated/mg. protein/hr for the preparations from *B. megaterium* and *B. stearothermophilus* respectively: these activities are appreciably greater than those that have been reported for *Staphylococcus aureus* and *Micrococcus lysodeikticus*.

Anti-microbial Drugs and Pulse-labelled RNA. By J. GRINSTED (*Subdepartment of Chemical Microbiology, Department of Biochemistry, University of Cambridge*)

The effects of proflavine and ethidium bromide on the pulse-labelled RNA of a thermophilic *Bacillus* were examined, and compared with the effects obtained using actinomycin D.

Actinomycin D can reveal decay of pulse-labelled RNA because it prevents RNA synthesis, which normally masks the breakdown. It follows that other drugs which selectively inhibit nucleic acid synthesis should give the same effect; Woese *et al.* ((1963) *Biochem. Biophys. Res. Commun.* **11**, 435) have demonstrated decay of pulse-labelled RNA in *Escherichia coli* using proflavine.

All experiments were carried out with cultures growing exponentially at 55°. Pulse length in all cases was 30 sec.; 5-^{3}H-uridine was added to the cultures and 30 sec. later the pulses were terminated by the addition of drug. The label went almost exclusively into RNA.

A decay of pulse-labelled RNA was observed with either proflavine or ethidium. The half-life of decay was concentration dependent and an approximately linear relationship between half-life and drug concentration (relative to cell density) was established for both drugs. The percentage of the total labelled RNA which was stable varied in a similar way, increasing from a minimum at low drug concentrations to almost 100 % at high drug concentrations.

Extensive experiments with actinomycin D over a concentration range of 2–75 μg./ml. revealed no significant differences in half-life of decay or in the stable percentage. The half-life of decay with actinomycin was about 30 sec., which was approximately the same as the minimum half-life observed with either proflavine or ethidium.

The different effects observed with actinomycin on the one hand, and proflavine and ethidium on the other, could be explained by the fact that the latter two drugs bind to RNA; this binding could inhibit enzymic degradation of RNA, explaining the concentration depend-

ence of the half-life of decay. Drug binding to RNA also inhibits protein synthesis, and this could explain the protection from decay conferred on the pulse-labelled RNA by high concentration of proflavine or ethidium.

Thiol-disulphide Interaction in Yeast Cell Wall. By D. K. KIDBY and R. DAVIES (*Subdepartment of Chemical Microbiology, Department of Biochemistry, University of Cambridge*)

Invertase is released from cells of *Saccharomyces fragilis* by 2-mercaptoethanol (Davies, R. & Elvin, P. A., (1964), *Biochem. J.* **93**, 8P; Weimberg, R. & Orton, W. L., (1966), *J. Bact.* **91**, 1–13). This paper reports on the probable mechanism of this and certain other thiol-induced effects on yeast cell walls.

Kinetic studies on invertase release from 2-mercaptoethanol treated cells of *S. fragilis* suggest that:

(1) Invertase release results from two consecutive but distinguishable processes.
(2) The first process is thiol-dependent.
(3) The second process is not thiol-dependent but is dependent upon the first process.
(4) The first process involves reduction of disulphide bridges in the cell-wall protein.
(5) The second process involves the movement of invertase through the thiol-modified cell wall.

Comparison of thiol-released invertase with invertase released by cell breakage reveals no obvious effect of the thiol upon the integrity of the enzyme.

On the basis of the proposed mechanism, it is possible to predict certain experimentally useful techniques for the study of yeasts and possibly other fungi.

The Interaction of Polyoma DNA with DNA Polymerase. By J. D. PITTS and M. FRIED (*Medical Research Council Experimental Virus Research Unit, Institute of Virology, and Institute of Biochemistry, University of Glasgow, Glasgow, W.* 2)

DNA polymerase activity is low and DNase activity undetectable in extracts of mouse embryo cells maintained in low serum medium (Eagle's medium supplemented with 0·5 % calf serum). After infection with polyoma virus, or treatment with medium containing 10 % serum, the level of polymerase increases 5–20-fold, while DNase remains undetectable. DNA polymerase from unpurified extracts of infected, or serum-treated, low serum cells binds to double-stranded polyoma DNA and the bound enzyme can be separated from the free enzyme and from other protein, by zone centrifugation through sucrose gradients. When the polyoma DNA concentration in the mixture is increased, all the polymerase activity from a cell extract sediments with the DNA. Polymerase also binds to purified, supercoiled, polyoma DNA and to native mouse embryo DNA.

Sedimentation of polyoma DNA alone through sucrose gradients separates 20s supercoiled component I from a 14–16s mixture of open-circular component II and linear component III. Addition of an extract of polyoma infected, or serum-treated, low serum cells to polyoma DNA converts component I to a slower sedimenting form (about 17s) but has little effect on the sedimentation rate of a mixture of components II and III. Breakage of the polynucleotide strands of component I (e.g. by DNase action) would reduce the sedimentation rate, but results indicate that the DNA extracted from the complex has both strands intact.

Identification of Closely Linked Loci Controlling UV Sensitivity and Refractivity to Colicin E2 in *Escherichia coli* K 12. By E. J. THRELFALL and I. B. HOLLAND (*Department of Genetics, University of Leicester, England*)

Unlike classical antibiotics colicins apparently do not penetrate sensitive bacteria nor do they cause gross damage to the cytoplasmic membrane. The colicin protein rather acts indirectly from the cell surface, its effect being mediated by some form of 'pathway' linking the

extracellular colicin to its intracellular target. To study the more distal steps in this pathway which presumably involves one or more membrane proteins, mutants, refractory to E2 (Ref-II), were isolated which nevertheless retained the E2-surface receptors. Since a final step in the action of E2 is the 'activation' of a DNase which causes rapid degradation of DNA, Ref-II mutants were screened for any that were now UV sensitive. This anticipated that such mutants might be defective in some DNase activity. A few such, Ref-II, UV^s mutants were observed and the uv^s locus was found to map at the same position, 3 min. counter-clockwise to *thr*, as the *ref*II locus. Previous physiological studies indicated that the UV sensitivity of the Ref-II, UV^s mutants was of the Rec type and moreover previous workers (Van de Putte, P., Zwenk H. & Rörsch, A., (1966), *Mutation Res.* **3**, 381; Wood, W. B. (1966), *J. molec. Biol.* **16**, 118) have indicated the location of rec^- and r^-m^- (restriction modification) loci also close to *thr*. In crosses with male strains the Ref-II, UV^s mutants do in fact behave as Rec mutants, giving reduced recombination frequencies. The possibility is therefore being investigated that the *ref*II locus constitutes part of an operon concerned with chromosomal metabolism and that UV sensitivity appears in certain Ref-II mutants as a result of a polar mutation or deletion. An alternative possibility will be discussed that the pleiotropic effects of these mutants is due to a change in some membrane protein whose integrity may be essential for the binding and normal activity of certain repair and recombination enzymes including that specific to E2 action.

Transfer of Irradiated *colI* Factors and Indirect Induction. By MARILYN MONK (*M.R.C. Microbial Genetics Research Unit, Hammersmith Hospital, London, W.* 12)

A recipient cell lysogenic for phage λ may be induced as a result of mating with an irradiated *colI* donor. Induction is initiated early after cell contact, with kinetics similar to the transfer of *un*-irradiated *colI* factors. Using a non-lysogenic recipient it was shown that there is some lethality and inhibition of division of recipient cells mated with the irradiated donor, these effects similarly being initiated extremely early after cell contact. However, with UV doses to the donor optimum for indirect induction, there is no detectable transfer of viable (colicin-producing) *colI* factors, except in rare instances where transfer is considerably delayed (up to 40 min.). Also, indirect induction and *colI* transfer following irradiation are susceptible to DNA repair processes in the donor, but not in the recipient. The question was thus raised as to whether these effects of mating with an irradiated donor follow completion of transfer to the recipient of inviable defective *colI* factors, or reflect a block in transfer. Autoradiographic experiments designed to detect possible material transfer of heavily irradiated non-viable *colI* factors to the recipient cell suggest that UV has little effect on transfer of *colI* DNA, thus favouring the former alternative.

Temperature Sensitive Mutants of *Bacillus subtilis* Defective in DNA Synthesis. By D. KARAMATA (*M.R.C. Microbial Genetics Research Unit, Hammersmith Hospital, London, W.* 12)

About 800 unselected temperature sensitive mutants of *Bacillus subtilis* have been isolated. The mutants specifically affected in DNA synthesis at high temperature (DNA^- t.s. mutants) have been picked by measuring the variation of the ratio of ^{14}C leucine to ^3H thymine incorporation. The genetic analysis of 30 independent DNA^- t.s. mutants reveals that they fall into four small linkage groups which are located in four different regions of the *B. subtilis* chromosome. The basic physiological properties of the four groups will be presented and their possible function will be discussed.

A New Class of Donor-specific Filamentous Phages. By G. G. MEYNELL and A. M. LAWN (*Guinness-Lister Research Unit and Department of Electron Microscopy, Lister Institute, London, S.W.* 1)

The filamentous phages so far described all adsorb to the F sex pili determined by F and other plasmids like ColV, ColB and fi^+ drug-resistance (R) factors. Col I^+ cells form a different sex pilus—the 'I pilus' (Meynell, G. G. & Lawn A. M. (1967), *Genet. Res., Camb.* **9**, 359)—whose formation is also determined by fi^- R factors (Lawn, A. M., Meynell, E., Meynell,

G. G. & Datta, N. (1967) *Nature, Lond.* **216**, 343). F phages do not adsorb to I pili. However, filamentous I phages have now been isolated from sewage which adsorb to I pili but not to F pili. The method of selection consisted of incubating sewage with *Salmonella typhimurium* carrying a de-repressed mutant *fi*⁻ R factor (Meynell, E. & Datta N. (1967), *Nature, Lond.* **214**, 885), followed by plating on *E. coli* K 12 carrying the same mutant R factor. The receptors for many phages are provided by the bacterial somatic antigens but, as these differ in *Salmonella* and in *Escherichia*, such phages enriched on *Salmonella* fail to plate on K 12. In the event, not only were two isolates of I phages obtained (If1 and If2) for five flagellar phages were also found, all apparently the same as that isolated by Sertic & Boulgakov in 1937 (Edwards, S. & Meynell, G. G. (1968), *J. gen. Virol.* in press).

I and F filamentous phages resemble each other in their marked heat-resistance and in the absence of a clear-cut rise period in one-step growth experiments. The I phages have a modal length of 1·3 μ, about 50 % longer than the F phages, fd, M13 and EC9. Phages If1 and If2 are antigenically identical, as are fd and EC9. Anti-I sera do not neutralize fd or EC9, although antisera to the latter neutralize the I phages. Nevertheless, microscopy shows that anti-I sera contain antibody combining with fd or EC9. There is no cross-neutralization between M13 and the I phages.

The I phages have proved valuable in classifying sex pili (Lawn *et al.* (1967)) and also in demonstrating sex factors unlinked to easily recognizable characters like colicinogeny. Thus, ColEla proves to consist of two linkage groups; *ColEla*, the determinants of colicin Ela; and a sex factor which is I-like by phage-sensitivity and other criteria (Meynell, G. G. & Lawn, A. M. (1967), *Genet. Res., Camb.*, **10**, 323; Edwards, S. & Meynell, G. G. (1967) *Microbial Genetics Bulletin*, (Nov.).).

Further Observations of The Structure of Influenza Viruses. By K. Apostolov and T. H. Flewett (*Wellcome Research Laboratory, Beckenham and Regional Virus Laboratory Birmingham*)

Evidence will be presented that particles of Influenza C virus possessing the reticular surface structure previously described are not contaminants and are found in more than one strain.

The walls of Influenza A, B and C viruses and Sendai virus seen in thin section possess three layers: a surface layer of 'spikes'; an electron-transparent layer; and an inner electron-dense section.

The internal structures correspond in their dimensions to those found in negatively stained preparations. The internal component of Influenza C resembles in size and distribution that of Influenza A and B. In all the influenza viruses this component appears to be hollow and is possibly helical.

The Role of ATP in the Control of Energy Metabolism in Growing Bacteria. By D. E. F. Harrison and P. Maitra (*Water Pollution Research Laboratory, Stevenage and Tata Institute for Fundamental Research, Bombay*)

Previous attempts to measure and interpret ATP levels in growing micro-organisms (Polakis, E. S. & Bartley, W. (1965), *Biochem. J.* **99**, 521; Wimpenny, J. W. T. (1967), *Biochem J.* **102** 34P) have failed to appreciate the very high rate of turnover of ATP in growing cells. For the conditions used in this work, i.e. a glucose-limited chemostat culture of *Klebsiella aerogenes* with a growth rate of 0·2 hr^{-1}, the calculated turnover time for the total ATP content of the cells is of the order of 1 sec. Clearly, the ATP 'pool' is not an energy reserve and measurement of the steady-state level by itself can reveal nothing of the control systems in the cell. However, the response in ATP level to perturbations of the steady state can provide information about control mechanisms.

For this work a rapid sampling technique was used which, it is estimated, allowed less than 0·05 sec. from the time cells leave a chemostat until they are extracted in 6 % perchloric acid. Adenosine phosphates were estimated enzymically by the methods of Maitra & Estabrook ((1964), *Anal. Biochem.* **7**, 472).

The respiration rate of *Klebsiella aerogenes* may be stimulated by lowering the oxygen tension below 2 mm. Hg (Harrison, D. E. F. & Pirt, J. (1967), *J. gen. Microbiol.* **46**, 193). Samples taken during such a stimulation of respiration showed that the ATP level did not increase with the respiration rate but remained just below the steady state level, indicating either a reduction in the P/O ratio or an increase in ATP turnover rate. The sudden addition, to the glucose-limited cultures of excess substrate in the form of glucose or succinate caused initial changes in ATP level, but the level was quickly brought back to its steady-state value. The results demonstrated a very tight control of ATP level within the growing cell.

The Lipase of a Rumen Bacterium. By C. HENDERSON and P. N. HOBSON (*The Rowett Research Institute, Bucksburn, Aberdeen*)

Since the isolation of lipolytic bacteria was reported (Hobson, P. N. & Mann, S. O. (1961) *J. gen. Microbiol.* **25**, 227) other strains of these anaerobic bacteria have been isolated and one (strain 5s) has been used for a study of the lipolytic enzymes produced. Some of this work has been described (Hobson, P. N. & Summers, R. (1966), *Nature, Lond.* **209**, 736).

In the present work the bacteria were grown in carbohydrate-limiting conditions using glycerol as an energy source. The lipase was assayed using either mono-, di or triglycerides, and the liberated fatty acids extracted and titrated with sodium hydroxide.

Studies of enzyme production in batch culture showed the release of enzyme into the medium during the logarithmic phase of cell growth. Concentration of the lipase by ultrafiltration or freeze drying of the culture supernatant was destructive, but about 25 % of the activity was recovered by half-saturating with ammonium sulphate and adding 2 % cellulose powder. After stirring for 3 hr at 1° the cellulose was collected by centrifugation, made into a column, and the lipase eluted by phosphate buffer. Further purification was possible by chromatography on Sephadex G-100 from which the lipase was totally excluded.

Studies using this enzyme have shown that the activity is dependent on substrates being finely emulsified and long chain acids are preferred. Tributyrin was not hydrolysed.

No triglyceride-hydrolysing enzyme was detected attached to cells or cell particles but using naphthyl esters as substrates a cell-bound enzyme was found which hydrolysed 2-naphthyl acetate faster than 2-naphthyl laurate.

The Cultivation and Some Properties of the Rumen Ciliate *Entodinium simplex*. By G. S. COLEMAN (*Biochemistry Department, Agricultural Research Council Institute of Animal Physiology, Babraham, Cambridge*)

The purpose of this communication is to report the successful cultivation of the rumen ciliate *Entodinium simplex*. This organism which is the smallest Entodiniomorphid protozoon found in the ovine rumen, was separated from larger ciliates by differential centrifugation and inoculated into growth medium. Subsequently only those cultures which contained small non-caudate protozoa of uniform size were selected to form stock cultures. These ciliates have now been cultured anaerobically, dividing at least every third day, for 18 months on a medium of salts, bicarbonate/CO_2 buffer, cysteine, autoclaved rumen fluid, wholemeal flour and dried grass. Fresh wholemeal flour had to be added to the medium each day and the cultures were normally diluted with an equal volume of fresh medium twice each week. Under these conditions the maximum number of *Entodinium simplex* was approximately 40,000/ml. The wholemeal flour was replaceable by a mixture of rice starch and washed bran but not by rice, wheat or maize starch or by washed bran alone. Replacement of the autoclaved rumen fluid in the medium by autoclaved clear rumen fluid supernatant fluid decreased the number of protozoa by 50 % and omission of any rumen fluid material decreased the numbers to only 10–30 %.

By the use of ^{14}C-labelled *Escherichia coli*, washed suspensions of *Entodinium simplex* have been shown to engulf bacteria progressively for over 4 hr. However, the maximum rate was only 200 bacteria/protozoon/hr compared with 12,000 bacteria/protozoon/hr for *Entodinium caudatum* (Coleman, G. S. (1964), *J. gen. Microbiol.* **37**, 209). After continuous engulfment of bacteria for 1 hr only 0·5 % of the *Escherichia coli* engulfed by *Entodinium simplex* was still viable.

Some Aspects of the Control of Encystment of an Amoeba *Hartmanella castellanii*. By A. J. GRIFFITHS and S. M. BOWEN (*Department of Microbiology, University College of South Wales and Monmouthshire Cardiff*)

The encystment of the soil amoeba *Hartmanella castellanii* may be induced by replacing the growth medium with water containing 10^{-2}M-$MgCl_2$. Under these conditions, and depending on the physiological state of the organisms, about 90 % of the amoebae form cysts within 64 hr. During encystment one of the most important events is the synthesis of cellulose which forms one of the layers of the cyst wall and also constitutes about 25 % of the dry weight of the cyst. The measurement of cellulose in the cysts also facilitates the evaluation of encystment.

Encystment is inhibited by tetracycline and to a lesser extent by chloramphenicol which suggests that the synthesis of new enzymes may be required for cyst formation. Encystment was also inhibited by arsenate, arsenite and iodoacetate but not by 2,4-dinitrophenol, malonate or sodium fluoride. The presence of glucose or α-ketoglutarate in the encystment medium results in a greater degree of cellulose synthesis and glucose also exerts a sparing effect on the dry-weight loss which normally occurs during encystment. Glutamate or histidine however are inhibitory to encystment and bring about cell death within 24 hr. Other carbon and nitrogen sources do not appear to affect encystment in any way.

Encystment is well suited to biochemical analysis and recent work involving the measurement of levels of certain enzymes will be discussed. This system also has the attraction of affording a comparison with other differentiating systems in which morphogenesis, metabolism and genetics play interacting roles in amoebae. These include the more complex sporulation phenomenon of the cellular slime-moulds.

The Metabolism of Hydrocarbons by Soil Micro-organisms. By J. G. JONES (*Department of Microbiology, University College, Cathays Park, Cardiff*)

A recent report by Jones, J. G. & Edington, M. A. ((1968), *J. gen. Microbiol.* (in press)) demonstrates the ability of local populations of soil micro-organisms to adapt themselves to the metabolism of hydrocarbons. Of the four samples studied one contained a considerably higher concentration of hydrocarbons. The adaptation to hydrocarbons of the microflora of this sample has been demonstrated by respirometric and population studies. It has been very difficult to establish reproducible results in the Warburg apparatus and the methods used to obtain such results will be discussed. An adaptation of the buried slide technique (Cholodny, N. (1930), *Arch. Microbiol* **1**, 620–52) has shown that the metabolic patterns observed with *n*-aliphatic hydrocarbons in the range C_8 to C_{16} hold true for the metabolism of *n*-Eicosane (C_{20}), a hydrocarbon which is solid at the experimental temperatures used. Very little is known about the effect of varying soil factors on the rate of hydrocarbon degradation in soils and considerable care is needed in interpretation of the results obtained in the respirometric studies. Further studies on the disappearance of hydrocarbons from the soils was followed by gas chromatography and provides further information on the rates of breakdown as determined by 'zero time' analysis. The relevance of the results to contamination by crude oil of natural environments will be briefly discussed.

Chemical and Oxidative Properties of Coupled Mitochondria from *Aspergillus niger*. By K. WATSON and J. E. SMITH (*Department of Applied Microbiology, University of Strathclyde, Glasgow, Scotland*)

The preparation and properties of mitochondria from animal tissues, e.g. rat-liver and beef-heart, is now well documented. Comparatively recently there has been an increasing number of reports of the preparation of tightly coupled mitochondria from plants, e.g. mung beans, corn, potato tubers, etc.

Although yeast was one of the first organisms shown to contain cytochromes it was not until 1964 that Duell, E. A. Inove, S. and Utter, M. F. ((1964), *J. Bact.* **88**, 1762) and Ohnishi, T. & Hagihara, B. ((1964), *J. Biochem.* **56**, 484) described the preparation of tightly coupled mitochondria extracted from yeast by the digestion of the cell wall with snail enzyme. Recently coupled mitochondria have been isolated from *Aspergillus niger* by Watson, K. &

Smith, J. E. ((1967) *Biochem. J.* **104**, 332) and from *Neurospora crassa* by Hall, D. O. & Greenawalt, J. W. ((1967), *J. gen. Microbiol.* **48**, 419).

Properties of extracted mitochondria from *Aspergillus niger* are extremely similar to those of intact yeast and mammalian mitochondria. Oxidation, with respiratory control, of a wide range of substrates of TCA cycle and of exogenous NADH was obtained. The extracted mitochondria contained cytochromes $a+a_3$, b, and $c+c_1$ as well as ubiquinone and NAD. Electron micrographs indicated relatively homogeneous mitochondrial fractions but also showed some lack of cristae. This latter phenomenon appeared to be related to the presence or absence of bovine serum albumin.

Induction of Asexual Sporulation in *Aspergillus niger* in Submerged Liquid Culture. By J. GALBRIATH and J. E. SMITH (*Department of Applied Microbiology, University of Strathclyde, Glasgow, Scotland*)

Variation of the nature and concentration of the nitrogen source, which is known to affect reproduction in many fungi, showed that sporulation of *Aspergillus niger* occurred in the complete absence of or at very low nitrogen concentrations regardless of the source. At higher concentrations, ammonium was inhibitory but nitrate allowed prolific sporulation. Twenty amino acids were provided as nitrogen sources at a concentration of nitrogen which was inhibitory in the case of ammonium. Sporulation occurred in all except cysteine, valine, ornithine, methionine and phenylalanine. Most amino acids were able to overcome the inhibitory effect of ammonium on sporulation when added to an an ammonium nitrate medium. Exceptions were cysteine, ornithine, methionine, asparagine and aspartic acid. Intermediates of the Kreb's cycle also promoted sporulation in the presence of ammonium. Malate, fumarate, citrate and α-ketogluterate were most effective, acetate and succinate least effective. Glyoxylate had an effect similar to the first group. Enzymes forming branch points between Kreb's cycle and amino acid synthesis have been examined i.e. glutamic dehydrogenase, glutamic-oxaloacetic transaminase and glutamic-pyruvic transaminase and the results will be discussed.

Glucose Metabolism in *Aspergillus nidulans*. By B. L. A. CARTER and A. T. BULL (*Department of Microbiology, Queen Elizabeth College, London, W.* 8)

Aspergillus nidulans has been grown in a chemostat at various growth rates with glucose as the growth limiting nutrient and nitrate as sole N-source. At low growth rates the Q_{O_2} was depressed by inhibitors of glycolysis to a greater extent than at high growth rates. Similarly, changing levels of enzymes of the hexose monophosphate pathway (HMP) and glycolysis suggests that glycolysis is the major pathway of glucose catabolism at the lowest growth rate while the HMP plays an increasing role as the growth rate increases. This conclusion was confirmed by radiorespirometry (Wang *et al.* (1956) *J. Am. Chem. Soc.* 78, 1869; (1958) *J. Bact.* 76, 216) which showed that glycolysis accounted for 78 % of the utilized glucose at a dilution rate of 0·034 hr^{-1} but only for 58 % at a dilution rate of 0·07 hr^{-1}.

Chemostat-grown cultures were subjected to various controlled oxygen tensions (ranging from 156 to 1 mm. Hg) at a constant growth rate ($D = 0{\cdot}05\ hr^{-1}$). Results of inhibition experiments and enzyme assays suggested that the HMP was the major route of glucose catabolism at low oxygen tensions. Radiorespirometric analyses demonstrated that the percentage of glucose flowing through the HMP increased from 25 at air saturation to 54 at 1·75 mm. Hg. In all experiments we have found that high glycolytic activity is characteristic of intensely melanized mycelium, whereas non-pigmented mycelium was distinguished by its enhanced HMP activity.

The critical oxygen tension for *A. nidulans* (that point at which oxygen uptake rate is no longer independent of oxygen tension) had the unusually low value of 1·75 mm. Hg. Below this tension nitrite accumulated in the medium, indicating the involvement of a dissimilatory nitrate reductase in terminal electron transport under conditions of very low oxygen concentration.

In batch culture, the amount of glucose catabolised via the HMP increased throughout logarithmic growth, then decreased markedly at the onset of the stationary phase.

Mode of Action of Viomycin on *Rhizobium meliloti*. By D. C. JORDAN, M. E. MCKAGUE and Y. YAMAMURA (*Microbiology Department, Ontario Agricultural College, University of Guelph, Canada*)

When legume root-nodule bacteria become resistant to certain compounds, including viomycin (Schwinghamer, E. A. (1964), *Can. J. Microbiol.* **10**, 221) they become ineffective in nitrogen fixation. Viomycin was found to enter both sensitive and resistant cells of *Rhizobium meliloti* within 15 min. of its addition and, although none was located in the cell-wall fraction of disrupted cells, a large amount was bound to the ribosomes, from which it could be released in an active form by inorganic cations. Such cations also protected intact cells from growth inhibition, any lag in growth being proportional to the time interval between antibiotic addition and cation addition. This antibiotic also bound to isolated DNA from both resistant and sensitive strains of *R. meliloti*.

Thirty min. after adding viomycin to log. phase cells the viable count ceased increasing and thereafter rapidly declined. Uptake of ^{14}C-glutamate into a purified wall fraction WBS only affected after 60 min., whereas the synthesis of total cellular protein and nucleic acids showed inhibition only after 120 min.

Resistance to viomycin in this bacterium is not related to the antibiotic's ability to enter the cell. Furthermore, since no marked alteration was noted either in the active uptake of amino acids by growing and resting cells or in cellular leakage from pre-loaded cells it appears unlikely that a loss in N-fixing ability is a function of a generalized alteration in the permeability of viomycin-resistant cells.

It is suggested that viomycin possesses multiple modes of action, the first being an inhibition in cellular division, perhaps by interference in septum formation, and the second being an inhibition of protein and nucleic acid synthesis. The extensive binding of this basic polypeptide antibiotic to the negatively-charged ribosomes and DNA is undoubtedly directly related to the secondary mode of action.

The Deoxyribonucleic Acid Base Composition in Micrococci. By J. BOHÁČEK (*Institute of Biophysics, Czechoslovak Academy of Sciences*), M. KOCUR and T. MARTINEC (*Czechoslovak Collection of Microorganisms, J. E. Purkyně University, Brno, Czechoslovakia*)

The present report gives the results of studies on the % guanine-cytosine (GC) content in DNA of 70 strains hitherto considered to be members of the genus *Micrococcus*. For the determination of the % GC content in DNA, three different methods were used: (i) determination of the % GC content from the T_m value, (ii) determination of the % GC content according to the ratio E 260/280 at pH = 3, and (iii) paper chromatography.

On the basis of the % GC content in DNA, the strains were divided into several separate groups. The most numerous group included strains in which the % GC content ranged from 66 to 75; only such strains are considered to be true micrococci.

Of the other groups, the group of obligate halophilic cocci with their 57–61 % GC is of special interest. As it differs significantly in its GC content from the true micrococci, it is recommended to place it in a separate genus—*Halococcus*. Another interesting group includes flagellated cocci with 40–51 % GC. These cocci can be subdivided into two clusters containing a mean of 40 and 50 % GC respectively. It is suggested that these strains should be classified in the genus *Planococcus*. Some strains described as micrococci had a GC content in DNAs similar to that of the species of the genus *Staphylococcus*.

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