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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)

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The following books may be found useful:

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

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

S. T. Cowan, *A Dictionary of Microbial Taxonomic Usage* (1968). Edinburgh: Oliver and Boyd.

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

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Amended Nomenclature for Strains Related to *Mycoplasma laidlawii*

By D. G. ff. EDWARD*
Wellcome Research Laboratories, Langley Court, Beckenham, Kent
AND E. A. FREUNDT
Institute of Medical Microbiology, University of Aarhus, Aarhus, Denmark

(*Accepted for publication 6 March 1970*)

SUMMARY

In an earlier paper it was proposed that a second family be re-established within the Mycoplasmatales for those strains not requiring sterol, and the nomenclature of Sabin, who first made this proposal, was followed in the belief that although unsuitable it was valid. It has now been learnt that the generic name *Sapromyces* is illegitimate, as it is the name of a genus of fungi. It is therefore proposed that the genus be named *Acholeplasma* in the family Acholeplasmataceae.

Sabin (1941*a*) proposed a classification of the organisms of the pleuropneumonia group in which those organisms, believed to be saprophytes and represented by the sewage isolates of Laidlaw & Elford (1936), were classified into a separate genus and family from the parasitic species. The genus was named *Sapromyces* and the family Sapromycetaceae (Sabin, 1941*b*). The later classification of Edward & Freundt (1956) recognized only a number of species of one genus *Mycoplasma* belonging to the order Mycoplasmatales, the sewage and similar strains being named *Mycoplasma laidlawii*. Recently Edward & Freundt (1969) proposed the re-establishment of a second genus and family for those strains like *M. laidlawii* which differ from other members of the Mycoplasmatales in not requiring sterol for growth. The proposal was made to facilitate further classification and in the belief that dependence on sterol was a fundamental property. It has received further support from the finding that two species of the new genus have a larger genome size than several species of *Mycoplasma* (Bak, Black, Christiansen & Freundt, 1970).

The names given to the second genus and family were those proposed by Sabin, in the belief that although inappropriate they were valid. Following the publication of the paper it has been brought to our attention that *Sapromyces* is illegitimate, as it is the valid name of a genus of fungi of the order Leptomitales (Fritsch, 1893).

It is therefore proposed that the genus to contain those species of the Mycoplasmatales which do not require sterol for growth be named *Acholeplasma* gen. nov. (*a* Gr. = not; *chole* Gr.n. = bile; *plasma* Gr.n. = anything moulded or formed), type species *Acholeplasma laidlawii* (Sabin) comb. nov., and that the genus should be the type of a new family Acholeplasmataceae. The name suggests lack of a requirement for an important constituent of bile, namely cholesterol. The ending -plasma has

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been chosen in the hope that it will set a precedent for future naming of new genera of Mycoplasmatales, which are now recognized to belong to a distinct biological class of micro-organism, the Mollicutes (Edward & Freundt, 1967; Edward *et al.* 1967). Since the species at present classified as *Mycoplasma granularum* belongs to the new family, it must be renamed *Acholeplasma granularum*.

We wish to thank Dr G. C. Ainsworth and Professor L. E. Hawker for so quickly pointing out to us the illegitimacy of the first name, and all those who have helped us in arriving at an alternative name for the genus.

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Effect of Peptidic Groups Isolated from Enzymic Casein Hydrolysate on Growth and Toxinogenesis of *Clostridium welchii* (*perfringens*)

By KATEŘINA NEKVASILOVÁ, J. ŠÍDLO AND J. HÁZA
Institute of Sera and Vaccines, Praha 10, Czechoslovakia

(*Accepted for publication 20 January 1970*)

SUMMARY

Enzymic casein hydrolysate, which is a suitable nitrogenous basis of the medium for alpha-toxin production by *Clostridium welchii* (*perfringens*), was fractionated by column chromatography on Sephadex G25 gel, using ammoniacal buffer. Combined peptidic fractions were able to support microbial growth, though toxin production was minimal. Toxinogenic substances were retained on the gel and could be eluted with weak acetic acid. This active substance represented about 0·5 % of the original hydrolysate and consisted of a mixture of low molecular weight peptides with free amino acids and a mineral portion (calcium, magnesium and iron). When column chromatography of enzymic casein hydrolysate was done with Sephadex G25 and 0·05 M-ammonium hydrogen carbonate buffer, toxinogenic substances were not retained on the gel. The procedure resulted in a group separation of peptides according to their molecular weight and simultaneously an adsorption effect was observed of aromatic amino acids on the gel. Four fractions were obtained exhibiting different effects on growth and toxinogenesis by *C. welchii*.

The fraction containing the largest molecules and the major part of total nitrogen (90 % approx.) proved to stimulate microbial growth, though only minimal α-toxin production. The other three low molecular fractions exhibit a stimulating effect on α-toxin production; their amino acid compositions were different.

The most active of the low molecular weight fractions contained about 1·8 % of the initial nitrogen and could be fractionated with DEAE-Sephadex A25 into a further four groups of which the most remarkable was peptide-bound tyrosine.

Gel chromatography on Sephadex columns resulted in an unequal distribution of Fe ions among the fractions; this was allowed for in performing comparative microbiological tests.

INTRODUCTION

It has been shown repeatedly that some synthetic media gave good growth of *Clostridium welchii* (*perfringens*) without any α-toxin production (Taylor & Stewart, 1941; Boyd, Logan & Tytell, 1948). The production of toxin was determined or strongly stimulated by the presence of muscular extracts (Macfarlane & Knight, 1941; Logan, Tytell, Danielson & Griner, 1945) or by the presence of enzymic protein hydrolysates (Adams, Hendee & Pappenheimer, 1947; Boyd *et al.* 1948; Murata, Yamada & Kameyama, 1958). The distinct nature of these toxinogenic nitrogenous substances was not clarified. Rogers & Knight (1946) concluded that the active substance obtained from an extract of horse meat had the character of an amino sugar. Experiments

aimed at isolating toxinogenic substances from enzymic digests of casein and gelatin using different separation methods revealed their presence in several fractions; the active substances were dialyzable (Adams *et al.* 1947). Jayko & Lichstein (1959) showed a stimulating capacity of some of the 29 synthetic dipeptides tested, especially glycyl-L-asparagine. Murata *et al.* (1958) isolated from a dialysate of commercial peptone a so-called 'methanol-insoluble factor' which contained different peptides as well as other unidentified components. Stavri, Bittner & Ficiu (1964) obtained best toxin yields by using meat hydrolysates by extreme enzymic digestion. The above results obviously indicated that there probably exist several toxinogenic factors of peptide nature, all being low-molecular dialysable compounds. We expected that a fractionation of the peptides contained in enzymic casein hydrolysate based on the principle of different molecular weight might contribute to a more accurate characterization of toxinogenic factors. This is why enzymic digests were fractionated by gel chromatography on Sephadex columns, followed by a study of the effect of the fractions obtained on the growth and toxinogenesis of *C. welchii*. It was the aim of this study to give a biochemical characterization of the fractions, in particular of the group exhibiting the most pronounced toxinogenic effect. Special attention was paid to the adsorption of the stimulating substances on gel.

METHODS

Culture medium. Semi-defined medium was used according to Adams & Hendee (1945) containing in a 100 ml. volume: pancreatic casein digest, 50 ml.; $Na_2HPO_4.12H_2O$, 0·584 g.; KH_2PO_4, 0·05 g.; $MgSO_4.7H_2O$, 0·016 g.; soluble starch, 0·22 g.; pH 7·6 to 7·7. For the preparation of the digest the methods by the above authors were adopted, the sole difference being that the hydrolysis of casein proceeded with continuous stirring. In experimental media casein digest was replaced by peptide fractions obtained by gel chromatography. Freeze-dried fractions were dissolved in distilled water and made up to the initial volume of the fractionated hydrolysate. The media were filled 35 ml. into wide test-tubes, after adding pieces of cotton-wool sterilized in steam for 40 min.

Organism. Clostridium welchii (*perfringens*) BP6K type A preserved on tryptically digested meat broth containing 0·5 % glucose, in two passages (18 and 8 h.) at 37°. The second passage was used for inoculating semi-defined medium.

Cultivation. After cooling the medium to 37°, 0·5 % glucose was added together with 2 % (v/v) inoculum from the second passage of the strain. Incubation was done in two parallel series for 8 h. at 37°. Lecithinase content was determined in the supernatant fluid of the cultures obtained by 20 min. centrifuging at 3000 *g*.

Growth. This was determined by measuring extinction after diluting the suspension with physiological saline to 1/50. Pulfrich colorimeter, blue filter, light path 5 cm. E_{5cm} values were converted by a calibrating curve to 10^9 organisms/1 ml. bacterial suspension.

Lecithinase determination. Determinations were done *in vitro*, using the lecitho-vitelline test (van Heyningen, 1941; Kémeny & Illés, 1952) at 0·2 u.α level (Lv); the results were converted to 1 u.α level. As standard, purified horse serum globulin was used, its titre being determined by a comparison with international standard anti-perfringens serum from Copenhagen.

Fractionation of pancreatic casein digest. Gel chromatography was done in two ways. (*a*) In alkaline medium according to Phillips & Gibbs (1961). A 50 ml. volume of

pancreatic casein digest (PCD) was filtered through a column of Sephadex G25 medium (Pharmacia, Uppsala); size of the column 5 × 50 cm.; elution buffer 0·2 M-ammonia; fractions were collected in an automatic collector, flow rate 5 to 6 ml./10 min.; line shift 10 min. Colorimetric evaluation was done in 0·1 ml. from each fraction by the Folin method modified by Lowry *et al.* (1951). Measurements were made with a 'Prema' photometer of Czechoslovak make, at 520 nm.; light path 1 cm.; results are expressed in % of light transmitted. The fractions forming the individual peaks were freeze-dried. (*b*) In weakly alkaline medium, 50 ml. of PCD were deposited on a 5 × 100 cm. column of Sephadex G25, elution with 0·05 M-ammonium hydrogen carbonate (pH 7·6). The fractions were collected automatically, flow rate about 45 drops/min., line displacement 20 min. From each of about 150 fractions, 0·1 ml. samples were withdrawn and evaluated as described above. Fractions from each peak were freeze-dried. Fractionation was at 4°.

Fractionation of active peptide and amino acid groups on ion exchange gel. Fractionations were made by gradient elution with a linear pH change according to Carnegie (1961). A glass column, size 1 × 20 cm., was filled with DEAE-Sephadex A25 in acetate form and brought to equilibrium with 0·1 M-collidine acetate buffer (pH 7·8 to 7·9). 12·1 g. collidine distilled *in vacuo*, were dissolved in about 750 ml. distilled water, pH adjusted to 7·8 to 7·9 with 10 % acetic acid and the volume made to 1000 ml.

Freeze-dried fractions obtained by PCD fractionation on Sephadex G25 (elution buffer 0·05 M-NH_4HCO_3) were dissolved in 20 ml. physiological saline and 2 ml. of the solution (containing 1·2 mg. total N) were applied to the column. Elution was effected with collidine acetate buffer, started at pH 7·8; pH gradient was obtained by gradual mixing with 0·1 M-acetic acid, while M-acetic acid was used from the 49th test-tube on. The fractions were taken by an automatic collector, line displacement 10 min., volume of fractions 2·5 ml. About 120 fractions were collected. Detection was made in 1 ml. samples from each test-tube, with ninhydrin reagent, using Rosen's modification (1957) of Moore & Stein's technique. The colour was measured with a Zeiss spectrometer, light path 1 cm., 520 nm. and expressed as % of light transmitted.

Paper chromatography of peptides and amino acids. For preparatory fractionations Whatman no. 3 paper was used. Several equal samples were spotted side by side and eluted in a descending way in an *n*-butanol + acetic acid + water solvent system (100 + 9 + 30 by vol.). The outer strip of paper was cut off and developed by 0·2 % solution of ninhydrin in *n*-butanol. The remaining strips of paper were cut into several zones. The paper strips showing an identical group of ninhydrin-positive substances were stitched together and eluted with water as described by Vaněček (1961). The eluate was concentrated on silicon-coated watch glasses and subjected to paper chromatography.

Analytical chromatography was done on Whatman no. 1 paper, in a descending one-dimensional way, in an *n*-butanol + acetic acid + water solvent (100 + 9 + 30 by vol.) and in a phenol + water solvent in NH_3 atmosphere. Detection with 0·2 % ninhydrin in *n*-butanol. The identity of amino acids was verified with the following specific reagents: tryptophane with Ehrlich's reagent in Sprince's modification (1960), tyrosine with Pauly's agent (Hais & Macek, 1959), arginine according to Takaguchi (Hais & Macek, 1959), cysteine with a solution of Na nitroprusside + NaCN, methionine with potassium iodoplatinate.

Hydrolysis of peptides. The samples were hydrolysed with 6 N-HCl in sealed vials, at 105° for 18 h.; HCl excess was removed by repeated evaporation on silicon-coated watch glasses.

Total nitrogen was determined by the Kjeldahl method.

Amino nitrogen was determined by using the iodometric technique according to Schroeder, Kay & Mills (1950).

Iron determination. After incineration with sulphuric and perchloric acids, Fe was reduced with ascorbic acid to Fe^{2+}. The pink colouring resulting from the formation of a complex with *O*-phenanthrolin was measured colorimetrically at 498 nm.

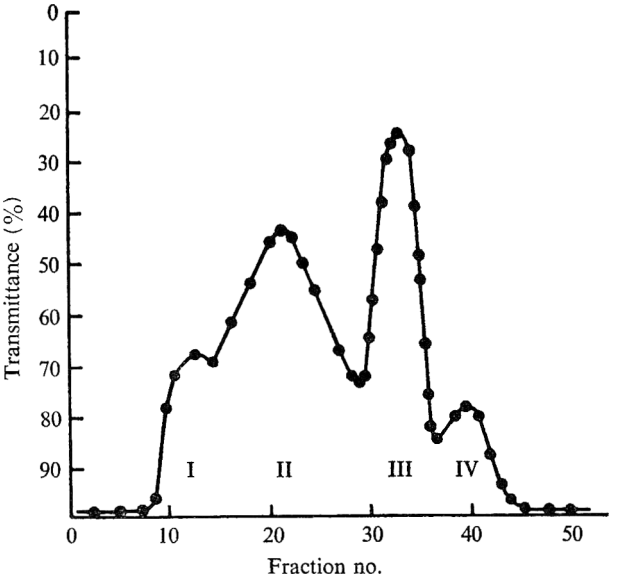

Fig. 1. Pancreatic casein digest filtered through a column (5 × 50 cm.) of Sephadex G25. Sample applied 50 ml., elution with 0·2 M-NH_3, fractions collected at a flow rate of about 60 ml./h. Detection by the Folin–Ciocalteu reagent according to Lowry *et al.* (1951), absorbancy at 520 nm.

RESULTS

Fractionation of pancreatic casein digest (PCD) in ammoniacal medium. The fractionation of the digest which represented the only nitrogenous material of the medium was done, in the first experimental series, on Sephadex G25 columns with an ammoniacal eluant by the method of Phillips & Gibbs (1961). The separation of peptides, though somewhat imperfect, was meant to provide orientating information on the effect of different groups on α-toxin production (Fig. 1).

Effect of fractions on growth and toxinogenesis. In the culture medium PCD was replaced with various mixtures of freeze-dried peptide fractions. Experiments made by eliminating procedures were unsuccessful; the growth as well as production of α-toxin in all media appeared to be weaker than in the control medium and even a mixture of all four fractions did not induce the initial degree of toxin formation (Table 1). The above results changed the direction of the experimental work, showing that the decrease of growth and toxinogenesis must be sought in adsorption of active factors on gel.

Retardation of toxinogenic substances on gel. The retarded substances were eluated after the passage of the last ninhydrin-positive components with 4 l. of 0·1 M-acetic acid. The acidic eluate was concentrated in vacuum and freeze-dried. From 100 ml. initial PCD 55 to 60 mg. dry matter were obtained, representing about 0·5 % of the dry matter contained in PCD. On addition of the acetic eluate to the combined NH_3-fractions nos. I to IV α-toxin titres were about doubled, but did not attain the titres of the control cultures. A stimulating effect was also observed on the growth of the strain (Table 2).

Table 1. *Growth and α-toxin production by Clostridium welchii in media prepared from fractions obtained by gel chromatography of pancreatic casein digest (PCD) on Sephadex G 25 columns, eluant 0·2 M-NH_3*

Nitrogen source	Total N g./100 ml.	Microbial count $\times 10^{-9}$/ml.	Lv/u.α/ml.
PCD (control)	0·64	1·2	5·0
Fraction I+II+III+IV	0·61	4·9	1·6
I+II+III	0·58	4·5	1·2
I+III+IV	0·50	5·0	1·2
I+III+IV	0·35	3·1	< 1·0
I+II+IV	0·36	2·8	< 1·0

Table 2. *Effect of gel-retarded substances on growth and α-toxin production*

Sephadex G 25 column, eluant 0·2 M-NH_3, subsequent elution with 0·1 M-acetic acid. Results of 2 Expts. A and B, given as average of 2 replicas.

Nitrogen source	Total N g./100 ml.		Microbial count $\times 10^{-9}$/ml.		Lv/u.α/ml.		Lv/u.α/10^9 microbes	
	A	B	A	B	A	B	A	B
PCD (control)	0·66	0·73	11·5	11·5	8·0	6·4	0·69	0·55
Fractions I–IV	0·58	0·68	4·5	6·5	1·6	1·2	0·35	0·18
Fractions I–IV + acetic eluate	0·61	0·73	6·0	8·9	3·2	3·2	0·53	0·36

Table 3. *Effect of acetic eluate and its mineral component on growth and α-toxin production*

Fractionation of PCD on Sephadex G 25, elution buffer 0·2 M-NH_3, extraction of adsorbed substances with 0·1 M-acetic acid.

Nitrogen source	Iron μg./ml.	Microbial count $\times 10^{-9}$/ml.	Lv/u.α/ml.	Lv/u.α/10^9 microbes
PCD (control)	3·2	13·2	6·2	0·47
Fractions I–IV	1·0	6·2	< 1	< 0·1
Fractions I–IV + acetic eluate	1·4	8·1	3·2	0·4
Fractions I–IV + mineral component of acetic eluate	1·3	8·7	2·0	0·23
Fractions I–IV + Fe^{2+}	3·1	10·0	2·0	0·20

Analysis of the substances retarded on gel. The incineration of the combined freeze-dried acetic eluates from 20 column fractionations revealed that 55 mg. dry matter from 100 ml. initial PCD contained 30 mg. ash. As PCD contained a total of 1·1 to 1·2 % (w/v) mineral substances this result meant that about 2·5 % (w/w) of the initial quantity of mineral substances remained adsorbed on the gel after elution with 0·2 M-NH_3. Analysis of the ash showed the presence of magnesium, calcium and iron. Of

the original quantity of 7·8 μg. iron/ml. PCD, only 2·4 μg. were found in 1 ml. of combined NH_3 fractions (I to IV) and 2·9 μg. in 1 ml. acetic acid eluate. Iron values found in the medium were usually lower (Table 3) than those which corresponded to the concentrations in the eluates, because of Fe losses during the preparation of media due to adsorption on flocculating phosphates which were removed by filtration.

Freeze-dried acetic acid eluate contained 1·9 % total N and was ninhydrin- and Folin-positive. By preparative chromatography on Whatman no. 3 paper in an *n*-butanol + acetic acid + water solvent peptides and amino acids were separated into 11 ninhydrin-positive substances. The six separated zones were eluated, hydrolysed with 6 N-HCl and chromatographed on Whatman no. 1 paper (Pl. 1). The peptides were split by acid hydrolysis into several amino acids with a marked content of aspartic acid, serine + glycine, glutamic acid, threonine, alanine and other amino acids in all zones. Several aromatic acids were found, such as tyrosine, phenylalanine and tryptophane before hydrolysis. The nitrogenous proportion of the eluate obviously represents a mixture of peptides and amino acids.

The effect of the mineral and organic compounds of acetic acid extract on toxinogenesis. The effect was studied of the mineral component of acetic acid eluate and compared with the action of complete eluate. In another group of media the mineral component was replaced with 0·1 % $FeSO_4 . 7H_2O$ solution in a quantity corresponding to the deficient Fe concentration (Table 3). The results indicated that the stimulating effect of the acetic acid eluate was due in part only to its mineral component, i.e. essentially to Fe ions. Identification of the active substance in so small a quantity of the complex mixture of ninhydrin-positive substances was extremely tedious; therefore, in the following experiments, a fractionation technique was adopted which enabled group fractionation of peptides without undesirable retardation of toxinogenic substances on gel.

Fractionation of pancreatic casein digest (PCD) in a weakly alkaline medium. Quantities of 50 ml. PCD were fractionated on Sephadex G25 column, elution with 0·05 M-ammonium hydrogen carbonate (pH 7·6). Freeze-dried fractions were used for biochemical and microbiological characterization. The elution curve is shown in Fig. 2.

Effect of fractions on growth and toxinogenesis. The results of the tests with media prepared of variously combined fractions showed (Table 4) that the mixed four fractions gave a toxin production equal to the control medium. The first fraction caused a good growth but slight toxin production only; it contained about 85 % of the substances present in the PCD. Its admixture with fractions II + III increased the titres to the initial values. The fourth fraction stimulated growth but only a slight increase of toxin production. Low-molecular fractions II to IV could be tested only in admixture with fraction I which represented a nutritional nitrogen source for microbial multiplication. In all media the Fe content was somewhat decreased, though its 1 μg. Fe/ml. was sufficient for full toxinogenesis.

To eliminate a potential influence of different Fe values (Pappenheimer & Shaskan, 1944; Shankar & Bard, 1952) further experimental series were done in media the Fe contents of which were adjusted to the value of the control medium. The results (Table 5) indicated that the medium prepared from fraction I showed again a low α-toxin formation, although the difference was less marked than in the above experiments (Table 4). Each of the low-molecular weight groups was capable of stimulating toxin production, most notably fraction III; the absence of fractions II and IV exerted no

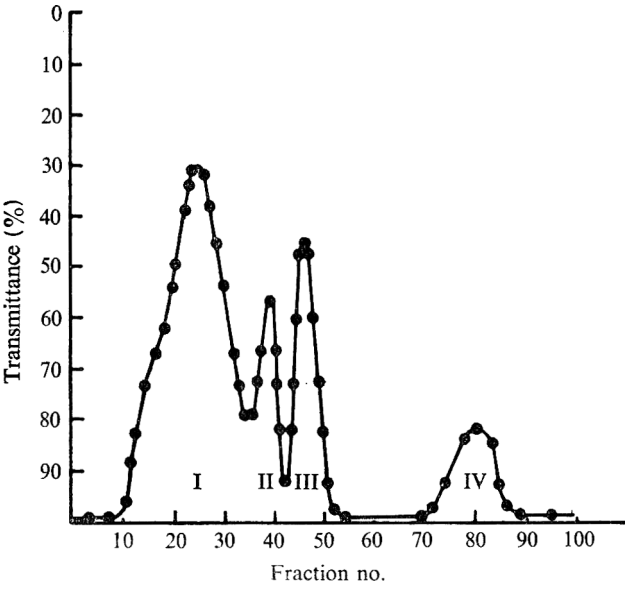

Fig. 2. Pancreatic casein digest filtered through a column (5 × 100 cm.) of Sephadex G25. A 50 ml. sample was applied, eluant 0·05 M-NH_4HCO_3, fractions collected at a flow rate of about 60 ml./h. Detection—see Fig. 1.

Table 4. *Effect of peptidic fractions on growth and toxin production by Clostridium welchii*

Fractionation of PCD on Sephadex G25, elution buffer 0·05 M-NH_4HCO_3.

Nitrogen source	Iron μg./ml.	Microbial count × 10^{-9}/ml.	Lv/u.α/ml.	Lv/u.α/10^9 microbes
PCD	4·5	10·9	5·0	0·46
Fractions I+II+III+IV	1·1	9·8	5·0	0·51
Fraction I	1·3	8·4	1·6	0·19
Fractions I−II+III	1·0	9·8	5·0	0·51
Fraction I+IV	1·3	10·4	2·4	0·23

Table 5. *Effect of peptidic fractions of PCD on growth and α-toxin production by Clostridium welchii*

Fractionation of PCD on Sephadex G25, elution buffer 0·05 M-NH_4HCO_3. Iron levels in media were made up to the value of the control experiment.

Nitrogen source	Iron μg./ml.	Microbial count × 10^{-9}/ml.	Lv/u.α/ml.	Lv/u.α/10^9 microbes
PCD (control)	3·4	13·7	6·2	0·45
Fraction I	3·4	9·2	2·0	0·22
I+II	3·4	11·8	4·0	0·34
I+III	3·3	8·6	6·2	0·72
I+IV	3·0	9·3	3·2	0·34
I+II+III	3·0	9·8	6·2	0·63
I+II+III+IV	3·1	11·1	5·0	0·63

influence on titre values. These results are shown best by expressing the amount of toxin produced per 10^9 organisms. The different fractions obtained by gel chromatography, in particular the most active of them, were characterized biochemically.

Contents of dry matter, nitrogen and amino-nitrogen. The determinations were made after mixing identical peak material from 5 gel preparations. Table 6 shows that the major part of the total N was concentrated in the first peak. The most active, i.e. the third fraction, contained only about 2 % of the initial total N.

Table 6. *Contents of dry matter and nitrogenous substances in PCD fractions obtained by gel chromatography on Sephadex G25 medium, elution buffer 0·05 M-NH_4HCO_3*

		Total nitrogen		Amino-nitrogen		
Fraction no.	Dry matter mg./100 ml.	mg./100 ml.	%	mg./100 ml.	After HCl hydrolysis mg./100 ml.	Increase after hydrolysis (%)
I	9·45	1220	88·34	444·7	833·3	+87·3
II	0·90	119	8·62	63·0	83·1	+31·9
III	0·25	24	1·74	14·4	17·3	+20·1
IV	0·15	18	1·30	7·4	6·8	− 8.1
Sum	10·75	1381	100·0	529·5	940·5	+77·6
Initial PCD	11·02	1340	—	532·7	953·9	+79·0

Table 7. *Iron contents in PCD fractions obtained by gel chromatography on Sephadex G25 medium, elution buffer 0·05 M-NH_4HCO_3*

Fe^{2+} determination with *O*-phenanthrolin.

		Fe^{2+}	
Fraction no.	Dry matter g./100 ml.	μg./ml.	μg./g. dry matter
I	9·45	4·60	48
II	0·90	0·66	73
III	0·25	1·18	472
IV	0·15	1·56	1040
Initial PCD	11·2	7·80	69

Data on the sizes of the peptides in the different fractions were obtained by amino-N determination before and after acid hydrolysis. Resulting amino-N increase indicated that the fractions successively passing through the column contained decreasing quantities of cleavable peptide bonds. The decrease of amino-N in the 4th fraction was associated with its tryptophan content, being destroyed by acid hydrolysis.

Iron content of the fractions. The preceding experiments showed a cation retention on gel and therefore the presence of iron in the different fractions was studied. Table 7 shows that all initially present iron left the column. Fraction I contained more than half the initial amount; but a calculation in reference to the dry matter revealed a higher % of iron leaving with the low-molecular weight groups. Irregular distributions of ions among the various peaks could distort the results of the cultivation tests with the peptide fractions, therefore an appropriate balancing of iron values in the culture media was indispensable.

Chromatographic analysis of the individual fractions. Paper chromatography showed that fractions I and II contained about 12 well separated ninhydrin-positive substances each; fraction III formed one long smear from the start towards tyrosine, with a separated spot in the phenylalanine position (Pl. 2). Fraction IV contained tryptophan

Table 8. *Paper chromatography of amino acids in different fractions of pancreatic casein hydrolysate after HCl hydrolysis*

Fraction no.	Cys	Lys	His	Arg	Asp	Ser + Gly	Glu	Thr	Ala	Pro	Tyr	Met	Val + Phe	Leu + Ile
I	++	++	+++	++++	++++	++++	++++	+++	++	++	−	++	+++	+++
II	−	−	+	++++	++++	++++	+++	++	++	++	+	+	+	++
III	−	−	−	−	++	+++	++	++	+	−	++++	−	++	+
IV	−	−	−	+	++	++	++	+	+	−	±	−	+	+

Solvent: *n*-butanol + acetic acid + water (100 + 9 + 30), detection: 0·2% ninhydrin in *n*-butanol, Pauly (tyr, his), nitroprusside-NaCN (cys), iodoplatinate (met). Whatman no. 1, one-dimensional.

detectable with Ehrlich's reagent. Table 8 gives a schematic picture of the amino acids in the different fractions. It was characteristic that fraction I contained, apart from other amino acids, also amino acids of the sulphur group (cystine, methionine) but no tyrosine or tryptophan. The second group had a closely similar composition to the preceding fraction, but it contained no cystine or lysine though, in contrast to fraction I, traces of tyrosine were present. Fraction III consisted mainly of a Pauly-positive spot which disintegrated during acid hydrolysis into tyrosine and some other amino acids.

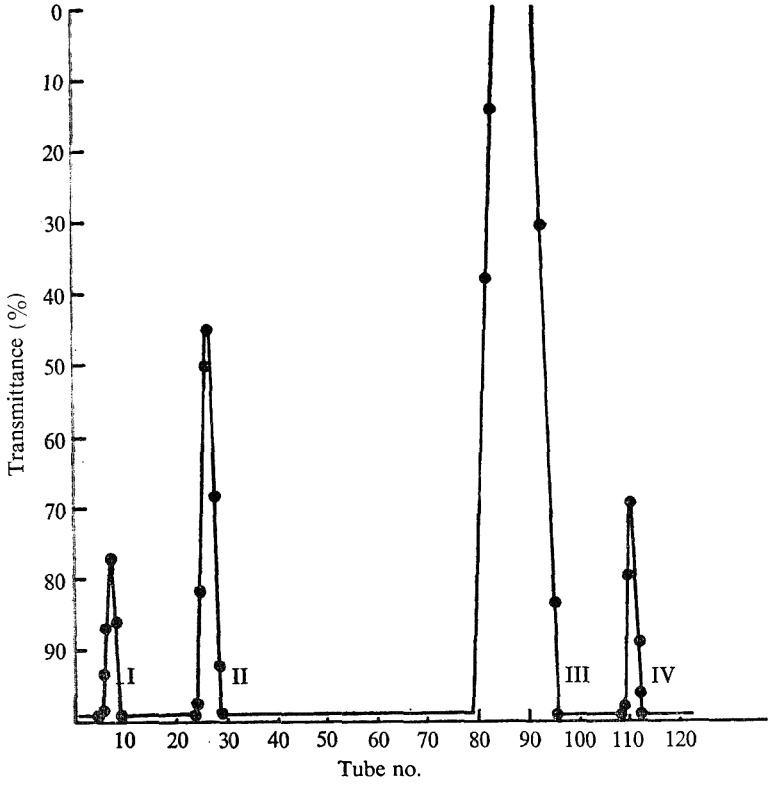

Fig. 3. Separation on Sephadex ion exchanger of the toxin-promoting 'third' fraction obtained by gel filtration of pancreatic casein digest through Sephadex G25, elution with 0·05 M-NH_4HCO_3 (see Fig. 2). Column of DEAE-Sephadex A25 (1 × 20 cm.) in the acetate form. A 2 ml. sample containing 1·2 mg. total N was applied on the bed. Elution with 0·1 M-collidine in acetate buffer, pH 7·8, gradient in pH 0·1 M-acetic acid up to the 48th test-tube, then 1 M-acetic acid. Flow rate 15 ml./h. approx. Detection with Moore–Stein ninhydrin reagent as modified by Rosen (1957), transmittance at 520 nm.

Fractionation of peak III on DEAE-Sephadex. Fraction III obtained by fractionation of 50 ml. PCD was freeze-dried, dissolved in 20 ml. physiological saline and fractionated on DEAE-Sephadex A25 column, by Carnegie's technique (1961). On the column 2 ml. samples of fraction III solution were spotted, containing about 1·2 mg. total N Fractionation was done by gradient elution with collidine+acetate buffer, with pH line shift. Fig. 3 shows that the peptide group was again fractionated into four components which were analysed by paper chromatography. Paper chromatography of the individual groups, before and after acid hydrolysis, in an *n*-butanol+acetic acid+

water solvent gave the following picture after detection with ninhydrin, Pauly's and Ehrlich's reagents: Peak I exhibited prior to HCl hydrolysis one Ehrlich-positive spot, tryptophan, which disappeared on hydrolysis, and serine + glycine appeared; peak II contained mainly free phe, traces of ser + gly, glu ac and tyr; peak III showed a long Pauly-positive smear, after HCl hydrolysis marked spots of tyr, ser + gly, asp ac, glu ac and one Pauly-positive spot under tyr, probably a product of tyrosine decomposition; peak IV contained free amino acids: asp ac, ser + gly and glu ac.

It is evident that the stimulating group consisted mainly of aromatic amino acids, with a predominant content of tyrosine and its peptides; free phenylalanine, a small quantity of peptide-bound tryptophan and to a lesser degree some free amino acids were also present.

DISCUSSION

The above-described results with *Clostridium welchii* show that fractionation of enzymic casein hydrolysate by molecular sieving can provide information about toxinogenic components in media. It seems promising to use for the separation of peptides the advantages offered by a highly cross-linked Sephadex gel, the choice of eluant being limited by the requirement of its easy elimination by freeze-drying.

The first series of experiments showed that the fractionation method applied by Phillips & Gibbs (1961) to show the stimulating effect of some peptide fractions on the growth of *Lactobacillus casei* could not be used for cultivating *Clostridium welchii*. The culture medium prepared from a mixture of all fractions obtained by using ammonium hydroxide buffer as an eluant was capable of inducing growth, but toxin production was markedly inhibited. The reason for this failure could be sought in a retardation or adsorption of toxinogenic substances on the Sephadex. The adsorbed substances extractable by a weak acid showed a stimulatory effect on α-toxin production. The organic portion of the extracts played an important part in the stimulatory action, but it represented a complex mixture of ninhydrin-positive aliphatic and aromatic substances, the separation and growth-testing of which would mean a laborious task which could not be resolved within a reasonable time. In the extracted active substance also calcium, magnesium and iron were found, but growth tests showed that a partial depletion of the nutrient medium of some cations did not offer a sufficient explanation for the toxin-promoting nature of the acetic acid extract. The quantity of these toxinogenic substances represented only a very small proportion of the original enzymic hydrolysate and seemed to be of a biocatalytic rather than of a nutritional nature.

We presume that the choice of an eluant which does not cause retention of toxinogenic substances on gel may facilitate the localization and definition of the active substances. Fractionation with ammonium hydrogen carbonate as an eluant did not induce retention of the substances required for full growth and toxin production. The first of four fractions obtained, consisting of the largest molecules and containing 85 to 90 % of nitrogenous substances present, was able to induce full growth of the strain, but the production of toxin was depressed, proving again that the growth-promoting substances were not identical with the substances stimulating the formation of potent α-toxin. Each of the further low-molecular groups was capable of increasing toxin titres, especially the third fraction which passed through the column. These results indicated the presence of different stimulating substances with less or more pronounced toxinogenic capacities.

The question arises whether the stimulating effect is associated with peptides of a certain size only which can be easily metabolized by the microbe, or rather with their specific composition. Biochemical characterization of the obtained peptidic groups provides a partial explanation at least for that question. It must be emphasized that chromatographic analyses showed that separation of peptide groups by gel filtration did not proceed on the basis of molecular size only, but also according to the chemical nature of the substances. The known adsorption effect of gel on aromatic amino acids (Gelotte, 1960; Determann & Walter, 1968) played a part here.

Folin-positive peptide groups which first passed through the column contained not only the largest molecules but also almost all the essential amino acids except tyrosine and tryptophan. This might lead to a conclusion that it is the absence of the two aromatic amino acids which is responsible for the weak toxinogenesis. It should not be forgotten, however, that the second fraction containing no tryptophan and only traces of tyrosine exhibited an enhancing effect on toxin production, though it differed from fraction I mainly by the size of molecules. Most active appeared to be the third fraction containing the most tyrosine, both free and peptide-bound. This fraction mixed with fraction I fully restored the toxinogenic capacity of the strain. The last of the low-molecular fractions, containing mostly tryptophan, was also capable of slightly stimulating toxin formation, but it was dispensable, as shown by the high toxin titres obtained in media prepared from fractions I + III exclusively. This finding was in conformity with the results of experiments on the cultivation of *Clostridium welchii* in Boyd's semi-defined medium (Boyd *et al.* 1948); in this medium, containing acid hydrolysate enriched with cystine + tryptophan, we did not obtain α-toxin production, while the addition of enzymic casein digest to the acid hydrolysate resulted in a marked lecithinase production (unpublished data). In this case, too, tryptophan was not an efficient factor for toxin formation.

Further fractionation of the third group of ion-exchange gel fractions showed that this group was a mixture of amino acids and peptides with a prevailing quantity of aromatic substances. When judging the potential toxinogenic effect of the different substances obtained by ion-exchange technique, the presence of a small quantity of tryptophan could be neglected since it was not indispensable for toxin formation. Phenylalanine detected in the active fraction did not seem to provoke stimulation of the whole third fraction; it was present in the first fraction from the Sephadex G25 column, though its toxinogenic effect was very low. The quantitatively strongest substance to which a stimulatory effect could be ascribed was peptide-bound tyrosine. Similarly, as in many other cases (Kihara & Snell, 1960; Prescott, Peters & Snell, 1953; Stone & Hoberman, 1953; Mikeš, Kakol, Zbrozyna & Šorm, 1960), we can presume that the peptides influenced microbial metabolism more strongly than the free amino acids they contained. Peptide-bound amino acids are often better utilizable by the microbe for transpeptidation and proteosynthesis than free amino acids (Fry, 1955).

When comparing the chemical character of the toxinogenic substances retarded on gel during elution by an ammoniacal buffer with the nature of the active third fraction obtained on elution with ammonium hydrogen carbonate, we found that both were low molecular weight substances containing aromatic amino acids. The experiments so far do not permit us to draw definite conclusions about the identity of the two active components obtained by the different fractionation techniques.

Returning to the question about the nature of the active substances, it should be

pointed out that, while the fraction with the larger tyrosine content showed a strong stimulation, there were several other low-molecular peptide groups which promoted toxin production. The toxinogenic effect of some substances resulting from enzymic splitting of casein was related primarily to the binding of amino acids in small peptides and secondly only to the specific amino acid composition. This finding explains, in part at least, the heterogeneity of the active substances found by several authors (Rogers & Knight, 1946; Adams *et al.* 1947; Murata *et al.* 1958; Jayko & Lichstein, 1959). It can be assumed that the difficulties accompanying the attempt to obtain high α-toxin yields in media from acid casein hydrolysate may be attributed to the absence of peptide-bound essential amino acids. Attempts are being made to replace the third active group with synthetic tyrosine peptides.

In the present work our attention was concentrated on the effect of nitrogenous products of casein cleavage. The influence of the quantitative cation distribution among the different peptide fractions should not be neglected. A higher percentage of cations leave the Sephadex column with the low molecular weight groups. The above experiments suggested that growth tests of peptide fractions must take into consideration the irregular distribution of cations, and their effect on growth and toxin production must be eliminated by an appropriate adjustment of nutrient media; but an influence of further trace cations cannot be excluded.

The authors want to acknowledge the skilful performance of chromatographs by Mrs Šindelářová, and the continual interest and support of Ing. Petera.

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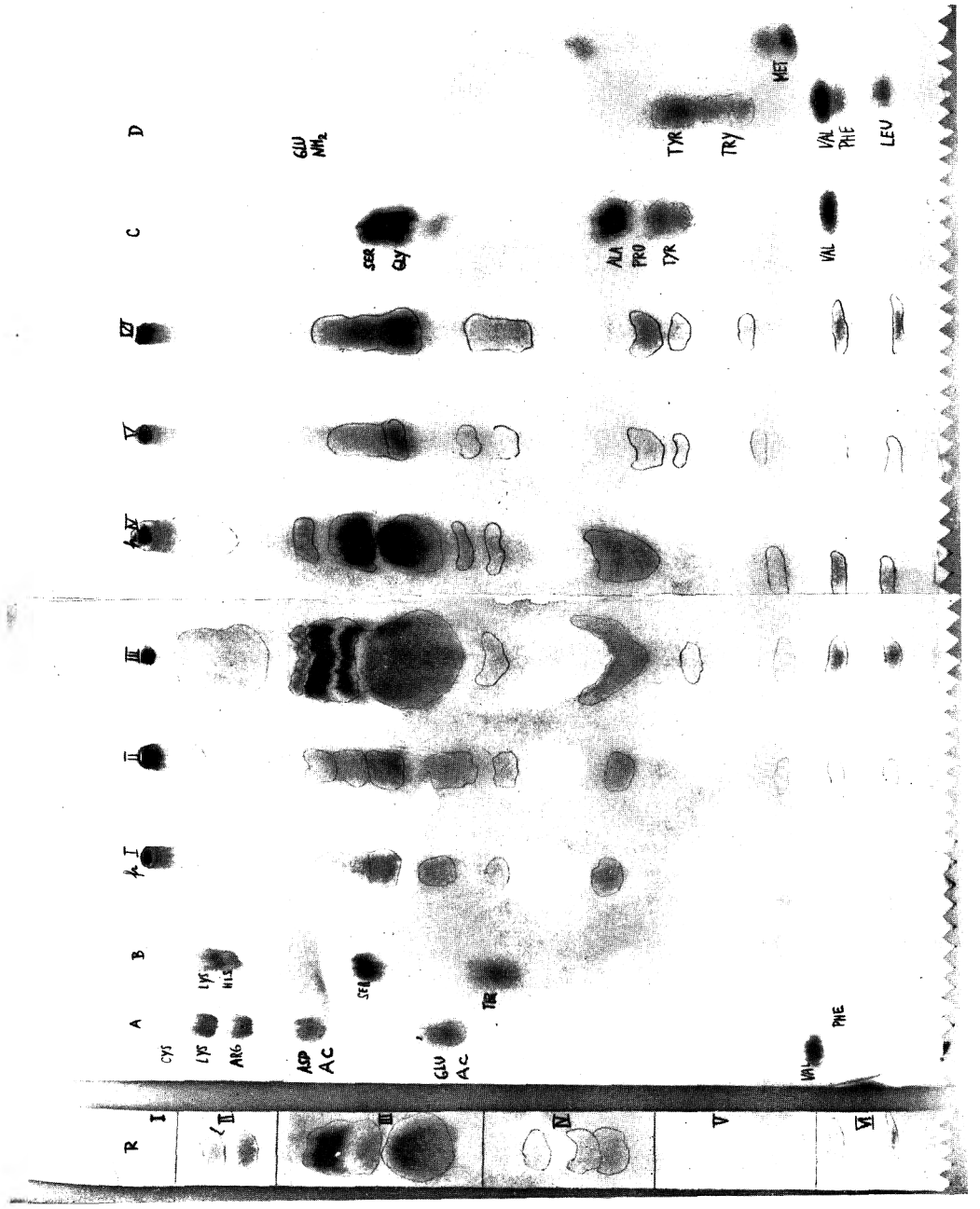
EXPLANATION OF PLATES

PLATE 1

Paper chromatography of the active substance adsorbed on Sephadex G25 bed, eluant 0·2 M-NH_3, subsequent elution with 0·1 M-acetic acid. The acetic effluent separated on Whatman no. 3 paper is shown to the left. The paper strip was cut into 6 sections I–VI and peptide groups were eluated with water, then HCl hydrolysed and rechromatographed on Whatman no. 1 paper. A–D are standard mixtures of amino acids. Solvent: *n*-butanol + acetic acid + water (100 + 9 + 30); detection: 0·2% (w/v) ninhydrin solution in *n*-butanol.

PLATE 2

Paper chromatography of fractions I–IV obtained by gel filtration of pancreatic casein digest through Sephadex G25, elution with 0·05 M-NH_4HCO_3. Whatman no. 1 paper, solvent system *n*-butanol + acetic acid + water (100 + 9 + 30), detection with 0·2% (w/v) solution of ninhydrin in *n*-butanol. Fraction IV before and after HCl hydrolysis is shown to the right. Detection of tryptophan with *p*-dimethylaminobenzaldehyde. (Samples after HCl hydrolysis are designated with an index 'H'.)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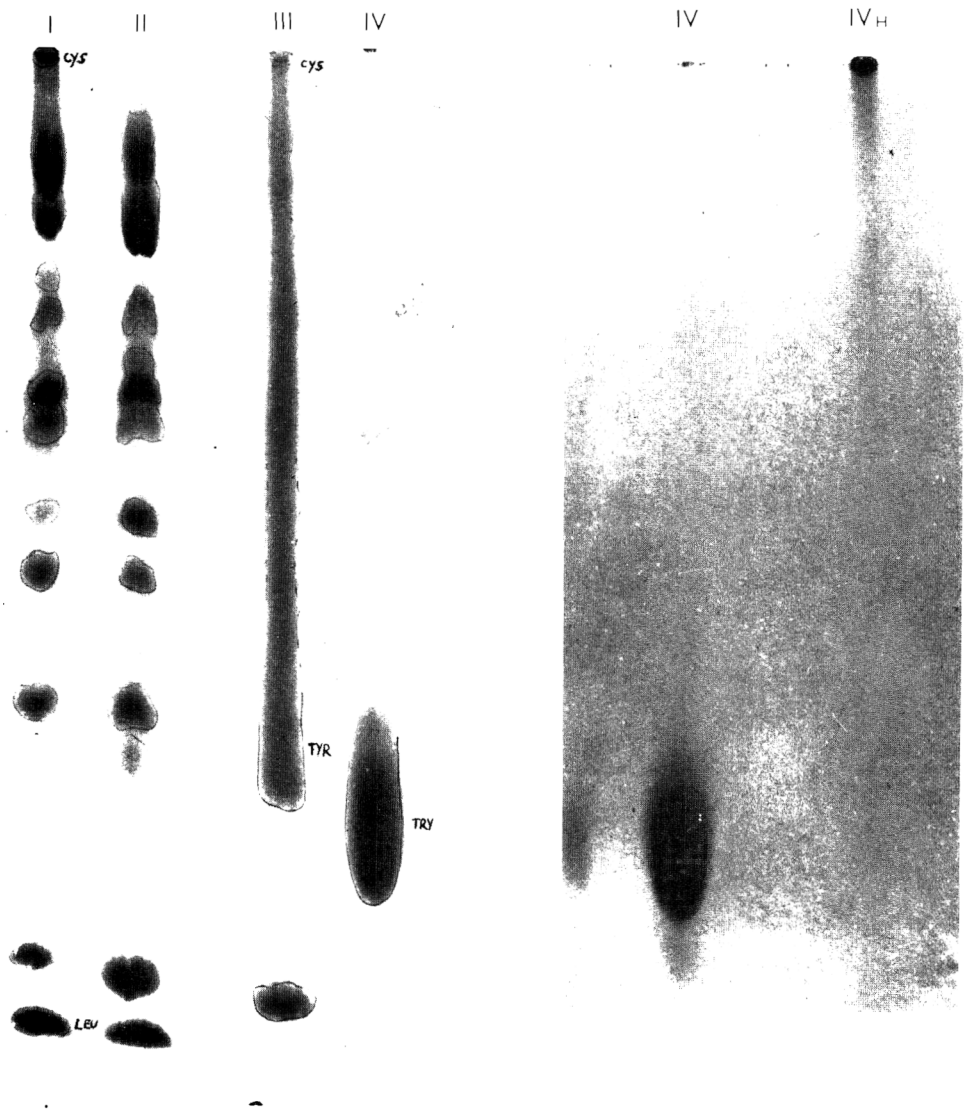

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Studies on the Mechanism of Inhibition of Growth of *Vibrio cholerae* by Erythrose

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SUMMARY

Erythrose strongly inhibits the growth of *Vibrio cholerae* and *Vibrio eltor*. The inhibition can be reversed by washing the cells free from erythrose with sterile normal saline. The respiration of *V. cholerae* cells in the presence of glucose is markedly inhibited by erythrose but the oxygen uptake of cell-free extract under the same conditions is not affected. From the results of experiments on the uptake of [^{14}C]glucose and [α-^{14}C]methylglucoside, it may be concluded that erythrose inhibits the transport of glucose across the cell membrane and thereby inhibits the growth of the organism.

INTRODUCTION

It has been reported (Roy Chowdhury & Datta, 1965) that erythrose has a marked inhibitory effect on the growth of *Vibrio cholerae*, when grown on synthetic medium containing glucose. This growth inhibitory effect of erythrose could be demonstrated even when glucose was replaced by different hexoses or pentoses. The growth inhibitory effect of erythrose was found to be proportional to the concentration of erythrose in the medium; it could also be demonstrated in experiments *in vivo* (Bhattacharya, Roy Chowdhury & Datta, 1965). This paper presents a more detailed biochemical study on the mechanism of this growth-inhibitory effect of erythrose.

METHODS

Organisms and media. Vibrio cholerae (Inaba type) or *Vibrio eltor* were grown for 18 h. at 37° on a synthetic medium of Finkelstein & Lankford (1955) containing glucose instead of sucrose, as the sole carbon source. Glucose was added aseptically, according to the concentration needed, as a 50 % (w/v) solution sterilized by steaming for three periods of half an hour each. Growth was assayed by measuring the turbidity increase in 18 h. using a Klett Summerson photoelectric colorimeter at 540 nm. For this purpose 50 ml. conical flasks, with optically matched sidearms to fit in the colorimeter, were used.

Respiration studies. The vibrios were grown on the synthetic medium and washed by centrifuging three times in cold normal saline (pH 7·8). Oxygen consumption was measured in a Warburg apparatus by the conventional method (Umbreit, Burris & Stauffer, 1959) at 37° using air as the gas phase. The results have been corrected for endogenous respiration.

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Preparation of cell-free extract. The vibrios were grown in 500 ml. conical flasks containing 100 ml. of synthetic medium with the requisite amount of glucose, on a rotary shaker at 35°. Organisms from two to three flasks were pooled and washed three times in cold normal saline by centrifugation at 3500*g* in a Sorvall refrigerated centrifuge. The packed cells were transferred to a chilled mortar with a minimal volume of cold 0·05M-K phosphate buffer (pH 7·4) and homogenized with an equal weight of chilled glass-powder for 1 to 2 min. The mixture was then extracted with a minimal volume of the same buffer, quickly transferred to a chilled centrifuge tube, and the glass powder and cell debris removed by centrifugation at 10,000*g* for 10 min. The resulting supernatant (cell-free extract) was dialysed for 2 h. against deionized water and used for the respirometric experiments. The whole operation was carried out at 0 to 4°.

Assay of different enzymes. Undialysed cell-free extract of *Vibrio cholerae*, prepared as stated above, was used. Hexokinase (Racker, 1947), glucose-6-phosphate dehydrogenase (Kornberg & Horecker, 1955) and phosphohexoseisomerase (Slein, 1955) were assayed by measuring the increase in absorbance at 340 nm. in a Zeiss spectrophotometer, using 1 cm. light-path cuvettes. Protein concentrations were estimated by the method of Lowry, Rosebrough, Farr & Randall (1951) with a bovine serum albumin (Sigma Chemicals) as standard.

[^{14}C]*Glucose uptake. Vibrio cholerae* was grown overnight in synthetic medium, harvested in a refrigerated centrifuge, washed twice with five volumes of cold normal saline and finally suspended in two volumes of cold normal saline. Usually 0·5 ml. of this cell suspension (0·25 g. organisms, wet wt) was incubated with 550 μmoles of [^{14}C] glucose (36,000 counts/min.) at 37° for 30 min. either in the presence or absence of 50 μmoles of erythrose. After incubation, the bacteria were washed three times with five volumes of cold normal saline and finally bacteria suspended in 1 ml. of distilled water: 0·5 ml. of this suspension was dried on an aluminium planchet by means of an infrared lamp for measurement of radioactive counts. In addition a suitable sample of the suspension was used for determination of the protein content.

[α-^{14}C]*Methylglucoside.* This radiochemical was prepared according to the method of Bollenback (1963) using uniformly labelled [^{14}C]glucose and methanol in the presence of a cation exchange resin.

^{14}*Carbon counts* were measured in a Tracer Lab Super scaler Gas-flow counter, Model SC 18 A.

Chemicals. The following were obtained from Sigma: glycolaldehyde, erythrose, glyceraldehyde, sodium pyruvate, DL-glyceraldehyde-3-phosphate (diethyl acetal barium salt), glucose-6-phosphate, 6-phosphogluconate, ATP, NAD, NADP and tris (hydroxymethylaminomethane). Analar (British Drug Houses, Poole, England) glucose, 2,4-dinitrophenol (DNP) and erythritol were used in these experiments. Erythrose was obtained from L. Light & Co., Colnbrook, England. D-Threose was kindly supplied by Dr Perlin, and erythrose-4-phosphate was a gift from Professor B. L. Horecker. [^{14}C]Glucose (uniformly labelled) was supplied by the Bhabha Atomic Research Centre, India.

RESULTS

Inhibition of growth

When it was observed that five- and six-carbon sugars did not inhibit the growth of *Vibrio cholerae* (Roy Chowdhury & Datta, 1965), sugars of shorter carbon-chain length and their derivatives were tested using the same procedure as described earlier. They were particularly chosen to provide either terminal groups identical with those of erythrose, or only one terminal group similar to erythrose. The compounds tested were threose, erythritol, glyceraldehyde, glyceraldehyde-3-phosphate, erythrose-4-phosphate and glycolaldehyde: all except erythritol inhibited growth (Table 1). The inhibitory compounds have the common characteristic of a free aldehyde group on the first carbon. Erythritol, in contrast to the rest of the compounds, does not have this reducing group on the first carbon and it may be noted that it did not produce any inhibitory effect on the growth of *V. cholerae*. Erythrose similarly inhibited the growth of *V. eltor*, the causative organism of a closely related disease to cholera. Under the same experimental conditions as described in Table 1, the turbidity change due to growth of *V. eltor* with glucose as sole carbon source was 300 whereas in presence of glucose and erythrose the turbidity change was only 20.

Since the experiments *in vivo* (Bhattacharya *et al.* 1965) indicated that erythrose is only vibriostatic and not vibriocidal, an attempt was made to study the same phenomenon *in vitro*. The inhibitory effect of erythrose was found to be a reversible process and could be removed by merely washing the vibrios. When the organisms were incubated in synthetic medium containing erythrose, no turbidity increase occurred in 18 h. If these inhibited vibrios were centrifuged, washed aseptically three times in sterile normal saline and then transferred to fresh medium containing glucose, growth occurred during the next 18 h. The increase in turbidity was similar to that of organisms which had not been exposed to erythrose. This alternate inhibition and restoration of growth after washing could be repeated at least three successive times with the same culture.

Growth of *Vibrio cholerae* on peptone broth medium instead of the synthetic medium was not inhibited by erythrose, and the addition of a mixture of 20 amino acids to the synthetic medium completely prevented inhibition by erythrose. Cysteine alone could reverse the inhibitory effect of erythrose but could not support the growth of the organism when added as sole carbon source (Table 2). Cysteine, by itself, has very little effect on the growth of *V. cholerae*.

The growth-inhibitory effect was thus specific for the aldehyde group at the carbon-one position of the sugar molecule; it was not bactericidal and could be removed by washing the organisms.

Inhibition of glucose metabolism

The glucose respiration of non-dividing *Vibrio cholerae* was almost completely inhibited in the presence of erythrose (Fig. 1). Although there was a small oxygen consumption with erythrose alone as substrate, the oxygen consumption was almost nil when both glucose and erythrose were added together. With cell-free extracts of *V. cholerae* using glucose as substrate, however, there was no appreciable inhibition in the presence of erythrose (Fig. 2). Attempts to abolish the initial lag in the uptake of oxygen by the addition of ATP and almost all the known cofactors were unsuccessful. When

Table 1. *Effect of sugars and sugar derivatives on the growth of Vibrio cholerae*

The increase in turbidity (in Klett units) was determined after 18 h. incubation at 37° in 10 ml. synthetic medium + 550 μmoles glucose + 50 μmoles of additional sugar or derivative.

System	Turbidity increase
Glucose	200
Glucose + erythrose	0
Glucose + threose	0
Glucose + glyceraldehyde	0
Glucose + erythrose-4-phosphate	0
Glucose + glyceraldehyde-3-phosphate	0
Glucose + erythritol	195

Table 2. *Effect of cysteine on the growth inhibitory effect of erythrose on Vibrio cholerae*

Turbidity change (in Klett units) determined after 18 h. at 37° in 10 ml. synthetic medium + 550 μmoles glucose; cysteine and erythrose (50 μmoles of each) added as indicated.

System	Turbidity increase
Glucose	198
Glucose + cysteine	210
Glucose + erythrose	0
Glucose + erythrose + cysteine	195
Cysteine	45

6-phosphogluconate and pyruvate, the primary reactants of the hexose monophosphate shunt pathway and the tricarboxylic acid cycle, were used as substrates, oxygen uptake by cell-free extract was also not inhibited by erythrose.

It has been reported by Racker, Klybas & Schramm (1959) and Fluharty & Ballou (1959) that D-threose 2,4-diphosphate strongly inhibits glyceraldehyde-3-phosphate dehydrogenase. Furthermore, Grazi, De Flora & Pontremoli (1960) and subsequently Venkataraman & Racker (1961) have shown that erythrose-4-phosphate strongly inhibits phosphohexose-isomerase. Hence studies were made with cell-free extract of *Vibrio cholerae*, in which the activities of certain enzymes were assayed spectrophotometrically in the presence and absence of erythrose. The activities of hexokinase, glucose-6-phosphate dehydrogenase, phosphohexoseisomerase and glyceraldehyde-3-phosphate dehydrogenase were not inhibited significantly by erythrose.

Inhibition of oxygen consumption of whole cells by erythrose and failure to produce such inhibition in experiments with cell-free extracts led us to investigate the effect of erythrose on the uptake of glucose by whole cells.

Uptake of glucose

When *Vibrio cholerae* was grown overnight (18 h.) in a synthetic medium in the presence and absence of erythrose together with [^{14}C]glucose, parallel effects by erythrose on inhibition of growth and inhibition of [^{14}C]glucose uptake were found (Table 3).

Since in this experiment glucose uptake by *Vibrio cholerae* cells was measured during an 18 h. growth period, with the possibility of glucose being metabolized during that long period of incubation, short-term incorporation studies were made using resting

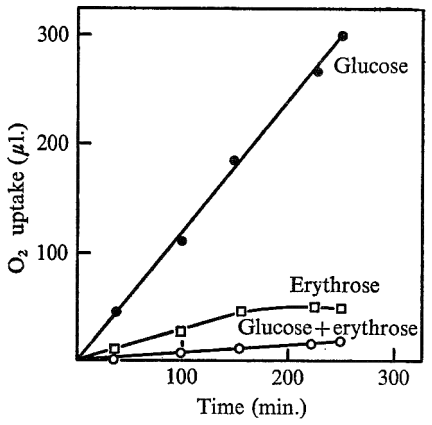

Fig. 1

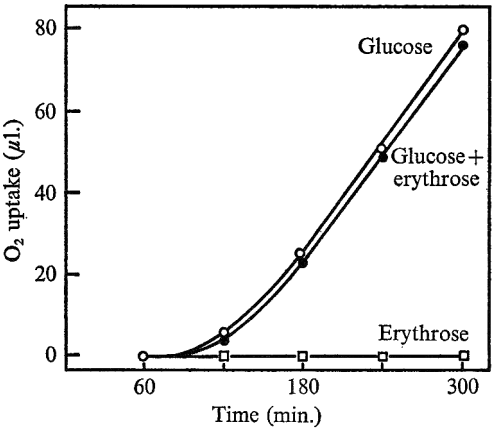

Fig. 2

Fig. 1. Effect of erythrose on the oxygen uptake of non-dividing *Vibrio cholerae*. Final volume 3 ml., containing 150 µmoles K phosphate buffer, pH 7·4; 10 µmoles glucose; 1 µmole erythrose (where indicated) and 1·0 ml. bacterial suspension (6 mg. protein). The centre well contained 0·15 ml. 30% KOH. Gas phase, air; temp. 37°.

Fig. 2. Effect of erythrose on the oxygen uptake of cell-free extracts of *Vibrio cholerae*. Final volume 3 ml. containing 150 µmoles K phosphate buffer, pH 7·4; 10 µmoles glucose; 3 µmoles ATP; 1 µmole erythrose and 1·0 ml. of cell-free extract (4·5 mg. protein). The centre well contained 0·15 ml. of 30% KOH. Gas phase, air; temp. 37°.

Table 3. *Effect of erythrose on* [^{14}C]*glucose uptake during growth of Vibrio cholerae*

Flasks containing 560 µmoles of unlabelled glucose, 1 µCi of [^{14}C]glucose and 50 µmoles of erythrose (where required) in a final volume of 10 ml. synthetic medium were inoculated with *V. cholerae* and incubated at 37° for 18 h. Growth was measured in terms of increase in turbidity (Klett units). After 18 h. the organisms were washed twice with normal saline and once with distilled water, suspended in 1 ml. of distilled water and 0·5 ml. of this suspension used for counting the radioactivity. Protein was measured on the remaining suspension and the results are expressed as counts/min. mg. protein.

	Uptake of ^{14}C		Growth	
System	counts/min./ mg. protein	Inhibition (%)	Turbidity increase in 18 h.	Inhibition (%)
Glucose	1012	—	145	—
Glucose+erythrose	150	85	20	86

suspensions of *V. cholerae*. The results in Table 4 confirm that erythrose effectively inhibited the uptake of [^{14}C]glucose by *V. cholerae*. It may be recalled (see Table 2) that the inhibitory effect of erythrose on the growth of the organism was reversed by the addition of cysteine. From Table 4 it may be seen that the inhibition of [^{14}C]glucose uptake by the *V. cholerae* in the presence of erythrose was also reversed by cysteine which was without effect in the absence of erythrose. Table 4 also shows that the inhibition of glucose uptake by 2,4-dinitrophenol was accentuated by erythrose.

Cohen & Monod (1957) have shown that α-methylglucoside, normally a non-

metabolizable glucose derivative, is useful in the study of the glucose permeation mechanism in bacteria. When α-methylglucoside was added to the synthetic medium in place of glucose, it did not support growth of *Vibrio cholerae* indicating that it is not measurably metabolized by the organism. The compound accumulated inside the cell, however, at a linear rate for 2 h. (Fig. 3). Glucose at a concentration of 10^{-4} M considerably inhibited the uptake of [α-^{14}C]methylglucoside (Fig. 3).

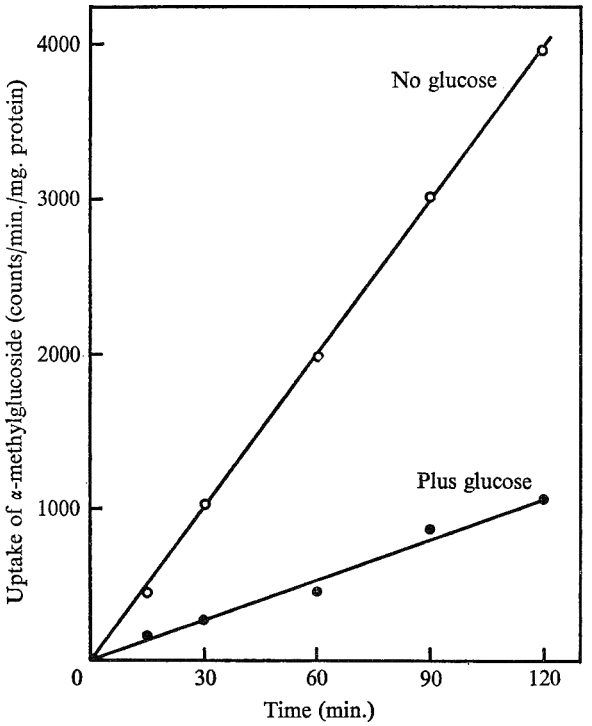

Fig. 3. Effect of glucose on the uptake of [α-^{14}C]methylglucoside by *Vibrio cholerae*. Incubation mixture contained 0·5 ml. bacterial suspension, 10 μmoles [α-^{14}C]methylglucoside (1000 counts/min./μmole) and 0·1 μmole glucose in a final volume of 1·0 ml. The reaction mixtures were incubated for different periods of time and uptake measured as Table 3.

Table 4. *Effect of erythrose on the uptake of* [^{14}C]*glucose by non-growing Vibrio cholerae*

The incubation mixture contained 550 μmoles of [^{14}C]glucose (36,000 total counts/min.) and 0·5 ml. bacterial suspension in a final volume of 10 ml. normal saline with, where indicated, erythrose, 50 μmoles; 2,4-dinitrophenol, 25 μmoles; cysteine, 10 μmoles. After 30 min. incubation at 37° the organisms were centrifuged, washed and treated as described in Table 3.

System	Uptake of ^{14}C (counts/min./ mg. protein)
Glucose	3030
Glucose + erythrose	218
Glucose + 2,4-dinitrophenol	607
Glucose + 2,4-dinitrophenol + erythrose	101
Glucose + cysteine	3010
Glucose + cysteine + erythrose	2800

To check whether [α-^{14}C]methylglucoside was transported intact into the bacteria, *Vibrio cholerae* was exposed to the glucoside and then collected by centrifugation. After washing twice with 5 ml. normal saline the packed organisms were extracted with 3 × 5 ml. portions of hot water. The combined washings were concentrated to about 1·0 ml. and 0·05 ml. samples were chromatographed on Whatman no. 1 filter paper using butanol:acetic acid:water (60:30:10). Radioautograms of these extracts showed a single radioactive spot with and R_F value identical to that of authentic α-methylglucoside. Furthermore, 80 % of the total radioactivity applied to the paper could be eluted from the α-methylglucoside region and when co-chromatographed with authentic [α-^{14}C]methylglucoside gave one single radioactive spot. The above observations suggest that α-methylglucoside was transported into *V. cholerae* as free α-methylglucoside through the mediation of the permease responsible for the entry of glucose.

Table 5 shows that erythrose inhibited the uptake of [α-^{14}C]methylglucoside by *Vibrio cholerae*. Attempts were made to check whether the inhibition of α-methylglucoside uptake by erythrose was of competitive nature by plotting the reciprocals

Table 5. *Effect of erythrose on the uptake of* [α-^{14}C]*methylglucoside by non-growing Vibrio cholerae*

The incubation mixture contained 0·5 ml. bacterial suspension, 10 μmoles of [α-^{14}C]methylglucoside (1000 counts/min./μmole) in a final volume of 1·0 ml. normal saline. Erythrose, 1 μmole. Incubation for 30 min. at 37°, and then the uptake of ^{14}C measured as in Table 3.

System	Uptake of ^{14}C (counts/min./mg. protein)
α-Methylglucoside	1058
α-Methylglucoside + erythrose	328

of the observed uptakes of α-methylglucoside at different concentrations of the glucoside in the presence and absence of erythrose. Results of these experiments were erratic and no definite conclusion could be made.

DISCUSSION

The growth inhibitory effect described was dependent on the presence of a free terminal aldehyde group in a sugar molecule with a chain length of not more than four carbons: the lack of inhibition by pentoses or hexoses may be due to the fact that when in a ring form their aldehyde groups are masked.

The results indicate that erythrose does not inhibit any one of the major pathways of glucose catabolism (e.g. glycolytic, shunt pathway and tricarboxylic acid cycle) in cell-free extracts of *Vibrio cholerae*, whereas it does inhibit very strongly the oxygen uptake of whole organisms with glucose as substrate. This effect is explained by the marked inhibitory effect of erythrose on the uptake of [^{14}C]glucose and [α-^{14}C]methylglucoside by *V. cholerae*, an effect demonstrable even with bacteria in the growth phase. Since transport of substrates across bacterial membranes is believed to be mediated by stereospecific proteins, or 'permeases' (Cohen & Monod, 1957), the growth inhibitory effect of erythrose on *V. cholerae* might be on the 'gluco-permease' system. Such systems are likely to be depressed in the presence of a typical uncoupling agent

like 2,4-dinitrophenol which markedly reduced the glucose uptake by non-growing organisms, and this inhibitory effect was increased by erythrose.

Results presented in Table 3 to 5 strongly suggest the inhibitory effect of erythrose to be on the transport of glucose across the bacterial cell membrane. The concluding experiment, which was done to establish this 'glucose-permease' inhibition by erythrose, was to study the uptake of [α-^{14}C]methylglucoside in non-growing *Vibrio cholerae*. This glucoside appears to be transported into bacteria by the same 'permease mechanism as is needed for glucose (Cohen & Monod, 1957) and so the rate of uptake of α-methylglucoside by bacteria can be used as an indirect measure of the 'glucose-permease' activity of an organism. It was very interesting to note that erythrose caused 70 % inhibition of the uptake of α-methylglucoside (Table 5). This inhibition of α-methylglucoside uptake confirms the mode of inhibition by this four-carbon sugar on *V. cholerae* to be on the permeation of glucose into the organism.

Recently Együd & Szent-Györgyi (1966*a*) reported that methylglyoxal, a ketoaldehyde, inhibits strongly the growth of *Escherichia coli* as well as the synthesis of protein in the same organism (Együd & Szent-Györgyi, 1966*b*). The inhibition of protein synthesis by methylglyoxal can be reversed by the addition of cysteine (Együd & Szent-Györgyi, 1966*b*). Szent-Györgyi, Együd & McLaughlin (1967) suggested that the methylglyoxal inhibition could be due to a stoichiometric reaction between the ketone aldehyde and certain sulphydryl groups and the reversal by cysteine could be due to regeneration of these sulphydryl groups. In our system, growth inhibition by erythrose could be due to an interaction between the four-carbon sugar and sulphydryl groups, of the gluco-permease of *Vibrio cholerae* and the reversal by cysteine could be due to the regeneration of these groups.

In conclusion, it may be said that erythrose primarily inhibits the glucose uptake by *Vibrio cholerae*, which, secondarily, leads to the inhibition of growth of this organism.

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Growth Inhibition of Soil Fungi by Insecticides and Annulment of Inhibition by Yeast Extract or Nitrogenous Nutrients

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SUMMARY

Seventeen fungal species from Wisconsin prairie soils were grown on Czapek nutrient media that had been treated with the insecticides aldrin, lindane, parathion, phorate or carbaryl. All five insecticides inhibited to some extent the growth of most fungal species; this inhibition was a result of a particular insecticide–fungus combination. Threshold concentrations of insecticides, at which no decrease in growth of *Aspergillus fumigatus* or *Fusarium oxysporum* occurred, differed for each insecticide and also for each of the two fungi. Since most of the insecticides had some fungicidal effect, it was not surprising that none of the 17 fungi was able to utilize any of the insecticides as a carbon or phosphorus source. Carbaryl or aldrin at 20 μg./ml. inhibited growth of *F. oxysporum* by 37 to 44 %. However, the addition of yeast extract, asparagine, ammonium sulphate, ammonium nitrate or ammonium sulphamate to the culture media resulted in a complete suppression of the growth inhibitory effect of carbaryl and that of aldrin to a large extent Replacement of yeast extract with a vitamin mixture had no effect; fungal growth was still inhibited.

INTRODUCTION

The use of agricultural chemicals over the last 25 years has presented problems pertaining to the interaction of residual pesticides of long persistence with biological systems in the environment. A considerable amount of pesticides, even if not applied directly to soils, ultimately ends in the upper soil layers. Hence the interaction of pesticides with soil micro-organisms has drawn considerable attention from many workers (Engst & Kujawa, 1968; Matsumura, Boush & Tai, 1968; Kearney, Kaufman, von Endt & Guardia, 1969; Sethunathan & MacRae, 1969). Many of these studies dealt with the problem of reducing the concentration of insecticidal residues in soils. Efforts were made to isolate species of micro-organisms that would be able to degrade and to detoxify pesticidal residues, especially those of a more persistent nature; also the effects of pesticidal residues on micro-organisms in soil are of importance (Ko & Lockwood, 1968). Richardson & Miller (1960) tested the toxic effects of eight chlorinated hydrocarbon insecticides on mycelial growth of some fungi; they concluded that the water solubility and vapour pressure of a particular insecticide were major factors responsible for the degree of toxicity to fungi as determined by the diameter of

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mycelia produced. In the present study an attempt has been made to investigate the toxic effects of some chlorinated hydrocarbon, organophosphorus and carbamate insecticides on soil fungi as affected by nutritional factors.

METHODS

Organisms. Fungi used in this study were isolated by Orpurt & Curtis (1957) from the upper inch layer of Wisconsin prairie soils, which had not been disturbed by grazing or ploughing. Because of their similarities to Wisconsin agricultural soils, prairie soils were chosen as the source of the fungal species. Seventeen fungi were selected on account of their high frequency in these soils; nine of these fungal species belonged to the genus *Penicillium*.

Culture media. The basal culture medium used was a modified Czapek nutrient agar (Ainsworth, 1960) in which glucose was the carbon source.

The insecticides used were two chlorinated hydrocarbon compounds (aldrin, lindane), two organophosphorus compounds (parathion and phorate) and one carbamate (carbaryl or Sevin®). They were all of analytical grade. Ethanol (95 %, v/v in water) was used as solvent for the insecticides.

Yeast extract, thiamine, riboflavin, pyridoxine, niacin, pantothenic acid, biotin, inositol, choline, folic acid and vitamin B_{12} were added to some culture media to test the potential interaction of insecticides with various growth-promoting substances. The effects of asparagine, ammonium sulphate, ammonium nitrate and ammonium sulphamate were also investigated.

Treatment and inoculation of culture media. Culture media were sterilized by autoclaving for 20 min. at 12·0 lb. pressure. After the media had cooled to 45 to 50° and while still liquid they were treated with different ethanolic solutions of the insecticides. The concentration of ethanol never exceeded 0·2 % (v/v) in the medium. Culture media that had been treated only with ethanol served as controls. The various growth substances of nitrogenous materials were added in aqueous solution before sterilization of the culture media.

About 20 ml. of each medium were poured into each of 4 Petri dishes, to give 4 replicates. Plates were inoculated by transferring a small agar block containing hyphal tips from a standard culture of one of the fungi which previously had been grown on the basal culture medium. Plates were then incubated as described for each experiment.

Evaluation of insecticidal effects. At the end of the incubation period relative sporulation was evaluated by visual inspection, and the diameter of each fungus colony was recorded. The colonies were harvested for dry weight determinations by melting the agar at 100° and transferring to a boiling water bath. The colonies were then washed for 2 min. in three successive baths to remove agar adhering to the mycelia. Each mycelial mat was then placed on an oven-dried and tared filter paper in a Buchner funnel and washed with warm water. The washed colonies and the filter paper were dried overnight at 80 to 90° before the final weight determinations. It was felt that determination of the dry weight was the most reliable criterion for measuring insecticide toxicity. All data were analysed with the t-test.

Basically, the following four experiments were conducted.

Experiment 1. Effects of insecticides on growth of soil microfungi. This experiment was done as an initial screening test for establishing toxic effects and the selection of

specific fungi for future investigations. Basal culture media were treated at the relatively high dosage of 40 ug./ml. with either aldrin, lindane, parathion, phorate or carbaryl. These media were then inoculated, giving 85 combinations of 5 insecticides and 17 fungi. Depending on the growth rate of each species the fungi were incubated at $25 \pm 1°$ for 7 to 12 days. Relative amounts of sporulation were then determined and growth measurements (diameter, weight) were made.

Experiment 2. Threshold concentrations of insecticides affecting growth of two microfungi. Tests were made to determine the highest insecticide concentration (threshold concentration) at which no significant (1 % level, t-test) decrease of fungal growth occurred. For this *Aspergillus fumigatus* and *Fusarium oxysporum* were exposed to increasing concentrations (1, 2, 5, 10, 20, 40 μg./ml.) of each of the five insecticides. These fungi were selected because of their relatively rapid growth rate and their susceptibility to all five insecticides. After inoculation of the culture media, these were incubated for 5 days at $34 \pm 3°$. The dry weight of each fungus colony was then determined.

Experiment 3. Effects of insecticides on fungi, grown on media lacking glucose or inorganic phosphate. Culture media were prepared that lacked glucose or inorganic phosphate (K_2HPO_4). The glucose-deficient media were treated with one of the five insecticides at 40 μg./ml. while the potassium phosphate-deficient media were treated with parathion or phorate at 40 μg./ml. Media deficient in glucose or inorganic phosphate, but treated with ethanol only, served as controls. Results (growth and sporulation measurements) were then compared with data obtained after fungi had also grown on complete media, previously treated with either ethanol or with an insecticide in ethanol.

Experiment 4. Counteraction of insecticidal effects. (*a*) With yeast extract. An experiment identical to the tests for the determination of threshold concentrations (Expt. 2) was made, except that 0·15 % (w/v) yeast extract ('Difco') was added to the culture media. This was done to determine whether the presence of yeast extract counteracted inhibitory effects of the insecticides on the growth of *Aspergillus fumigatus* and *Fusarium oxysporum*. Production of fungal dry weight was used as a criterion for the evaluation of the effects of yeast extract and insecticides. Culture media + yeast extract but no insecticide served as controls; results were also compared with those obtained in the absence of yeast extract (Expt. 2).

(*b*) With vitamins. Experiments were also made with components of yeast extract (e.g. vitamins) with *Fusarium oxysporum* as test organism. This fungus, a very characteristic species of prairie soils, was selected because of its general sensitivity to the insecticides. It was grown on aldrin or carbaryl-treated (20 μg./ml.) culture media to which yeast extract (control) or vitamins had been added.

Vitamins were used to determine whether the action of insecticides in decreasing the growth rate of the fungus was related to the function of certain vitamins within the mycelium. Aqueous solutions of the vitamins were prepared to give (per l.) 2 mg. thiamine, 10 mg. riboflavin, 5 mg. pyridoxine, 30 mg. niacin, 30 mg. pantothenic acid, 1 mg. biotin, 0·75 g. inositol, 0·75 g. choline, 1 mg. folic acid and 15 mg. vitamin B_{12}. One ml. of this solution was added to 100 ml. of basic medium, from which four agar plates were poured. The concentration of these vitamins was slightly higher than the amounts that would be present in yeast extract at a concentration of 0·15 % (w/v).

(*c*) With various nitrogenous compounds. Since the nature of the nitrogen source

in the culture media might also be a factor that influences the degree of growth inhibition by insecticides, four nitrogen-containing compounds were used in addition to the $NaNO_3$ which was a component of the basal culture medium. For this purpose, media were prepared to which asparagine, $(NH_4)_2SO_4$, NH_4NO_3 or $NH_4SO_3NH_2$ were added to 0·15 % (w/v). These four different media were then treated with aldrin or carbaryl at 20 μg./ml., followed by inoculation. Colonies of the fungi were incubated and handled as described in Expt. 2.

RESULTS AND DISCUSSION

Experiment 1. Effects of insecticides on growth of soil microfungi. Results on the effect of aldrin, lindane, parathion, phorate or carbaryl on growth and sporulation of 17 soil microfungi are summarized in Table 1. They are expressed as the dry weight of each fungus from insecticide-treated media as a percentage of the dry weight that was produced in the absence of insecticides. All five insecticides inhibited to some extent the growth of most fungal species although various degrees of growth inhibition could be noticed.

Table 1. *Effect of five insecticides (at 40 μg./ml. in basic culture medium) on the growth and sporulation of various soil microfungi*

	Dry Weight as % of Control (culture medium + 0·2 % ethanol)				
Fungus	Aldrin	Lindane	Parathion	Phorate	Carbaryl
Acrostalagmus sp.	97 ± 10·7[a]	109 ± 13·3	94 ± 10·2	89 ± 3·1[c]	79 ± 7·0[b]
Aspergillus fumigatus	76 ± 4·2[b]	75 ± 10·3[c]	58 ± 2·7[b]	62 ± 16·3[b]	52 ± 10·6[b]
A. terreus	99 ± 8·6	29 ± 5·7[b d]	52 ± 9·0[b]	57 ± 3·0[b d]	35 ± 3·8[b]
Emericellopsis sp.	45 ± 15·4[b]	66 ± 4·6[c]	14 ± 1·0[b]	100 ± 1·0	25 ± 2·7[b]
Fusarium oxysporum	45 ± 4·7[b]	34 ± 6·9[b]	18 ± 3·5[b]	14 ± 2·2[b]	27 ± 3·6[b]
Myrothecium strigtosporum	100 ± 14·3	12 ± 1·0[b]	20 ± 3·2[b]	42 ± 3·7[b]	32 ± 4·8[b]
Thielaviopsis sulphurellum	82 ± 4·0	49 ± 2·6[b]	19 ± 1·4[b]	99 ± 2·5	27 ± 3·1[b]
Penicillium janthinellum	78 ± 5·4[b e]	45 ± 4·5[b d]	38 ± 3·2[b]	92 ± 3·0[c]	43 ± 3·9[b]
P. javanicum	70 ± 4·8[b]	77 ± 11·8[c]	55 ± 3·0[b]		
P. lilacinum	96 ± 1·8	56 ± 8·1[b]	60 ± 5·9[b]	65 ± 3·2[b]	44 ± 3·5[b]
P. nigricans	89 ± 5.8[e]	40 ± 3·3[b d]	62 ± 2·2[b d]	87 ± 1·8[b d]	58 ± 3·5[b d]
P. restrictum	84 ± 5·0[c]	67 ± 1·1[b]	55 ± 3·2[b]	102 ± 4·5	72 ± 6·5[b]
P. roseo-purpureum	87 ± 7·4[d]	34 ± 5·3[b d]	34 ± 2·6[b]	94 ± 2·4[c]	61 ± 3·1[b]
P. simplicissimum	83 ± 2·2[c e]	92 ± 9·2	69 ± 3·9[b d]	62 ± 2·8[b]	77 ± 4·1[b]
P. thomii	102 ± 2·5[d]	66 ± 6·8[b d]	52 ± 4·7[b e]	40 ± 1·8[b e]	6 ± 0·3[b d]
P. variabile	93 ± 4·9	49 ± 2·7[b]	61 ± 3·2[b e]	87 ± 4·1[b d]	51 ± 5·4[b]
Paecilomyces marquandii	101 ± 3·7[c]	26 ± 3·2[b]	39 ± 4·3[b e]	86 ± 2·1[b d]	98 ± 3·9

[a] = Standard deviation; [b] = inhibition significant at 1 % level; [c] = inhibition significant at 5 % level; [d] = decreased sporulation as compared to control; [e] = increased sporulation as compared to control.

Based on the number of species inhibited, aldrin was the least toxic since the growth of only 7 out of 17 species was significantly affected. Lindane inhibited the growth of 15 fungi, parathion of 16, phorate of 13 and carbaryl of 15 species. Based on the % decrease in dry weight, aldrin and phorate were least toxic, since the growth of only 2 and 3 fungi respectively was inhibited by more than 50 %. Lindane, parathion and carbaryl, however, caused a growth inhibition of more than 50 % with 7 to 9 fungal species. Since there was no species which was affected to the same degree by all insecticides and since there was no insecticide that affected all fungi in the same manner, the observed data are results of particular insecticide–fungus combinations.

Experiment 2. Threshold concentrations of insecticides affecting growth of two microfungi. After *Aspergillus fumigatus* and *Fusarium oxysporum* had been exposed to the insecticides, the dry weight of each mycelial mat was expressed as % dry weight of fungi grown on control media (ethanol-treated). Figure 1 summarizes the results obtained. Two conclusions can be drawn: (i) the highest concentration of the chemicals (Fig. 1 T) at which no significant (at the 1 % level) decrease of fungal growth occurred differed for each insecticide; (ii) this threshold concentration was also a function of

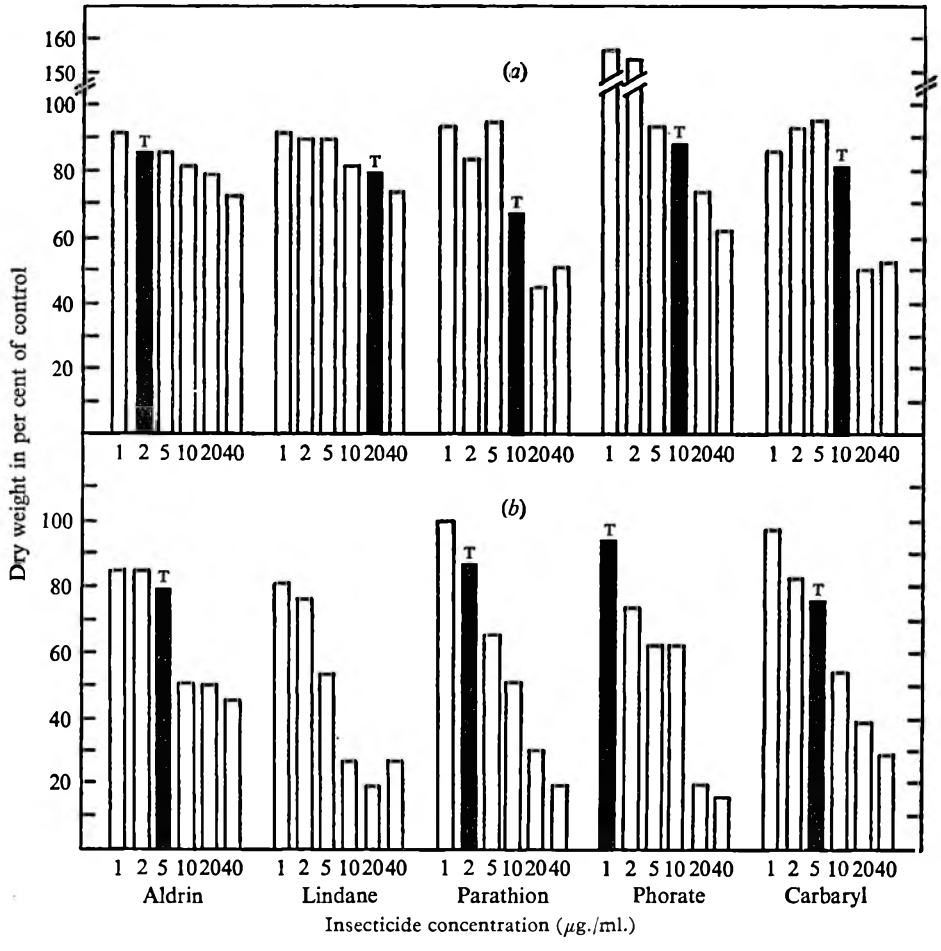

Fig. 1. Effects of five insecticides on the growth of *Aspergillus fumigatus* (*a*) and *Fusarium oxysporum* (*b*). T = Highest insecticide concentration (threshold) at which no significant (1 % level) decrease of fungal growth occurred.

the test organism, and was different for each of the two fungi. In general, growth of *A. fumigatus* (Fig. 1 *a*) was less affected by the insecticides than was the growth of *F. oxysporum* (Fig. 1 *b*). *Aspergillus fumigatus* was, however, very susceptible to aldrin, which at both 2 and 5 μg./ml. caused a 14 % decrease of fungal growth. Because the variation between replicates was relatively large at 2 μg./ml., this 'inhibition' was not significantly different from the control. The concentration of 2 μg./ml. of aldrin was

therefore regarded as the threshold that did not inhibit fungal growth. Phorate at 1 and 2 μg./ml. caused a significant (1 % level) growth stimulation of *A. fumigatus* while its threshold concentration was 10 μg./ml. With *F. oxysporum*, lindane inhibited its growth even at a concentration of 1 μg./ml. The threshold concentration of this insecticide, therefore, was below 1 μg./ml. Phorate inhibited the growth of this fungus at 2 μg./ml.

However, the threshold concentration as defined above (highest insecticide concentration at which no inhibition of fungal growth occurred) is not synonymous with the 'no effect' level. This is particularly shown with *Aspergillus fumigatus* in the presence of phorate (Fig. 1*a*). The threshold concentration of this insecticide was 10 μg./ml. However, a significant (1 % level) growth stimulation was observed with phorate at 1 and 2 μg./ml., resulting in a 57 and 54 % weight increase of the respective mycelia.

Experiment 3. Effects of insecticides on fungi grown on media lacking glucose or inorganic phosphate. Since most of the insecticides had some fungicidal effects, it was not surprising that none of the 17 fungi was able to utilize any of the insecticides as a carbon or phosphorus source. In the absence of glucose, fungi were still able to grow slightly (Pl. 1). With the exception of aldrin, the addition of insecticides to glucose-deficient media further inhibited the growth of the fungal colonies. Plate 1, fig. 1 shows the effect of lindane on the growth of *Penicillium lilacinum* on complete media (CK) and glucose-deficient media (−C), while Pl. 1, fig. 2 presents the effect of parathion on the growth of *Fusarium oxysporum* in both glucose-deficient media (−C) and phosphate-deficient media (−P). As shown in these examples, the absence of glucose resulted in decreased fungal growth which was further inhibited by lindane or parathion. Omission of inorganic phosphate, however, resulted in a 90 % inhibition of growth of *F. oxysporum*; the addition of parathion to the phosphate-deficient medium had no effect.

Since aldrin in nutrient-deficient media did not further inhibit fungal growth, it is possible that the concentration of this insecticide (40 μg./ml.) was too low to serve as a nutrient source.

Table 2. *Effect of additions to the basic culture medium*[a] *on the growth-inhibitory action of insecticides (at 20 μg./ml.) on Fusarium oxysporum*

Additions to basic medium[a]	Insecticide treatment: None	Carbaryl: Dry weight (mg.)	Carbaryl: % CK[b]	None	Aldrin: Dry weight (mg.)	Aldrin: % CK
None	39·8 ± 4·27[c]	22·3 ± 1·93	56	27·3 ± 2·19	17·1 ± 1·69	63
Vitamins[d]	39·9 ± 0·93	22·0 ± 1·14	55	26·5 ± 2·68	15·1 ± 2·20	57
Yeast extract	75·8 ± 3·60	74·8 ± 5·05	99[e]	90·5 ± 2·64	76·7 ± 1·62	85[e]
None	36·3 ± 1·53	21·5 ± 3·57	59	40·3 ± 2·57	26·3 ± 1·62	65
Asparagine	68·7 ± 2·03	63·2 ± 3·10	93[e]	73·5 ± 5·22	59·7 ± 4·90	81[e]
None	43·6 ± 3·25	28·4 ± 5·00	65	45·4 ± 4·20	26·9 ± 1·78	59
$(NH_4)_2SO_4$	56·4 ± 4·16	50·9 ± 3·01	90[f]	54·5 ± 3·67	39·4 ± 4·65	72[f]
NH_4NO_3	68·1 ± 3·28	67·6 ± 3·82	99[e]	63·7 ± 1·84	47·6 ± 0·75	75[e]
$NH_4SO_3NH_2$	60·0 ± 2·77	55·3 ± 3·27	92[f]	58·7 ± 2·45	41·0 ± 2·13	70[e]

[a] Basic culture medium contains $NaNO_3$ as the only nitrogen source; [b] % CK = insecticide-treated as % of untreated (none); [c] standard deviation; [d] thiamine, riboflavin, pyridoxine, niacin, pantothenic acid, biotin, folic acid, vitamin B_{12}, inositol, choline; [e] significantly different at the 1 % level (t-test) from medium not containing additives; [f] significantly different at the 5 % level (t-test) from medium not containing additives.

Experiment 4. Counteraction of insecticidal effects. (*a*) With yeast extract. The addition of yeast extract to the culture media decreased or counteracted the growth inhibitory effects of the insecticides and increased fungal growth in general (Pl. 2). Threshold concentrations of the insecticides were usually higher when yeast extract was included in the media. These concentrations, as measured with *Fusarium oxysporum*, increased from below 1 to 2 μg./ml. with lindane (Pl. 2, fig. 3), from 2 to 5 μg./ml. with parathion, and from 1 to 10 μg./ml. with phorate, and from 5 to 20 μg./ml. with carbaryl. The threshold concentration of aldrin was not affected, although the actual growth inhibition at higher concentrations was less pronounced with yeast extract than it was on media that did not contain it (Pl. 2, fig. 4).

(*b*) and (*c*) With vitamins and nitrogen nutrients. Table 2 summarizes the results obtained after *Fusarium oxysporum* had been grown on carbaryl or aldrin-treated media to which yeast extract, vitamins or different sources of nitrogen had been added. The insecticides by themselves inhibited fungal growth by 37 to 44 %. However, yeast extract completely suppressed the inhibition by carbaryl and that by aldrin to a large extent. Replacement of the yeast extract with the vitamin mixture had no effect, since fungal growth was still inhibited by 45 and 43 %. It appears, therefore, that the growth inhibitory effects of carbaryl and aldrin were not related to interference with the function of these vitamins in *F. oxysporum*.

Addition of asparagine, ammonium sulphate, ammonium nitrate or ammonium sulphamate to the insecticide-treated media had an effect similar to that observed with yeast extract. The nitrogenous compounds counteracted or neutralized the toxic effects of the insecticides on growth of *Fusarium oxysporum*. This was especially the case with carbaryl. The toxic effects of aldrin were also somewhat decreased through the presence of these compounds.

It thus appears that a reduced inorganic form of nitrogen, or an organic form, is a more favourable nitrogen source for *Fusarium oxysporum* than is nitrate nitrogen. Carbaryl and aldrin apparently inhibited the growth of *F. oxysporum* by interfering with the utilization of nitrate nitrogen. It also appears that there are other factors involved, since inhibition with aldrin was not completely overcome by the addition of these nitrogen sources. Carbaryl could have interfered with the absorption of nitrate or its subsequent metabolism in cells, while reduced or organic nitrogen compounds were not affected by this insecticide. Aldrin, on the other hand, could have interfered with the uptake or metabolism of both nitrate and reduced nitrogen, but to a greater degree with that of nitrate.

The data presented show that the five insecticides affect the growth of some soil microfungi in culture, and threshold studies show that the inhibitory effects occur at concentrations that might be encountered under field conditions. However, because of adsorption and metabolism phenomena in soils (Lichtenstein, 1966; Lichtenstein, Fuhremann & Schulz, 1968), these insecticides will have different effects in field soils.

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EXPLANATION OF PLATES

CK = Effects of insecticide in complete culture media; −C = effects of insecticide in glucose-deficient media; −P = effects of insecticide in phosphate-deficient media. Media to the right are insecticide-treated.

PLATE 1

Effect of insecticides on growth of fungi.

Fig. 1. *Penicillium lilacinum* on lindane-treated media.

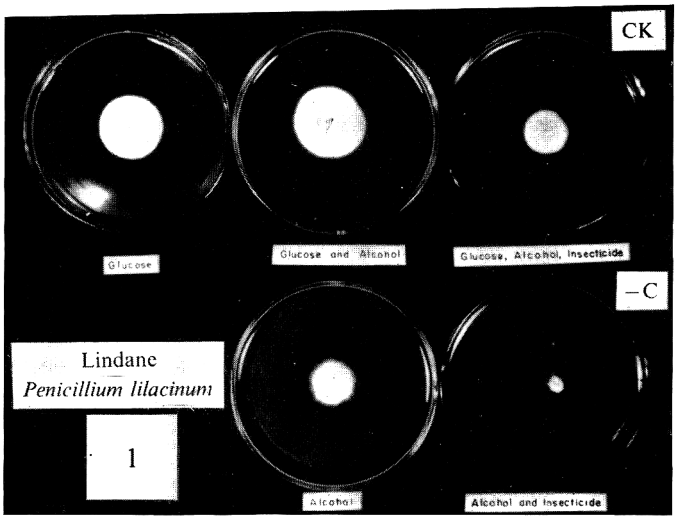
Fig. 2. *Fusarium oxysporum* on parathion-treated media.

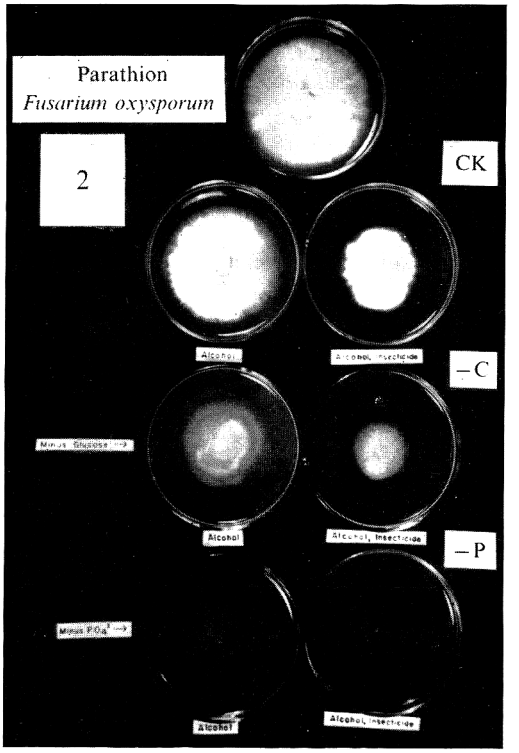
PLATE 2

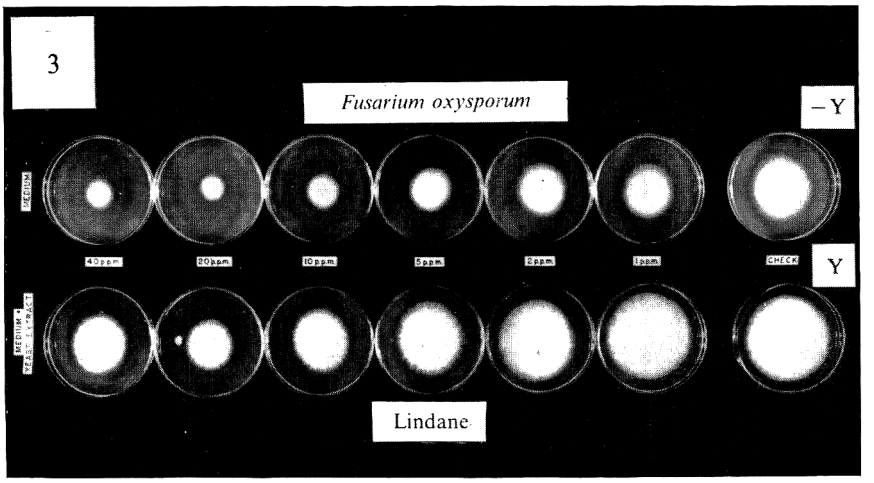
Effects of insecticides and yeast extract on *Fusarium oxysporum* grown on culture media with (Y) or without (−Y) yeast extract.

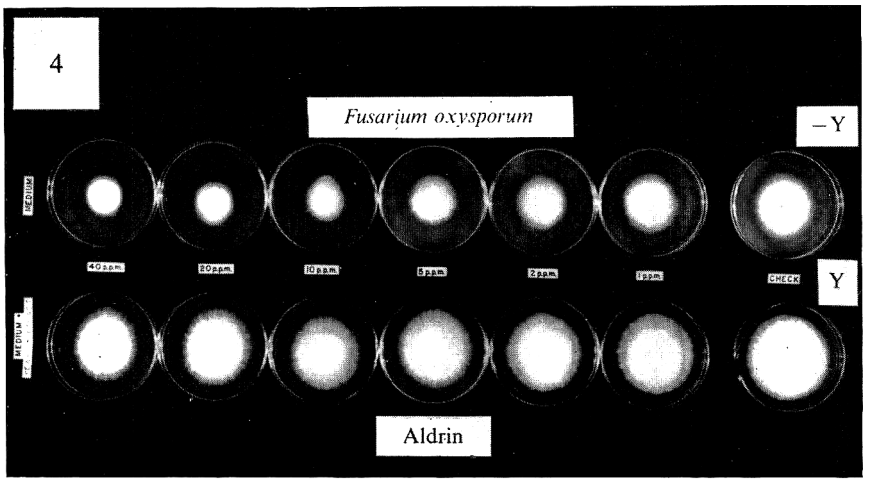
Fig. 3. Media treated with lindane.

Fig. 4. Media treated with aldrin.


CK
Glucose
Glucose and Alcohol
Glucose, Alcohol, Insecticide
−C
Lindane
Penicillium lilacinum
1
Alcohol
Alcohol and Insecticide


Parathion
Fusarium oxysporum
2
CK
Alcohol
−C
Alcohol
Alcohol, Insecticide
−P
Alcohol
Alcohol, Insecticide


3
Fusarium oxysporum
−Y
MEDIUM
40 p.p.m.
20 p.p.m.
10 p.p.m.
5 p.p.m.
2 p.p.m.
1 p.p.m.
CHECK
Y
Lindane


4
Fusarium oxysporum
−Y
MEDIUM
40 p.p.m.
20 p.p.m.
10 p.p.m.
5 p.p.m.
2 p.p.m.
1 p.p.m.
CHECK
Y
Aldrin

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Enhanced CO_2 Production by Yeast Exposed to Elevated Temperatures

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SUMMARY

After starvation, yeast exposed to elevated temperatures produced CO_2 twice as fast as unexposed organisms. The lag which preceded linear CO_2 production by starved yeast was essentially eliminated by heat treatment. Uptake and retention of sorbose was greater in heated yeast. Heating was accomplished by brief immersion of the organisms in heated solutions and by growth for 2 h. at 35°. Short heat treatments increased the production of CO_2 when glucose was included in the suspending medium, whereas heating in water or in growth medium without glucose resulted in a decreased production of CO_2.

INTRODUCTION

Temperature changes can markedly alter or completely inactivate biological processes (Wood, 1956; Ingraham, 1962; Farrell & Rose, 1967). Availability of nutrient (Sherman, 1959*a*; Begue & Lichstein, 1963) and age of culture (Rosenberg & Wood, 1957) have significant effects upon heat-induced changes in yeast, though the critically affected reactions have not been identified. Even small increases in temperature may affect control mechanisms and thus have far-reaching effects on organelle formation (Sherman, 1959*b*) and protein synthesis (Hartwell & McLaughlin, 1969; Schiebel, Chayka, DeVries & Rusch, 1969).

The effects of elevated temperatures on the anaerobic production of CO_2 by yeast were studied because this process and the enzymes involved are well known.

METHODS

Preparation of yeast samples. Cultures of *Saccharomyces cerevisiae* were grown at 28·5° as described previously (Spoerl & Doyle, 1968*a*). The organisms were harvested during exponential growth at 2×10^7/ml. and washed twice with distilled water by centrifugation. Samples for control tests were resuspended in distilled water for starvation or in one of the buffer solutions (see below) for measurement of CO_2 production or sorbose uptake. For starvation, suspensions (4×10^7 organisms/ml.) were shaken aerobically for 21 h. at 28·5°. This procedure was carried out as aseptically as was practicable; suspensions were examined microscopically and discarded if contaminated. For measurements of CO_2 production, sorbose uptake or sorbose efflux after starvation, samples were again washed twice with water and resuspended in the appropriate buffer solution.

Methods of heat treatment. Yeasts were exposed to increased temperatures for brief periods by resuspending in an appropriate fluid (*a*) at the desired temperature

in a water bath, or (*b*) at room temperature after which the samples were placed in a water bath at the desired temperature. In the latter case, yeast suspensions reached 45° in 3·5 min. The suspensions were then cooled rapidly to room temperature by a brief immersion in an ice bath, washed again with water (either by two centrifugations or after filtration onto a membrane filter (Millipore, type RA)) and finally resuspended either in a buffer solution for the measurement of CO_2 output and sorbose uptake, or in water for starvation.

Yeasts were also exposed to elevated temperatures by growing cultures at 35°. Culture flasks were moved from the normal shaker bath at 28·5° to one at 35° 2 h. before the yeast was due to be harvested. Other flasks were moved to a shaker bath at 21° for 2 h. After being harvested and washed, the yeasts were resuspended in buffer solution or in water as appropriate.

Manometric experiments. CO_2 production was measured at 30° under N_2 by standard Warburg procedures (Umbreit, Burris & Stauffer, 1949). Yeasts were suspended in 0·06 M buffer solution, pH 7·0 (except for Table 3), containing 0·1 M-glucose as substrate.

Uptake and efflux of sorbose. Yeasts were sampled from aerobic suspensions maintained at 30° and handled as described by Spoerl & Doyle (1968*c*). They were suspended in 0·02 M buffer solution (pH 5·6) containing 0·1 M-[^{14}C]-L-sorbose (uniformly labelled; 3 μCi/mole), a nonmetabolized sugar, for uptake measurements, and were washed and resuspended in 0·02 M buffer solution (pH 7·0) for efflux measurements. Efflux time for the second stage of exit was calculated from an initial value obtained by extrapolating the curve of efflux as a function of time back to zero time (Spoerl & Doyle, 1968*b*).

Buffer solutions. Solutions of mixtures of KH_2PO_4 and K_2HPO_4 were employed; they contained 2 mM-$MgCl_2$.

RESULTS

Effect of brief exposures of yeast to elevated temperatures. In the first experiments yeasts were rapidly resuspended in water or in growth medium at the desired temperature. CO_2 production was measured immediately after heat treatment and again after starvation (Table 1). For organisms which had been heated in growth medium at 40 or 45° and then starved, the Q_{CO_2} was increased. In contrast, heating in water at 45° decreased the Q_{CO_2} after starvation. Heating at 50° decreased CO_2 production by both starved and unstarved organisms. Starved yeast produces CO_2 at about 35 % the rate of unstarved controls (Spoerl & Doyle, 1967) so the heat-induced increases noted after starvation did not exceed the rate characteristic of fresh yeast.

In later experiments samples were heated at 45° in a variety of suspending media by immersing them in a water bath and holding them there for 1 min. after they reached 45° (total time 4·5 min.). The Q_{CO_2} of yeast heated in growth medium was doubled (Table 2) compared with unheated control yeast, and the lag in CO_2 output, characteristic of starved yeast (Spoerl & Doyle, 1967), was shortened. Heating in solutions of glucose (0·005 to 0·05 M) increased the Q_{CO_2} by 40 % compared with unheated organisms. Heating in 0·1 M-glucose, a concentration which reduces glycolysis and causes a loss, or 'excretion', of 260 nm. absorbing materials during incubation (Doyle & Spoerl, 1968), increased CO_2 production less than the lower concentrations of glucose, but the lag was more effectively shortened. Though a 21 h. incubation in

0·001 M-glucose increases CO_2 production (Spoerl & Doyle, 1968*a*), heating in such a glucose solution reduced the Q_{CO_2}, as did heating in water. Heating briefly in maltose and in mannitol solutions did not change the Q_{CO_2}, though incubation for 21 h. in 0·2 M-mannitol enhances CO_2 production (Spoerl & Doyle, 1968*a*). Heating in growth medium without glucose markedly reduced the Q_{CO_2}. The Q_{CO_2} also decreased when 0·01 M-glucose replaced the usual 0·22 M-glucose; 0·22 M-mannitol in the medium did not protect the yeast.

The Q_{CO_2} of yeast samples suspended in growth medium or glucose solutions and

Table 1. *CO_2 production by yeast heated at different temperatures and by yeast starved after heat treatment*

Samples of growing yeast cultures were washed and resuspended in buffer solution. CO_2 production was measured after heating and compared with unheated controls. Starved samples were starved for 21 h. before Q_{CO_2} measurements were made.

			Q_{CO_2} (% of control)							
			Period of heating (min.)							No. of
Test medium	Tem-perature	Type	0·5	1	2	4	8	16	32	experi-ments
H_2O	45°	Unstarved	103	99	102	105	—	—	—	2
H_2O	45°	Starved	54	35	36	9	—	—	—	3
Growth medium	40°	Starved	119	129	131	153	151	186	237	4
Growth medium	45°	Unstarved	103	96	99	96	84	—	—	3
Growth medium	45°	Starved	129	146	159	115	—	—	—	5
Growth medium	50°	Unstarved	78	73	64	—	—	—	—	3
Growth medium	50°	Starved	40	27	8	—	—	—	—	4

Table 2. *CO_2 production by starved yeast previously heated at 45° or held at 28·5° in various media*

After heat treatment, the yeast was starved for 21 h. before measuring Q_{CO_2} values. Lag period measured in min. The control Q_{CO_2} was 101 and its lag was 102 min. (average of 11 values). Values listed are averages of 3 to 6 separate measurements (more in the case of the 206% growth medium value).

Heated at 45° in	Q_{CO_2} (% of control)	Lag (% of control)
Growth medium	206*	3
K-phosphate buffer (0·06 M, pH 7·0)	71	79
H_2O	44*	100
Growth medium minus glucose	5*	—
Growth medium minus glucose + 0·22 M-mannitol	12*	—
Growth medium minus glucose + 0·01 M-glucose	83	30
0·1 M-Glucose	119	32
0·05 M-Glucose	138*	38
0·03 M-Glucose	143*	46
0·005 M-Glucose	141*	62
0·001 M-Glucose	66*	97
0·1 M-Maltose	110	100
0·2 M-Mannitol	92	90
Held at 28·5° in		
Growth medium	159*	20
0·1 M-Glucose	144*	43
0·01 M-Glucose	130*	48
0·005 M-Glucose	110	91

* Differs from 100 at the 5% level of significance.

held for 4·5 min. at 28·5° increased compared with samples resuspended directly in water and starved (Table 2). Heating at 45° enhanced the Q_{CO_2} more effectively, but evidently also produced some injury. That is, CO_2 production was significantly increased by suspending yeast in 0·1 M-glucose at 28·5° but not at 45°. The lower Q_{CO_2} of organisms heated at 45° may have resulted from an increased 'excretion' response (see above).

The Q_{CO_2} of yeast heated in growth medium and tested without starvation was 97 % of the control. Heating did not increase immediate CO_2 production in any environment, and decreases in Q_{CO_2} were small except with some samples heated in glucose solutions. The excretion of 260 nm. absorbing materials caused by glucose (Lewis & Stephanopoulos, 1967; Doyle & Spoerl, 1968) may account for the lower Q_{CO_2} of organisms heated in glucose solutions.

Effect of growth at 35°. Yeasts suspended for 32 min. in medium at 40° produced CO_2 at high rates after starvation (Table 1). Because growth and a variety of syntheses could have occurred during this period of time, experiments were carried out in which the yeast was grown for 2 h. at 35°. The generation time decreased from 1·5 h. at 28·5° to 1·2 h.; the immediate Q_{CO_2} did not change (Table 3). After starvation, the Q_{CO_2} of yeast grown at 35° was double that of organisms grown at 28·5°, and the lag was shortened (Table 3). CO_2 production by yeast grown at 35° was similar at pH 4·5 and 7·0, whereas it was lower at pH 4·5 than at pH 7·0 when the yeast was grown at 28·5°. The Q_{CO_2} of organisms grown at 21° for 2 h. did not differ greatly from that of organisms grown at 28·5°.

Table 3. *CO_2 production by yeast grown at different temperatures*

The Q_{CO_2} of yeasts grown continuously at 28·5° or shifted for 2 h. of growth at 35° or 21° was measured at the listed pH immediately after harvesting and after 21 h. of starvation. No lag occurs with unstarved yeast. Values are averages of 4 to 9 separate measurements; the ratio column lists average values for Q_{CO_2} divided by the Q_{CO_2} at 28·5°.

		Starved yeast			Unstarved yeast	
Growth temperature	pH	Q_{CO_2}	Lag (min.)	Ratio	Q_{CO_2}	Ratio
28·5°	7·0	127	74	—	323	—
28·5°	4·5	84	72	—	324	—
35°	7·0	258	14	2·08*	340	1·03
35°	4·5	275	18	2·72*	334	1·03
21°	7·0	103	80	0·87	232	0·73*
21°	4·5	101	78	1·28	282	0·87

* Differs from 1·0 at the 5 % level of significance.

Uptake and efflux of sorbose. The enhancement of CO_2 production by heat treatments is similar to an enhancement brought about by incubation of yeast in solutions of certain sugars and polyols (Spoerl & Doyle, 1968*a*). Sorbose uptake and efflux are also affected by incubation in sugar solutions (Spoerl & Doyle, 1968*b*), so the effect of heating yeast on these processes was examined.

Table 4 shows that more sorbose was taken up by starved organisms previously grown at 35°, and by starved organisms which had been heated at 45° in growth medium, than by those grown at 28·5°. Heating at 45° in mannitol did not increase sorbose

uptake and may have been deleterious. Uptake of sorbose by the yeast immediately after heat treatment did not differ significantly from that of unheated yeast.

Sorbose efflux from these yeasts initially occurs at a fast rate which then slows for a second stage of exit (Spoerl & Doyle, 1968*b*; Spoerl, 1969). Efflux during the second stage of exit was slower from heat-treated than from control yeast (Table 4). This difference in rate was not entirely consistent and did not occur every time. Initial efflux rates were alike (data not shown). Thus a slowed exit and an increased uptake of sorbose occurred in heat-treated yeast, though neither was as marked as those which occur in organisms incubated in sugar solutions (Spoerl & Doyle, 1968*b*).

Table 4. [^{14}C]-*L-Sorbose uptake and efflux from yeast exposed to elevated temperatures*

Percentages are averages of 3 to 9 measurements of μg. sorbose/mg. yeast dry wt after 90 min. of uptake and of the time for sorbose content to fall to one-half its initial value during the second stage of exit (see text). Controls were grown at 28·5°.

	Uptake (% of control)		Efflux (% of control)	
Treatment	Starved	Unstarved	Starved	Unstarved
Grown at 35° for 2 h.	116*	97	135	93
Heated at 45° in growth medium	126*	112	143	110
Heated at 45° in 0·01 M-glucose	109	108	144	99
Heated at 45° in 0·2 M-mannitol	88	106	—	—

*Differs from 100 at the 5% level of significance.

DISCUSSION

The temperatures employed in these experiments are not lethal and generally do not affect the appearance, growth or viability of *Saccharomyces cerevisiae* (Burns, 1956; Sherman, 1959*a*, *b*; Louderback, Scherbaum & Jahn, 1961). Starvation may influence various cellular processes (Mandelstam, 1960), and it functioned in these experiments to reveal heat-induced changes not observable in unstarved yeast.

Brief exposures to elevated temperatures, because they provide too limited a time for syntheses to occur, presumably caused a rearrangement of some constituents of the yeast so that the capacity to produce CO_2 was preserved. Because growth at 35° also resulted in an increased Q_{CO_2} after starvation, changes caused by such growth appear to be similar to those which occurred quickly when the yeast was exposed briefly to 45°. Exposure to higher temperatures or for longer times (Table 1) caused additional changes which resulted in less CO_2 production. If such additional effects occurred during brief exposures at 45°, they were overridden by the changes which produced the striking enhancement of CO_2 production.

Brief exposures to elevated temperatures enhanced the Q_{CO_2} when glucose was available, but not in mannitol or maltose solutions. Because use of maltose must be induced, this suggests that the changes at elevated temperatures which ultimately increase CO_2 production only occur quickly if an energy source is available. Moreover, heating in solutions which do not contain an energy source can reduce the Q_{CO_2}. Heating in growth medium which combines glucose with medium salts produced the highest Q_{CO_2}. The salts, perhaps by counteracting an 'excretion' response, prevented the decrease in the Q_{CO_2} which was caused by high concentrations of glucose alone, although the salts by themselves caused injury when glucose was not present.

Unheated yeast resuspended briefly in growth medium or in glucose solutions at 28·5° before starvation (Table 2) also showed an increased Q_{CO_2} compared with yeast taken directly from a growing culture at 28·5° and starved. The resuspension procedure, presumably by interrupting exponential growth and resupplying an energy source, affected metabolic processes so that the capacity to produce CO_2 did not decrease as usual during starvation. Though this procedure was involved in the results obtained with brief exposures, yeasts grown at 35° were not resuspended and the higher Q_{CO_2} was the result of the elevated temperature alone.

Both uptake and retention of sorbose are increased when CO_2 production is enhanced by incubation of yeast with glucose (Spoerl & Doyle, 1968*b*). The heat-treated organisms showed similar responses, but less consistently and to a lesser degree. Because maximal enhancement of CO_2 production did not differ greatly between incubation and heat experiments, the lesser uptake of sorbose in the heat experiments indicates that this response may be independent of the CO_2 response. Moreover, though heating in glucose solutions increased retention of sorbose, neither uptake nor CO_2 production was increased, as it was after heating in growth medium. Thus the mechanisms responsible for uptake and retention also may be affected independently. Other studies of sorbose retention have indicated that retention may be a function of the yeast vacuole (Spoerl, 1969); therefore, increased temperatures may affect vacuolar membranes.

Although a simple explanation for these heat effects is not at hand, studies of enhanced CO_2 production obtained by incubating yeast with sugars or polyols have indicated that the increased output of CO_2 did not involve changes in concentration of enzymes or cofactors, in viability, or in the loss of metabolites (Spoerl & Doyle, 1968*a*), and it was suggested that they might be due to changes in the yeast membranes. The capacities of membranes to transport and to bind sugars, as well as to form compartments for metabolites and reactions, include several functions which could be critically involved in the enhancement of CO_2 production.

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Demonstration of Succinic Dehydrogenase in the Mesosomes of the Mycelial Phase of *Paracoccidioides brasiliensis*

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SUMMARY

In the hypha of *Paracoccidioides brasiliensis* the succinic dehydrogenase activity is localized in the mesosomes, plasma membrane and mitochondria. Short permanganate fixation after incubation enhances the positive cytochemical reaction. The higher activity is localized in mesosomes and it is observed as high electron-dense zones and concentric ring-like patterns. The membrane shows a spotty reaction and the mitochondrion shows a positive zone in the intracristae space.

INTRODUCTION

Mesosomes or intracytoplasmic membrane systems have been demonstrated in several fungi (Edwards & Edwards, 1960; Moore & McAlear, 1961; Carbonell & Rodriguez, 1968). Light microscope demonstrations of reductases have been done in *Saccharomyces cerevisiae* (Reiss, 1967*a*), *Neurospora crassa* (Reiss, 1967*b*, 1968) and *Paracoccidioides brasiliensis* (Carbonell & Kanetsuna, 1966). Different types of oxido-reductase have been demonstrated cytochemically and biochemically in the mesosomes of Gram-positive bacteria (Vanderwinkel & Murray, 1962; van Iterson & Leene, 1964; Sedar & Burde, 1965; Ferrandez, Chaix & Ryter, 1966; Cesari, Rieber & Imaeda, 1969; Reaveley & Rogers, 1969). Morphologically, the mesosomes of fungi and bacteria are very much alike and the similarities could be confirmed if they were shown to have similar cytochemical characteristics. The purpose of the present work is to demonstrate succinic dehydrogenase activity in the mesosomes of *Paracoccidioides brasiliensis*.

METHODS

The mycelial phase of *Paracoccidioides brasiliensis* (IVIC-Pb9) was inoculated into a GGY medium (glycine 1 %, glucose 2 %, yeast extract 0·2 %) in Erlenmeyer flasks which were placed on a reciprocal shaker at 100 oscillations/min., stroke amplitude 5 cm., at 20 to 22°.

Four-day-old samples were harvested by low speed centrifugation and incubated for 30 to 50 min. in the following mixture: 3·5 ml. 0·8 M-sodium succinate, tetranitro blue tetrazolium (TNBT) 1 mg./ml., which had been previously dissolved in *N,N*-dimethylformamide (Sedar, Rosa & Tsou, 1962; Sedar & Burde, 1965). For controls, sodium malonate (0·5 g.) was added to the incubated mixture as a competitive inhibitor; the material was incubated without substrate; and the enzyme was inactivated

with glutaraldehyde, or by treating at 65° to 80° for 10 min. The material was then fixed.

As previously shown (Carbonell & Kanetsuna, 1966), the intrinsic factors which reduced the tetrazolium salt are eliminated by treating the samples with cold acetone for 10 min. before incubation, and washing in a mixture of 0·2 M-phosphate buffer (pH 7·4) and Ringer solution (1:9, v/v). Following incubation, the samples were harvested by low speed centrifugation. Acetate–veronal buffer of Michaelis, pH 6·1 (Kellenberg, Ryter & Séchaud, 1958), was used for preparing the fixative, for washing and for the agar solution.

After incubation, different fixation procedures were used: (*a*) 1 % osmium tetroxide at 20 to 22° for 12 h.; (*b*) 5 % glutaraldehyde at 4° for 12 h.; (*c*) fixation as in (*b*), and postfixed as in (*a*), for 3 h.; and (*d*) permanganate fixation for 10 min. or less at 4° following the technique of Kellenberg *et al.* (1958). To facilitate manipulation, the fixed material was embedded in 2 % agar. It was then dehydrated with ethanol and embedded in Maraglas (Freeman & Spurlock, 1962). Sections were cut with diamond knives and examined with a Hitachi II B electron microscope. For morphological details, samples were also fixed in glutaraldehyde and postfixed in osmium tetroxide, embedded in Maraglas and stained with lead nitrate and uranyl acetate.

RESULTS

The fine structure of the untreated mycelia of *Paracoccidioides brasiliensis* has been reported (Carbonell & Rodriguez, 1968). The following brief description will deal only with the features relevant to the present topic. The structure of the plasma membrane varied according to the sectioning angle. When attached to the cell wall it appeared as a wide electron-dense layer, or as a three-layered structure (an outer wide electron-dense layer, a low-density middle space and a very narrow inner electron-dense layer). The invaginations of the plasma membrane are interpreted as the beginning of mesosomes. This membranous system, formed by the apposition of the infolded plasma membrane, underwent additional invaginations, forming multivesicular or lamellar structures that are interpreted as tubular infoldings of the plasma membrane seen in different sectioning angles (Pl. 1, fig. 1; Pl. 3, fig. 7; Pl. 4, fig. 11). In addition to the plasmalemma and mesosomes, a cell wall with septum, septal bodies (Woronin bodies), nuclei, mitochondria, glycogen and vacuoles was identified (Pl. 1, fig. 1; Pl. 3, fig. 6, 7).

In media containing succinate, TBNT and buffer, TBNT showed reduction within a few minutes. When malonate was present and when enzymes were inactivated, the reduction of the TBNT was minimal.

The best fixation procedures used were glutaraldehyde + osmium and $KMnO_4$ fixations. Fixation with glutaraldehyde before incubation almost completely destroyed the enzymic activity (Seligman *et al.* 1967) and there was no increase in morphological details.

Deposits of TBNT-formazan (TNF) were found in the plasma membrane (Pl. 1, fig. 2; Pl. 2, fig. 5; Pl. 3, fig. 6) and mesosomes (Pl. 1, fig. 2, 3; Pl. 2, fig. 4; Pl. 3, fig. 8). In the plasma membrane they appeared scattered as electron-dense lines. The positive reaction of the plasma membrane was present near the mesosomes and in the membrane that embraced it (Pl. 1, fig. 2; Pl. 2, fig. 5). In the mesosomes, TNF appeared

as a homogeneous confluent electron-dense deposit (Pl. 2, fig. 4; Pl. 4, fig. 11) or as a concentric ring-like pattern (Pl. 2, fig. 4; Pl. 3, fig. 8). In some mesosomes one or other of the two patterns predominated. When the concentric ring-like pattern appeared it seemed that the reaction was not completely positive in the whole structure (Pl. 1, fig. 2; Pl. 2, fig. 4). Some mesosome structures showed heterogeneity in their reaction, as demonstrated by a clear positive zone near to a completely negative area (Pl. 4, fig. 11). In rare instances a deposit of needle-like microcrystals lying outside the cell wall was seen. Their significance cannot yet be explained. The reaction of the mitochondria in the material studied was localized in the intracristae space and in the outer membrane (Pl. 4, fig. 12). The small number of cristae in the mitochondria is noteworthy.

With malonate and the incubation mixture without substrate, or with inactivation of the enzyme with heat or fixation, some electron-density persisted in the mesosome (Pl. 4, fig. 9, 10).

DISCUSSION

The results show that the succinic dehydrogenase (SDH) activity of *Paracoccidioides brasiliensis* was mainly found in the mesosomes and less in the plasma membrane and mitochondria. The fine structure and the localization of the positively reacting zones near the septum and the cell wall identify these as mesosomes (Carbonell & Rodriguez, 1968). The positive reaction observed in the mesosome of a hypha of *P. brasiliensis* was due to SDH activity, since it appeared only when the incubation mixture had succinate as substrate and was not observed when the enzyme was inactivated or inhibited, or when the incubation mixture was used without substrate, as shown in Pl. 4, fig. 9, 10. Incubation with succinate followed by shorter permanganate fixation than the one commonly used (Kellenberg *et al.* 1958; Mollenhauer, 1959) proved to be the best procedure to demonstrate SDH activity. It was better than glutaraldehyde + osmium fixation because the electron-dense zones were more clearly delimited and the electron density was higher.

We presume that the high electron density is due to three factors: the electron density given by the TNF (Seligman *et al.* 1967), by the potassium permanganate when used alone (Bradbury & Mek, 1960; Hopwood, 1969) and by the oxidation of formazan by potassium permanganate. The most important factor is the enhancement of the reaction when permanganate is used. Due to the fact that mesosomes are made by complex infoldings of the plasma membrane, and the SDH is associated with the infoldings, the electron densities vary with the concentration of SDH in a given area.

The heterogeneity of mitochondria with respect to SDH activity has been shown by Seligman *et al.* (1967) and Ogawa & Barnett (1964). The first authors saw an all-or-none reactivity of the mitochondrion, while Ogawa & Barnett (1964) observed heterogeneity of the reaction inside the same mitochondrion. Dense zones and negative areas were seen in our material, indicating heterogeneity of reaction.

It is interesting to note that the hypha of *Paracoccicioides brasiliensis* has mitochondria and mesosomes (Carbonell & Rodriguez, 1968) and that SDH activity is found in both membranous systems, but much more in the mesosomes than in the mitochondria. Carbonell & Rodriguez (1968) demonstrated a close ultrastructural relationship between the mesosome and the formation of the septum which is part of the cell

wall. Since SDH activity was found in mesosomes, it is possible that respiratory activity present in the mesosome has something to do with the building of the cell wall.

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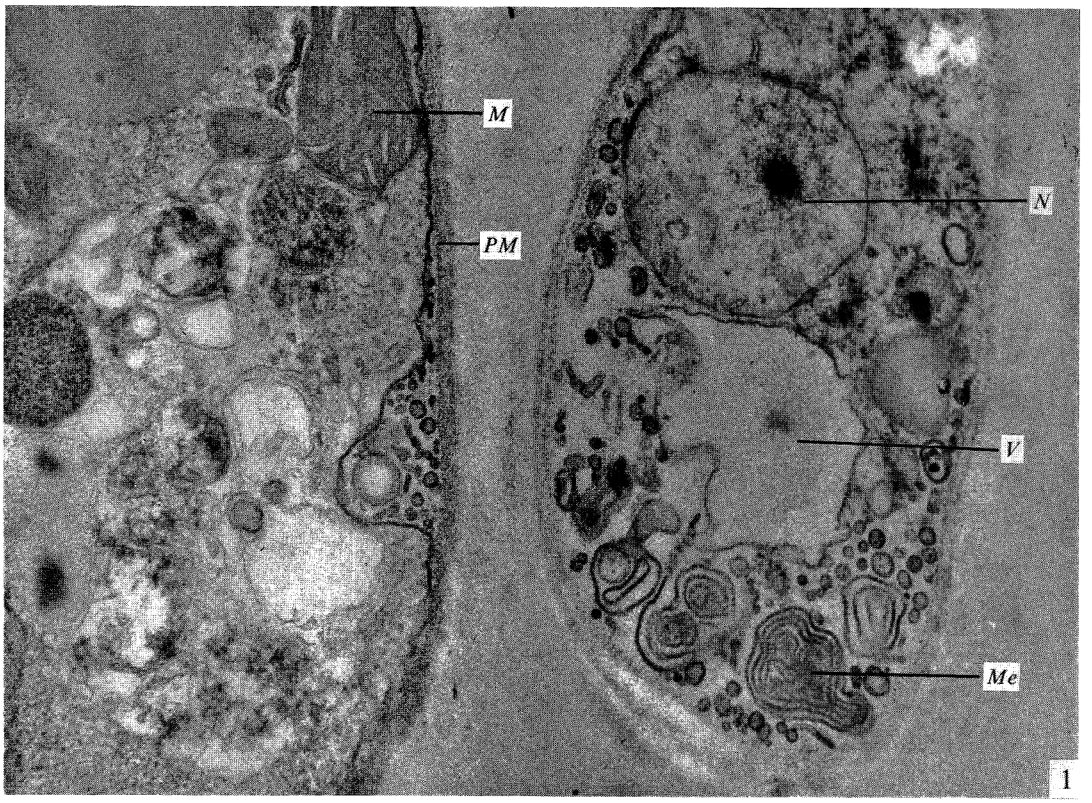
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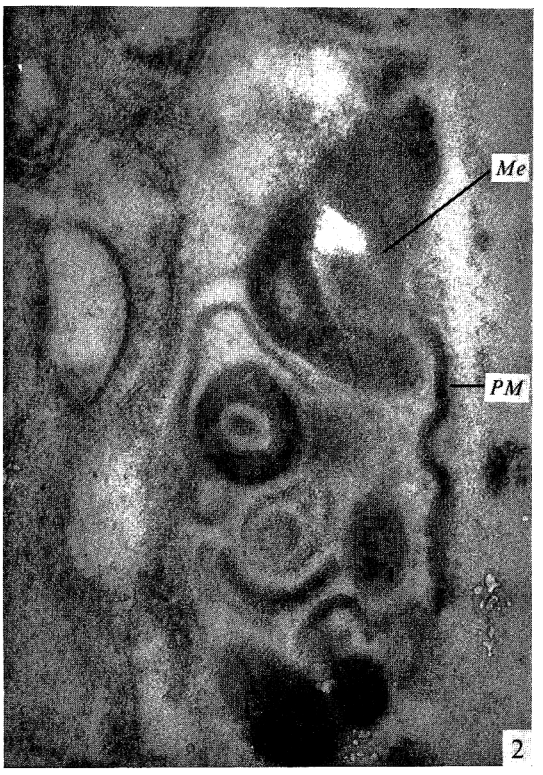
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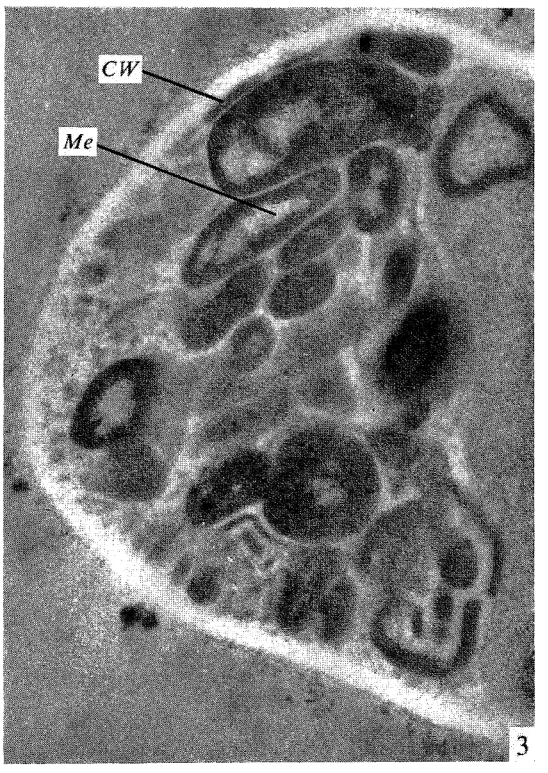
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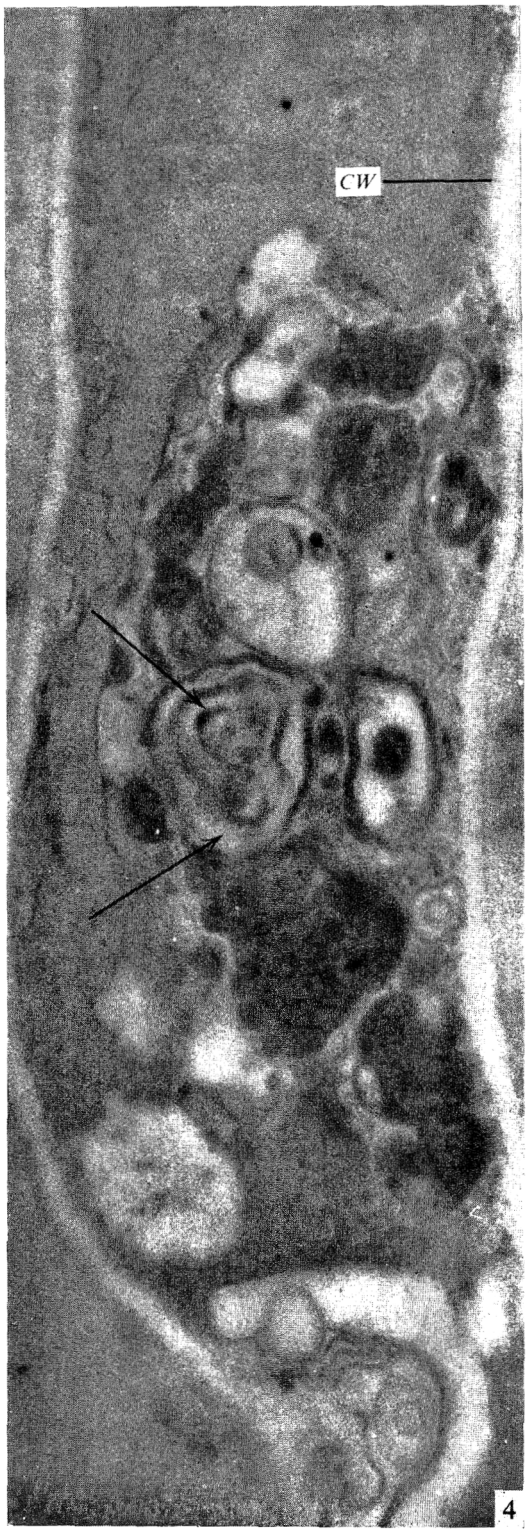

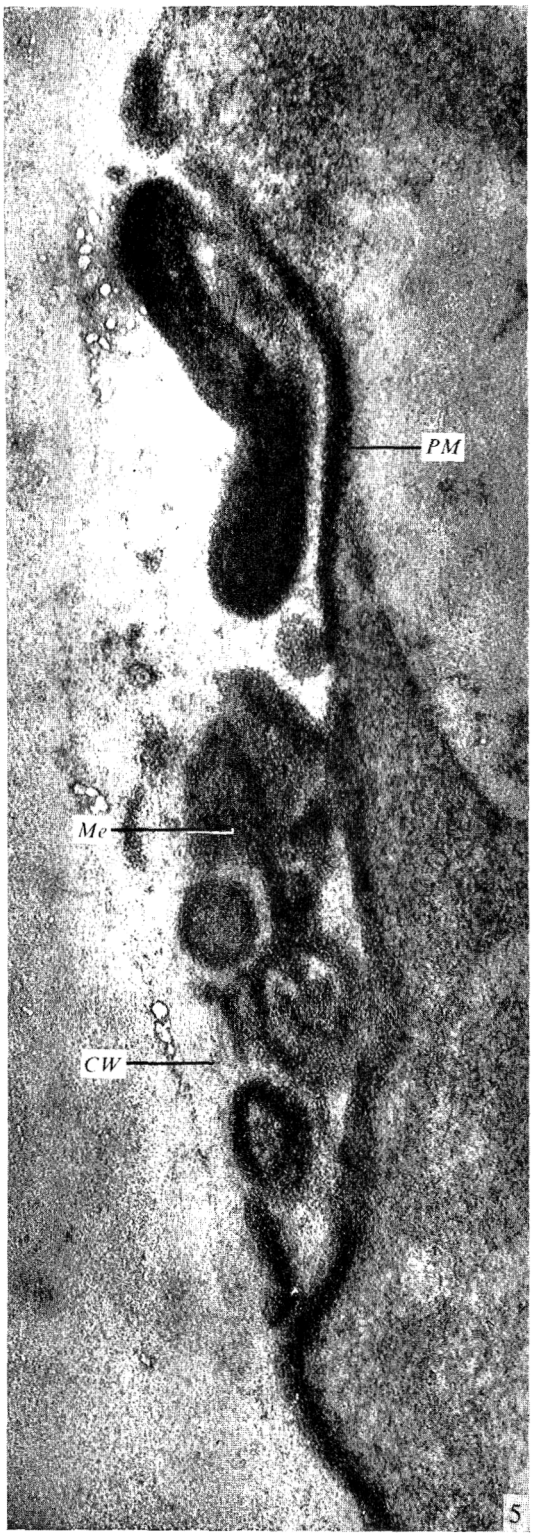

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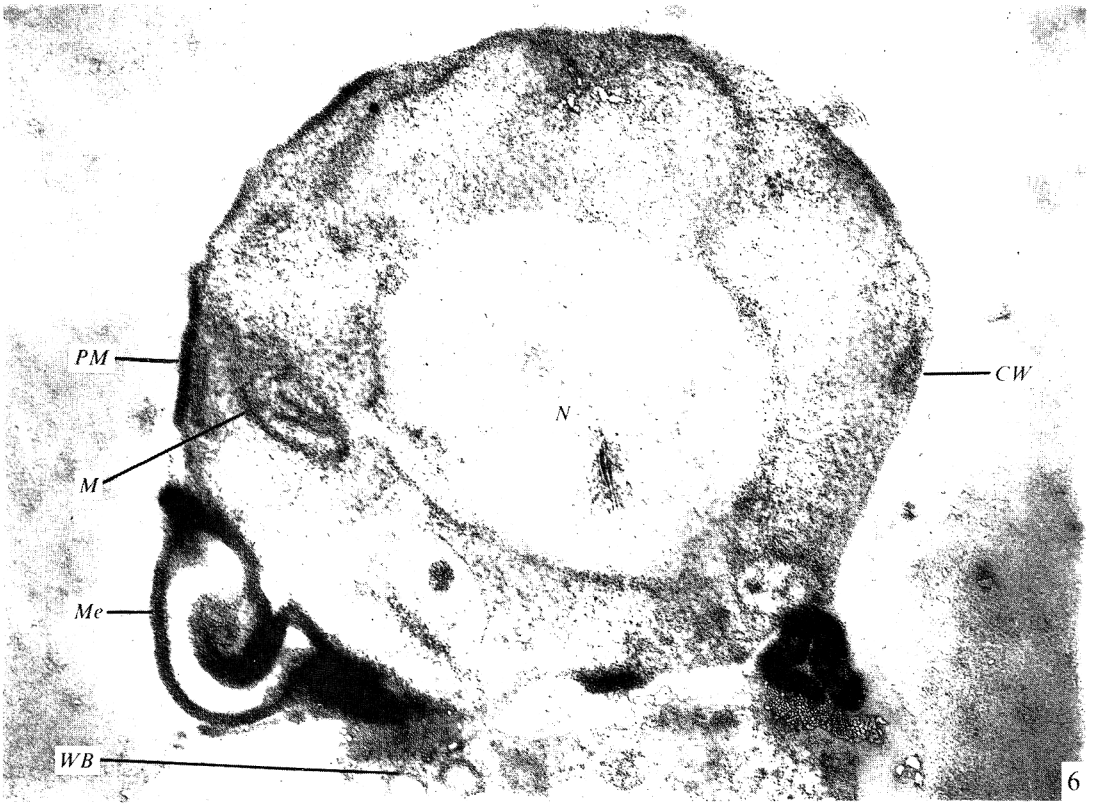
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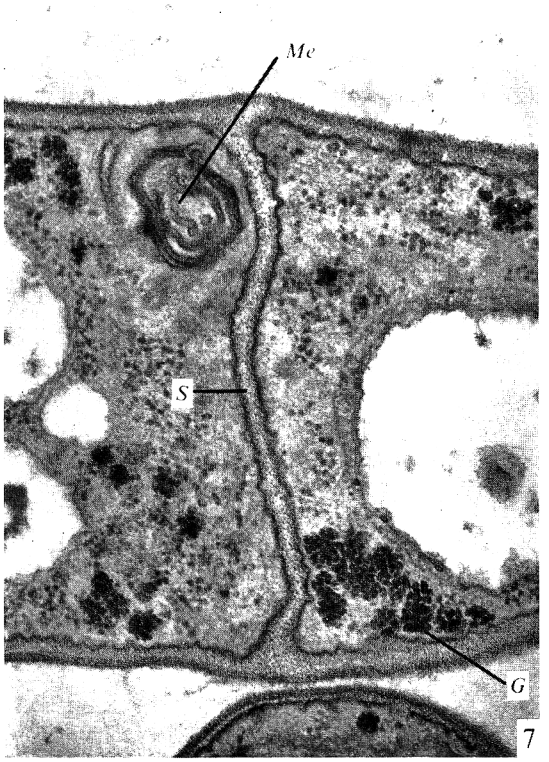
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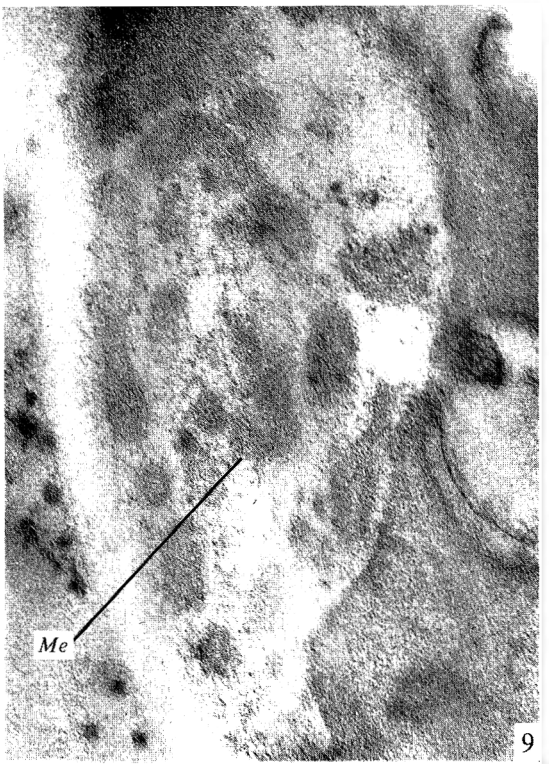

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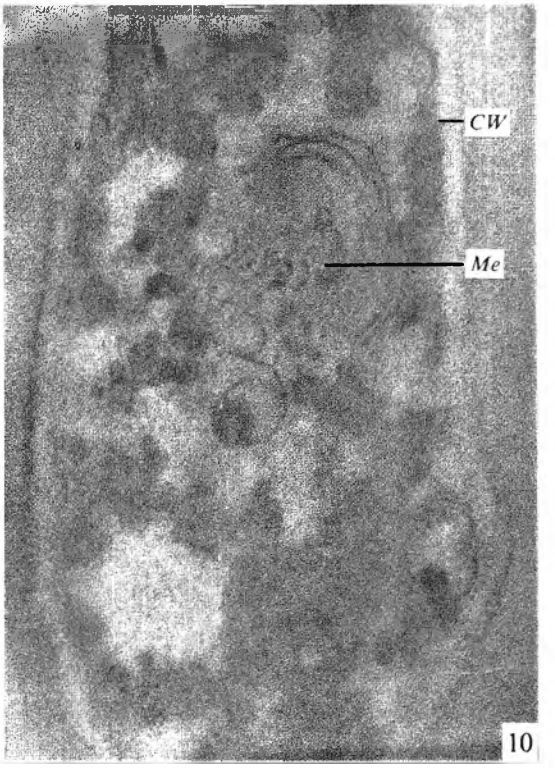

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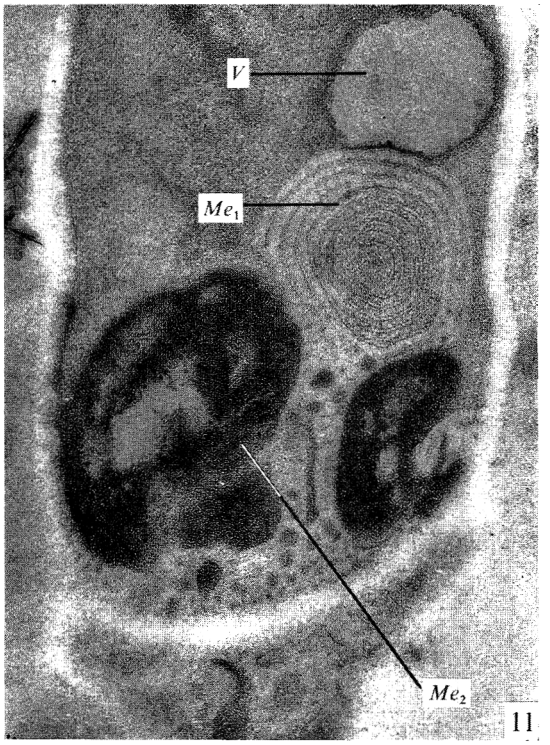

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EXPLANATION OF PLATES

In the following figures, *G* signifies glycogen; *N*, nucleus; *M*, mitochondria; *V*, vacuole; *PM*, plasma membrane; *CW*, cell wall; *Me*, mesosomes; *S*, septum; *WB*, Woronin bodies.

PLATE 1

Fig. 1. Mycelial phase of *Paracoccidioides brasiliensis*. Observe the nucleus, mitochondria, vacuoles plasma membrane, cell wall and mesosomes which are formed by a whorl of membranes and tubulo-vesicular structures. Glutaraldehyde + osmium tetroxide fixation with lead nitrate + uranyl acetate stain. × 24.000.

Fig. 2. Observe deposits of TNF indicating succinic dehydrogenase activity in the mesosome and a portion of plasma membrane. Incubation mixture, $KMnO_4$ fixation without stain. × 76,000.

Fig. 3. Mesosomes at the tip of a hypha. Observe the heterogeneous reactivity of the mesosome, the electron-dense zones in contrast with others of less electron density. Note the low electron density of the cell wall. Incubation mixture, $KMnO_4$ fixation without stain. × 68,000.

PLATE 2

Fig. 4. A large mesosome with many positively and negatively reacting areas. Note (arrows) that in some of the concentric ring-like structures the membrane has different electron densities. Incubation mixture, glutaraldehyde + osmium fixation, without stain. × 40,000.

Fig. 5. This shows a well-defined and positive reaction at the mesosome, and at the plasma membrane which delimited it. The cell wall is completely negative. Incubation mixture, $KMnO_4$ fixation without stain. × 100,000.

PLATE 3

Fig. 6. Septal mesosomes. Portions of the plasma membrane show an electron density similar to that observed in the mesosomes. Nucleus, mitochondria and three Woronin bodies are also seen. Incubation mixture, $KMnO_4$ fixation without stain. × 60,000.

Fig. 7. Septal mesosome in which a clearly delimited membrane is seen. Glutaraldehyde + osmium fixation with stain. × 45,000.

Fig. 8. In this mesosome note high electron density of each whorl. Incubation mixture, $KMnO_4$ fixation without stain. × 144,000.

PLATE 4

Fig. 9. Observe the very low electron density in the mesosome. Incubation mixture without substrate; $KMnO_4$ fixation without stain. × 90,000.

Fig. 10. Observe a very low electron density in the mesosome. Incubation mixture without substrate. Glutaraldehyde + osmium fixation without stain. × 60,000.

Fig. 11. Heterogeneity of the reaction in the mesosome structure. Compare well delimited structure with negative reaction (Me_1) with other (Me_2) which shows a positive reaction. Incubation mixture, $KMnO_4$ fixation without stain. × 70,000.

Fig. 12. A mitochondrion with positive reaction located in the outer membrane and in the intracristae space. Incubation mixture, $KMnO_4$ fixation without stain. × 80,000.

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Sporulation in *Bacillus subtilis*. Theoretical and Experimental Studies in Continuous Culture Systems

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SUMMARY

A theoretical treatment of growth and sporulation of *Bacillus subtilis* Marburg strain 168 in continuous culture is given. Sporulation is considered as a probability event, and a specific rate constant for its initiation in vegetative cells is introduced. The processes of growth and sporulation have been assumed to compete with each other in the vegetative organism. Equations are given which allow calculation of the rate constants characterizing growth and sporulation in steady-state continuous-flow systems. The relationship between the specific rate of initiation of sporulation and the growth rate of vegetative cells was determined with glucose-limited cultures and was approximately linear; the spore initiation rate increased with decreasing growth rate. This result was used to obtain equations relating the incidence of refractile spores to the dilution rate of a continuous culture maintained in a steady state. A number of possible models of the process of spore initiation have been considered; two were consistent with the linear relationship between spore initiation rate and growth rate determined by experiment.

INTRODUCTION

Continuous cultivation techniques have been applied by numerous workers to the study of spore or cyst formation by bacteria. Málek (1952) and Macura & Kotková (1953) investigated the growth phases of Azotobacter in multistage continuous cultures; similar techniques were employed by Málek, Chaloupka, Vosyková & Vinter (1953) to study sporulation in *Bacillus pumilis*. Sporulation of *Bacillus cereus* has been used by Řičica (1969) as an example of product formation in multistage continuous cultures. In single stage chemostats a number of attempts to study sporulation have been made, for example, in *Bacillus megaterium* (Aubert, Millet & Castoriadis-May, 1961; Aubert, Millet & Schaeffer, 1965); in *Bacillus subtilis* (Kerravala, Srinivasan & Halvorson, 1964; Dawes & Mandelstam, 1969; Dawes, Kay & Mandelstam, 1969); in *Clostridium thermosaccharolyticum* (Hsu & Ordal, 1969). Ierusalimskii (1958) and Ierusalimskii & Rukina (1959) introduced a procedure whereby organisms growing in continuous culture could be viewed microscopically and thereby studied the reversibility of sporulation of clostridia and bacilli.

Despite this interest in spore formation in continuous culture, we are not aware of any attempt to develop a theoretical treatment of bacterial differentiation in continuous culture analogous to those carried out for vegetative bacterial growth, e.g.

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Monod (1950), Novick & Szilard (1950), Herbert, Elsworth & Telling (1956), Herbert (1959, 1961), among others. This neglect may be due partly to the greater complexity of the system and partly to practical problems which have been encountered in continuous cultivation of spore-forming organisms. These include the selection of asporogenous mutants (Aubert *et al.* 1961), the failure of some spore-forming bacteria to attain a steady state (Kerravala, *et al.* 1964) or the germination of all but about 10% of the spores formed in single stage systems (E. O. Powell, personal communication).

This paper examines sporulation in continuous cultures in which growth is a function of the concentration of a single growth-limiting substrate; the possible dependence of sporulation on growth rate is investigated. The treatment is based on proposed models of sporulating systems; these models are evaluated in terms of experimental data obtained from steady state continuous culture systems.

THEORY

General remarks concerning sporulation

To describe formally a culture of bacteria undergoing spore formation it is first necessary to consider the nature of the sporulation process and to categorize the various populations of bacteria present in a culture undergoing both vegetative growth and sporulation.

Initiation of vegetative organisms to sporulation is thought to occur when certain nutrients necessary for growth become depleted (Büchner, 1890; Knaysi, 1945; Grelet, 1946, 1952, 1957). However, Aubert *et al.* (1961) and Schaeffer, Millet & Aubert (1965) showed that, in certain bacilli, sporulation occurred to some extent in minimal media, even when the bacteria were growing exponentially at their maximum rate. This observation led Schaeffer *et al.* (1965) to propose that initiation of a bacterium to spore formation could be considered as a probability event, an hypothesis confirmed by experiments with continuous culture in which Dawes & Mandelstam (1969) found that bacteria were initiated to form spores with a probability dependent on the dilution rate.

Once initiated to spore formation the organisms pass through a sequence of morphological and biochemical changes including the production of protease, antibiotic, alkaline phosphatase, glucose dehydrogenase and dipicolinic acid and the appearance of refractility and heat resistance of the spores, etc. (Murrell, 1967; Mandelstam, 1969).

Microbiologists, particularly those interested in continuous culture, describe cultures in terms of 'organism' as the basic unit; that is, in terms of 'recognizably separate entities' (Powell, 1956). For a sporulating system, however, there is no *a priori* argument for adopting the organism level as the unit for quantitative representation of initiation to spore formation. In fact, members of the genus *Bacillus* can be found either as unicellular organisms, or as organisms containing more than one cell (up to multicellular filamentous forms). We have found that the majority of *Bacillus subtilis* (Marburg strain 168), under the conditions of continuous culture specified in this paper, are unicellular, some are bicellular and few occur as filamentous forms. Under favourable conditions every cell can form a spore (see Robinow, 1960) regardless of the number of cells in each organism. Under less favourable conditions

cells within a filamentous organism may behave differently with respect to initiation of sporulation.

For these reasons the cultures have been described in terms of cell as the sporulating unit in this paper.

Cell populations present in sporulating systems

To analyse a sporulating bacterial culture, we assume it consists of three populations: vegetative cells (population X) which have not been initiated to form spores and are undergoing the normal process of cell division (all members of X are assumed competent to form spores under appropriate conditions); population Y, which consists of cells that have become initiated to form spores, but which have not reached that stage of development in the sporulation sequence which is arbitrarily used as the criterion of spore formation (in this study the criterion is the appearance of refractility; once initiated, cells are presumed incapable of further division and do not contribute to increases in cell numbers); population Z, which have reached refractility or which have developed further (this class contains all free spores in addition to cells containing observable spores).

We use n_x, n_y and n_z to denote the numbers per unit volume of culture of the cell types X, Y and Z, respectively.

The spore initiation process

Little is known of the mechanism of initiation to sporulation of vegetative cells, particularly those in a growing culture. The theory developed in this paper is based on a treatment of spore initiation which resulted in the simplest mathematical description. Other feasible models (not given here) have been treated and lead to similar expressions.

Vegetative cells are treated as a single population which can undergo one of two competing processes: growth or sporulation

$$X \xrightarrow{\nu} 2X \quad \text{(growth)},$$

$$X \xrightarrow{\kappa} Y \quad (\longrightarrow Z) \quad \text{(sporulation)},$$

in which ν and κ are first-order rate constants. Once a cell has entered the spore formation sequence, we assume it always forms a mature spore at some later time and that there are no cells in the culture which are undergoing neither vegetative growth nor spore formation.

Next, the probability of initiation of a cell to sporulation before it divides is defined by ϕ. As would be expected, the relationship of the probability (ϕ) to the rate constants (ν and κ) depends upon the details of the particular treatment of the process of spore initiation chosen, although different models lead to similar expressions to that derived from the simple model outlined above, i.e.

$$\phi = \frac{\kappa}{\nu+\kappa}. \tag{1}$$

Kinetics of growth and initiation to sporulation

The growth of non-sporulating bacteria in terms of cell number n_x is usually described by the rate equation

$$\frac{dn_x}{dt} = \nu n_x, \tag{2}$$

where ν is the specific number growth rate.

Aubert *et al.* (1961) showed that bacteria formed spores during exponential growth, and that spores increased in proportion to the cell population. Thus a first order specific rate constant κ for the process of initiation of vegetative cells to sporulation may be defined such that:

$$\text{Rate of cell initiation to spore formation} = \kappa n_x. \tag{3}$$

Since we assume that growth and initiation are exclusive, the rate of increase of vegetative cells in a sporulating culture is given by

$$\frac{dn_x}{dt} = (\nu - \kappa)\, n_x. \tag{4}$$

Here ν is the specific growth rate of the cells if no sporulation were taking place. Increase in cell numbers is still exponential, but the effective growth rate is now $(\nu - \kappa)$.

Kinetics of spore germination

Most studies of the germination kinetics of spores have been carried out in cultures where an agent promoting germination has been applied suddenly (see Sussman & Halvorson, 1966). In such systems, Woese & Morowitz (1958) found that germination can be approximately represented by first order kinetics. In the chemostat, however, spores are continually exposed to any germinant which may be present, and are heterogeneous with respect to germination rate (see experimental section).

For a system composed of n spore fractions, each fraction Z_i germinating with first-order rate constant α_i, and comprising a proportion λ_i of the total spore population, the rate equation for the change in total spore concentration, n_z, at time t, due to germination is

$$\frac{d}{dt}[n_z(t)] = -\left[\sum_{i=1}^{n} \alpha_i \lambda_i(t)\right] n_z(t). \tag{5}$$

In a steady-state chemostat culture the proportion λ_i will be constant at a given dilution rate and an overall first-order rate constant α can be used to describe germination, i.e.

$$\alpha = \left[\sum_{i=1}^{n} \alpha_i \lambda_i\right]. \tag{6}$$

The values of α vary to some extent with dilution rate since the λ_i values vary with dilution rate.

Spore germination leads not only to a loss of spores from the culture, but contributes towards the vegetative cell population. There is a time lag between commencement of the germination process and outgrowth with the formation of a vegetative cell. This means that the rate of increase of vegetative cells at time t is not given by $\alpha n_z(t)$, but by $\alpha n_z(t - t_g)$ multiplied by a factor describing the loss of spores from the culture during the preceding time interval t_g (where t_g is the interval between the commencement of germination and cell outgrowth). For analysis of steady-state chemostat cultures it is reasonable to neglect this correction for washout since we found that at high dilution rates germination did not significantly contribute to any increase of vegetative cells, while at low dilution rates, where αn_z is significant, the correction for t_g is not important.

Growth and sporulation in the chemostat

The theoretical aspects of bacterial growth in single-stage homogeneous continuous cultivation systems given by Herbert *et al.* (1956) have been extended in the following treatment to the case of spore-forming bacteria.

Changes in the concentration of cell species. The completely mixed continuous culture system consists of a suitable vessel in which the culture is maintained at constant volume v. Sterile growth medium is fed into the vessel at constant flow rate f, and the vessel contents stirred so that there is uniform and rapid mixing. The dilution rate D is defined by

$$D = \frac{f}{v}. \tag{7}$$

The time dependence of the vegetative cell (number) concentration, n_x, is given by

$$\frac{dn_x}{dt} = (\nu - \kappa n_x) - Dn_x + \alpha n_z. \tag{8}$$

The number of initiated cells at time t, $n_y(t)$, is related to the values of κ and n_x during the previous time interval $t - t_0$ to t, since there is a finite time interval between initiation of sporulation and appearance of a recognizably refractile spore. This time interval t_0 we call the sporulation time. At time $t-\tau$, the rate of initiation to sporulation from equation (3) is

$$\kappa(t-\tau)\, n_x(t-\tau).$$

Of those cells initiated at time $t-\tau$, a fraction will wash out of the culture before reaching time t, i.e. before the cells reach an 'age from initiation' of τ. This fraction of cells washed out is given by the exponential factor

$$\exp\left(-\int_{t-\tau}^{t} D(\xi)\, d\xi\right).$$

Combining these last two expressions, at time t the number of initiated cells with ages between τ and $\tau + d\tau$ is

$$\kappa(t-\tau)\, n_x(t-\tau) \exp\left\{-\int_{t-\tau}^{t} D(\xi)\, d\xi\right\}. d\tau$$

and $n_y(t)$ is given by:

$$n_y(t) = \int_0^{t_0} \kappa(t-\tau)\, n_x(t-\tau) \exp\left\{-\int_{t-\tau}^{t} D(\xi) d\xi\right\} d\tau. \tag{9}$$

Finally, the equation governing n_z, the number concentration of entities recognizable as spores, is required. Cells are not recognized as containing spores until they have reached an age from initiation of t_0, hence the rate balance equation for $n_z(t)$ is given by

$$\frac{dn_z}{dt} = \kappa(t-t_0)\, n_x(t-t_0) \exp\left\{-\int_{t-t_0} D(\xi)\, d\xi\right\} - Dn_z - \alpha n_z. \tag{10}$$

Changes in the concentration of substrate. Monod (1942) showed, for non-sporulating bacteria, the existence of an empirical relationship between the rate of bacterial growth and the rate of substrate utilization:

$$\frac{dn}{dt} = -y\frac{ds}{dt}, \tag{11}$$

where n is the concentration of bacteria and y is known as the yield constant. This yield constant describes the rate of consumption of substrate by cells undergoing vegetative growth (Monod, 1950; Herbert *et al.* 1956).

In a culture undergoing sporulation as well as vegetative growth, growth-limiting substrate is undoubtedly consumed by cells which have been initiated to spore formation. However, the rate of consumption of substrate by initiated cells will depend on the age τ of the initiated cells. A function $\mathscr{C}(\tau)$ describing the rate of consumption of substrate by initiated cells $N_y(t, \tau)\,d\tau$ with ages between τ and $\tau+d\tau$ can be defined by letting the rate of consumption of substrate be

$$\mathscr{C}(\tau)\, N_y(t, \tau)\, d\tau. \tag{12}$$

From this, the total rate of change of substrate concentration by initiated cells is

$$\frac{ds}{dt} = D(s_R - s) - \frac{1}{y}\cdot\frac{dn_x}{dt} - \int_0^{t_0} \mathscr{C}(\tau)\, \kappa(t-\tau)\, n_x(t-\tau)\, \exp\left\{-\int_{t-\tau}^{t} D(\xi)\, d\xi\right\} d\tau. \tag{13}$$

The steady state

In the following equations the practice of Herbert *et al.* (1956), who use a tilde to denote steady-state values, will be followed.

When steady-state conditions obtain, $(d/dt)(n_x)$, $(d/dt)(n_z)$ and (ds/dt) are zero, and ν, κ, and D are time invariant, and equations (8), (9) and (10) simplify respectively to

$$\nu - \kappa = D - \alpha\frac{\tilde{n}_z}{\tilde{n}_x}, \tag{14}$$

$$\tilde{n}_y = \frac{\kappa\tilde{n}_x}{D}[1 - \exp(-Dt_0)], \tag{15}$$

$$\tilde{n}_z = \frac{\kappa\tilde{n}_x}{D+\alpha}\exp(-Dt_0). \tag{16}$$

The total cell concentration, n_T, at steady state is given by:

$$\tilde{n}_T = \tilde{n}_x + \tilde{n}_y + \tilde{n}_z = \tilde{n}_x\left[1 + \frac{\kappa}{D}\left\{(1 - \exp(-Dt_0)\right\} + \frac{\kappa}{D+\alpha}\exp(-Dt_0)\right]. \tag{17}$$

Finally, an equation relating substrate concentration to species concentrations can be obtained from equation (13) if the function describing the rate of consumption of substrate by initiated cells is known. These aspects will not be developed further in this paper.

An observable characteristic of sporulating systems is the number of refractile spores as a fraction (θ) of the total population present in the culture, defined by

$$\theta = \frac{n_z}{n_x + n_y + n_z}. \tag{18}$$

From equations (14), (15), (16) and (18), the following relations can be derived;

$$\tilde{\theta} = \frac{\kappa\exp(-Dt_0)}{D + \kappa + \alpha\left[1 + \frac{\kappa}{D}\left\{1 - \exp(Dt_0)\right\}\right]}, \tag{19}$$

$$\kappa = \frac{D\tilde{\theta}(D+\alpha)}{D[\exp(-Dt_0) - \tilde{\theta}] - \alpha\tilde{\theta}[1 - \exp(-Dt_0)]}, \tag{20}$$

$$\nu = D + \kappa\left\{1 - \frac{\alpha\exp(-Dt_0)}{D+\alpha}\right\}. \tag{21}$$

The probability of initiation of a vegetative cell to spore formation before it can undergo cell division has been assumed to be given by equation (1). Experimentally it is possible to measure $\bar{\theta}$, α and D, and t_0 can be obtained by examining the transient variation of θ with time on making a shift in dilution rate, e.g. Dawes *et al.* (1969). Thus application of equations (20), (21) and (1) allows experimental evaluation of κ, ν and ϕ, without the use of any assumptions concerning the detailed dependence of κ and ν on the substrate concentration, s, provided $\bar{\theta}$ values can be measured at particular dilution rates. However, in order to predict values for $\bar{\theta}$, κ, ν, and ϕ given D alone, it is necessary to determine the detailed dependence of κ on ν or of κ and ν on s. Moreover, these relationships must be available in order to begin to predict n_x, n_y, n_z, n_T and s as functions of D.

Variation of growth rate and spore initiation rate with substrate concentration. Some assumptions must now be made regarding factors affecting the specific rate constants ν and κ. We assume that both ν and κ depend only on the concentration of the limiting substrate at time t, and are not affected by the prevailing levels of n_x, n_y or n_z, or by the previous history of the culture. We further assume that the specific rate constant for the increase of cell numbers, ν (which describes the rate of division of the bacteria and not the rate of increase of vegetative cell numbers in the sporulating population), varies with the limiting substrate concentration as

$$\nu = \nu_m \left(\frac{s}{K_s+s}\right), \tag{22}$$

as was first demonstrated by Monod (1942) for non-sporulating systems; ν_m is the maximum specific growth rate of the bacteria at saturation levels of the limiting substrate, and K_s is a saturation constant.

It therefore remains to determine the variation of κ (or ϕ) with either ν or s. It has been shown that κ and ν can be obtained experimentally by measuring θ values at various dilution rates. It is therefore possible to determine empirically the variation of κ with ν and hence κ with s from equation (22).

METHODS

Organism. Bacillus subtilis (Marburg strain 168), auxotrophic for indole or tryptophan was stored as a spore suspension.

Continuous cultivation. The composition of the growth medium was as follows: 2·78 mM-glucose, 4·75 mM-sodium citrate, 0·1 mM-tryptophan, 9·5 mM-NH_4Cl, 20 mM-KH_2PO_4, 1 mM-$NaNO_3$, 1 mM-Na_2SO_4, 1 mM-KCl, 2 mM-$CaCl_2$, 0·0036 mM-$FeCl_3$, 0·041 mM-$MgCl_2$, 0·1 mM-$MnCl_2$; the pH was adjusted to 7·0 with NaOH. Growth under these conditions was limited by the concentration of glucose. Cultivation was carried out at 37° in a conical vessel of approximately 500 ml. capacity. The culture (about 100 ml.) was stirred by rapid aeration (1 l. $min.^{-1}$ of sterile-filtered humidified air). Problems of evaporation of the culture were avoided by saturation of the inlet air with water vapour. Sterile medium was fed into the vessel by means of a peristaltic pump. In order to maintain a near-continuous inflow of medium at low flow rates, an air-lock and a fine bore inlet were added to the feed line between the pump and inlet.

Inoculation of the chemostat was carried out as described previously (Dawes *at al.* 1969), and the culture allowed to reach equilibrium at various dilution rates. The

attainment of a steady state was determined by observing the turbidity of the culture at 600 nm. and by measuring the fraction of refractile spores in the culture.

Estimations. The incidence of refractile spores (θ) as a fraction of the total population was determined under the phase-contrast microscope, using cell samples taken from the chemostat and fixed immediately after sampling with formaldehyde. Samples were taken at intervals of about one generation time until there was no change in spore count; for these samples, sufficient cells were counted to include at least 100 spores: this corresponds to a 10% counting error.

The time taken for an initiated cell to form a refractile spore (t_0) was determined by observing the effect of a sudden decrease in dilution rate of the chemostat system, as described previously (Dawes *et al.* 1969).

No suitable technique was available for estimation of the specific germination rate (α) of spores under chemostat conditions. However, germination rates were obtained for various spore fractions (resolved by equilibrium density gradient centrifugation of a sample from a steady-state culture) by suspending the spores in the supernatant of a steady-state chemostat culture and determining at intervals thereafter the fraction of spores which appeared phase-dark in the phase-contrast microscope. This supernatant was obtained by rapidly filtering 50 ml. of culture through a membrane filter (Millipore, 0·65 μ pore size). From these data, plots of

$$-\ln \frac{n_z(t = t)}{n_z(t = 0)}$$

against time t were constructed for the various fractions and their germination rates determined from the slopes. The overall germination rate for the spore population was obtained from these rates and the proportions of spores present in each fraction using equation (6).

From equations (20), (21) and (1) it can be seen that small errors in measurement of dilution rates could lead to much larger errors in estimation of the parameters, κ, ν and ϕ. For this reason the flow rate of medium was obtained by measuring the time taken for 25 ml. of culture to flow from the chemostat. At least three series of measurements were taken at each steady state. The volume of culture in the chemostat vessel was measured directly after each experiment.

The maximum growth rate constant (ν_m) was determined by observing the rate of increase of bacterial mass (estimated by turbidity measurements) from a low inoculum into the chemostat vessel containing fresh sterile medium. Rates were estimated at relatively low bacterial concentrations (less than 0·1 mg. ml.$^{-1}$), the average of three determinations was taken as ν_m.

Turbidity of the culture was measured at 600 nm. using a SP 600 spectrophotometer. Measurements of pH, using an EIL 2320 pH meter, were taken as soon as possible after sampling from the culture.

Contaminants and asporogenous mutants were detected by plating samples of culture on various minimal-media plates. *Bacillus subtilis* 168 requires tryptophan for growth, hence contaminants were readily observed on tryptophanless minimal-media plates. Asporogenous mutants were detected as white colonies on minimal-agar plates containing tryptophan: the parent organism formed brown colonies after several days incubation at 37°.

Three spore fractions were resolved from a steady-state culture by density-gradient

centrifugation. An exponential gradient of Urografin was prepared over the density range 1·c to 1·25, and the cells and spores obtained from a steady-state continuous culture sample (50 ml.; D = 0·12 h.$^{-1}$) by centrifugation were suspended in 1 ml. of the culture supernatant and were layered onto the gradient. Fractions were then obtained by centrifugation at 45,000g for 30 min.; one fraction pelleted due to its greater density (> 1·25 g.cm.$^{-3}$) and was found to contain free spores on microscopic examination. Two other fractions containing spores were obtained in the gradient and found to consist of spores included in lytic cells (mixed with germinating phase-dark spores) and spores included in nonlytic cells.

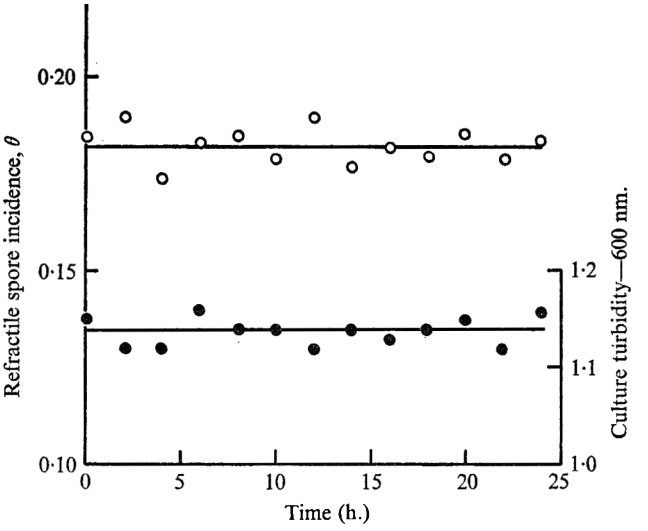

Fig. 1. Sporulation in steady-state continuous culture of *Bacillus subtilis* 168. A continuous culture of *B. subtilis* 168 was allowed to reach steady state under glucose limitation at a dilution rate of 0·11 h.$^{-1}$. Measurements of bacterial concentration (●) and refractile spore incidence (○) were taken at intervals over a 24 h. period, and were not found to differ significantly from straight lines of zero slope.

RESULTS

Stability of continuous cultures of Bacillus subtilis 168

Steady-state glucose-limited continuous cultures of *Bacillus subtilis* 168, undergoing sporulation as well as vegetative growth, were obtained over the range of dilution rates from 0·05 h.$^{-1}$ to 0·39 h.$^{-1}$. These steady states were not maintained indefinitely with respect to spore formation, since asporogenous mutants appeared spontaneously and began to replace the sporulating organism to a significant extent after the steady state had been established for about 15 days.

Figure 1 gives an example of a steady-state culture (in which the dilution rate, D, was 0·11 h.$^{-1}$) before the observable appearance of mutants; measurements were taken over a 24 h. period of the incidence of refractile spores (θ) and the turbidity of the culture. In general, therefore, experiments were not continued for longer than a week.

Parameters for treatment of data obtained from continuous cultures

Germination rate (α). Spores formed in continuous cultures were found, by examination, to be heterogenous with respect to their germination characteristics. Three spore fractions of differing densities were obtained and germination rates for these fractions each suspended in filtrate from a steady-state culture were obtained, as described in Methods. The plots of

$$-\ln\left[\frac{n_z(t)}{n_z(0)}\right] \text{ versus } t$$

are given in Fig. 2, the slopes of the straight lines in these plots were taken as estimates of the germination rates for the various spore fractions; these are given, together with the density range of the spores and the proportion of spores in each fraction, in Table 1.

Hence, as 'young spores' (those in nonlytic cells) germinate at faster rates than 'old spores', the specific germination rate for the population in the chemostat is not

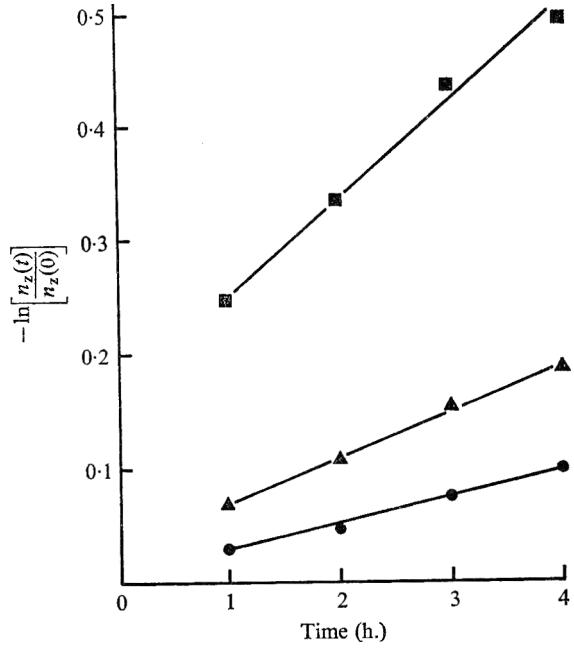

Fig. 2. Rates of germination of various spore fractions. Three spore fractions resolved from a chemostat spore population by equilibrium density-gradient centrifugation (see text) were each suspended in the rapid membrane filtrate of a steady-state culture and the refractile spore incidence determined at hourly intervals. The plots suggested in the text for estimating first-order germination rates for each of these fractions are given for: ● spores from the most dense fraction; ▲ spores from the intermediate density fraction, and ■ from the least dense fraction. $n_z(t)$ is the number of refractile spores per unit volume at time t.

constant for all dilution rates since it can be shown that the ratio of 'young spores' to 'old spores' varies with dilution rate. However, as was discussed in the theoretical section, the correction for germination is not important at higher dilution rates. Hence the value calculated for the above low dilution rate sample from the data in Table 1 and equation (6) of $\alpha = 0{\cdot}05$ h.$^{-1}$ has been taken as a reasonable approximation for

representation of the system. All data were treated using a range of α values in order to determine the effect of variation of this parameter on the interpretation of results.

The time for the formation of refractile spores (t_0). The effect of imposing a sudden decrease in the dilution rate (from 0·4 h.$^{-1}$ to 0·05 h.$^{-1}$) on the incidence of refractile spores in a steady-state chemostat culture is shown in Fig. 3. The incidence of refractile

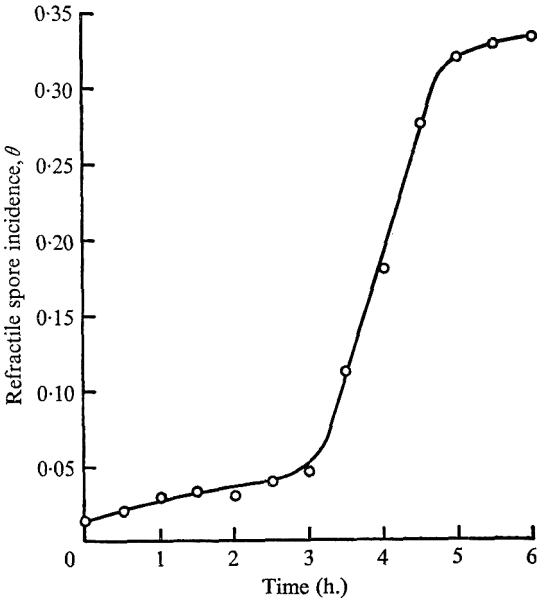

Fig. 3. The time for the formation of a refractile spore, t_0. At zero time the dilution rate of a steady-state culture at an initial value of 0·42 h.$^{-1}$ was changed to 0·05 h.$^{-1}$, and the incidence of refractile spores (○) followed with time. At 3 h. a sudden change in the spore incidence began which was complete at 5 h. The average time for the change to occur (4 h.) has been taken as the time (t_0) for the formation of a refractile spore.

Table 1. *The variation of germination rate of spore fractions from a continuous culture sample*

Three fractions of the total spore population of a steady-state continuous culture sample (D = 0·117 h.$^{-1}$) were obtained by equilibrium density gradient centrifugation. The first order germination rate for each fraction suspended in the filtrate of a steady state continuous culture (D = 0·123 h.$^{-1}$) were obtained. Methods have been given in the text.

Type of spore	Density of spores (g. cm.$^{-3}$)	Germination rate (h.$^{-1}$)	Proportion of spores
Spores included in non-lytic cells	1.17 to 1·22	0·092	0·39
Spores included in lytic cells	1·23 to 1·25	0·042	0·19
Spores free from parent cells	> 1·25	0·024	0·42

spores began to increase 3 h. after the change, and reached a new steady-state value approximately 5 h. after the change. The average time for the increase in refractile spores (4·0 h.) has been taken as the average time for formation of a refractile spore after initiation of a vegetative cell.

The maximum growth rate constant (ν_m). For balanced growth under batch conditions, the mass growth rate and the number growth rate, ν, must have the same value,

Table 2. *The effect of dilution rate on spore incidence in steady-state continuous culture of Bacillus subtilis 168*

The continuous culture apparatus and experimental details are described in the text. Measurements were taken at each dilution rate until steady state was reached, and each row of figures refers to a different steady state. Six different chemostat runs were necessary to obtain this data, and the values obtained from each run are denoted by the superscripts a, b, ...f.

Dilution rate (D) h.$^{-1}$	Refractile spore fraction (θ)	pH of culture pH units	Turbidity of culture 600 nm.
0·054^{c}	0·3060	7·9	1·18
0·055^{a}	0·3310	7·9	1·03
0·102^{e}	0·1995	7·7	1·00
0·111^{b}	0·1820	7·9	1·16
0·115^{b}	0·1750	7·9	1·04
0·116^{c}	0·1880	—	—
0·123^{d}	0·1690	7·8	1·05
0·132^{d}	0·1219	7·8	1·02
0·161^{a}	0·1190	7·9	0·98
0·194^{e}	0·0750	—	0·94
0·222^{b}	0·0675	7·5	0·86
0·257^{d}	0·0418	7·5	0·91
0·261^{b}	0·0501	7·4	0·86
0·269^{d}	0·0440	7·2	0·90
0·280^{c}	0·0347	7·6	0·94
0·299^{c}	0·0262	7·7	0·96
0·303^{d}	0·0295	7·6	0·91
0·307^{d}	0·0307	7·6	0·85
0·380^{f}	0·020	7·4	0·74
0·387^{b}	0·013	7·2	0·68

Table 3. *Calculated data for steady-state cultures of Bacillus subtilis 168*

Each row of figures refers to the values for the variables calculated from equations (1), (20) and (21) and the data in Table 2 for each different steady state.

Dilution rate (D) h.$^{-1}$	Specific growth rate (ν) h.$^{-1}$	Specific initiation rate (κ) h.$^{-1}$	Probability of initiation (ϕ)
0·054	0·099	0·0721	0·422
0·055	0·107	0·0844	0·440
0·102	0·156	0·0698	0·309
0·111	0·165	0·0679	0·291
0·115	0·169	0·0673	0·284
0·116	0·178	0·0762	0·300
0·123	0·181	0·0705	0·280
0·312	0·174	0·0496	0·222
0·161	0·217	0·0644	0·229
0·194	0·237	0·0477	0·167
0·222	0·272	0·0547	0·167
0·261	0·311	0·0527	0·145
0·257	0·296	0·0414	0·123
0·269	0·314	0·0481	0·133
0·280	0·318	0·0399	0·111
0·299	0·331	0·0335	0·092
0·303	0·341	0·0393	0·103
0·307	0·347	0·0422	0·108
0·380	0·423	0·0437	0·094
0·387	0·415	0·0287	0·065

Hence the mean value (of three observations) obtained for the maximum mass growth rate as described in the Methods section (0·487 h.$^{-1}$) was taken as the maximum growth rate constant, ν_m.

Quantitative data for steady-state cultures at various dilution rates

The steady-state data obtained from six separate runs in the same chemostat are summarized in Table 2, and the calculated values of κ, ν and ϕ for each steady-state sample are given in Table 3 and are plotted against the dilution rate (D) in Fig. 6, 7 and 8. Fig. 5 gives the values of θ against D. For calculation, the values $\alpha = 0{\cdot}05$ h.$^{-1}$ and $t_0 = 4{\cdot}0$ h. were used. A plot of κ versus ν is given in Fig. 4. This was found to be approximately linear.

Regression analysis gave the relationship

$$\kappa = 0{\cdot}091 - 0{\cdot}143\nu. \qquad (23)$$

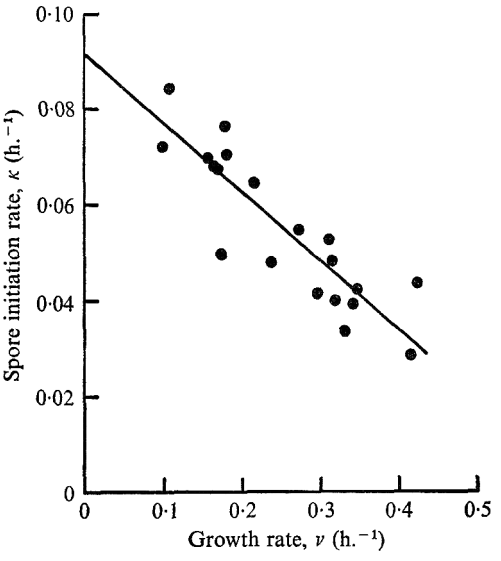

Fig. 4

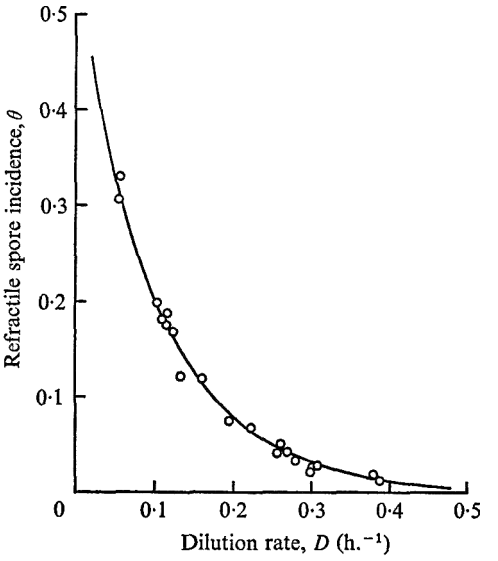

Fig. 5

Fig. 4. Variation of the rate of sporulation initiation with growth rate of *Bacillus subtilis* 168 in steady-state continuous culture. Values for the rate of sporulation initiation (κ) and the growth rate (ν) obtained experimentally from glucose-limited continuous cultures at different steady-state dilution rates were plotted against each other. The continuous line is that obtained from a linear regression analysis of κ on ν, and is represented by equation (23).

Fig. 5. The relationship between the refractile spore incidence at steady state in a continuous culture and dilution rate. Data for the refractile spore incidence given in Table 2 are plotted against dilution rate for comparison with the theoretical relationship predicted from equations (19), (21) and (23). Open circles represent experimental points, each point represents the refractile spore incidence in the culture after a particular steady state had been established. The continuous line represents the theoretical curve.

Test of the theoretical treatment

In addition to the calculation of κ, ν and ϕ values using the observed values for α and t_0, various combinations of α values (from 0 h.$^{-1}$ to 0·1 h.$^{-1}$) and t_0 values (from 3·0 h. to 5·0 h.) were used in treatment of the data in Table 2 in order to evaluate the

effect of errors in measurement of these parameters on the interpretation. If an α value of 0·005 h.$^{-1}$ or less is used in the calculation of κ and ν, the resulting values of κ are almost constant over the range of dilution rates employed. This situation is, however, unlikely since germinating spores have been observed in steady-state cultures by phase-contrast or electron microscopy in numbers which indicate that the α value must be close to that used to calculate the data in Table 3, i.e. α = 0·05 h.$^{-1}$.

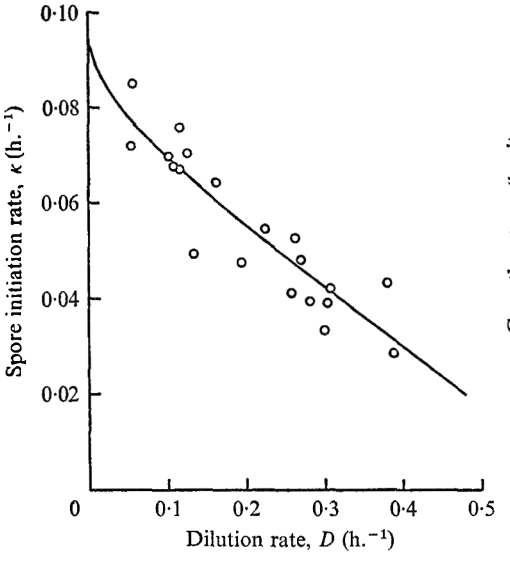

Fig. 6

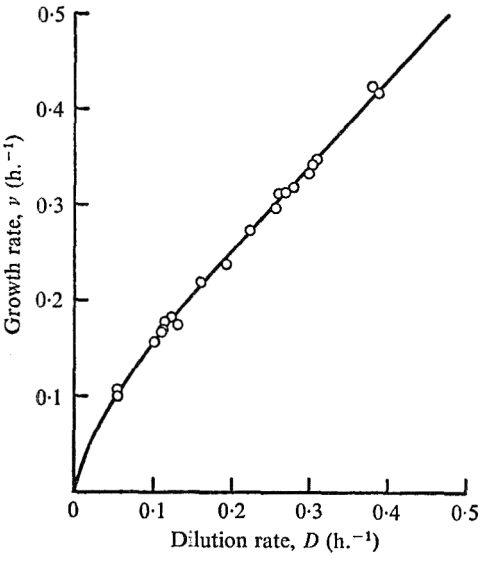

Fig. 7

Fig. 6. The quantitative relationship of the rate of sporulation initiation to dilution rate of a steady-state continuous culture of *Bacillus subtilis* 168. Values for the sporulation initiation rate (κ) calculated from the experimental data as described in the text are plotted against dilution rate (D). The open circles correspond to measurements taken on a culture after a particular steady state had been established (see Table 3). For comparison, the theoretical curve obtained from equations (21) and (23) is given as the continuous line.

Fig. 7. The quantitative relationship of the growth rate to the dilution rate of a steady-state continuous culture of *Bacillus subtilis* 168. Values for the growth rate (ν) calculated from the experimental data as described in the text are plotted against the dilution rate (D). Each open circle corresponds to a particular steady state, and the theoretical curve obtained from equations (21) and (23) is given as the continuous line.

Prediction of θ, κ, ν and ϕ values. With knowledge of the two constants arising from the linear relationship between κ and ν, it is possible to predict $\tilde{\theta}$, κ, ν and ϕ values, given the dilution rate D, without knowing the values for either the yield constants for substrate utilization or the saturation constant for growth rate (K_S) using equations (21) (23), (19) and (1). By substitution of equation (23) in equation (21), ν can be obtained as a function of D and the constants α and t_0 ,and from this the variation of κ, $\tilde{\theta}$ and ϕ follow directly from the above equations.

Theoretical curves for $\tilde{\theta}$, κ, ν and ϕ against D have been obtained from α = 0·05 h.$^{-1}$, t_0 = 4·0 h., ν_m = 0·487 h.$^{-1}$, and using equation (23). These have been plotted in Fig. 5 to 8 together with the experimental data. It can be seen that good quantitative agreement exists between the theoretical predictions and the experimental values.

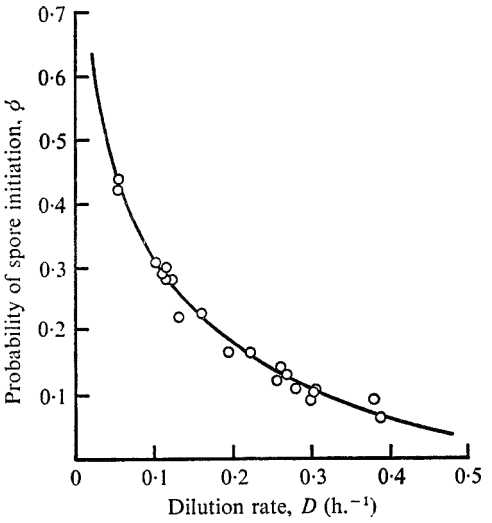

Fig. 8. The quantitative relationship of the probability of initiation of a vegetative cell to sporulation to the dilution rate of a steady-state continuous culture of *Bacillus subtilis* 168. Values for the probability of spore initiation (ϕ) calculated from the data of Table 2 as described in the text are plotted against the dilution rate (D). Open circles correspond to particular steady states, and the continuous line represents the theoretical curve obtained from equations (1), (21) and (23).

DISCUSSION

In the sporulating system, it is clear that a single yield constant will not, in principle, be sufficient to describe the substrate utilization of the very heterogeneous population. A theoretical basis for a description of substrate utilization in a sporulating culture has been briefly discussed, although in the absence of good data concerning substrate levels prevailing in the culture vessel, these aspects of the problem were not pursued further. The concentration of limiting substrate was not measured due to the difficulty of obtaining accurate data at low dilution rates; such a difficulty was encountered in experiments with glycerol limitation in chemostat cultures of *Aerobacter cloacae* (Herbert *et al.* 1956). We must state at this point that this lack of data about substrate concentrations led us to assume (without direct experimental verification) that Monod's relation, equation (22), describes the dependence of growth rate (ν) on the substrate concentration (s). This assumption does not affect the conclusions drawn concerning the linear dependence of the specific rate of spore initiation on growth rate, nor is it implicit in the treatment of the second model of spore initiation which appears in the following discussion. Nor is this assumption required for prediction of the observed variation of κ, ν, θ and ϕ with dilution rate. The assumption has been made in the first model of spore initiation.

A new and important parameter in the present treatment is the specific rate constant κ, which describes the process of initiation of vegetative cells to spore formation. Assumptions which simplify the relation of κ to growth and probability of sporulation were chosen in order to keep the mathematics simple, and those assumptions leading to more complicated mathematics added nothing.

With all these assumptions (all of which can be criticized) we have shown that for *Bacillus subtilis* 168 cultures under the conditions specified here, the relationship between the spore initiation rate κ and the cell division rate ν is approximately linear and is of the form of equation (23)

$$\kappa = a - b\nu,$$

where $a = 0{\cdot}091$ h.$^{-1}$ and $b = 0{\cdot}143$.

Models of the spore initiation process

The empirical relationship found between the spore initiation rate and the growth rate provides a method for testing models of the spore initiation process. We have found two very different models of spore initiation to predict the observed behaviour and these are set out briefly below.

Model 1. Repression model. There have been several suggestions made in the literature that sporulation in bacilli is controlled by the level of intracellular metabolite(s), see Schaeffer *et al.* (1965), Mandelstam (1969), Hsu & Ordal (1969). Schaeffer *et al.* (1965) drew an analogy between sporulation control and catabolite repression. The following repression system is analogous to the mechanism of control of the synthesis of an inducible enzyme postulated by Jacob & Monod (1961). Its postulates are:

(i) Spore formation is initiated by a repression system in which an 'effector' metabolite (E) reacts wtih a target, T_1 (say a protein)

$$E + T_1 \rightleftharpoons T_1E$$

and the molecule T_1E reacts with a second target T_2 (say a site on the chromosome)

$$T_1E + T_2 \rightleftharpoons T_1T_2E.$$

(ii) Both reactions are reversible and in equilibrium.

(iii) The concentration of the effect or metabolite is a function of the limiting substrate concentration, s. The concentrations of the 'targets' ($[T_1]$ and $[T_2]$) do not vary with s.

(iv) The probability of initiation of a cell to spore formation is proportional to the fraction of T_2 target sites unoccupied at any particular instant, i.e.

$$\phi = \frac{[T_2]}{[T_2] + [T_1T_2E]}.$$

(v) The concentration of effector metabolite $[E]$ is proportional to the limiting substrate concentration, s.

By straightforward application of these postulates the probability ϕ can be shown o vary with substrate concentration as

$$\phi = \frac{a+b}{a+bs},$$

where a, b are constants derived from the concentrations of the targets and the equilibrium constants for the reactions postulated in (i) and $b \rightleftharpoons 1$.

From this, by means of equations (1) and (22), the linear relationship, equation (23), of κ to ν can be derived.

Model 2. Initiation occurs at constant frequency in cells containing nonreplicating chromosomes. This model was suggested by Professor J. Mandelstam (personal communication) as an interesting alternative to repression models. Its postulates are:

(i) Cells can be initiated to spore formation only when they contain nonreplicating

chromosomes. (This assumption leaves open the question of what control mechanism prevents expression of the spore genes when DNA-replication is occurring.)

(ii) The 'time gap' between successive rounds of DNA replication increases linearly with generation time for generation times in excess of a minimum DNA-replication time τ_{rep}. Initiation occurs at constant rate in cells containing nonreplicating chromosomes.

Using these postulates it can be shown that

$$\kappa = \gamma(1 - 1{\cdot}44\tau_{rep}\nu),$$

i.e. a linear relationship of the form (equation (23)):

$$\kappa = a - b\nu.$$

If this model were correct, the constants (a and b) determined empirically would enable one to estimate the minimum DNA-replication time τ_{rep}. From the data in the experimental section τ_{rep} would have a value of 1·09 h. (cf. 0·7 h. for *Escherichia coli* found by Lark, 1966, and Helmstetter & Cooper, 1968).

Neither of the above models may in fact represent the actual process of spore initiation. At the moment, evidence obtained from other studies tends to support hypotheses based on the involvement of 'inhibition' of sporulation by small molecule metabolites. For example, growth limitation of bacilli in continuous culture by means of an auxotrophic requirement or an essential ion leads to much lower incidences of spores than that found under conditions of carbon or nitrogen limitation (Aubert *et al.* 1965; Dawes & Mandelstam, 1969). However, it is possible that 'time gaps' between DNA replications vary differently with growth rate when different substrates limit growth. No conclusive evidence in support of either hypothesis (or any other) is currently available. The results presented here do, however, provide a method for testing the validity of hypotheses concerning the control of sporulation initiation.

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Further Investigations of Actinomycetes by Scanning Electron Microscopy

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SUMMARY

To improve results obtained when examining actinomycetes by scanning electron microscopy, the microscope was operated under conditions giving higher resolution with a reduced but sufficient depth of focus. In addition, fixation and dehydration of specimens before coating reduced the distortion caused by desiccation under vacuum. By these means, representatives of the following genera were examined: *Actinobifida*, *Actinoplanes*, *Actinopycnidium*, *Actinosporangium*, *Microbispora*, *Microechinospora*, *Microellobosporia*, *Planomonospora*, *Sporichthya*, *Thermoactinomyces* and *Thermomonospora*. The study revealed points of similarity between *Actinopycnidium* and *Actinosporangium*; *Microellobosporia* and *Microechinospora*; and *Actinobifida* and *Thermoactinomyces*.

INTRODUCTION

The potential usefulness of scanning electron microscopy in studies of actinomycetes was demonstrated by Williams & Davies (1967). The technique has now been successfully applied to a variety of microbes. Studies include an ever-increasing number on fungi (Barnes & Neve, 1968; Greenhalgh & Evans, 1968; Hawker, 1968; Hawker & Gooday, 1968; Jones, 1968; Old & Robertson, 1969). Bacillus endospores have been examined by Murphy & Campbell (1969), bacteria in soil by Gray (1967) and Hagen, Hawrylewicz, Anderson, Tolkacz & Cephus (1968), blue-green algae by Echlin (1968), and protozoa by Small & Marszalek (1969). Dietz & Matthews (1969) have recently studied spore morphology of various Streptomyces species.

Since the initial work on actinomycetes by Williams & Davies (1967), a large number of strains have been examined and further information on the range of form in this group obtained. In addition, improved specimen preparation and instrument operation have resulted in better resolution at higher magnifications. Some of the results obtained are reported here.

METHODS

Strains used. These were: *Actinobifida alba* (CUB 432), *A. dichotomica* (CUB 339), *Actinoplanes* sp., *Actinopycnidium caeruleum* (RIA 729), *Actinosporangium violaceum* (RIA 655), *Microbispora rosea* (RIA 477), *Microechinospora grisea* (LIA 0442), *Microellosbosporia flavea* (IMRU 3858), *Planomonospora venezuelense* (ATCC 23865), *Sporichthya polymorpha* (IMRU 3913), *Thermoactinomyces vulgaris* (CUB) and *Thermomonospora viridis* (CUB). (RIA = U.S.S.R. Research Institute for Antibiotics, Moscow; LIA = Research Institute of Antibiotics, Leningrad; IMRU = Institute of Microbiology,

Rutgers, New Brunswick, U.S.A.: ATCC = American Type Culture Collection; CUB = Department of Biological Sciences, University of Bradford.)

Media. Actinobifida alba, *Thermoactinomyces vulgaris* and *Thermomonospora viridis* were grown on Oxoid nutrient agar; *Actinoplanes* sp., *Planomonospora venezuelense* and *Sporichthya polymorpha* on potato–carrot agar (Cross, Lechevalier & Lechevalier, 1963); and the rest on oatmeal agar (Waksman, 1961).

Culture methods. Each strain was inoculated both directly on to the surface of the medium and on to coverslips embedded in the medium (Williams & Davies, 1967). All were incubated at 25° except *Actinobifida alba*, *A. dichotomica*, *Thermoactinomyces vulgaris* and *Thermomonospora viridis*, which were grown at 45°, and *Microechinospora grisea*, which was incubated at 37°.

Preparation for examination. Material grown on both the medium and on coverslips was used, and fixed material as well as untreated was examined. Fixation was carried out by immersing a coverslip or a block of medium (5 $mm.^3$) with adherent growth in a 1% solution of osmium tetroxide in buffer for 2 h. at room temperature. After washing three times with distilled water, the material was dehydrated in 50 and 70% (30 min. each) and absolute ethanol (60 min.)

Coverslips and agar blocks with fixed and untreated growth were attached to metal specimen stubs with adhesive. They were then coated under vacuum with a thin film (about 30 nm.) of gold–palladium alloy.

Examination with scanning electron microscope. Specimens were examined over a range of working magnifications from 5000 to 50,000 times using a 'Stereoscan' electron microscope (Cambridge Scientific Instruments Ltd). The instrument was operated at 20 kV with a beam-specimen angle of 45° and a working distance of 9 to 11 mm. High lens condenser currents (0·7 to 0·9 A) were used to reduce the final probe diameter, i.e. to increase resolution. Resulting noise levels on the visual screen were minimized by use of a large (200 μm.) final aperture. Remaining noise was removed when photographing by having a long exposure time (200 or 400 sec.). Subjects were photographed using Ilford HP 4 film.

RESULTS

Comparison of preparation methods. Fixation and dehydration of specimens prior to coating eliminated or greatly reduced the distortion of cells caused by desiccation under vacuum which was observed by Williams & Davies (1967). Comparison of treated and untreated material revealed no changes in surface structures induced by the treatments, although some were observed in Streptomyces spore surfaces (Williams & Sharples, 1970). Sometimes partial collapse of untreated specimens emphasized surface detail; the firm ridges on spores of Actinobifida and Thermoactinomyces (Pl. 4, fig. 18) were more prominent in untreated material. Some strains, such as *Sporichthya polymorpha*, were unaltered by desiccation under vacuum and satisfactory results were obtained with untreated material (Pl. 5, fig. 23 to 27).

Information provided by coverslip and agar block preparations varied with the growth habit of the strain. The agar blocks were most useful when fruiting was sparse on coverslips, as for example with *Actinoplanes* sp. Growth on the blocks was often too dense for accurate observation. Therefore, with some exceptions, best results were obtained from fixed-dehydrated growth on coverslips.

Examination of genera. Genera studied included a number which formed sporangia. These were *Actinoplanes*, *Microechinospora*, *Microellobosporia* and *Planomonospora*. In *Actinoplanes*, both sessile and stalked sporangia were present (Pl. 1, fig. 1, 2). Sessile sporangia developed on a mound raised above the surface of the medium which was composed of hyphae; these were probably analogous with the palisade hyphae observed in sporangial genera by Couch (1955). The arrangement of spores within the sporangium appeared either columnar (Pl. 1, fig. 3) or spiral (Pl. 1, fig. 2), but the former could usually be shown to be spiral when viewed from another angle. A young ruptured sporangium (Pl. 1, fig. 5) showed the spiral arrangement of the sporongenous hypha which was in association with small pieces of material possibly representing the intersporal substance observed by Lechevalier & Holbert (1965). Young spores were irregular in shape with occasional pitting of their surface (Pl. 1, fig. 4). The sporangial envelope, while present as a close investment of the developing spores (Pl. 1, fig. 3), was not visible in mature sporangia. At all stages it was possible to see the spores through it; this was not possible with the envelope of *Streptosporangium* (Williams & Davies, 1967) and the other sporangia-forming genera studied here. *Microellobosporia* is characterized by the formation of a short row of two to five spores in a club-shaped sporangium (Cross *et al.* 1963). A two-spored sporangium is illustrated in Pl. 1, fig. 6, with its wrinkled sporangial envelope. A similar genus, *Microechinospora*, differing from *Microellobosporia* by having a sporangium with spines on its wall and usually containing only one spore, was described by Konev, Tsyganov, Minbaev & Morosov (1965). Examination of *Microechinospora grisea* did not reveal any ornamentation of the mostly one-spored sporangia (Pl. 1, fig. 7).

Another form of sporangium occurs in a number of recently described genera. This is an elongated structure that releases its spore or spores through the tip, and occurs in *Dactylosporangium* (Thiemann, Pagani & Beretta, 1967*a*), *Planomonospora* (Thiemann, Pagani & Beretta, 1967*b*) and *Planobispora* (Thiemann & Beretta, 1968). These genera differ mainly in the number of spores formed in the sporangium. The example studied here, *Planomonospora*, was characterized as having only one spore in the sporangium. Double rows of closely packed sporangia were formed, the sporangia arising directly from a trailing basal hypha (Pl. 2, fig. 8, 9). A number of the sporangia appeared to be invested by a more transparent outer sheath that possibly originated from the outer component of the hyphal wall. Tips of the sporangia were at first beaked (Pl. 2, fig. 8) but eventually the ends disappeared leaving a distinct pore (Pl. 2, fig. 11). The pore obviously allowed the release of the elongated spore (Pl. 2, fig. 10) but the only evidence of this was obtained when an irregular mass of material, possibly a ruptured spore, emerged from the pore (Pl. 2, fig. 12). Spore emergence was observed by Thiemann *et al.* (1967*b*).

The genus *Actinosporangium* was described as forming 'pseudo-sporangia' lacking an envelope (Krasilnikov & Tsi-Shen, 1961). These were formed by the repeated branching and intertwining of a number of hyphae (Pl. 2, fig. 13) which eventually resulted in the formation of large, localized masses of spores and hyphal fragments (Pl. 3, fig. 14, 15).

The genus *Actinopycnidium* was characterized by the formation of pycnidia, 70 to 100 μm. in diameter, containing spores (Krasilnikov, 1962). The pycnidium wall was said to be formed of interwoven hyphae; this, unlike other aggregate hyphal structures, such as sclerotia, was easily ruptured (Baldacci, Locci & Rogers Locci, 1966).

Observations here indicated that the 'pycnidium' consisted of a dense mass of spores, formed by the localization of spore production in a manner similar to *Actinosporangium*. The surface of these masses appeared to consist of spores adhering closely together (Pl. 3, fig. 16, 17).

The production of polygonal spores by *Thermoactinomyces vulgaris* was observed by Williams & Davies (1967). Further examination of this and other thermophilic genera has shown that the spores have ridges in *Thermoactinomyces* and *Actinobifida* (Pl. 4, fig. 18), but not in *Thermomonospora* (Pl. 4, fig. 19). The silhouettes of *Actinobifida chromogena* from the study by Krasilnikov & Agre (1965) also suggest a ridged structure. Cross, Walker & Gould (1968) demonstrated the presence of ridges overlaid by an outer sporangial envelope in sections of *T. vulgaris* and *Actinobifida dichotomica* and also found that such spores had a high degree of heat resistence. Similar structured endospores are formed by certain Bacillus species and those of *Bacillus polymyxa* were examined with the scanning electron microscope by Murphy & Campbell (1969). In young spores of both *Actinobifida* and *Thermoactinomyces* the ridges were not visible, spores being evenly covered by a wrinkled outer envelope. (Pl. 4, fig. 20).

The characteristically paired spores of *Microbispora* were attached to their sporophore in an unusual manner. The tip of the sporophore was expanded to form a platform and the basal spore was invaginated to fit over this in a ball and socket arrangement (Pl. 4, fig. 21, 22). This structure represents the collar observed by Lechevalier & Lechevalier (1957) in a silhouette of such a spore.

The genus *Sporichthya*, described recently by Lechevalier, Lechevalier & Holbert (1968), has a life cycle unlike that of any other known actinomycete. Detached cells, with a basal collar (Pl. 5, fig. 23) divided to form vertical piles of cells of varying height (Pl. 5, fig. 24). These were formed on the surface of the substrate (in this case glass), the basal cell being slightly inflated to form an attachment to the surface (Pl. 5, fig. 25). The piles broke into single cells, or occasionally a fish-shaped outgrowth from a cell was formed (Pl. 5, fig. 26). Branching hyphae were observed infrequently (Pl. 5, fig. 27). When grown on agar media, the same structures were observed and little penetration of the substrate occurred. The stages seen conform to the life cycle pattern suggested by Lechevalier *et al.* (1968).

DISCUSSION

Many applications of scanning electron microscopy to biological material have not involved working magnifications in excess of 5000 times. By making use of the large depth of focus provided by this instrument, much information has been obtained at this and much lower magnifications. However, for microbes as small as actinomycetes magnifications below 5000 times provide limited information, and greater magnification is often needed. To obtain informative pictures of actinomycetes at higher magnifications specimens must be prepared by appropriate methods, and it must be borne in mind that the reactions of different strains to fixation and dessication under vacuum are not uniform. In addition, the microscope should be operated under conditions that provide high resolution combined with a reduced but sufficient depth of focus.

The results described here illustrate further the considerable range of form of the actinomycetes. Many of their structures have analogues in the fungi. Thus the sporangium of *Actinoplanes* is similar to that of fungi in the order Chytridales whilst that of

Planomonospora is not unlike the operculate ascus of certain Discomycetes. The predominance of structural features in the classification of actinomycetes seems likely to continue, although they can be successfully allied with information from other techniques such as cell wall analysis (Lechevalier & Lechevalier, 1965; Williams, Davies & Cross, 1968). Scanning electron microscopy in addition to giving more accurate information about known characters also provides new ones.

The observations of some of the genera in this study raise questions about their taxonomic status. Both *Actinopycnidium* and *Actinosporangium* produce chains of spores or fragments and are peculiar only in the tendency for these to be concentrated in local sites. Cell wall analysis data of Becker, Lechevalier & Lechevalier (1965) and Yamaguchi (1965) place these genera in the same group as *Streptomyces*, and the observations made here are consistent with this suggestion. The genera *Microellobosporia* and *Microechinospora* spp. appear to be very closely related, differing only in the number of spores in the sporangium, and *Microechinospora* spp. could be reclassified as a species of *Microellobosporia*. Spore shape indicates an affinity between *Actinobifida* and *Thermoactinomyces*. These genera have a number of other common features including formation of spores on both substrate mycelium and aerial mycelium, development of spores within a sporangium, heat resistance of the spores and resistance to novobiocin (Cross *et al.* 1968). In morphology and life cycle, *Sporichthya* is unlike any previously described actinomycete. From its morphological features alone it is difficult to place, having similarities with *Streptomyces* (chains of cells on aerial growth), *Nocardia* (transitory mycelial phase) and certain sporangium-forming genera (flagellate cells). Lechevalier *et al.* (1968) placed it in the family Streptomycetaceae, finding its cell wall composition to be similar to *Streptomyces*.

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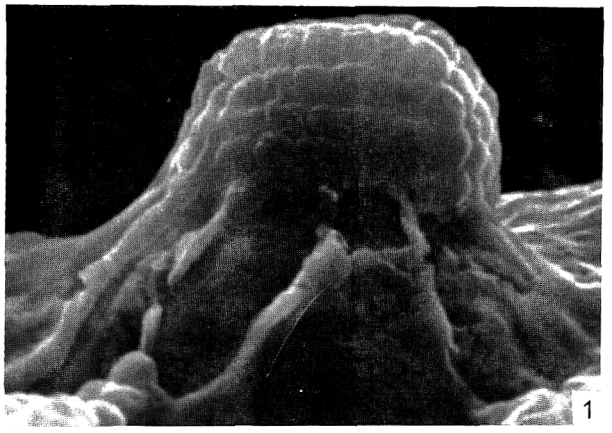
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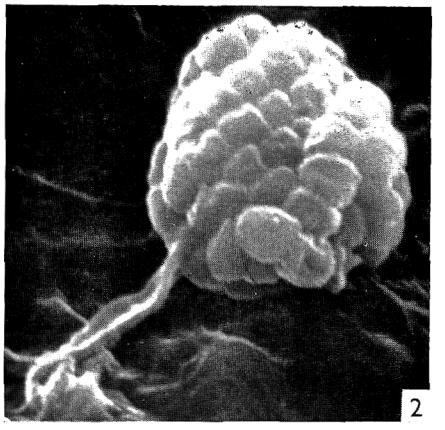
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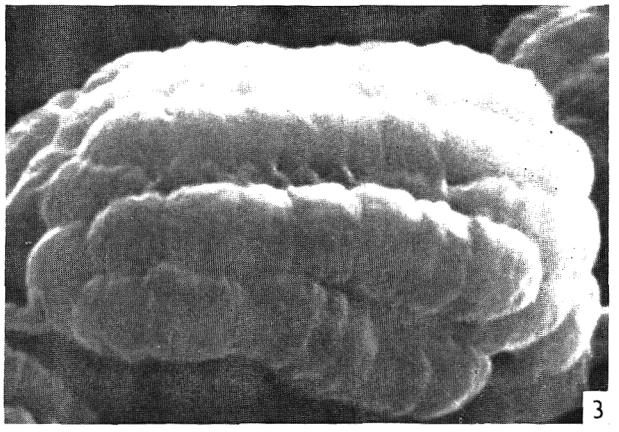
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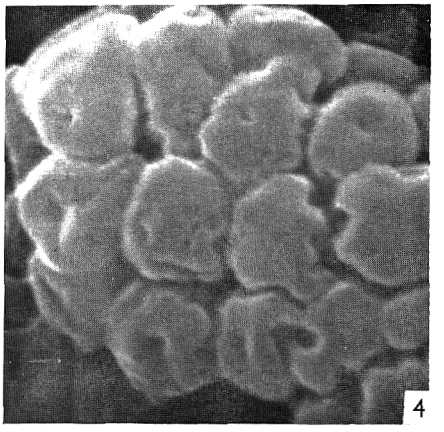
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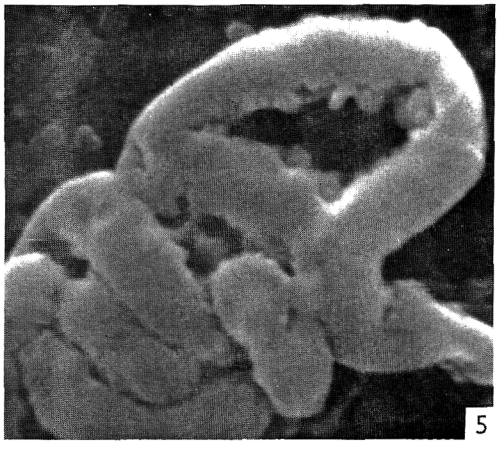
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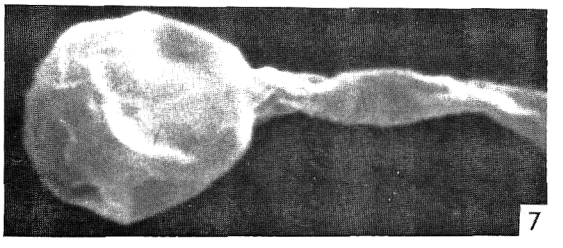
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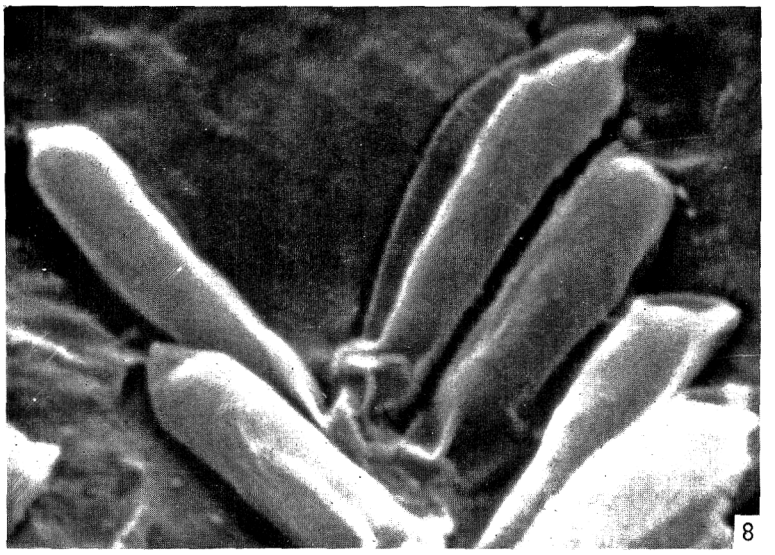
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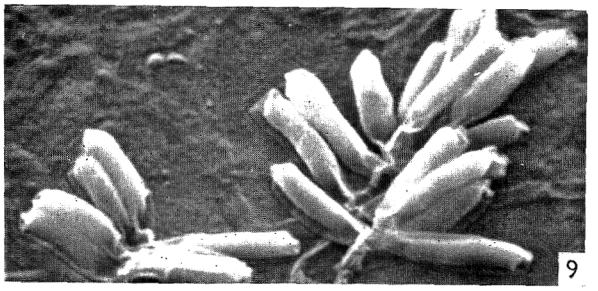
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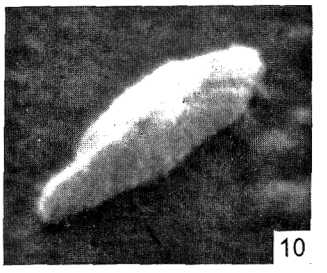
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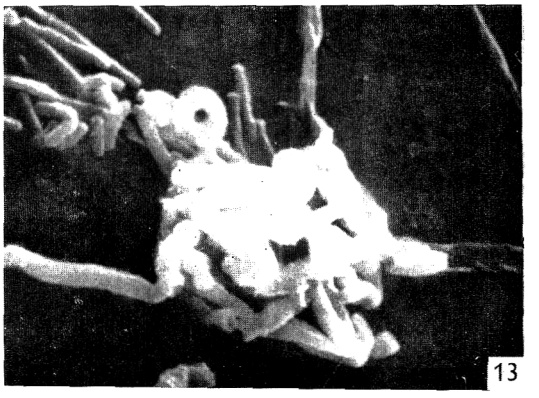
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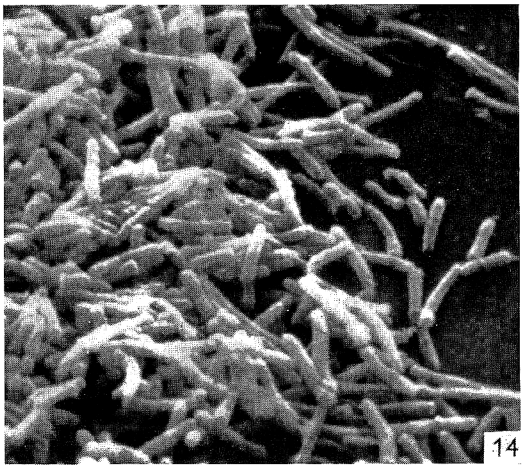
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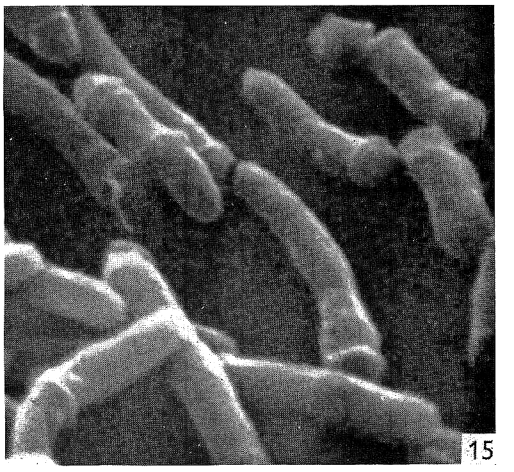
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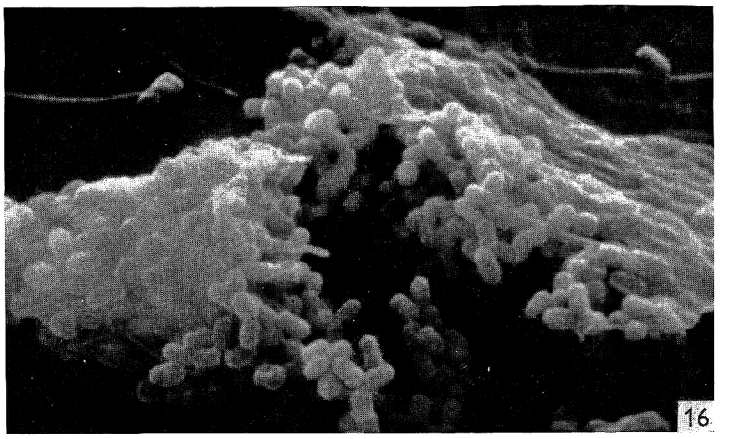
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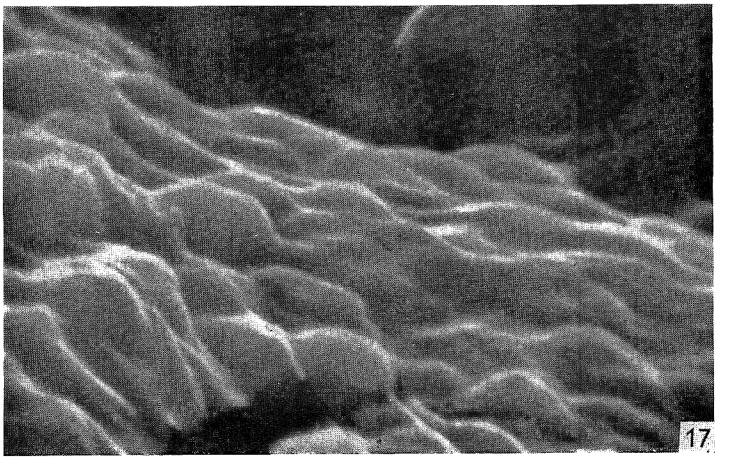
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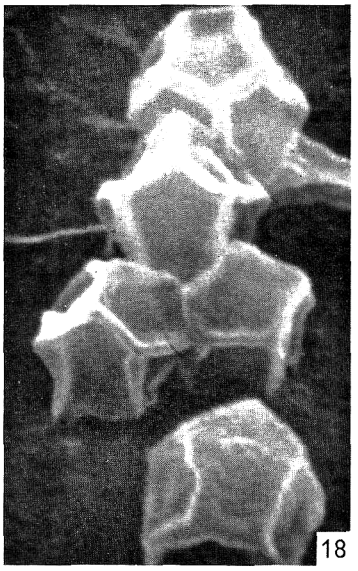
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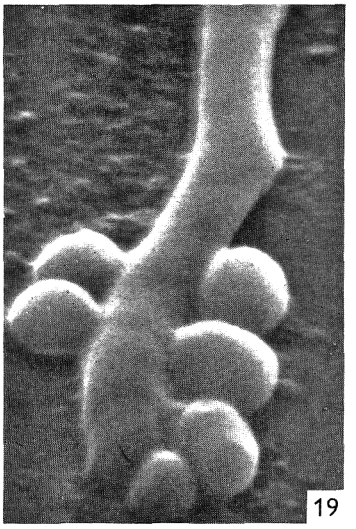
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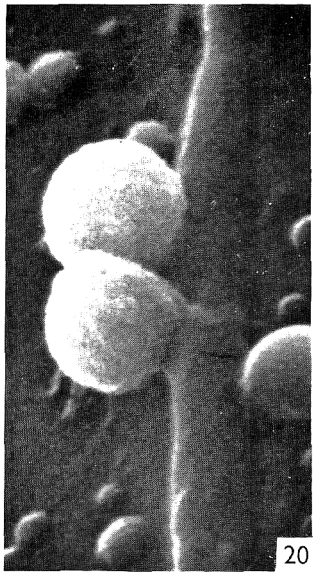
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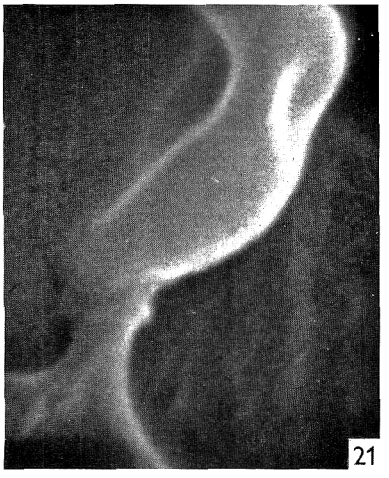
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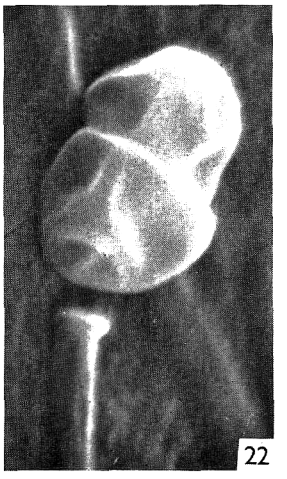
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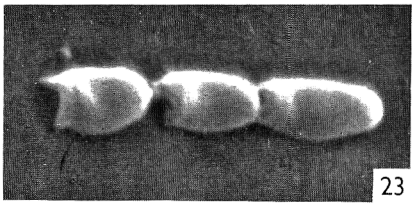
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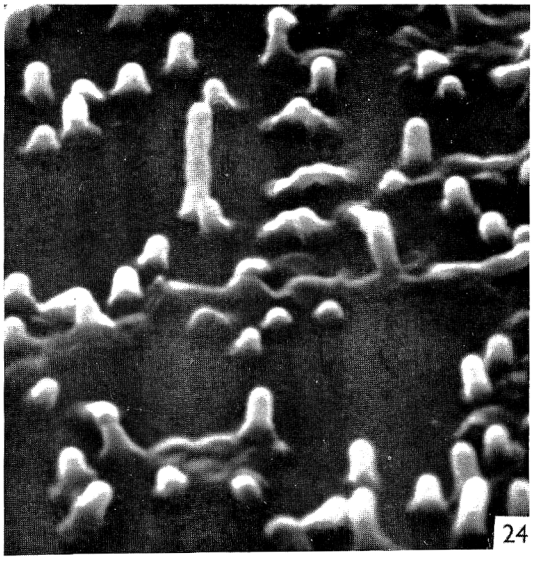
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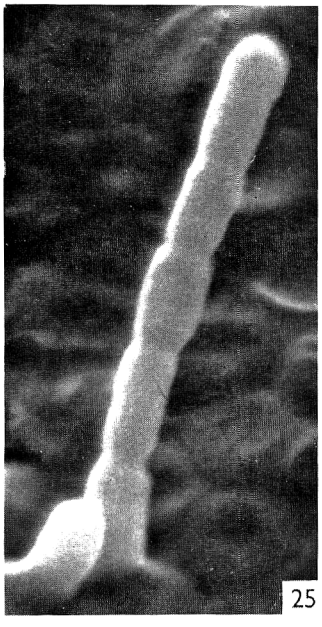
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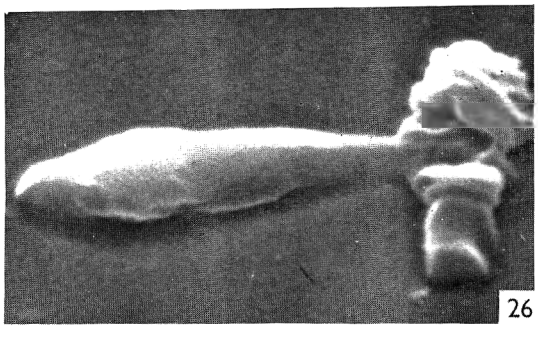
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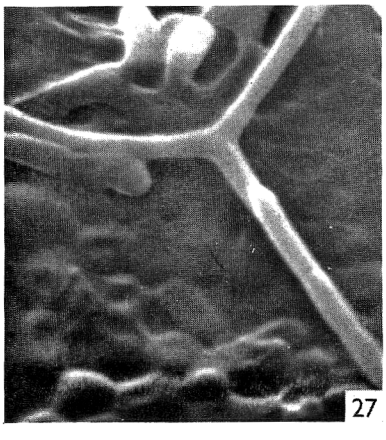
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EXPLANATION OF PLATES

PLATE 1

Fig. 1. *Actinoplanes* sp., a sessile sporangium formed on a mound of hyphae (fixed and dehydrated). × 5000.

Fig. 2. *Actinoplanes* sp., a sporangium borne on a short sporangiophore (fixed and dehydrated). × 5500.

Fig. 3. *Actinoplanes* sp., apparently linear arrangement of spores in sporangium (fixed and dehydrated). × 12,000.

Fig. 4. *Actinoplanes* sp., young spores with irregular shape and surface pitting (fixed and dehydrated). × 15,000.

Fig. 5. *Actinoplanes* sp., a spiral sporogenous hypha from a disrupted sporangium (fixed and dehydrated). × 13,000.

Fig. 6. *Microellobosporia flavea*, a two-spored sporangium with wrinkled outer envelope (untreated). × 12,000.

Fig. 7. *Microechinospora grisea*, a single-spored sporangium with unornamented outer envelope (untreated). × 20,000.

PLATE 2

Fig. 8. *Planomospora venezuelense*, part of a double row of elongated sporangia showing open and unopen tips (fixed and dehydrated). × 24,000.

Fig. 9. *Planomonospora venezuelense*, groups of closely packed sporangia (fixed and dehydrated). × 6000.

Fig. 10. *Planomonospora venezuelense*, a released spore (fixed and dehydrated). × 24,000.

Fig. 11. *Planomonospora venezuelense*, a sporangium with an apical pore (fixed and dehydrated). × 24,000.

Fig. 12. *Planomonospora venezuelense*, sporangium releasing material, possibly a disrupted spore (fixed and dehydrated). × 24,000.

Fig. 13. *Actinosporangium violaceum*, initiation of pseudosporangium by intertwining of branches from several hyphae (fixed and dehydrated). × 5000.

PLATE 3

Fig. 14. *Actinosporangium violaceum*, part of mass of spores and fragments forming a pseudosporangium (fixed and dehydrated). × 4000.

Fig. 15. *Actinosporangium violaceum*, fragments from a pseudosporangium (fixed and dehydrated). × 20,000.

Fig. 16. *Actinopycnidium caeruleum*, a ruptured 'pycnidium' (fixed and dehydrated). × 5000.

Fig. 17. *Actinopycnidium caeruleum*, surface of a 'pycnidium' (fixed and dehydrated). × 20,000.

PLATE 4

Fig. 18. *Actinobifida dichotomica*, ridged spores (untreated). × 30,000.

Fig. 19. *Thermomonospora viridis*, sessile pores with smooth surface (fixed and dehydrated). × 19,000.

Fig. 20. *Thermoactinomyces vulgaris*, young sessile spores with wrinkled sporangial envelope but no ridges visible (fixed and dehydrated). × 24,000.

Fig. 21. *Microbispora rosea*, base of attached spore showing collar (fixed and dehydrated). × 33,000.

Fig. 22. *Microbispora rosea*, released spore showing inflated tip of sporophore and basal invagination of spore (fixed and dehydrated). × 20,000.

PLATE 5

Fig. 23. *Sporichthya polymorpha*, short chain of dispersed cells with basal collar (untreated). × 21,000.

Fig. 24. *Sporichthya polymorpha*, general view of developing upright cells (untreated). × 9000.

Fig. 25. *Sporichthya polymorpha*, a chain of cells with basal one slightly enlarged to act as a holdfast on the substrate (untreated). × 24,000.

Fig. 26. *Sporichthya polymorpha*, a pisciform element developed from one cell of a chain (untreated). × 21,000.

Fig. 27. *Sporichthya polymorpha*, branching hyphae (untreated). × 12,000.

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Ribonucleic Acid Synthesis During Fungal Spore Germination

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SUMMARY

Spores of several fungi were examined in an effort to understand the role of RNA in the biochemical events occurring early in germination. RNA synthesis appeared to be an essential requirement for germination in *Neurospora crassa* and *Aspergillus nidulans* but not in *Alternaria solani* and *Peronospora tabacina*. Gross changes in the relative amounts of the various molecular species of RNA were not detected during germination of *P. tabacina* and *N. crassa* spores. Pulse-labelling of the germinating spores revealed that, in *N. crassa*, ribosomal and soluble RNAs were the major species being synthesized, whereas in *P. tabacina* label was incorporated into soluble RNA and an unstable heterodisperse RNA, but apparently not into ribosomal RNA. This pattern of RNA synthesis in germinating *P. tabacina* spores is similar to that found in cleaving embryos of certain animals where development is also rapid and, like germinating *P. tabacina* spores, can occur when RNA synthesis is inhibited.

INTRODUCTION

Protein synthesis appears essential for the germination of fungal spores (Gottlieb, 1966). However, studies with *Peronospora tabacina* conidia revealed that RNA synthesis, although occurring before germ-tube emergence, was not required for germination (Hollomon, 1969). Although several workers have examined RNA synthesis in germinating fungal spores (for review see Gottlieb, 1966), the importance of this synthesis in the germination of species other than *P. tabacina* is largely unknown. The importance of this synthesis in the germination of spores of five fungi has been assessed. Results show that species differ not only in their need to synthesize RNA, but also in the types of RNA being synthesized.

METHODS

Preparation and germination of spores

Peronospora tabacina Adam. Conidia were collected and prepared as described previously (Hollomon, 1969) except that chloramphenicol (100 μg./ml.) was added to the germination medium to limit bacterial contamination. (The rate of germination was unaltered by this concentration of chloramphenicol.) Conidial suspensions ($2{\cdot}0 \times 10^5$/ml.) were incubated at 15° and germ tubes first appeared after 60 min.

Neurospora crassa Shear and Dodge. Using cold sterile distilled water, conidia were washed from 4 day cultures grown on agar slopes of the medium described by Ryan (1950). Mycelial fragments were removed by filtration through glass wool, and when conidial suspensions (1×10^6/ml.) were incubated at 30° in Ryan's medium, germ tubes first appeared after 2 h.; after 8 h. 80% of the conidia had germinated.

Aspergillus nidulans (Eidam) Wint. Using 0·02 % (v/v) Teepol, conidia were washed from 7 day cultures grown on agar slopes of Czapek–Dox medium (pH 6·8) modified by the addition of biotin (10 μg./l.) and trace element solution (Sussman, 1966) (1·0 ml./l.). Suspensions were filtered to remove mycelial fragments and washed once with cold sterile distilled water. When suspensions (4×10^6 conidia/ml.) were incubated at 37° in the modified Czapek-Dox medium, germ tubes appeared after 4 h.; at 8 h. 80 % of the conidia had germinated.

Alternaria solani (Ellis and Martin) Sorauer. Sporulation of this fungus is stimulated by alternating periods of light and dark (Lukens, 1963). Cultures were, therefore, grown at 24° on potato glucose agar and placed on a windowsill where they received natural daylight. Conidia were washed from 14 day cultures with cold sterile distilled water and mycelial fragments removed by filtration through glass wool. Conidial suspensions (2×10^5/ml.) were incubated at 26° in the modified Czapek–Dox medium used for *Aspergillus nidulans*; germ tubes first appeared after 60 min.; at 6 h. 90 % of the conidia had germinated.

Puccinia graminis Pers. f. sp. *tritici* Eriks and E. Henn. Uredospores (strain ANZ 34-1,2,3,6,7 kindly supplied by Dr N. H. Luig, University of Sydney, N.S.W., Australia) were stored under vacuum in a refrigerator. To initiate germination 5 mg. of uredospores were heat-shocked for 5 min. at 40° (Bromfield, 1964) and then suspended in 2·5 ml. 0·01 % (v/v) Tween 20. When shaken at 26° in 50 ml. conical flasks germ tubes appeared within 30 min.; maximum germination, which never exceeded 60 %, occurred after 2 h.

Germination was considered complete when the first sign of a germ tube was noted and, unless stated otherwise, all species were germinated without shaking in 5·0 cm. diameter Petri dishes containing a final volume of 2·5 ml. Except for the *Peronospora tabacina* medium, which was not sterilized, all media were autoclaved at 117° for 15 min.

RNA extraction from germinating Peronospora tabacina and Neurospora crassa conidia

Germination was stopped by cooling with ice and conidia were collected on membrane filters (Oxoid). Bentonite (7·0 mg.) was added and the conidia were then transferred to a chilled mortar containing solid CO_2, sand, and sufficient 20 % (w/v) sodium dodecyl sulphate (SDS) to give a final concentration of 1 % (w/v) SDS. Conidia were broken by grinding (microscopic examination showed breakage always exceeded 80 %) and extracted with 3·0 ml. 0·05 M-tris + HCl (pH 8·0) containing 5·0 mM-$MgCl_2$. The broken spore suspension was extracted three times with tris-saturated phenol containing 0·05 M-EDTA and 0·5 % (w/v) SDS, the first phenol extraction being carried out by shaking for 3 min. at 60°. Subsequent phenol extractions and further purification were carried out as described previously (Hollomon, 1969).

Fractionation of RNA by polyacrylamide gel electrophoresis

Nine cm. polyacrylamide gels (2·4 % w/v) were prepared in Perspex tubes (1/4 in. internal diameter × 5 in. long) by the method of Loening (1967) except that 10 % (v/v) glycerol and 1 % (w/v) agarose were added to facilitate slicing the gels. RNA samples were layered directly on to the gels in tris + acetate buffer (final concentration 0·045 M-tris + HCl; 0·02 M-Na acetate; 2 mM-sodium EDTA; acetic acid was used to adjust to pH 7·3. at 24°) containing 15 % (v/v) glycerol, and electrophoresis was carried

out for 150 min. at 5 mA/gel and 10 V/cm. using tris + acetate buffer containing 10 % (v/v) glycerol.

After electrophoresis gels were stained in 0·025 % (w/v) Azure B in 0·065 M-citrate + phosphate buffer (pH 4·6), de-stained overnight in distilled water, and scanned in a Joyce Loebl Chromoscan with a red (620 nm.) filter. Under these conditions direct proportionality between absorbance and amount of nucleic acid did not exist, and therefore quantitative estimation of the RNA was not possible. After scanning, gels were frozen with solid CO_2 and sliced into 1 mm. sections with a McIlwain gel slicer (Mickle Engineering, Gomshall, Surrey). Two sections were placed into each scintillation vial and hydrolysed with 0·5 ml. hyamine for 30 min. at 50°. To accommodate the water present in the samples six drops of Triton X 100 were added to each vial from a Pasteur pipette and the radioactivity was counted in a Packard Tri-carb Liquid Scintillation Spectrometer using 2,5 diphenoxazole (6·0 g./l. toluene) as scintillant.

Preparation of ribosomes from Peronospora tabacina conidia

Germinated conidia were augmented with dormant conidia to provide sufficient material for manipulation. Conidia were collected on a membrane filter and transferred to a chilled mortar containing solid CO_2, sand and 28 mg. sodium deoxycholate. Conidia were broken by grinding and upon thawing were extracted with 5·0 ml. buffer (0·01 M-tris + HCl (pH 7·8); 0·01 M-Mg acetate; 0·06 M-KCl; 0·006 M-mercaptoethanol (KMT buffer)). Sand was sedimented from this extract by centrifugation for 1 min. at 100 *g* and the supernatant fluid incubated at 37° for 20 min. to permit digestion of messenger RNA. Mitochondria and larger particles were sedimented by centrifugation for 10 min. at 10,000 *g* and the supernatant fluid further centrifuged for 2 h. at 117,000 *g*. The ribosomal pellet was suspended in 2·0 ml. KMT buffer and centrifuged for 90 min. at 117,000 *g* to yield washed ribosomes. These ribosomes gave a characteristic u.v. spectrum with a $E^{260}_{1\,cm.} : E^{230}_{1\,cm.}$ of 1·4, indicating the presence of about 20 % contaminating protein (Peterman, 1964). Dialysis for 90 min. against 0·05 M-tris + HCl (pH 7·8) containing 0·01 M-sodium EDTA resulted in dissociation of the ribosomes into two subunits which could be fractionated on a 5 to 20 % (w/v) sucrose density gradient by centrifugation for 2 h. at 37,000 rev./min. (Spinco S.W. 39 rotor).

RNA present in these ribosome preparations was fractionated by polyacrylamide gel electrophoresis using the procedure described above, except that in the gels and the electrophoresis buffer glycerol was replaced by 0·2 % (w/v) SDS. Ribosomes were disrupted in KMT buffer (without mercaptoethanol) containing 0·5 % (w/v) SDS and 10 % (w/v) sucrose and layered on the gels. After electrophoresis (150 min., 5 mA/gel, 10 V/cm.) gels were rinsed with distilled water for 30 min. and scanned, using transmitted u.v. irradiation by the method of Loening (1969) (except that the *p*-dimethylaminobenzaldehyde filter was omitted). Gels were then sectioned and prepared for radioactive counting as described earlier.

Radioisotopes. Reconstituted [^{14}C]yeast protein hydrolysate was purchased from Schwarz Biochemicals Inc., Orangeburg, N.Y., U.S.A. [^{3}H]uridine (13 Ci/m-mole) and [^{3}H]cytidine (30·2 Ci/m-mole) were purchased from the Radiochemical Centre, Amersham, Buckinghamshire. Carrier-free [^{32}P] as orthophosphate (10 to 25 Ci/mg.P) was purchased from the Australian Atomic Energy Commission, Lucas Heights, Sydney, N.S.W., Australia.

RESULTS

RNA and protein synthesis requirements during germination

The effect of proflavine on RNA and protein synthesis in *Alternaria solani*, *Aspergillus nidulans*, *Neurospora crassa* and *Peronospora tabacina* is shown in Fig. 1. Addition of proflavine was followed, 10 min. later, by either [^{3}H]uridine or [^{14}C]-labelled amino

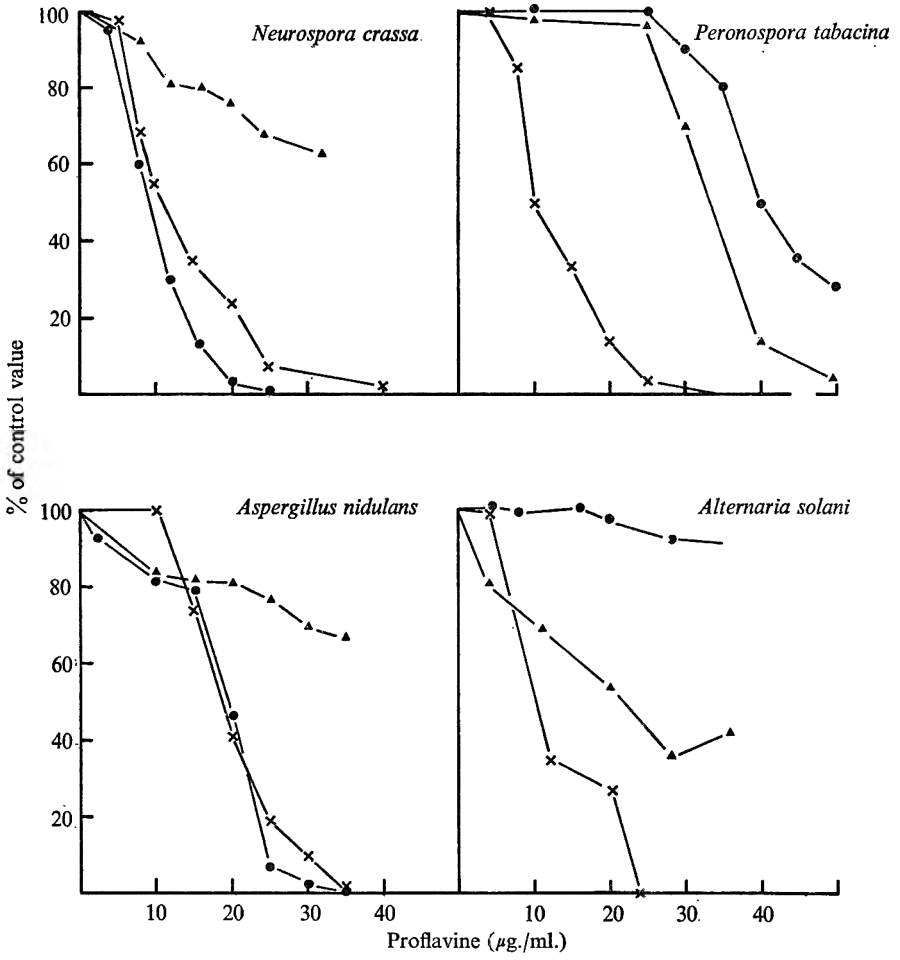

Fig. 1. Effect of proflavine on RNA synthesis, protein synthesis, and germination in *Peronospora tabacina*, *Neurospora crassa*, *Aspergillus nidulans*, and *Alternaria solani*. For RNA and protein synthesis proflavine was added to conidia of *A. solani* and *P. tabacina* at the start of germination, and to *N. crassa* and *A. nidulans* conidia after 135 and 225 min. incubation, respectively. In all cases, addition of proflavine was followed 10 min. later by the addition of either [^{3}H]uridine or [^{14}C]-labelled amino acids. Conidia were incubated in the presence of the isotope for 15 min., except for *A. solani* where uptake was for 120 min. Incorporation was stopped by adding 0·5 ml. cold 30% (w/v) TCA and conidia were then treated as described previously (Hollomon, 1969). For germination proflavine was added to all conidia at the start of germination and the percentage germination was assessed after 4 h. at 15° for *P. tabacina*, 8 h. at 30° for *N. crassa*, 8 h. at 37° for *A. nidulans*, and 6 h. at 26° for *A. solani*. Results are presented as % of control where control represents the value obtained in the absence of proflavine. ●——●, Germination; ×——×, RNA synthesis; ▲——▲, protein synthesis.

acids. Uptake of these isotopes was stopped by adding 0·5 ml. cold 30% (w/v) trichloracetic acid (TCA), and incorporation into TCA-insoluble material was taken as a measure of RNA and protein synthesis, respectively. As in other organisms (Waring, 1966) proflavine inhibited RNA synthesis but reduced protein synthesis only at higher concentrations. Nevertheless, proflavine had a differential effect on germination (Fig. 1). With *A. nidulans* and *N. crassa* inhibition of germination by proflavine closely

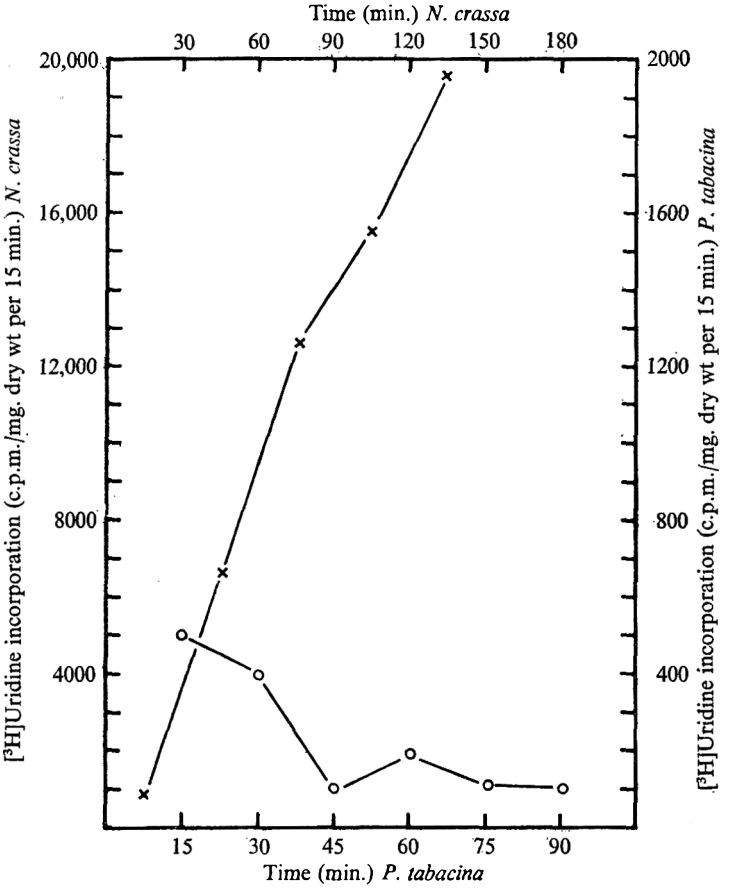

Fig. 2. The incorporation of [³H]uridine into RNA at intervals during germination. *Neurospora crassa* and *Peronospora tabacina* conidia were labelled for 15 min. pulses with 0·4 μCi/ml. [³H]uridine and 2·0 μCi/ml [³H]uridine respectively. Incorporation was stopped by addition of 0·5 ml. 30% (w/v) TCA and conidia were treated as described previously (Hollomon, 1969). ×———×, *Neurospora crassa*; ○———○, *Peronospora tabacina*.

paralleled its effect on RNA synthesis. However, germination in *A. solani* and *P. tabacina* was unaffected by proflavine concentrations which severely inhibited RNA synthesis, but was inhibited at higher concentrations where proflavine inhibited protein synthesis. These results suggest that in *A. solani* and *P. tabacina* RNA synthesis is not required for germination, whereas in *A. nidulans* and *N. crassa* RNA synthesis is an essential requirement for germination. Similar experiments with *Puccinia graminis* were less satisfactory. Although uredospores germinated at pro-

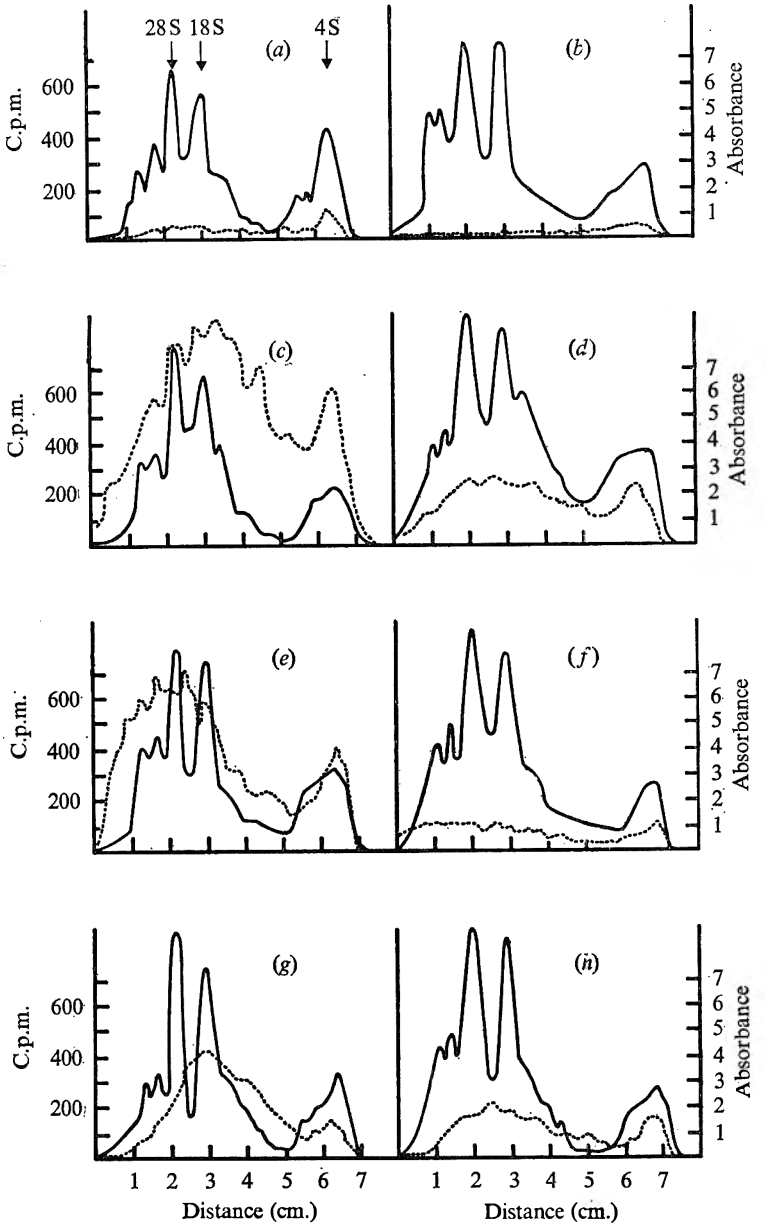

Fig. 3. RNA synthesis in germinating *Peronospora tabacina* conidia. For each treatment, 3.2×10^7 conidia were germinated in a medium without phosphate, in eight 15·0 cm. diameter Petri dishes each containing a final volume of 20 ml. Conidia in one dish only were labelled for 20 min. with [^{32}P] (10 to 12 μCi/ml.). After incorporation conidia from all dishes were collected together on a membrane filter either immediately or after a further 100 min. incubation in the presence of excess non-radioactive phosphate (chase). RNA was extracted and fractionated as described in the methods section. 100 μg. RNA were applied to each gel. (*a*) 0 to 20 min. pulse; (*b*) 0 to 20 min. pulse, 100 min. chase; (*c*) 20 to 40 min. pulse; (*d*) 20 to 40 min. pulse, 100 min. chase; (*e*) 40 to 60 min. pulse; (*f*) 40 to 60 min. pulse, 100 min. chase; (*g*) 60 to 80 min. pulse; (*h*) 60 to 80 min. pulse, 100 min. chase. – – –, Counts/min.[^{32}P]; ———, absorbance 620 nm. (arbitrary scale).

flavine concentrations which inhibited RNA synthesis in the other four species, uredospores failed to incorporate [^{3}H]uridine into RNA before germ-tube emergence, and the effect of proflavine on RNA synthesis could not be determined.

It would seem, therefore, that differences exist between species in their requirement for RNA synthesis during germination. These differences were examined in greater detail using *Peronospora tabacina* and *Neurospora crassa*. To determine the rate of RNA synthesis prior to germ-tube emergence, conidia were labelled with 15 min. pulses of [^{3}H]uridine given at 15 min. intervals for *P. tabacina*, and at 30 min. intervals for *N. crassa*. Although initially low, the rate of incorporation of [^{3}H]uridine into *N. crassa* increased as germination proceeded (Fig. 2). However, the rate of incorporation of [^{3}H]uridine into *P. tabacina* conidia was not only much lower, but was fairly constant throughout.

RNA synthesis in germinating Peronospora tabacina conidia

Because of the low rate of incorporation, sufficient radioactivity could not be applied to gels to permit satisfactory resolution of RNA labelled during germination with [^{3}H]uridine. This problem was overcome, however, by labelling with [^{32}P] rather than

Table 1. *Specific activity and RNAase susceptibility of* [^{32}P]*-labelled RNA from germinating Peronospora tabacina conidia*

Details of the method used to label conidia are given in Fig. 3. Susceptibility to RNAase was determined by incubation of 100 μg. pulse-labelled RNA with 10 μg. RNAase for 2 h. at 37° and precipitation of residual radioactivity on to membrane filters with cold 5% TCA.

Time after start of germination at which [^{32}]P pulse was given (min.)	Specific activity (counts/min./mg.RNA)		Radioactivity remaining after RNAase treatment of pulse-labelled RNA (%)
	Pulse	Chase	
0 to 20	$0{\cdot}49 \times 10^5$	$0{\cdot}36 \times 10^5$	28
20 to 40	$3{\cdot}42 \times 10^5$	$1{\cdot}12 \times 10^5$	8
40 to 60	$2{\cdot}21 \times 10^5$	$1{\cdot}25 \times 10^5$	7
60 to 80	$1{\cdot}94 \times 10^5$	$0{\cdot}51 \times 10^5$	3

[^{3}H]uridine. Conidia were labelled with 20 min. pulses of [^{32}P] given at 20 min. intervals during the first 80 min. of germination. In some experiments, this pulse was followed by further incubation for 100 min. in the presence of excess non-radioactive phosphate (chase). At the end of each pulse and each chase period, RNA was extracted with hot (60°) phenol to ensure extraction of the large molecular weight RNA. RNA was fractionated by polyacrylamide gel electrophoresis stained with Azure B and sectioned for radioactive counting. Any degradation occurring during extraction was monitored by the recovery of [^{3}H]-labelled Neurospora RNA which was added to the conidia at the time of grinding. Recovery of this labelled RNA varied between 60 and 80% of that initially added. Although some degradation undoubtedly occurred, this was similar in each preparation so that comparisons between preparations were possible.

Five major RNA bands were revealed when *Peronospora tabacina* extracts were stained with Azure B (Fig. 3): a fast-migrating soluble RNA band, two ribosomal RNA bands which were coincident with the ribosomal RNA bands of *Neurospora crassa*, and two bands migrating more slowly than ribosomal RNA. During the first

180 min. of germination no changes in the position or intensity of any of these bands could be detected. Although a constant amount of RNA was extracted throughout the germination period, [^{32}P] incorporation was not constant and was least during the first 20 min. (Table 1). Furthermore, 28 % of this early [^{32}P] incorporation was ribonuclease-resistant. After electrophoresis of this early RNA some radioactivity was detected in the anode buffer, suggesting incorporation into low molecular weight compounds, such as polyphosphate or simply [^{32}P] orthophosphate carried through in RNA preparation.

Much of the [^{32}P] incorporated into RNA early in germination was into soluble RNA (Fig. 3*a*). However, when germinated in the presence of ethidium bromide (50 μg./ml.), an RNA synthesis inhibitor (Waring, 1966), 20 % of the [^{32}P] incorpora-

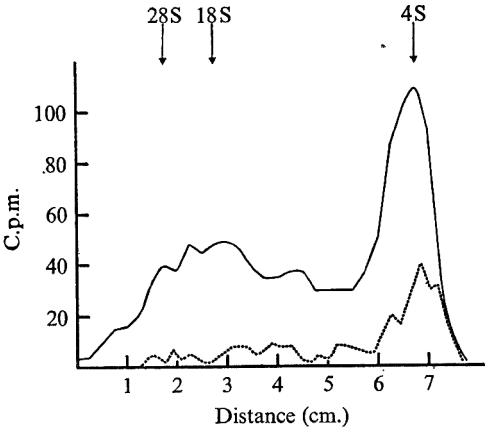

Fig. 4

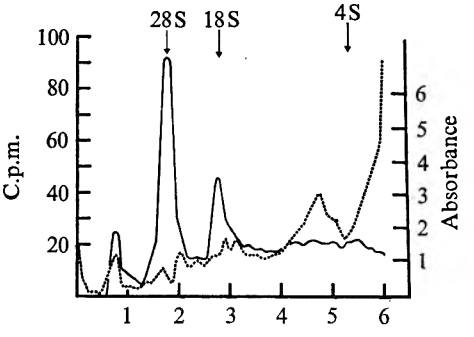

Fig. 5

Fig. 4. Effect of ethidium bromide (50 μg./ml.) on the incorporation of [^{32}P] into RNA by germinating *Peronospora tabacina* conidia. 4·0 × 10^6 conidia were labelled with [^{32}P] (11 to 12 μCi/ml.) during the first 20 min. of germination. Uptake was stopped by rapidly cooling to 0° and conidia were collected on a membrane filter together with 2·8 × 10^7 ungerminated conidia. RNA was extracted and fractionated as described in the Methods section, and 100 μg. RNA were applied to the gel. ———, Control; – – – –, ethidium bromide.

Fig. 5. Polyacrylamide gel electrophoresis of RNA from ribosomes of germinating *Peronospora tabacina* conidia. Ribosomes were extracted from conidia germinated in the presence of [^{32}P] as described in Table 3. 265 μg. ribosomes were disrupted with 0·5 % (w/v) SDS and layered on to the gel and electrophoresed as described in the Methods section. – – – –, Counts/min. [^{32}P]; ———, absorbance 620 nm. (arbitrary scale).

Table 2. *The effect of ethidium bromide on [^{3}H]cytidine and [^{3}H]uridine incorporation into RNA in Peronospora tabacina*

Conidia (2·0 × 10^5/ml.) were incubated for 40 min. in the presence of either [^{3}H]cytidine (2·0 μCi/ml.) or [^{3}H]uridine (2·0 μCi/ml.). Incorporation was stopped by adding 0·5 ml cold 30 % (w/v) TCA, and conidia then treated as described by Hollomon (1969) except that the hot TCA wash was omitted.

Treatment	Disintegration/min. incorporated into RNA	
	[^{3}H] cytidine	[^{3}H]uridine
Control	12,693	5,314
Ethidium bromide (50 μg./ml.)	9,913	436
Inhibition by ethidium bromide (%)	22	92

ted into RNA during the first 20 min. of germination remained, and was almost entirely in soluble RNA (Fig. 4). Whereas [^{3}H]uridine incorporation early in germination was inhibited by ethidium bromide, much of the [^{3}H]cytidine incorporation was not (Table 2). These results, together with the observation that much of the [^{32}P] incorporated into soluble RNA was metabolically unstable and was lost during the chase incubation (Figs. 3*b*, *d*), strongly suggest that [^{32}P] was incorporated into the three terminal nucleotides (*pCpCpA*) as well as into newly synthesized soluble RNA. In addition to soluble RNA, [^{32}P] was also incorporated into heterodisperse RNA, although this was largely lost during the chase incubation (Fig. 3). Longer electrophoresis increased the separation of the ribosomal RNA bands without showing any particular association of these bands with radioactivity, which suggests that perhaps ribosomal RNA synthesis did not occur early in germination.

Table 3. *Incorporation of* [^{32}*P*] *into ribosomes during germination of Peronospora tabacina conidia*

Conidia ($8{\cdot}0 \times 10^6$) were incubated for 60 min. with carrier-free [^{32}P] (4·9 μCi/ml.) followed by a further 100 min. incubation in the presence of excess non-radioactive phosphate. Conidia were collected on a membrane filter and augmented with $4{\cdot}0 \times 10^7$ ungerminated conidia. Ribosomes were isolated by the procedure already described.

Fraction	Counts/min. incorporated by $8{\cdot}0 \times 10^6$ conidia
Total incorporated	$455{\cdot}92 \times 10^4$
× 10,000 *g* ppt. (mitochondria, nuclei)	$7{\cdot}50 \times 10^4$
× 117,000 *g* supernatant fluid (soluble fraction)	$430{\cdot}84 \times 10^4$
× 117,000 *g* ppt. (washed ribosomes)	$3{\cdot}42 \times 10^4$
Washed ribosomes after ppt. with 5% TCA	$2{\cdot}26 \times 10^4$
Washed ribosomes after dialysis against 0·05 M-tris + HCl (pH 7·8) + 0·01 M-sodium EDTA	$2{\cdot}25 \times 10^4$

Conidia of *Peronospora tabacina* were incubated with [^{32}P] during the first 60 min. of germination followed by a further 100 min. incubation in the presence of excess non-radioactive phosphate, thus reducing incorporation into metabolically unstable heterodisperse RNA. Ribosomes were then isolated and incorporation into the different fractions is given in Table 3. Much of the [^{32}P] incorporated by the conidia was contained in the soluble fraction obtained after centrifugation for 2 h. at 117,000 *g*, whereas little [^{32}P] was incorporated into nuclei, mitochondria, or ribosomes. Further washing of the ribosomal pellet with KMT buffer did not remove radioactivity, yet only 66% of this incorporation into ribosomes could be precipitated with 5% (w/v) TCA and was presumably RNA. Dialysis for 90 min. against 0·05 M-tris + HCl (pH 7·8) containing 0·01 M-sodium EDTA resulted in a 34% loss of radioactivity from the ribosomes. When washed ribosomes were disrupted with 0·5% (w/v) SDS and electrophoresed, no radioactivity was associated with ribosomal RNA, which confirms that no detectable ribosomal RNA synthesis occurred in *P. tabacina* during the first 60 min. of germination (Fig. 5).

RNA synthesis in germinating Neurospora crassa conidia

Neurospora crassa conidia readily incorporated [^{3}H]uridine during germination (Fig. 2). As a result, conidia were labelled with 60 min. pulses of [^{3}H]uridine given at hourly intervals during the first 4 h. of germination. Conidia were germinated with

constant shaking at 30° in flasks containing 20 ml. of medium. Following each pulse, RNA was extracted, fractionated by polyacrylamide gel electrophoresis, and stained with Azure B. As can be seen from Fig. 6 there was little evidence of degradation and four major RNA bands were detected: two ribosomal RNA bands (28S and 18S; Rifkin, Wood & Luck, 1967); two fast-migrating bands, one being soluble RNA and

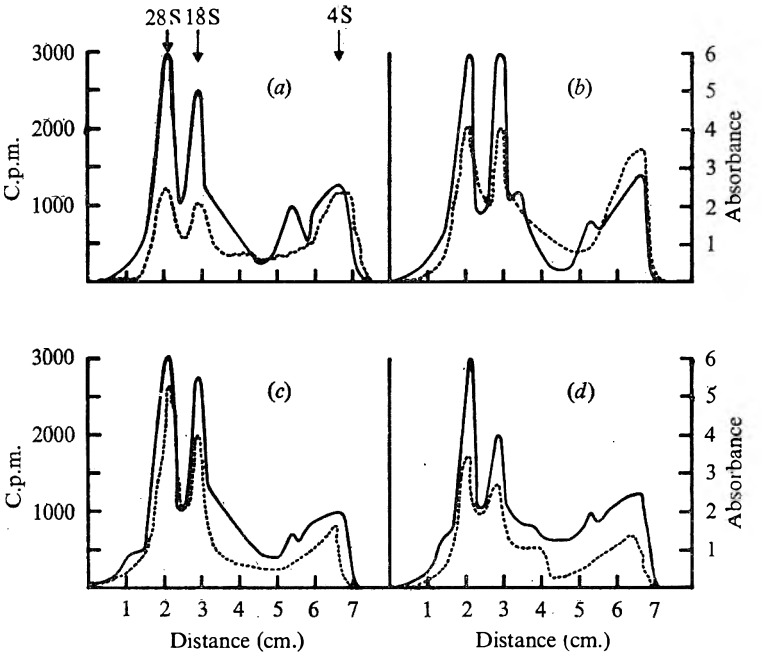

Fig. 6. RNA synthesis in germinating *Neurospora crassa* conidia. Conidia were germinated with constant shaking in five 500 ml. conical flasks each containing a final volume of 20 ml. Conidia were labelled for 60 min. with [^{3}H]uridine (1·25 μCi/ml.) given at 60 min. intervals after the start of germination. After incorporation conidia were collected together on membrane filters and RNA was extracted and fractionated. 23 μg. RNA were applied to each gel. (*a*) 0 to 60 min.; (*b*) 60 to 120 min.; (*c*) 120 to 180 min.; (*d*) 180 to 240 min.; – – – –, Counts/min. [^{3}H]; ———, absorbance 620 nm (arbitrary scale).

Table 4. *Specific activity of* [^{3}*H*]*uridine labelled RNA from germinating Neurospora crassa conidia*

Details of the conditions used to label conidia are given in Fig. 6

Time after start of germination at which [^{3}H]uridine was given (min.)	Specific activity (counts/min./mg. RNA)
0 to 60	$6{\cdot}96 \times 10^5$
60 to 120	$7{\cdot}26 \times 10^5$
120 to 180	$7{\cdot}68 \times 10^5$
180 to 240	$7{\cdot}44 \times 10^5$

the other perhaps 7S RNA. As with *Peronospora tabacina*, changes in the position or intensity of these bands during germination were not detected. The total amount of RNA extracted increased as germination proceeded whereas the specific activity remained fairly constant (Table 4). At first [^{3}H]uridine was incorporated equally into

ribosomal and soluble RNA but, by the third hour, in contrast to the situation found in *P. tabacina* conidia, ribosomal RNA synthesis predominated.

DISCUSSION

Germination of spores used in these experiments was inhibited by actidione, an inhibitor of protein synthesis, indicating that protein synthesis was essential for germination (unpublished results). RNA synthesis, however, was not required for germination of *Peronospora tabacina* conidia (Hollomon, 1969), *Alternaria solani* conidia, and perhaps also *Puccinia graminis* uredospores. On the other hand, proflavine inhibited the germination of *Neurospora crassa* and *Aspergillus nidulans* conidia at concentrations which also inhibited RNA synthesis, suggesting that germination of these two species required the synthesis of RNA. Some fungal species seem, therefore, to differ in their requirements for RNA synthesis during germination.

Some RNA is, nevertheless, synthesized by germinating *Peronospora tabacina* conidia, and a comparison of this synthesis with the essential RNA synthesis in germinating *Neurospora crassa* conidia revealed further differences between the two organisms. Less [^{3}H]uridine was incorporated into RNA by *P. tabacina* conidia than was incorporated by *N. crassa* conidia, despite a fivefold increase in the [^{3}H]uridine concentration (Fig. 2). The RNA synthesized by *N. crassa* conidia was largely ribosomal with some soluble RNA (closely resembling the pattern of synthesis in germinating *Aspergillus oryzae* (Ono, Kimura & Yanagita, 1966; Tanaka, Ono & Yanagita, 1966) whereas in *P. tabacina* ribosomal RNA synthesis was not detected. Instead, the RNA synthesized by *P. tabacina* at the start of germination was mainly soluble RNA, although some of this may represent the rapid end-labelling of the terminal *pCpCpA* sequence. After the first 20 min., but before germ-tube emergence, unstable heterodisperse RNA was synthesized in addition to the continuing synthesis of soluble RNA. Although this heterodisperse RNA may be messenger RNA, further work is required to confirm this. Early in germination a considerable proportion of the [^{32}P] was incorporated into RNAase-resistant material which is, presumably, a low molecular weight compound such as polyphosphate or unincorporated [^{32}P]orthophosphate.

During germination of both *Peronospora tabacina* and *Neurospora crassa* conidia changes in the position or intensity of the electrophoretically separated RNA bands were not detected; thus germination seemed to involve neither the appearance nor disappearance of RNA components. Although changes were not detected it is of interest to note, in view of the lack of ribosomal RNA synthesis in *P. tabacina*, two prominent bands which migrated more slowly than ribosomal RNA, and which were possibly precursor ribosomal RNA. Similar bands were barely detectable in *N. crassa* conidia where ribosomal RNA synthesis was more active.

Failure to synthesize ribosomal RNA is not unique to germinating *Peronospora tabacina* conidia. Ribosomal RNA synthesis does not occur in cleavage embryos of sea urchins (Nemer, 1963), amphibians (Brown & Gurdon, 1964), and echiuroid worms (Gould, 1969). As in germinating *P. tabacina* conidia, these systems are undergoing rapid development and, at least in sea urchins, this development also does not require the synthesis of new RNA (Spirin, 1966). Nevertheless, some RNA is synthesized by cleaving sea-urchin embryos (Wilt, 1964; Nemer & Infante, 1966), and the pattern of this synthesis is almost identical with that found in germinating *P. tabacina*

conidia. Early in cleavage radioactivity is rapidly incorporated into soluble RNA and this incorporation is largely into the three terminal nucleotides *p*C*p*C*p*A (Glisin & Glisin, 1964; Gross, Kraemer & Malkin, 1965). In addition, there is also synthesis of heterodisperse messenger-like RNA (Wilt, 1964; Nemer & Infante, 1966).

It is essential in a study of this type that RNA should be obtained with as little degradation as possible. Extraction of RNA with cold phenol + SDS from *Peronospora tabacina* conidia disrupted by ultrasonic treatment yielded degraded preparations. Undoubtedly some of this degradation was caused by the 20 min. ultrasonic treatment needed to disrupt the conidia and which has been shown to degrade *Escherichia coli* RNA (Das, Goldstein & Lowney, 1967; Fry & Artman, 1968). For this reason conidia were broken by grinding with solid CO_2 and sand. Also, it is essential during germination to prevent the growth of bacteria which occur in *P. tabacina* conidial preparations. Fortunately, bacterial growth is inhibited by chloramphenicol (100 μg./ml.) whereas germination is not. Failure to control bacterial contamination was responsible for the earlier report (Hollomon, 1969) of ribosomal RNA synthesis in *P. tabacina* conidia which conflicts with the results presented here.

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A Comparative Study of the Incorporation of [1-^{14}C]Acetate into Phospholipids by a Toxigenic and a Non-toxigenic Strain of *Aspergillus flavus*

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SUMMARY

The incorporation of [1-^{14}C]acetate into phospholipids by a toxigenic and a non-toxigenic strain of *Aspergillus flavus* was investigated, using a glucose salts medium (AM medium) and a sucrose yeast extract medium (YES medium) for growth and for the incorporation of radioactivity. YES medium yielded large amounts of aflatoxins. The toxigenic strain gave a higher incorporation of acetate in AM medium and a lower incorporation in YES medium than did the non-toxigenic strain. The growth and resuspension media strongly influenced the incorporation by the toxigenic strain. The specific activity obtained on AM medium was more than 10 times that on YES medium. The effects of the media on the non-toxigenic strain were less marked. The data indicate a close interrelation between the utilization of acetate for the synthesis of phospholipids and for the formation of aflatoxins.

INTRODUCTION

Aflatoxins, a group of toxic metabolites produced by *Aspergillus flavus*, have been attracting a large number of investigators because of their toxicity and carcinogenicity to a number of experimental animals, and because of the need to prevent their formation in agricultural products (Wogan, 1966; Goldblatt, 1967; Schoental, 1967.) Though a considerable amount of work has been done on their chemistry and biological effects and the factors affecting their formation, no systematic comparison of the metabolism of toxigenic and non-toxigenic strains of *A. flavus* seems to have been carried out. As a part of such a comparative study, the incorporation of [1-^{14}C]acetate into phospholipids by two strains of *A. flavus* was investigated, using two media which differed markedly in their ability to support the production of aflatoxins. The data obtained suggest a possible relation between lipid biosynthesis and aflatoxin formation.

METHODS*

Aspergillus flavus ATCC 15517, a toxigenic strain, and *A. flavus* ATCC 9643, a non-toxigenic strain were obtained from the Northern Regional Research Laboratory,

* The following abbreviations are used: AM medium, glucose salts medium; YES medium-sucrose yeast extract medium; PI, phosphatidyl inositol; PS, phosphatidyl serine; LPC, lysophos, phatidyl choline; LPE, lysophosphatidyl ethanolamine; PC, phosphatidyl choline; PE, phosphatidyl ethanolamine; PGP, polyglycerophosphatide; $NADPH_2$, reduced nicotinamide adenine dinucleotide.

Peoria, Illinois, U.S.A., and maintained as soil cultures. Sodium [1-^{14}C]acetate (specific activity 12·66 mCi/mM) was obtained from the Bhabha Atomic Research Centre, Bombay. The liquid media used had the following composition. Glucose salts medium (AM medium) (Adye & Mateles, 1964): Glucose, 50 g.; ammonium sulphate, 3 g.; $MgSO_4.7H_2O$, 200 mg.; ammonium molybdate $4H_2O$, 0·5 mg.; $CuSO_4.5H_2O$, 0·3 mg.; $MnSO_4.H_2O$, 0·11 mg.; $ZnSO_4.7H_2O$, 17·6 mg.; $Fe_2(SO_4)_3.6H_2O$, 10 mg.; $Na_4B_2O_7$, 0·7 mg./l.; pH 6·6. Sucrose yeast extract medium (YES medium) (Davis, Diener & Eldridge, 1966): Sucrose, 200 g.; Difco yeast extract, 20 g./l., pH 6·4.

Culture methods. The *Aspergillus* strains were grown first in glucose peptone agar slants and then in 50 ml. of the same medium in bottles. After 1 week, the spores from this growth were used to inoculate sufficient 100 ml. quantities of each of the two liquid media, AM and YES, in 500 ml. Erlenmeyer flasks which were then incubated on a rotary shaker for 3 days at 25°.

Incorporation of [1-^{14}C]acetate. The mycelium from each flask was separated by filtration under suction. Each mycelial mat was then washed well with distilled water, blotted and weighed. They were then resuspended in 50 ml. of sterile AM or YES medium, containing 10 μCi of sodium [1-^{14}C]acetate in 250 ml. Erlenmeyer flasks and incubated for 3 h. on a rotary shaker at 25°. Preliminary experiments indicated that maximum incorporation was obtained at 3 h. The mycelium was separated from the medium, washed well and the lipids extracted by grinding with sand and chloroform–methanol (2:1, v/v). Proteolipids were dissociated by the method of Folch, Lees & Stanley (1957) and the final lipid extract made up to a known volume in chloroform.

Separation and determination of phospholipids. Phospholipids were separated by thin layer chromatography on Silica gel G with chloroform–methanol–7M-ammonium hydroxide (46:18:3, by vol.) (Abramson & Blecher, 1964). The phospholipid spots were detected by exposure to iodine vapours, scraped out and eluted by a mixture of ethanol–chloroform–acetic acid–water (50:15:10:1, by vol.) as described by Biezenski (1967). The identity of the phospholipids was established by comparison with known phospholipid standards and by suitable spray reagents. The eluates were evaporated to a small volume under reduced pressure and a sample was used for the determination of phosphorus by the method of Bartlett (1959) as modified by Marinetti (1962). Another sample was dried in stainless steel planchets and the radioactivity determined in a Tracerlab Windowless gas-flow counter.

RESULTS

The total phospholipid content and the incorporation of radioactivity into total phospholipids are presented in Table 1. The total phospholipid content of the mycelia of the non-toxigenic strain grown in YES medium was less than that of mycelia grown in AM medium. No clear-cut differences were observed with the toxigenic strain.

When AM medium was used for both growth and resuspension, the total incorporation and specific activity obtained with the toxigenic strain were respectively 2·9 times and 2·3 times the values given by the non-toxigenic strain. On the other hand, with YES medium the toxigenic strain yielded values that were respectively 0·67 and 0·33 of those given by the non-toxigenic strain. The growth and resuspension media had a pronounced effect on the incorporation of [1-^{14}C]acetate into phospholipids in the case of the toxigenic strain, AM medium giving more than 10 times the incorporation

and specific activity obtained with YES medium. The differences were smaller with the non-toxigenic strain. When the different combinations of media given in Table 1 are considered, it is clear that the medium had a marked effect on the toxigenic strain and that there was a gradual decrease in specific activity in the order given in Table 1. With the non-toxigenic strain, somewhat higher specific activity was obtained when

Table 1. *Incorporation of [1-^{14}C]acetate into the total phospholipids by Aspergillus flavus*

Mycelia grown for 3 days on AM or YES medium were resuspended in one or other of these media containing 10 μCi of [1-^{14}C]acetate and incubated for 3 h. on a rotary shaker at 25°. All the experiments were performed in duplicate and average values are presented in this table.

Growth medium	Resuspension medium	Total phospholipids as μg. phosphorus/g. wet weight of mycelium	Radioactivity incorporated counts/min./g. wet weight of mycelium	Specific activity counts/min./μg. phosphorus
		Toxigenic strain ATCC 15517		
AM	AM	189	283,900	1,504
AM	YES	110	64,650	589
YES	AM	147	48,230	329
YES	YES	172	25,320	148
		Non-toxigenic strain ATCC 9643		
AM	AM	151	97,990	649
AM	YES	137	55,670	407
YES	AM	67	32,260	478
YES	YES	85	37,870	447

Table 2. *Incorporation of [1-^{14}C]acetate into individual phospholipids by the toxigenic strain Aspergillus flavus ATCC 15517*

Incorporation of [1-^{14}C]acetate was carried out as described in Table 1. Phospholipids were separated by thin layer chromatography on Silica gel G with chloroform–methanol–7 M-ammonium hydroxide (46:18:3, by vol.). All the experiments were performed in duplicate and average values are presented.

Growth medium	Resuspension medium	PI	PS	LPE	PC	PE	PGP
		Radioactivity incorporated counts/min./g. wet weight of mycelium					
AM	AM	8,879	34,650	72,400	72,870	84,360	10,770
AM	YES	4,283	7,173	10,903	25,740	13,530	3,019
YES	AM	2,299	8,881	5,218	18,910	11,830	1,094
YES	YES	1,042	2,188	4,104	9,134	8,274	579
		Specific activity counts/min./μg. phosphorus					
AM	AM	796	1,368	1,926	1,108	2,297	885
AM	YES	853	808	1,014	670	329	551
YES	AM	347	541	388	301	312	113
YES	YES	170	117	243	126	168	73

PI = phosphatidyl inositol; PS = phosphatidyl serine; LPE = lysophosphatidyl ethanolamine; PC = phosphatidyl choline; PE = phosphatidyl ethanolamine; PGP = polyglycerophosphatide.

AM medium was used for growth and resuspension. The specific activities given by the other media combinations did not differ much among themselves.

In general, the total incorporation and the specific activities of the individual phospholipids showed a pattern similar to that obtained for the total phospholipids (Tables 2, 3). For most phospholipids, the toxigenic strain yielded higher total counts and specific activities on AM medium and lower incorporation on YES medium than did the non-toxigenic strain. AM medium gave higher amounts of radioactivities with both strains, but the differences between the two media were much larger in the case of the toxigenic strain. Again, there was a decrease in the incorporation of [1-^{14}C]acetate into the individual phospholipids by the toxigenic strain on the different media combinations in the order given in Table 2. Such a regular change in radioactivity was not observed in the case of the non-toxigenic strain (Table 3).

Table 3. *Incorporation of [1-^{14}C]acetate into individual phospholipids by the non-toxigenic strain Aspergillus flavus ATCC 9643.*

Incorporation of [1-^{14}C]acetate and separation of phospholipids were carried out as described in Tables 1 and 2. All the experiments were performed in duplicate and average values are presented.

Growth medium	Resuspension medium	PI	PS	LPE	PC	PE	PGP
		Radioactivity incorporated counts/min./g. wet weight of mycelium					
AM	AM	10,150	13,070	17,010	33,460	22,210	2,093
AM	YES	1,221	12,150	7,065	13,010	19,310	2,914
YES	AM	1,311	2,980	3,663	11,360	12,380	572
YES	YES	1,549	2,920	6,277	12,280	14,150	791
		Specific activity counts/min./μg. phosphorus					
AM	AM	415	727	1,141	795	674	112
AM	YES	131	702	346	246	693	339
YES	AM	222	754	456	432	680	110
YES	YES	398	620	550	332	585	227

PI = phosphatidyl inositol; PS = phosphatidyl serine; LPE = lysophosphatidyl ethanolamine; PC = phosphatidyl choline; PE = phosphatidyl ethanolamine; PGP = polyglycerophosphatide.

In all the experiments, the highest incorporation was obtained in phosphatidyl choline (PC) and phosphatidyl ethanolamine (PE) and the next highest in lysophosphatidyl ethanolamine (LPE).

DISCUSSION

AM and YES media were chosen for this investigation, since the latter gives much higher yields of aflatoxins than AM medium. It was hoped that any relation between aflatoxin formation and phospholipid synthesis would be brought out clearly by a comparison of the two strains of *Aspergillus flavus* on these media.

The toxigenic strain was more efficient in synthesizing phospholipids and gave much higher specific activity than the non-toxigenic strain on AM medium (Table 1). But, under conditions of high toxin production on YES medium, it yielded one-third the specific activity obtained with the non-toxigenic strain, suggesting that [1-^{14}C]acetate was diverted to the formation of aflatoxins and hence the incorporation into phospholipids was reduced.

Both the growth and resuspension media had a very pronounced effect on the incorporation of acetate by the toxigenic strain (Table 1). Since their effect on the non-toxigenic strain was much less, the results obtained on the two media appear to arise from the differences in their ability to support toxin production. The gradual change in the specific activities yielded by the different combinations of media, presented in Table 1, indicate clearly that both the medium on which the fungus was grown and the one in which the incorporation was carried out had a marked effect on the extent of incorporation. AM medium, whether used as growth or resuspension medium, yielded higher specific activity than YES medium. In the case of the non-toxigenic strain, the differences between the different media combinations were not so clear cut, supporting the above reasoning, since this strain does not produce any aflatoxins.

The incorporation of [1-^{14}C]acetate into the individual phospholipids with different media combinations was similar to that into the total phospholipids, showing that the differences observed were not specific for any of the individual phospholipids (Tables 2, 3). PC and PE are the major phospholipids present in *Aspergillus flavus* and in most of the experiments they exhibited the maximum specific activity. LPE, which is probably an intermediate in the synthesis of PE, also incorporated considerable amounts of radioactivity. Lysophosphatidyl choline (LPC) was not detected in the lipid extract of this fungus.

Adye & Mateles (1964) have investigated the incorporation of different labelled substrates into aflatoxins and have shown that acetate is efficiently incorporated. The labelling patterns obtained with [1-^{14}C] and [2-^{14}C]acetate have also been worked out, and it has been suggested that acetate is first converted into a polyacetate compound, which by means of a series of ring closures and rearrangements gives rise to aflatoxins (Biollaz, Büchi & Milne, 1968). Since acetate is a precursor of a number of normal metabolites, competition may be expected between the production of such compounds and secondary metabolites, such as aflatoxins. Evidence that this process is under the control of regulatory mechanisms has accumulated in recent years (Tanenbaum, 1967). For example, an enzyme preparation from *Alternaria tenuis* incorporated radioactive precursors into lipids in media of low ionic strength in the presence of $NADPH_2$. In media of high ionic strength or in the absence of $NADPH_2$ there was practically no incorporation into lipids, but the secondary metabolite alternariol was labelled (Gatenbeck & Hermodsson, 1965). It is possible that a similar control mechanism operates in *Aspergillus flavus* and led to the differences observed in the incorporation of acetate into phospholipids by the toxigenic strain in different media.

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The Effect of Oxygen on Tetrathionate Reductase Activity and Biosynthesis

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SUMMARY

Cyanide (1 mM) strongly inhibited aerobic respiration, and 2,4 dinitrophenol (0·2 mM) apparently uncoupled oxidative phosphorylation, in non-growing Citrobacter. At these inhibitor concentrations, anaerobic tetrathionate reductase activity was not much affected. Aeration inhibited tetrathionate reductase activity; 0·2 mM-2,4 DNP did not influence oxygen inhibition, but 1 mM-KCN restored the reductase activity quantitatively. The process of aerobic respiration rather than the oxygen molecule itself therefore inhibits tetrathionate reductase activity. Induced synthesis of reductase required anaerobic conditions. Cyanide and 2,4 DNP allowed anaerobic synthesis of reductase; aeration prevented it. This effect of oxygen was abolished neither by KCN nor by 2,4 DNP. Oxygen therefore represses the synthesis of tetrathionate reductase directly.

INTRODUCTION

That some facultative anaerobic bacteria reduce tetrathionate to thiosulphate was discovered by Pollock and co-workers (Pollock, Knox & Gell, 1942; Pollock & Knox, 1943; Knox, Gell & Pollock, 1943). The reaction was quantitative:

$$S_4O_6^{2-} + 2H = 2S_2O_3^{2-} + 2H^+;$$

H^+ and thiosulphate accumulated in the medium. Later, Knox & Pollock (1944) demonstrated that the enzyme responsible for the reduction of tetrathionate was both specific and inducible, the sole known substrate and inducer being tetrathionate. Tetrathionate reduction is widespread among the facultative anaerobes and has some taxonomic and diagnostic value (Le Minor & Pichinoty, 1963; Nicolle & Le Minor, 1965; Le Minor, 1967; Papavassiliou, Samaraki-Lyberopoulou & Piperakis, 1969). Discussing the possible physiological role of tetrathionate reductase in bacteria, Pollock and co-workers concluded that it may be a form of anaerobic respiration analogous to the reduction of nitrate to nitrite. This analogy was supported by the finding (Pollock & Knox, 1943) that tetrathionate reductase activity, like nitrate reductase activity (Stickland, 1931), was reversibly inhibited by aeration. Pichinoty & Bigliardi-Rouvier (1963) found that oxygen not only inhibited the activity of tetrathionate reductase but also repressed its synthesis.

Our investigation attempts to clarify the mechanism of this dual action of oxygen in washed non-growing organisms. Tetrathionate reductase is not the sole bacterial enzyme which is inhibited and repressed by oxygen (Fowler, 1951; Pichinoty, 1965; Gray, Wimpenny, Hughes & Mossman, 1966; Gray, Wimpenny & Mossman, 1966),

thus results reported here are also a contribution to the understanding of a general phenomenon called the 'oxygen effect' (Pichinoty, 1965).

METHODS

Micro-organism and cultivation. The original strain isolated by Pollock (1946) and labelled '1433', recently identified by Sedlak & Slajsova (1967) as Citrobacter, was grown on a reciprocating shaker at 30° in the following medium (g./l. dist.H_2O): $Na_2HPO_4.12H_2O$, 3·57; KH_2PO_4, 0·98; $MgSO_4.7H_2O$, 0·03; NH_4Cl, 0·50; traces of $FeSO_4$ and $CaCl_2$; lactose, 2·0; yeast extract Difco, 0·5; Casamino acids Difco, 0·5; bacto-peptone Difco, 0·5. The pH was adjusted to 6·8 before sterilization for 20 min. at 127°; lactose was sterilized separately. If organisms possessing tetrathionate reductase activity were required, a solution of 1·0 g. $K_2S_4O_6$/l. (sterilized by filtration) was added as inducer. After 15 h. the bacteria (by then in the stationary phase) were harvested, washed twice and resuspended in distilled water.

Although aerobic cultivation represses the synthesis of tetrathionate reductase (Pichinoty & Bigliardi-Rouvier, 1963), organisms containing much tetrathionate reductase can be grown as described above because the concentration of dissolved oxygen decreased during growth of the culture and was practically zero at the high bacterial concentrations reached towards the end of the exponential growth phase. When the organisms were harvested, tetrathionate was still present in the medium indicating that inducer had been present throughout growth. This mode of cultivation had the advantage of a fourfold greater yield of active organisms compared with anaerobic cultivation.

Tetrathionate reductase activity. This was measured anaerobically as follows (Pollock & Knox, 1943; Kaprálek, Pichinoty & Riegrová, 1969): A buffer solution containing a source of hydrogen and electrons (e.g. glucose) and washed bacterial cells was warmed to 37° and $K_2S_4O_6$ was added at time zero (t_0). The composition of the test systems and the concentrations of components are given with the appropriate figures. This manner of static incubation of 20 ml. of thick bacterial suspension in an 18 mm. test-tube, provided adequately anaerobic conditions and was equivalent to incubation in a vacuum in a Thunberg tube or to incubation with bubbling O_2-free nitrogen through the solution. If aerobic conditions were required, 10 vol. of air/vol. medium/min. was sparged through the system. At intervals, 1 ml. samples were transferred to 3 ml. 96 % (v/v) ethanol or into 1 ml. of 5 % (w/v) trichloracetic acid to stop the reaction. Thiosulphate produced was then assayed iodometrically. From these data the initial velocity of tetrathionate reduction (v_t = μmole $K_2S_4O_6$ reduced/h./mg. dry wt) was calculated as the product of the first-order reaction rate constant and initial tetrathionate concentration (Kaprálek *et al.* 1969) and taken as the measure of tetrathionate reductase activity. The micro-organism used in our work did not reduce thiosulphate enzymatically, and therefore the thiosulphate found in the medium corresponded quantitatively to the tetrathionate reduced.

Induction of tetrathionate reductase. This was carried out in a similar manner to the assay above. Exact experimental conditions are mentioned under relevant figures.

Rate of oxygen uptake. This was measured using a Warburg apparatus at 37°, with air as gas phase. Results were expressed as Q_{O_2} (μl. O_2 consumed/h./mg. dry wt). The experiments with KCN were carried out using the method of Robbie (1946).

RESULTS

The inhibitory effect of oxygen on tetrathionate reductase activity

The inhibition of tetrathionate reductase activity by oxygen is demonstrated in Fig. 1. The inhibition disappeared immediately after aeration ceased. Nothing is known about the mechanism of this reversible inhibitory effect of oxygen. Three possibilities may be considered.

(1) Oxygen may directly and reversibly inactivate the tetrathionate reductase molecule. In this case its inhibitory effect would depend only on the concentration of oxygen in solution.

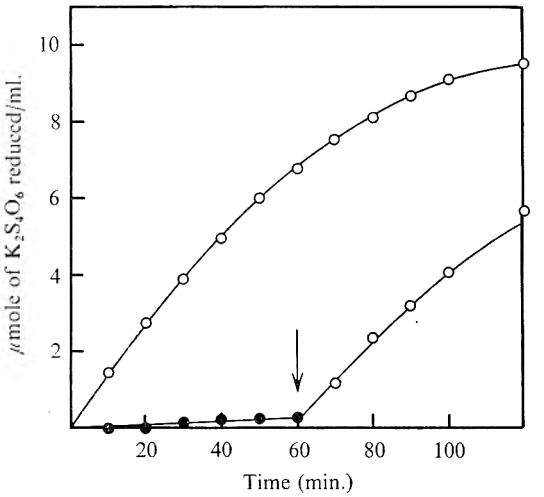


Fig. 1. Effect of aeration on the tetrathionate reductase activity of washed organisms previously grown in the presence of $K_2S_4O_6$. 0·16 M-phosphate buffer, pH 6·8; glucose 50 μmole/ml.; $K_2S_4O_6$ 25 μmole/ml.; chloramphenicol 50 μg./ml.; washed organisms equiv. 1·5 mg. dry wt/ml.; final volume 20 ml. ○——○, Anaerobically; ●——●, aerobically; arrow indicates the time the aeration was stopped.

(2) The oxygen itself may not be the inhibitor. It may act indirectly in that electrons are preferentially transferred to it for aerobic respiration. If so, the inhibitory effect would not depend on the dissolved oxygen concentration but on the organism's Q_{O_2}.

(3) Aerobic respiration brings about oxidative phosphorylation leading to an increased rate of ATP formation and decreased steady state level of ADP. Tetrathionate respiration might thus be inhibited in a manner analogous to the aerobic inhibition of glucose fermentation (the Pasteur effect), i.e. indirectly through the ratio of ATP to ADP. If so, inhibition by oxygen would depend neither on its concentration in solution nor on the Q_{O_2} activity, but on the ability of the organisms to perform aerobic oxidative phosphorylation.

Fig. 2 shows that 2,4 dinitrophenol did not influence the tetrathionate reductase activity at concentrations $< 0{\cdot}25$ mM, whereas from 0·01 mM it stimulated the respiratory activity as much as 40 %. At such concentrations 2,4 DNP uncouples phosphorylation from oxidation and thus usually causes an increase in the rate of oxygen consumption (James, 1953; Dolin, 1961; Slater, 1963). It is probable that the inhibitor acted similarly at 0·2 mM in our experiments. This conclusion is supported by growth experiments

(Fig. 2). The aerobic growth rate with 0·2 mM-2,4 DNP was equal to the rate of anaerobic growth. Nevertheless, no concentration of 2,4 DNP tested abolished the inhibitory effect of aeration on tetrathionate reductase activity (Fig. 2, the lowest curve), so it is probable that the inhibitory effect of oxygen on reductase activity was not determined by the ability of organisms to carry out aerobic oxidative phosphorylation. Thus the activity of tetrathionate reductase seems not to be controlled by the intracellular levels of ADP and ATP.

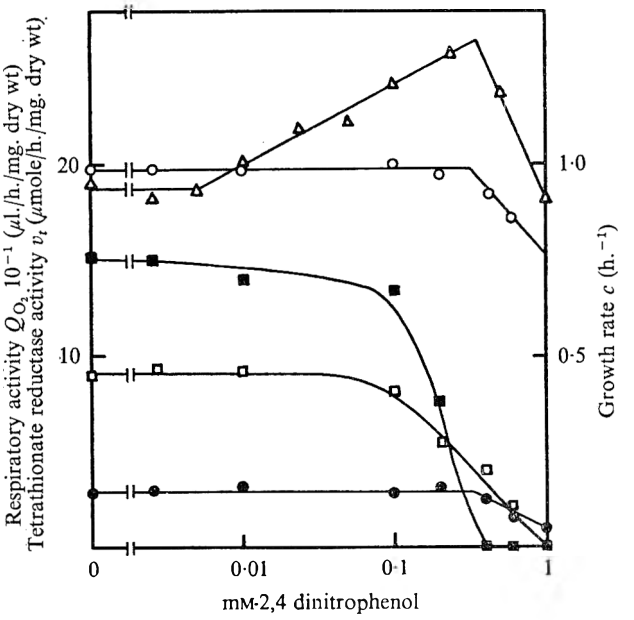


Fig. 2. Effect of 2,4 dinitrophenol on the respiratory activity Q_{O_2} (µl./h./mg. dry wt) (0·16 M-phosphate buffer, pH 6·8; glucose 50 µmole/ml.; chloramphenicol 50 µg./ml.; washed organisms equiv. 0·364 mg. dry wt/ml.), on the tetrathionate reductase activity v_t (µmole/h./mg.) (0·16 M-phosphate buffer, pH 6·8; glucose 50 µmole/ml.; $K_2S_4O_6$ 25 µmole/ml.; washed organisms equiv. 2 mg. dry wt/ml.; chloramphenicol 50 µg./ml.; final volume 20 ml.) and on the growth rate $c = (\log_2 x_2 - \log_2 x_1)/(t_2 - t_1)$ (h^{-1}) ($Na_2HPO_4 \cdot 12\ H_2O$, 3·57 g.; KH_2PO_4, 0·98 g.; $MgSO_4 \cdot 7\ H_2O$, 0·03 g.; NH_4Cl, 0·50 g; $FeSO_4$ and $CaCl_2$, traces; glucose, 2·0 g.; distilled water ad 1000 ml., pH 6·8). ○——○, Tetrathionate reductase activity anaerobically; ●——●, tetrathionate reductase activity aerobically; □——□, growth rate anaerobically (in O_2-free nitrogen); ■——■, growth rate aerobically (reciprocating shaker); △——△, respiratory activity Q_{O_2}.

Fig. 3. shows that KCN (up to 1 mM) did not influence the activity of tetrathionate reductase anaerobically but strongly inhibited the aerobic respiration. However, inhibition of tetrathionate reductase activity by aeration did not occur in the presence KCN (Table 1). Figure 4 shows a direct correlation between respiratory activity and tetrathionate reductase activity in the presence of oxygen. A graphical extrapolation (Dixon, 1953) of the data of this figure gave a dissociation constant (K_i) of 0·02 mM. Figure 5 shows the instantaneous (curve 2) and reversible (curves, 3, 3′) nature of the effect of cyanide.

Later experiments using carbon monoxide verified the results found with cyanide. Table 2 shows inhibition of respiration by CO: the sensitivity of aerobic respiration to CO was very low and Q_{O_2} values below 70% of the control were not obtained.

Table 3 shows that CO similarly to KCN reduced the inhibition of tetrathionate reductase by oxygen, but only partially, in line with its partial inhibition of the aerobic respiration. Tetrathionate reductase activity itself was not influenced by CO.

Thus cyanide and CO abolished the inhibition of tetrathionate reductase activity by oxygen by inhibiting aerobic respiration. Even in the presence of dissolved oxygen the

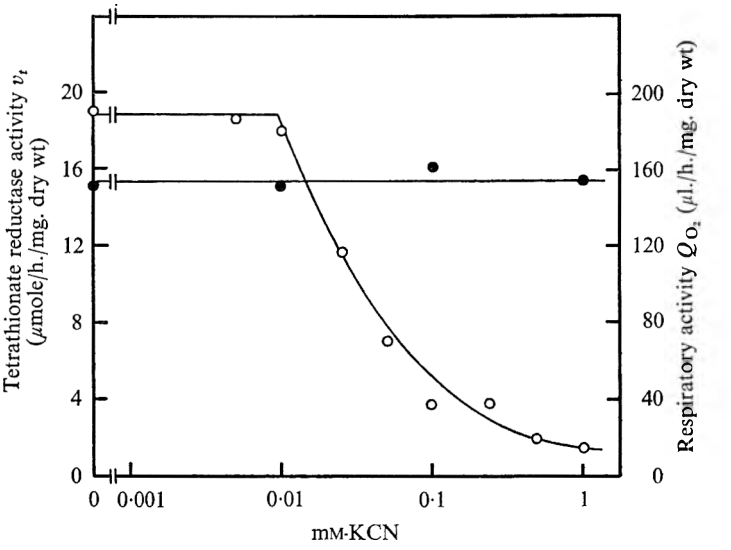


Fig. 3. Effect of KCN on the respiratory activity Q_{O_2} ○——○ (μl./h./mg.) (0·16 M-phosphate buffer, pH 6·8; glucose 50 μmole/ml.; chloramphenicol 50 μg./ml.; washed organisms equiv. 0·285 mg. dry wt/ml.) and on the tetrathionate reductase activity v_t ●——● (μmole/h./mg.) (0·16 M-phosphate buffer pH 6·8; glucose 50 μmole/ml.; $K_2S_4O_6$ 25 μmole/ml.; washed organisms equiv. 1 mg. dry wt/ml.; chloramphenicol 50 μg./ml.; final volume 20 ml.).

Table 1. *Inhibition of tetrathionate reductase activity of washed Citrobacter by oxygen in the presence of KCN*

0·16 M-phosphate buffer, pH 6·8; glucose 50 μmole/ml.; $K_2S_4O_6$ 25 μmole/ml.; washed organisms equiv. 1 mg. dry wt/ml.; chloramphenicol 50 μg./ml.; final volume 20 ml.

KCN mM	Tetrathionate reductase activity v_t (μmole/h./mg.)	
	Anaerobically	Aerobically
0	9·9	0·0
0·1	11·6	9·4
1·0	11·9	8·8

tetrathionate reductase system remained active because the transport of electrons to oxygen was blocked. Thus it was not oxygen itself, but aerobic respiration (the preferential transport of electrons to oxygen) that inhibited the tetrathionate reductase activity.

The question arose whether the reverse was true: could anaerobic respiration of tetrathionate inhibit aerobic respiration? Table 4 indicates an affirmative answer: the anaerobic respiration of tetrathionate inhibited the aerobic respiration of oxygen. It

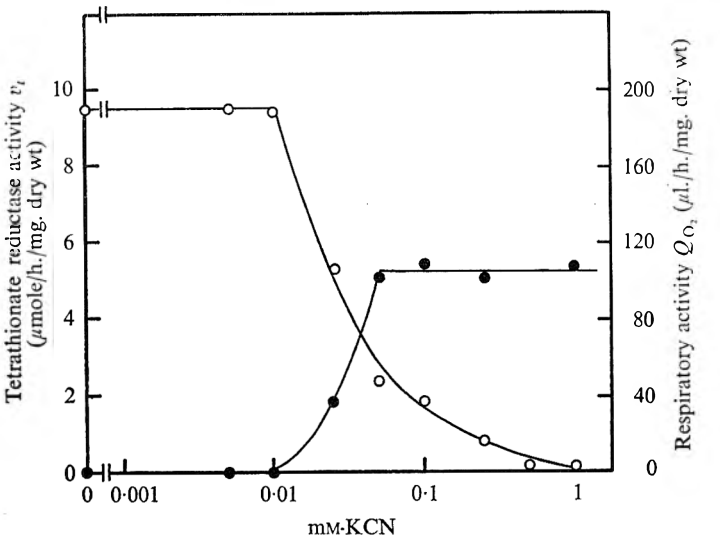


Fig. 4. Reversal by KCN of the inhibitory effect of oxygen on tetrathionate reductase activity as function of KCN concentration. ○——○, Respiratory activity Q_{O_2} (μl./h./mg): 0·16 M-phosphate buffer, pH 6·8; glucose 50 μmole/ml.; washed organisms equiv. 0·361 mg. dry wt/ml.; chloramphenicol 50 μg./ml. ●——●, Tetrathionate reductase activity v_t (μmole/h./mg.) under aerobic conditions: 0·16 M-phosphate buffer, pH 6·8; glucose 50 μmole/ml.; washed organisms equiv. 1 mg. dry wt/ml.; $K_2S_4O_6$ 25 μmole/ml.; chloramphenicol 50 μg./ml.; final volume 20 ml.

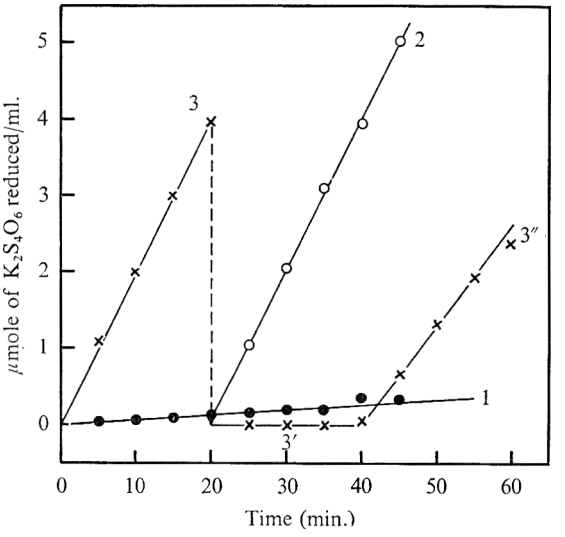


Fig. 5. Inhibitory effect of aeration on the activity of tetrathionate reductase and instantaneous and reversible removal of inhibition by cyanide. System 1: 0·16 M-phosphate buffer, pH 6·8; $K_2S_4O_6$ 25 μmole/ml.; washed organisms equiv. 1 mg. dry wt/ml.; glucose 50 μmole/ml.; chloramphenicol 50 μg./ml.; aerated. System 2: the same as system 1 (i.e. aerated) but at 20 min. 1 mM-KCN added. System 3: the same as system 1 (i.e. aerated) plus 1 mM-KCN; after 20 min. the organisms were washed and resuspended in the same medium but without KCN (3′); at 40 min. aeration was stopped (3″).

is not the tetrathionate itself which inhibits, since $K_2S_4O_6$ did not affect the Q_{O_2} of the organisms (B) lacking tetrathionate reductase activity. The lowering of Q_{O_2}, though significant, was not high and did not always occur. Its manifestation depended on the source of electrons and other factors such as the difference between A and C organisms. It was apparently conditioned by the proportions of aerobic and anaerobic respiratory activities in bacteria possessing both. The decisive fact is that oxygen may not fully inhibit the tetrathionate reductase activity in aerobic conditions.

Table 2. *Effect of CO on respiratory activity* Q_{O_2}

The composition of liquid phase in Warburg vessel: 0·16 M-phosphate buffer, pH 6·8; glucose 50 μmole/ml.; washed bacteria equiv. 0·31 mg. dry wt/ml.; chloramphenicol 50 μg./ml. The rate of chemical absorption of CO in KOH is subtracted, the endogenous respiration is not subtracted. The carbon monoxide was 99·995 % pure (L'air liquide, Paris).

Composition of gas phase (%)	N_2	—	95	—	90	—	85	—	80	—
	CO	Air	—	95	—	90	—	85	—	80
	O_2	—	5	5	10	10	15	15	20	20
Aerobic respiratory activity Q_{O_2} (μl./h./mg.)		206	127	91	169	125	200	172	202	183
Aerobic respiratory activity expressed in percentage of the control activity of (CO substituted by N_2)		—	100	71·6	100	74·0	100	86·0	100	90·6

Table 3. *Effect of CO on tetrathionate reductase activity in the presence and absence of oxygen*

The composition of liquid phase: 0·16 M-phosphate buffer, pH 6·8; $K_2S_4O_6$ 25 μmole/ml.; glucose 50 μmole/ml.; washed bacteria equiv. 1 mg. dry wt/ml.; final volume 20 ml. This solution was aerated with gaseous phases of the following composition.

The composition of gas phase (%)	N_2	—	100	89·3	50	—	—	—
	Air	No aeration	—	10·7	50	—	10·7	50
	CO	—	—	—	—	100	89·3	50
The composition of gas phase in partial pressure (atm.)	N_2	(Anaero-bically)	1·04	0·93	0·52	—	—	—
	Air	—	—	0·11	0·52	—	0·11	0·52
	CO	—	—	—	—	1·04	0·93	0·52
Tetrathionate reductase activity v_t (μmole/h./mg.)		10·3	10·3	1·8	0·0	10·3	8·7	0·5

The inhibitory effect of oxygen on tetrathionate reductase biosynthesis

The induction of tetrathionate reductase synthesis in washed non-growing organisms is shown in Fig. 6. With oxygen, as with chloramphenicol, induction did not take place. The repressive effect of oxygen on the tetrathionate reductase synthesis was reversible: after aeration ceased, induction occurred at practically the same rate as without previous aeration. The last curve excludes the possibility that inductive synthesis of reductase occurred during aeration but that its activity was inhibited by oxygen: after aeration ceased and anaerobic conditions were established in the presence of chloramphenicol (which did not influence the activity of enzyme already formed), no tetrathionate was reduced. Oxygen therefore repressed synthesis of tetrathionate reductase. Repression by oxygen did not involve ATP formed by oxidative phosphorylation in

Table 4. *Effect of tetrathionate on the aerobic respiratory utilization of oxygen*

Warburg vessels contained: 0·16 M-phosphate buffer, pH 6·8; glucose or lactate or formate 50 μmole/ml.; washed organisms A or B or C equiv. 0·666 mg. dry wt/ml.; chloramphenicol 50 μg./ml.; $K_2S_4O_6$ 25 μmole/ml. (omitted in control experiments). All values of Q_{O_2} are means from six parallel vessels. Tetrathionate reductase activity was assayed separately in a solution of the same composition.Organisms A were grown in the presence of $K_2S_4O_6$; organisms B in the absence of tetrathionate; organisms C were grown also without $K_2S_4O_6$, then washed, resuspended (2 mg. dry wt/ml.) anaerobically in a solution of phosphate buffer, pH 6·8 (0·16 M), $K_2S_4O_6$ (25 μmole/ml.), Casamino acids Difco (1 mg./ml.) and glucose (50 mole/ml.) and incubated at 30° for 1 h. (final volume 50 ml.). Under these conditions the induction of tetrathionate reductase in resting cells occurred.

Kinds of organisms	Reductant	$K_2S_4O_6$	Respiratory activity Q_{O_2} (μl./h./mg.)	Q_{O_2} (%)	Student's value t	Corresponding probability P	Tetrathionate reductase activity v_t (μmole/h./mg.) Anaerobically	Aerobically
A	Glucose	Absent	96 ± 5·349	100	5·16	0·007	9·9	0·8
		Present	83 ± 3·066	86				
A	Lactate	Absent	120 ± 1·673	100	7·63	0·002	6·5	1·1
		Present	108 ± 3·464	90				
A	Formate	Absent	65 ± 3·633	100	0·50	0·61	6·8	0·7
		Present	64 ± 3·225	98				
B	Glucose	Absent	70 ± 3·000	100	0·42	0·66	0·0	0·0
		Present	68 ± 2·191	97				
B	Lactate	Absent	134 ± 3·715	100	0·29	0·74	0·0	0·0
		Present	133 ± 7·681	99				
B	Formate	Absent	23 ± 1·732	100	0·00	1·00	0·0	0·0
		Present	23 ± 2·191	100				
C	Glucose	Absent	84 ± 5·020	100	0·00	1·00	4·3	0·3
		Present	84 ± 3·406	100				
C	Lactate	Absent	115 ± 5·138	100	0·93	0·40	3·9	0·3
		Present	113 ± 1·000	98				

aerobiosis since no aerobic synthesis of tetrathionate reductase took place at any concentration of 2,4 DNP (including 0·2 mM, which we showed earlier uncoupled oxidative phosphorylation from aerobic respiration) (Fig. 7). Fig. 8 shows that oxygen repression was not reversed by 1 mM cyanide which almost completely inhibited aerobic respiration (Fig. 3). It follows that aerobic respiration was not the factor inhibiting reductase synthesis: oxygen repression occurred whether aerobic respiration was present or not.

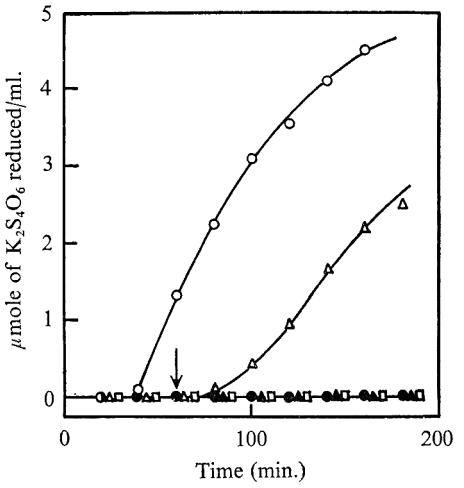


Fig. 6

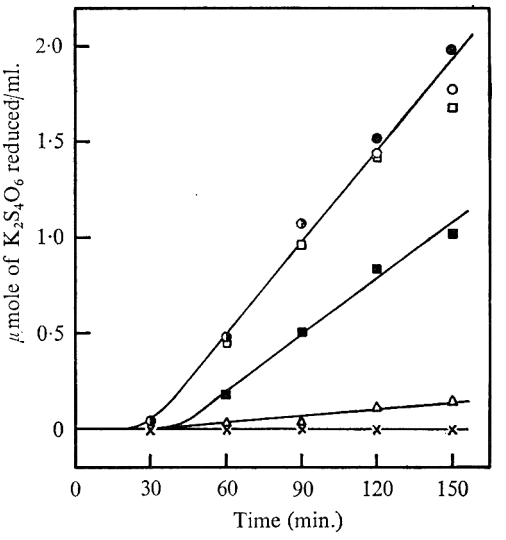


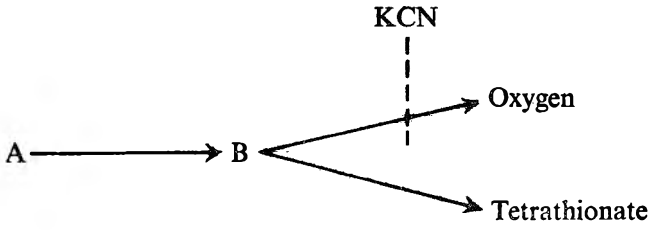
Fig. 7

Fig. 6. Induction of tetrathionate reductase synthesis in washed non-growing bacteria and the repressive effect of oxygen: 0·11 M-phosphate buffer, pH 6·8; glucose 50 μmole/ml.; washed organisms equiv. 2 mg. dry wt/ml.; $K_2S_4O_6$ 25 μmole/ml.; final volume 20 ml. ○——○, Anaerobically; □——□, aerobically; ●——●, anaerobically + chloramphenicol 50 μg./ml.; △——△, 60 min. aerobically, then anaerobically; ▲——▲, 60 min. aerobically, then anaerobically + chloramphenicol 50 μg./ml.

Fig. 7. Induction of tetrathionate reductase synthesis in washed non-growing bacteria anaerobically and aerobically in the presence of 2,4 dinitrophenol: 0·16 M-phosphate buffer, pH 6·8; glucose 50 μmole/ml.; $K_2S_4O_6$ 25 μmole/ml.; washed organisms equiv. 2 mg. dry wt/ml.; final volume 20 ml. ○——○, Anaerobically; ●——●, anaerobically + 0·01 mM—2,4 DNP; □——□, anaerobically + 0·1 mM-2,4 DNP; ■——■, anaerobically + 0·2 mM-2,4 DNP; △——△, anaerobically + 0·4 mM-2,4 DNP; ×——×, aerobically = anaerobically + chloramphenicol 50 μg./ml. = aerobically + 0·01 mM or 0·1 mM or 0·2 mM or 0·4 mM 2,4 DNP.

DISCUSSION

The results presented here support the idea that both terminal enzymes, the aerobic oxidase and the tetrathionate reductase, compete for electrons from a shared electron carrier:



The nature of this common carrier is unknown at present, but it is insensitive to cyanide. Aerobic respiration and oxygen, as the final electron acceptor, are preferred and aerobic respiration strongly inhibits tetrathionate respiration. A preliminary analysis of the reaction kinetics in a divergent linear chain of this type shows that behaviour in accordance with our experimental findings would be exhibited by a system in which either the concentration of aerobic oxidase were tenfold to 100-fold greater than the concentration of tetrathionate reductase (with the same Michaelis

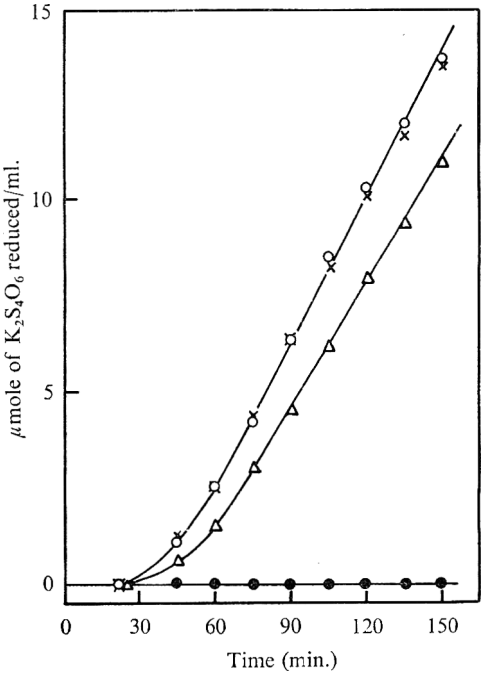


Fig. 8. Induction of tetrathionate reductase synthesis in washed non-growing bacteria anaerobically and aerobically in the presence of KCN: 0·11 M-phosphate buffer, pH 6·8; glucose 50 μmole/ml.; $K_2S_4O_6$ 25 μmole/ml.; washed organisms equiv. 2 mg. dry wt/ml.; final volume 20 ml. ○——○, Anaerobically; ×——×, anaerobically + 0·1 mM-KCN; △——△, anaerobically + 1 mM-KCN; ●——●, aerobically = anaerobically + chloramphenicol 50 μg./ml. = aerobically + 0·1 mM or 1 mM-KCN.

constants of both toward the common electron donor), or the Michaelis constant of aerobic oxidation toward common donor is tenfold to 100-fold lower than the Michaelis constant of tetrathionate reductase at the same concentrations of both enzymes. As the rates of oxygen consumption and of tetrathionate reduction measured separately and expressed in terms of electron equivalents transferred/h./mg. dry wt did not differ substantially (see Fig. 3), the second possibility is more probable. Nevertheless, the degree of mutual inhibition may depend on the relative concentrations of the competing terminal enzymes (Table 4). The mode by which oxygen inhibits tetrathionate reductase activity implies that competitive electron acceptors other than oxygen should decrease the rate of tetrathionate reduction.

A part of this work was conducted while one of us (F. K.) was a visiting fellow of

C.N.R.S. in Marseille under Professor J. C. Senez, whose interest and advice are highly appreciated. The authors are very grateful to Mrs J. Bigliardi for excellent technical assistance.

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Relationship Between Rhapidosome and Pyocin in *Pseudomonas fluorescens*

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SUMMARY

Rhapidosomal rods released from strain 3R of *Pseudomonas fluorescens* appear to be polymerized sheaths of pyocin which had a structure like that of T-even phage tails. This conclusion was based on the following observations: (1) Morphologically the rods have the same structure and diameter as the contracted sheath of R-type pyocin. (2) They resemble the polysheath of T-even phages. (3) Antigenically they are similar to R-type pyocin. (4) They are usually induced together with R-type pyocin but not with 28-type pyocin.

INTRODUCTION

Since the discovery of rod-shaped particles in Saprospira (Lewin, 1963), this kind of structure has been reported in several species of bacteria (Bradley, 1967; Yamamoto, 1967; Reichenbach, 1967; Ueda & Takagi, 1968; Clark-Walker, 1969). Such rods found within or liberated from bacterial cells have been given the name 'rhapidosomes' (Lewin, 1963). Pate, Johnson & Ordal (1967) suggested that they were degenerate forms of the membranous structure of host cells. Ueda & Takagi (1968) explained rods in Clostridia in the same way. Recently many such structures have been reported in mitomycin-induced lysates of several species of bacteria (Bradley, 1967: Iida & Inoue, 1968; Clark-Walker, 1969); these rhapidosomes often appeared together with bacteriocins which had the structure of phage tails, and their origin might be different from that of the first type (Reichenbach, 1967; Clark-Walker, 1969). Many members of the Pseudomonas group of bacteria are known to be pyocinogenic (Hamon, 1956). During the course of experiments on pyocin we discovered that some of them also produced the second type of rhapidosome. The relation between pyocin and rhapidosome was investigated and is reported in this paper.

METHODS

Bacteria. Strain 3R of *Pseudomonas fluorescens* isolated in our laboratory was used as the rhapidosome-producing organism. Activity of the released pyocin was titrated on the C14 strain of *P. aeruginosa* (from Dr Homma of Tokyo University, Institute of Medical Science), who also provided strains P28, C18 and K. The FP^+ strain was obtained from Dr T. Watanabe of Keio University, and other strains used in these experiments were isolated from patients at Kyushu University Hospital. All the strains were tested for the production of pyocin, rhapidosomes and phages.

Media and cultivation of bacteria. Bacteria were grown in peptone broth or heart infusion broth (Difco) with aeration. GS medium, a synthetic medium, contained (g./l.): $(NH_4)_2HPO_4$, 2·5 g; KH_2PO_4, 1·5; NaCl, 5·0; sodium glutamate, 3·0; glucose, 3·0; $MgSO_4.7H_2O$, 0·1; $CaCl_2$, 0·05; yeast extract, 1·0; pH 7·2.

For induction, mitomycin C (Kyowa Hakko Kogyo Co. Ltd, Tokyo, Japan) was added to the culture in logarithmic phase at a final concentration of 3 μg./ml. In experiments at temperatures other than 37°, cells growing logarithmically at 37° were held at the designated temperature for 1 h. and then induced by mitomycin.

Antisera and micro-agglutination technique. Antisera against pyocin were prepared as follows: Pyocin induced from strain 3R at 25° was partially purified by differential centrifugation, then, mixed with Freund's adjuvant, it was injected intradermally into rabbits. Two weeks later a second injection was given without adjuvant, and 10 days later blood was collected by heart puncture and serum was prepared. Antibodies against the components of the host cell were adsorbed with large amounts of host cells prior to use. The micro-agglutination technique of Hummeler, Anderson & Brown, (1962) was used: serum was diluted ten times with phosphate-buffered saline (pH 7·0) and mixed with an equal volume of concentrated pyocin prepared by concentrating the crude lysate of strain 3R by ultracentrifuging at 30,000 rev./min. for 1 h.; it contained R-type and 28-type pyocins and rhapidosomes. After standing at room temperature for 30 min., the mixture was centrifuged at 10,000 rev./min. for 30 min. The resulting pellets were resuspended in a small volume of 2% ammonium acetate solution and examined under the electron microscope.

Electron microscopy. Mitomycin-induced lysate was treated with desoxyribonuclease to reduce the viscosity due to host cell DNA, then after low-speed centrifugation to remove cell debris, pyocin and rhapidosomes were centrifuged down at 30,000 rev./min. for 1 h. Pellets were resuspended in 2% ammonium acetate solution and the suspension was negatively stained with neutral potassium phosphotungstate.

RESULTS

Effects of mitomycin C on the growth of strain 3R. To logarithmically growing cells at 37° 3 μg. of mitomycin C/ml. were added and cell growth was followed in terms of optical density at 660 nm. For 1 h. after induction density of the culture increased parallel to that of the control, and then decreased rapidly (Fig. 1).

Morphology of pyocin and rhapidosomes. Strain 3R produced two types of pyocin (Pl. 1, fig. 1). One, pyocin 28, had a structure similar to that of λ phage tails (Takeya, Minamishima, Amako & Ohnishi, 1967) and the other, pyocin R, resembled T-even phage tails (Ishii, Nishi & Egami, 1965). R-type pyocin had a contractile sheath with a diameter of 150 Å in the extended, and 180 Å in the contracted, state. Rhapidosomes were long rods 180 Å in diameter (Pl. 1, fig. 2, 3). Since phosphotungstate penetrated into the rhapidosomes they are thought to be hollow, of internal diameter about 80 Å. Their granular appearance suggests that they are constructed of small irregularly arranged subunits (Pl. 1, fig. 3).

Morphologically they resemble the polysheath of a T-even phage and might be polymerized sheath proteins of R-type pyocin.

Antigenic similarity of R-type pyocin and rhapidosomes. The suggestion of the last paragraph was investigated by specific micro-agglutination, as revealed by the electron

microscope (Hummeler *et al.* 1962). It was found that R-type and 28-type pyocin formed specific agglutinates, and that rhapidosomes agglutinated with R-type, but not with 28-type pyocins (Pl. 2, fig. 4, 5; Pl. 3, fig. 6). Antibody bridges were clearly seen between rhapidosome and R-type pyocin (Pl. 2, fig. 5 arrow) indicating antigenic similarity of the two.

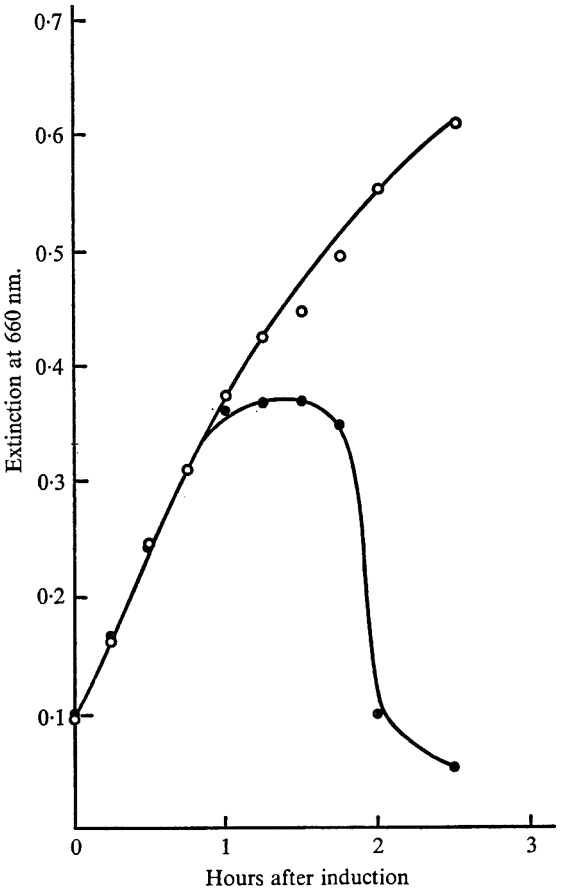

Fig. 1. Effect of mitomycin C on the growth of *Pseudomonas fluorescens* strain 3R at 37°. Mitomycin C was added to the logarithmic phase of the culture at a final concentration of 3 μg./ml. Growth was followed by observing changes in extinction (*E*) at 660 nm. Control, ○——○; mitomycin-treated, ●——●.

Conditions for rhapidosome production. The 3R strain was able to grow even in synthetic medium and to be lysed by mitomycin. Rhapidosome and pyocin production under various conditions is summarized in Table 1. When induction took place at low temperature (25°), 5 h. or more were required for complete cell lysis (Fig. 2) and no rhapidosomes were produced.

The lysate obtained at 40° contained small ring structures about 120 Å in diameter (Pl. 3, fig. 7) as well as short rods which might be polymerized forms of these rings.

Production of rhapidosomes in several strains of the Pseudomonas group. Data obtained so far suggest that the rhapidosome released from strain 3R is a polymerized

sheath protein of R-type pyocin. Accordingly, the relationship of rhapidosome and R-type pyocin production was investigated in several strains of the Pseudomonas group. Sixteen strains were induced with mitomycin C, the lysates were negatively stained, examined under the electron microscope, and the presence or absence of R-type and 28-type pyocin, rhapidosomes and phages was recorded.

Table 1. *Production of rhapidosomes and pyocin by mitomycin induction from Pseudomonas fluorescens strain 3R under various conditions*

Medium	Temperature	Cell growth	Pyocin production	Rhapidosome production
Heart infusion broth	40°	+	+	+
	37°	++	++	++
	28°	++	++	++
	25°	+	++	−
Peptone broth	37°	++	++	+
Synthetic medium (CS)	37°	+	++	+

Table 2. *Production of pyocin and rhapidosomes from several strains of the Pseudomonas group*

Strain	28-Type pyocin	R-type pyocin	Rhapidosome	Phage*
FP+	+	+	+	−
3R	+	+	+	−
K	+	+	+	−
118	+	+	+	+
A31	+	+	+	−
37	+	+	+	−
67	+	+	+	+
B10	+	+	+	−
A22	+	+	−	−
22	−	+	+	−
B62	−	+	−	−
C18	+	−	−	+
P28	+	−	−	−
A30	+	−	−	−
B46	+	−	−	−
C14	+	−	−	+
B52	−	−	−	+

* All phages induced from these strains are of λ phage type.

As shown in Table 2 rhapidosomes were produced concomitantly with R-type pyocin but not with 28-type pyocin. Only in two strains (A22, B62) which formed R-type pyocin could no rhapidosomes be seen, but as they were often scanty it is possible that they were missed. Phages induced in these strains all had the structure of λ phage.

DISCUSSION

It was concluded from these experiments that the rhapidosome induced from Pseudomonas is polymerized sheath protein of R-type pyocin. Morphologically it has the structure of the contractile sheath of the pyocin and it also resembles the polysheath which appeared in the lysate of a conditionally lethal mutant of T-even

phage (Epstein *et al.* 1963). Rhapidosomes always have the same diameter as the contracted, not extended, sheath. The polysheath of the T4 mutant is the polymerized sheath protein of the phage tail and also has the structure of the contracted sheath (Kellenberger & Boy de la Tour, 1964). Our micro-agglutination experiments showed that the rhapidosome and R-type pyocin have the same antigenicity, which supports the idea that the rhapidosome is made from the same protein as the sheath of R-type pyocin.

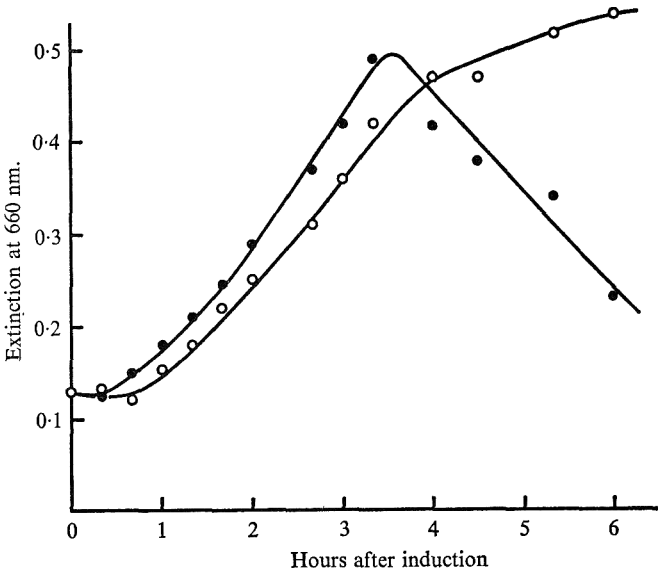

Fig. 2. Effect of mitomycin C on the growth of *P. fluorescens* strain 3R at 25°. Control, O——O; mitomycin-treated, ●——●.

In spontaneous lysates of older cultures of strain 3R no rhapidosomal structures were detected (cf. Lewin, 1963; Yamamoto, 1967), but they were readily found in mitomycin-induced lysates. Several strains of *Pseudomonas aeruginosa* or *P. fluorescens* induced by mitomycin produced rhapidosomes together with R-type but not with 28-type pyocin. Correll & Lewin (1964) analysed purified rhapidosomes obtained from Saprospira and showed that they too were uniform in size with a constant *S* value and consisted of RNA and protein.

At present all rod-shaped particles released from bacteria are called rhapidosomes, and from these observations it is clear that there are at least two types. However, the rhapidosomal structure whose origin has been proved to be phage-tail or bacteriocin should be called 'polysheath' and not 'rhapidosome'.

The ring structure observed in the lysate at 40° might be the sheath protein assembled into the ring form or a short rod. Why it is not assembled into complete pyocin has not yet been investigated.

To obtain more direct evidence for the mechanism of polysheath formation we are now trying to isolate sheath protein and reassemble it into polysheaths *in vitro*.

We thank Mr K. Takemori for the Pseudomonas strains at the Central Laboratory of Kyushu University Hospital, and Mr A. Takade for his excellent assistance in operating the electron microscope.

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. Pyocin induced from the 3R strain of *Pseudomonas fluorescens*. Two types of pyocin can be seen. One, R-type (R), has a structure like T-even phage-tails; the other, 28-type (28), resembles λ phage-tail. R-type pyocin has a contractile sheath. Negatively stained with neutral potassium phosphotungstate (PT). Scale marker represents 100 nm.

Fig. 2. Rhapidosome from strain 3R.

Fig. 3. Rhapidosomes and R-type pyocin from strain 3R. Rhapidosomes (RH) are long rods 180 Å in diameter. Their surface looks granular. Note penetration of PT into the rods.

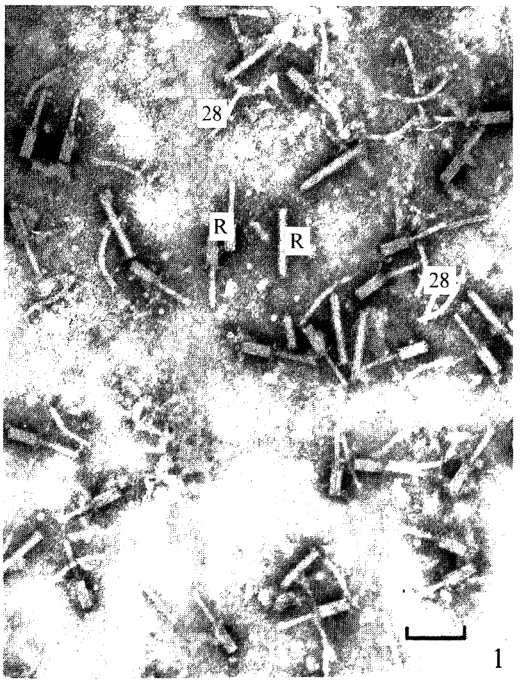
PLATE 2

Fig. 4, 5. Micro-agglutination between rhapidosomes and pyocins. R-type pyocins and 28-type pyocins form different specific agglutinates. Rhapidosomes agglutinate together with R-type pyocins. Antibody bridges can be seen between pyocin rods and rhapidosomes (arrow).

PLATE 3

Fig. 6. Micro-agglutination between rhapidosomes and pyocin. R-type and 28-type pyocin form different agglutinates.

Fig. 7. Lysate from strain 3R induced at 40°. A large number of small spherical particles can be seen, some of them arranged into short rods (arrows).



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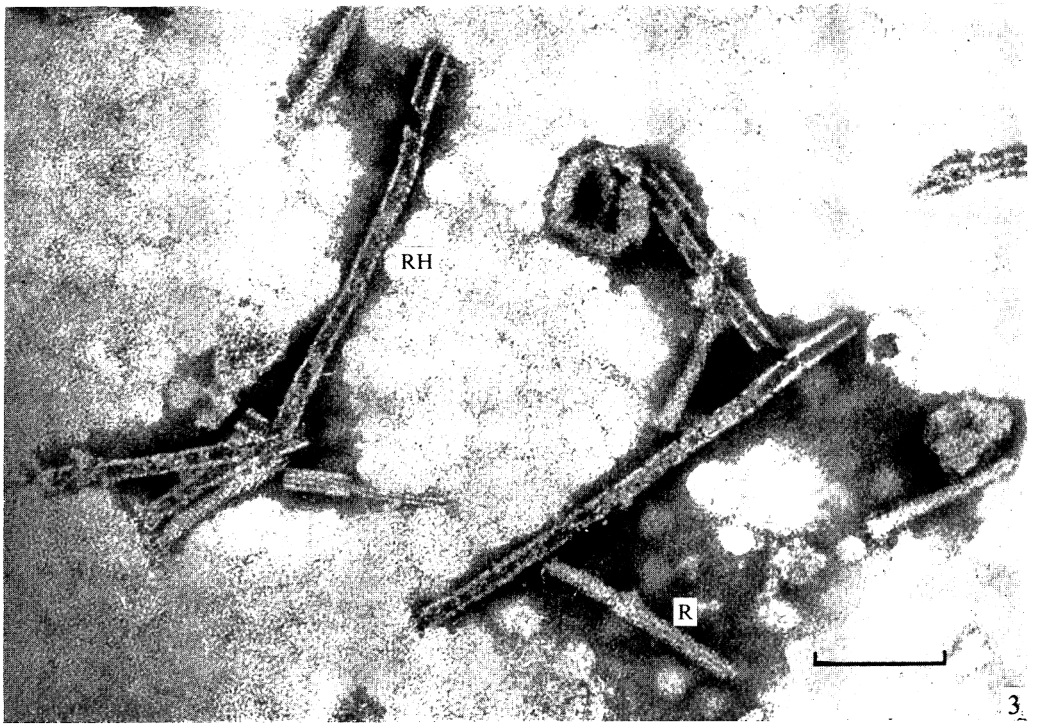

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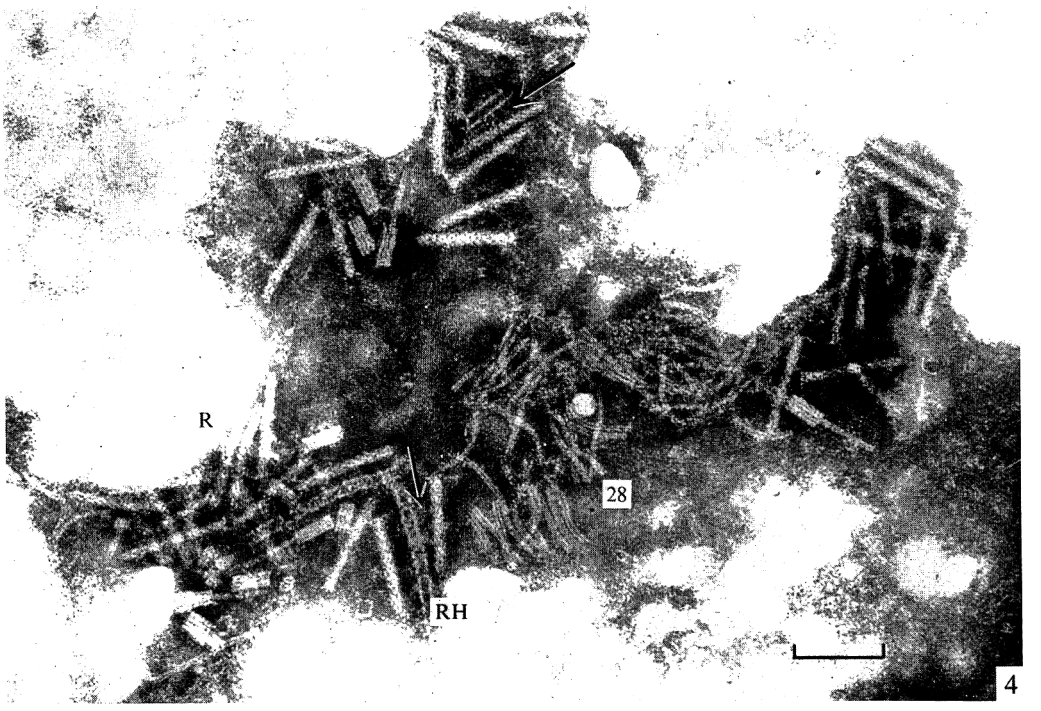

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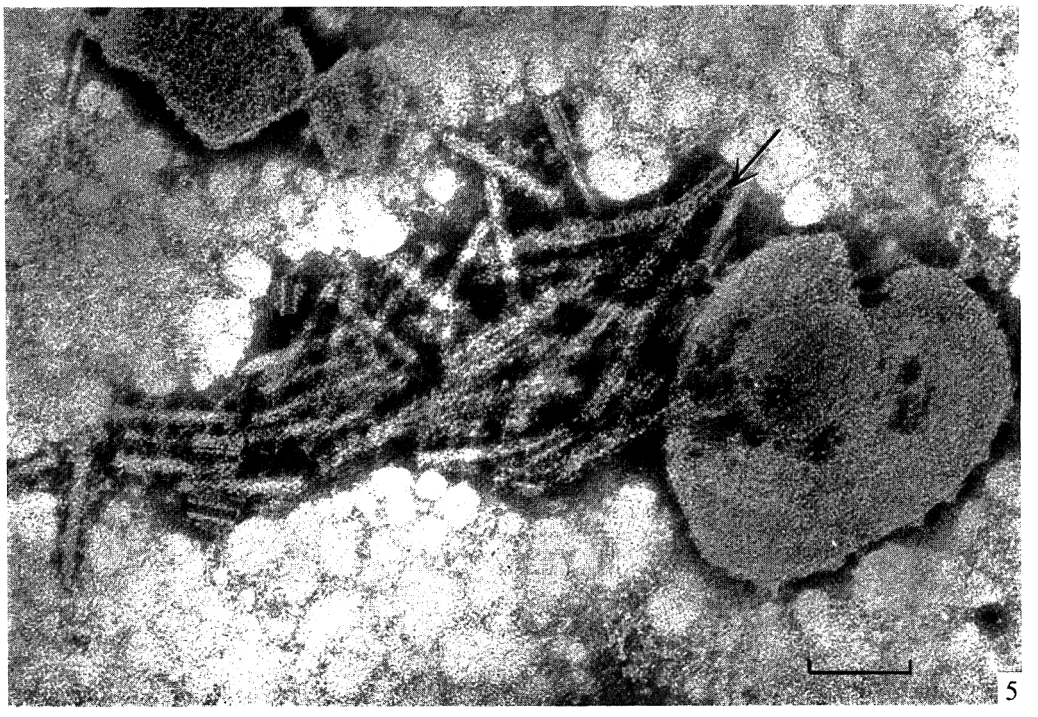

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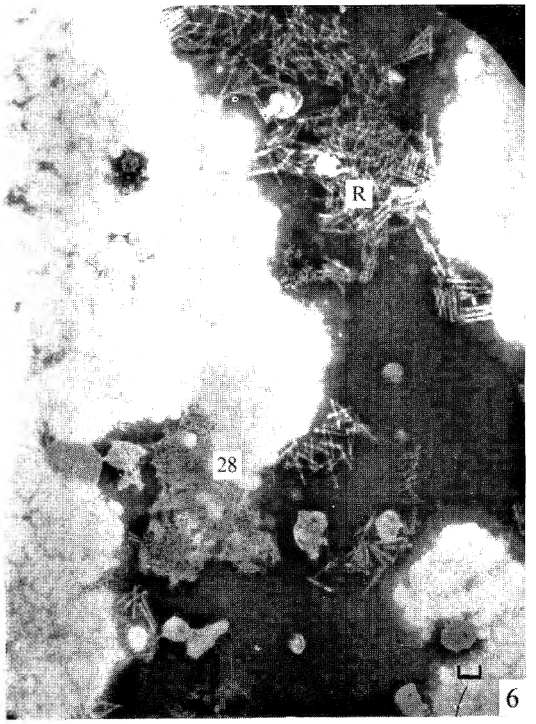

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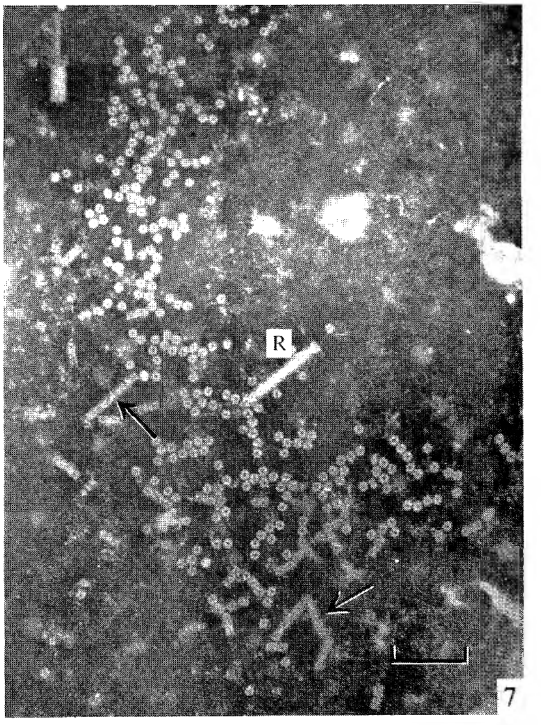

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Dictyostelium dimigraformum, *Dictyostelium laterosorum* and *Acytostelium ellipticum*: New Acrasieae from the American Tropics

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SUMMARY

Two new species of *Dictyostelium*, *D. dimigraformum* and *D. laterosorum*, and a new species of *Acytostelium*, *A. ellipticum*, are described. All three species were first isolated from the surface humus layer of tropical forest soils on the island of Trinidad, W.I. *D. dimigraformum* is distinguished from other species of the genus by its ability to form both stalkless and stalked migrating pseudoplasmodia. *D. laterosorum* is a member of the crampon-based Dictyostelia. It differs from other members of this group in bearing lateral, sessile sori along the terminal half of the sorophore. *A. ellipticum* is the second species in the genus *Acytostelium* to be described. It is distinguished from *A. leptosomum* by its elliptical spores, pattern of aggregation and development, and smaller size.

INTRODUCTION

As a part of my continuing investigation of forest soils for Acrasieae, I surveyed, during the summer of 1968, tropical forest soils in Puerto Rico, Trinidad and Tobago, West Indies, and Guyana, South America. Previous investigations indicated that a centre of diversity for Acrasieae exists in the tropics of Central and South America (Cavender & Raper, 1968; Cavender, 1969), as reflected in the several new Acrasieae which have been isolated from this area: *Dictyostelium rosarium*, *D. deminutivum*, *D. rhizopodium*, *D. lavandulum*, *D. coeruleo-stipes*, *D. vinaceo-fuscum* and *D. mucoroides* var. *stoloniferum*. Distribution studies have focused on the lowland seasonal evergreen forest which is the optimum forest habitat for Acrasieae in the tropics (Cavender & Raper, 1968).

On the island of Puerto Rico little such forest remains, consequently most collecting was done in the lower montane forest type prevalent in the Loquillo Experimental Forest. Only seven species and variants were isolated (Table 1). Two isolates, one resembling *Polysphondylium violaceum* in form but lacking pigment, and the other a member of the *Dictyostelium mucoroides* complex which produces exceptionally numerous sorocarps, are worthy of further study but have not yet been investigated. Possibly the less complex nature of the forests of Puerto Rico compared with those of the South American continent limits the number of Acrasieae species that survive there, or perhaps the island is simply too remote from the continent.

Floristically Trinidad resembles north-eastern South America, a consequence of long union with the continent. The forest vegetation has been studied extensively by J. S. Beard (e.g. 1946). Most of the forests sampled are seasonal evergreen; annual

rainfall 200 to 250 cm. with a dry season from January to May. Mora and crappo-guatecare (*Carapa–Eschweilera*) are the most widespread forest types. I collected from eight sites in the northern part of the island in the vicinity of, and on the lower slopes of, the Northern Range where elevations reach 500 m. Trinidad is an excellent area for Acrasieae. Fourteen species were isolated (Table 1), including the three species described here, which represent approximately 75% of the known soil Acrasieae. The adjacent island of Tobago supports fewer species of plants because of its greater isolation and smaller size. Less extensive collecting was done there. Only six species were found.

Table 1. *Species of Acrasieae isolated from tropical forest soils of Puerto Rico, Trinidad, Tobago and Guyana*

Species	Puerto Rico	Trinidad	Tobago	Guyana
Dictyostelium mucoroides	×	×	×	×
D. mucoroides variant I[1]	×	×	×	×
D. mucoroides variant II[2]	×	.	.	.
D. purpureum	×	×	×	×
D. polycephalum	×	×	.	×
D. rhizopodium	.	×	.	×
D. vinaceo-fuscum	.	×	.	.
D. aureum[3]	.	×	.	×
D. minutum[4]	.	×	.	×
D. laterosorum	.	×	×	.
D. dimigraformum	.	×	.	.
Polysphondylium pallidum	×	×	×	×
P. pallidum variant[5]	×	.	.	.
P. violaceum	.	×	.	.
Acytostelium leptosomum	.	×	×	×
A. ellipticum	.	×	.	×

[1] A delicate, hyaline-spored acrasian common in the tropics.
[2] Similar to the above but producing exceptionally numerous sorocarps.
[3] Golden-yellow sorocarps similar to *D. aureum* (E. Olive).
[4] In the tropics there are a variety of small hyaline-spored acrasians that resemble *D. minutum*.
[5] Unpigmented but resembling *P. violaceum* in form.

A limited area in Guyana was sampled—the forests located west of Bartica on the Essequibo River and along Moraballi Creek which are on either red or white sand. They are either mixed forests or consociations with mora, greenheart, morabukea and wallaba as the principal dominants (Davis & Richards, 1934). Ten species of Acrasieae were isolated (Table 1).

Two of the three Acrasieae described are considered new species of the genus *Dictyostelium* because they have unbranched cellular stalks. The third slime mould has an acellular stalk, consequently it is assigned to the genus *Acytostelium*.

METHODS

Soils were collected in small glass vials which hold about 30 g. Leaf mould, humus and some surface mineral soil were taken since these parts of the forest soil profile harbour the greatest number and kinds of Acrasieae (Cavender & Raper, 1965*b*). When I returned to the laboratory the samples were placed at 4°. The isolating technique of Cavender & Raper (1965*a*) was used. Developing Acrasieae were transferred

to dilute hay infusion agar media cross-streaked with *Escherichia coli* by touching a sorus with a needle and implanting the spores at the intersection of the bacterial streaks (see Raper, 1951). Further studies were carried out either by using this method of cultivation or by 'seeding' the surface of a dilute nutrient agar medium with a suspension of bacteria and spores which was then spread uniformly over the entire agar surface or in a broad band. This is a more favourable method for studying spore germination, aggregation and pseudoplasmodium formation (Raper, 1951). Other agar media used in this study, in addition to the hay infusion, were low nutrient media containing either lactose or dextrose and peptone (LP, DP) in varying amounts and non-nutrient agar supplemented with a pre-grown bacterial food supply.

The amount of growth per day of the slime mould and production of typical fruiting bodies were used as a basis for determining optimum conditions for cultivation. Temperature studies were carried out at 16, 20, 22, 25 and 30°. Five different bacteria, *Escherichia coli*, *Aerobacter aerogenes*, *Pseudomonas fluorescens*, *Serratia marcescens* and *Sarcina lutea* were tested as food for the Acrasieae using two types of media: (1) non-nutrient agar streaked with pre-grown bacterial samples, and (2) LP or DP agar media streaked with bacterial suspensions. Spores were implanted at the ends of the streaks. Most cellular slime moulds respond best to either *E. coli* or *A. aerogenes* and this was found to be true for the Acrasieae described here.

Dictyostelium dimigraformum Cavender, sp.nov.

Sorocarpi erecti, vel proni, non ramosi, magnitudine proportioneque varii; in nigritie pseudoplasmodia vaga, typice sine caulibus, in uniducto lumine phototropica, cum caulibus vel sine caulibus; sorophora incolora usque ad leviter flava, usque ad plerumque 3 ad 8 mm. in longitudinem; sori subglobosi usque ad citroformes, plerumque 200 ad 400 μm. in diametro, lactei usque ad citrini: spori elliptici usque ad reniformes, magnitudine maxime varii, plerumque 7·0 ad 12·0 × 2·5 ad 3·5 μm. sed interdum usque 26 × 5 μm.

Habitat: In foliari humo et summo solo, tropica humida silva, Trinidad, W.I.

Typica cultura: AR-5b.

Sorocarps erect or inclined, unbranched, variable in size and proportions, in the dark pseudoplasmodia wandering, typically without producing a stalk, in one-sided light phototropic, migration occurring with or without stalk production; sorophores unpigmented to yellowish, mostly 3 to 8 mm. in length; sori subglobose to citriform, mostly 200 to 400 μm. in diameter, white to lemon-yellow; spores elliptical to reniform, extremely variable in size, mostly 7·0 to 12·0 × 2·5 to 2·5 μm. but sometimes as large as 26 × 5 μm.

Habitat: In leaf mould and surface soil, tropical moist forest, Trinidad, W.I.

Type culture: AR-5b

ISOLATION AND CULTIVATION

The only isolate of *Dictyostelium dimigraformum* was obtained from the surface soil of a forest reserve in Trinidad located just above the Asa Wright Nature Centre on the south slope of the Northern Range along the road between Arima and Blanchisseuse. One clone of the organism appeared on the isolation plate.

Dictyostelium dimigraformum was characterized by robust growth but less so than *D. discoideum*, *D. purpureum* or some of the larger strains of *D. mucoroides*. It grew

well on a variety of media of low nutrient content. Those most often used were 0·1% LP and thin hay infusion agar (15 g. of dried, leached bluegrass per litre). Optimum growth on these media occurred with *Escherichia coli* although good growth was obtained with *Aerobacter aerogenes* and *Serratia marcescens*. The pigment of *S. marcescens*, prodigiosin, was not digested by the myxamoebae, consequently the slugs and sorocarps were pink to red in colour. Optimum temperature for growth and development was between 20 and 25°. At 16° growth and development were slow but normal, although much slime was left behind by the migrating slugs. No growth occurred at 30° but growth and differentiation proceeded normally at 28°.

Growth and morphogenesis

Spore germination. Germination took place in 4 to 5 h. when spores were spread on 0·1% LP medium. The germination process was similar to that described for *Dictyostelium discoideum* by Cotter & Raper (1968). The swelling spore (Pl. 1, fig. 1) in some cases approached spherical proportions before pressure from within caused the cellulose wall to split and allowed the myxamoeba to emerge (Pl. 1, fig. 2). The slime surrounding spores removed from a sorus dried very quickly making them difficult to spread on the agar surface without leaving clumps. Some such clumps of several hundred or more spores germinated *en masse* (Pl. 1, fig. 6). Aggregation and culmination followed without an intervening growth phase. However, this refruiting habit did not occur to the extent described for *D. mucoroides* var. *stoloniferum* (Cavender & Raper, 1968). An interesting characteristic found in *D. dimigraformum* was the range in spore size. Spores are elliptical in shape, occasionally reniform. Most spores are 7 to 12 × 2·5 to 3·5 μm. with a mean of 8·0 × 3·0 μm. Spores 15 to 18 μm in length were not uncommon (Pl. 1, fig. 4); larger spores were rare but one measured 26 × 5 μm. (Pl. 1, fig. 5). Spores of different size ranges are known for *D. discoideum* and for the crampon-based Dictyostelia (Raper & Fennell, 1967), but this is the first report of such extreme variation in spore size in Acrasieae. Sussman& Sussman (1962) found a relationship between ploidy and spore size in *D. discoideum*.

Vegetative growth. The myxamoebae appeared similar to those of *Dictyostelium discoideum* or *D. mucoroides* (Pl. 1, fig. 3). Variation in size was apparent from spore dimensions. Shape changed during the life-cycle: while feeding, myxamoebae were circular in outline with diameters averaging about 11 μm.; while moving, they elongated to about 20 × 10 μm. This elongation was even more exaggerated when the myxamoebae entered an aggregation stream.

Aggregation. In a culture prepared by inoculating the surface of 0·1% LP medium with a mixture of *Escherichia coli* and slime mould spores, aggregation began after available bacteria had been consumed. At 25° this period was about 40 h. for *Dictyostelium dimigraformum*. Centres were spaced at an average density of 6/cm.^{2} on dark-grown plates. In the light the density was greater. Almost 100% of the myxamoebae entered the aggregates. Streams appeared before actual centres could be detected and were often flat and sheet-like at this stage. Wheel-like aggregations soon developed, each with a definite centre or hub and well-defined streams or spokes (Pl. 1, fig. 7). A later stage had fewer but larger streams (Pl. 2, fig. 9). Usually one pseudoplasmodium or slug was formed from each centre. Where myxamoebae were very dense and large centres were formed, several slugs might emerge.

Pseudoplasmodia. When a culture plate was illuminated from above, sorocarps were

constructed vertically from the point of aggregation (Pl. 2, fig. 14). If grown with one-sided illumination, some slugs moved away from the point of aggregation and a prostrate stalk was formed as the slug migrated (Pl. 1, fig. 8). The occasional production of a stalkless slug (Pl. 2, fig. 11) distinguished this species from all those with stalk-forming slugs. The stalkless-slug left behind as a trail only the collapsed slime case that is continually secreted. This slug, identical in appearance and behaviour to its counterpart in the life-cycle of *Dictyostelium discoideum*, was especially abundant on 0·1 % LP medium and much less abundant on buffered hay infusion agar. In absolute darkness most slugs were stalkless. They might wander over the agar surface for a week or more before sorocarp formation occurred, but sorocarp formation could be initiated dramatically by exposing the culture to overhead light.* Slugs then stopped moving and within 1 to 2 h. became orientated on a vertical axis, and somewhat flattened to a pear shape (Pl. 2, fig. 12) as the internally formed stalk made contact with the agar surface (Pl. 2, fig. 10). As the stalk lengthened, the mass of cells left the substratum, perhaps partially through the individual efforts of the myxamoebae but principally through the force exerted by the lengthening stalk (Raper & Fennell, 1952).

A dark-grown slug might form a length of stalk and then cease stalk formation while continuing to migrate. The environmental factors which 'trigger' stalk formation in the dark are not known. Sorocarps that were formed were generally on the sides of the Petri dish, indicating that a decreasing relative humidity might act as a trigger as it does for other acrasians such as *Dictyostelium discoideum*.

Migrating slugs produced well-defined slime tracks often (especially at temperatures below 20°) leaving behind clumps of cells which occasionally formed small slugs or fruiting bodies (Pl. 1, fig. 8).

Sorocarps. The formation of sorocarps was not studied cytologically but appeared to follow closely the pattern described for *Dictyostelium discoideum* by Raper & Fennell (1952) except that a basal disc was not formed. Sorocarps varied greatly in size and orientation depending upon the density of myxamoebae and the availability and direction of light. Commonly, when produced on LP medium after a period of migration, they resembled the upright portions of sorocarps of *D. purpureum* or larger members of the *D. mucoroides* complex. The unbranched, tapering, upright stalk, 3 to 8 mm. high, usually had a short supporting horizontal portion surrounded by slime (Pl. 2, fig. 13). The terminal sori were globular or citriform, their diameters (usually proportional to the stalk length) most commonly 200 to 400 μm. The sori often developed an intense lemon-yellow colour derived from the pigmentation of the spores, but this did not always develop, and a gradation in colour from cream-white to lemon-yellow was found in most cultures.

Dictyostelium laterosorum Cavender, sp.nov.

Sorocarpi typice erecti vel proni, interdum procidui, non saepe ramosi, magnitudine proportioneque varii; sorophora caesia usque leviter violacea, saepe subbruna, plerumque 5 ad 10 mm. in longitudinem sed ex 1 mm. usque 20 mm. varia, sorophorum basi exiliter digitatae et instar claviculae; sori globosi, caesii usque leviter violacei, saepe subbruni, et ad laterales et terminales in positionibus, laterales sori plerumque minores, sessiles, 80 ad 150 μm. in diametro, 1 ad 10 in numero, terminales sori

* Light induction of sorocarp formation was recently discovered for *D. discoideum* by Newell, Telser & Sussman (1969).

130 ad 200 μm. in diametro; spori varii, elliptici, reniformes vel recurvi, 6·0 ad 13·0 × 2·5 ad 4·0 μm.

Habitat: In foliari humo et summo solo, tropica humida silva, Trinidad et Tobago, W.I.; Colombia, S.A.

Typica cultura: TB II-1

Sorocarps typically erect or inclined, sometimes prostrate, infrequently branched, variable in size and proportions, sorophores bluish grey to light violet, often with a brownish cast, usually 5 to 10 mm. in length but varying from 1 to 20 mm.; sorophore bases weakly digitate and crampon-like; sori globose, bluish grey to light violet often with a brownish cast, both lateral and terminal in position, lateral sori smaller, sessile 80 to 150 μm. in diameter, 1 to 10 in number, terminal sori 130 to 200 μm. in diameter; spores variable in shape and size, elliptical, reniform or recurved, 6·0 to 13·0 μm. × 2·5 to 4·0 μm.

Habitat: Leaf mould and surface soil, tropical moist forest, Trinidad and Tobago, W.I.; Colombia, S.A.

Type culture: TB II-1

Isolation and cultivation

Initially two isolations of this species were made, strain TB II-1 from semi-deciduous forest on the north slope of the Main Ridge, Tobago, along the road from Roxborough to Charlotville, and strain AE-4 from seasonal evergreen forest near Arena, Trinidad. Three other isolations have subsequently been made from Colombia. Excellent growth and development of *Dictyostelium laterosorum* was obtained at 22 to 25° on 0·1 % LP or hay infusion agar media in association with *Escherichia coli* or on non-nutrient agar streaked with pre-grown bacteria.

Growth and morphogenesis

Spores and myxamoebae. Spores gave rise to myxamoebae after 4 to 5 h. at 25°on LP medium. A large percentage failed to germinate. Spores of the two strains TB II-1 and AE-4 were originally different in shape and size; strain TB II-1 produced predominantly elliptical spores 6 to 9 × 2·5 to 4 μm. (Pl. 3, fig. 17); strain AE-4 produced larger spores, 9 to 13 × 3·5 to 4·5 μm., mostly reniform or recurved in shape (Pl. 3, fig. 16). After about eight months of laboratory cultivation diploidization apparently occurred. Strain TB II-1 began producing spores similar in size and shape to those of AE-4 (Pl. 3, fig. 18); other differences between the two strains remained constant.

The myxamoebae were distinguishable from those of *Dictyostelium dimigraformum* by their elongated triangular shape while moving (Pl. 3, fig. 19), the relatively broad front advancing, the tapering apex trailing behind. This posterior apex might be as long as the body of the myxamoeba, so narrow that it was just visible when magnified × 600, and occasionally it was forked. Up to five peripheral nucleoli were conspicuous in the nucleus. Feeding myxamoebae were circular in outline, their diameters mostly 10 to 15 μm.

On LP medium aggregation began after the bacteria had been consumed by the myxamoebal population. At 25° this period was *c.* 42 h. for strain AE-4 and *c.* 45 h. for strain TB II-1. The two strains differed somewhat in their patterns of aggregation. Myxamoebae of strain AE-4 tended to develop strong wheel-like centres without delay (Pl. 3, fig. 23), whereas those of strain TB II-1 did not form definite centres immediately

but first formed small clumps or mounds (Pl. 3, fig. 20) which gradually increased in size (Pl. 3, fig. 21). Some of these early aggregations developed short streams. After 72 h. certain centres had become dominant and attracted myxamoebae from most of the early aggregations (Pl. 3, fig. 22), though the radiate pattern exhibited by AE-4 did not often develop (Pl. 4, fig. 24) and the streams often broke up into numerous smaller centres, especially when aggrgeation occurred in the light.

Pseudoplasmodia and sorocarp formation. Dictyostelium laterosorum typically produced pseudoplasmodia that constructed stalks vertically or at an angle from the aggregation centre although the somewhat larger Colombian strains were prone to wander over the agar surface. The phototropic response was not particularly strong although one-sided illumination induced migration toward the light. Light is not essential for completion of the life-cycle.

The rising sorogen resembled that of *Dictyostelium mucoroides* during the early stage, but as the terminal half of the stalk was formed masses of myxamoebae were periodically abstricted from the posterior portion (Pl. 4, fig. 28). These myxamoebae differentiated into spore cells without concomitant formation of lateral stalks as in *Polysphondylium* (Pl. 4, fig. 26). The result was a series of sessile globular sori which gave the sorocarp a beaded appearance (Pl. 4, fig. 29). This method of increasing the efficiency of spore distribution was first discovered in the slime mould *Dictyostelium rosarium* (Raper & Cavender, 1968). *D. laterosorum* closely resembled this species in the nature and disposition of the sori. Occasionally a lateral branch bearing a sorus at the tip (Pl. 4, fig. 30) was seen. The masses of abstricted myxamoebae occasionally retained the capacity to construct a stalk. Sorocarps may have up to ten or more lateral sori, each 80 to 150 μm. in diameter, though the terminal sorus is slightly larger (130 to 200 μm.).

The pigmentation of the sorocarps approximated that of *Dictyostelium lavandulum* (Raper & Fennell, 1967), i.e. bluish grey deepening with age to light violet or lavender, often with a brownish cast.

The sorocarps were supported by a crampon base enveloped by a layer of supporting slime material (Pl. 4, fig. 27). The ramifications of the crampon, much less developed than in *Dictyostelium rhizopodium*, were short cellular extensions, often knob-like, protruding from the expanded base of the stalk (Pl. 4, fig. 25). Of other Acrasieae these crampons resembled most closely the expanded base type of *D. coeruleo-stipes* (Raper & Fennell, 1967).

Sorocarps have a broad size range. Lengths of mature stalks varied from 1 to 20 mm. but most were 5 to 10 mm. Strain AE-4 produced more large, rangy sorocarps with a greater proportion of stalk to spore mass than strain TB 11-1. Some of the Colombian strains also had this habit but produced more lateral sori than AE-4.

Acytostelium ellipticum Cavender, sp.nov.

Sorocarpi maxime delicati, solitarii vel gregarii, 200 ad 1000 μm. in altitudinem; sorophora incolora, acellularia, 1 ad 2 μm. in diametro attenuata usque $< 1{\cdot}0$ μm. in extrema parte; surgens sorogen ventricosa-rostrata; sori globosi, 18 ad 40 μm. in diametro; spori elliptici, $5{\cdot}5$ ad $8{\cdot}0$ μm. $\times$ $2{\cdot}0$ ad $3{\cdot}0$ μm.

Habitat: In foliari humo et summo solo, tropica humida silva, Trinidad, W.I.; Guyana, Colombia, S.A.

Typica cultura: AE-2

Sorocarps extremely delicate, solitary or gregarious, 200 to 1000 μm. in height; sorophores unpigmented, acellular, 1 to 2 μm. in diameter tapering to less than 1·0 μm. at the tip; rising sorogen ventricose-rostrate; sori globose, 18 to 40 μm. in diameter; spores elliptical, 5·5 to 8·0 μm $\times$ 2·0 to 3·0 μm.

Habitat: Leaf mould and surface soil, tropical moist forest, Trinidad, W.I.; Guyanas Colombia, S.A.

Type culture: AE-2

Isolation and cultivation

Acytostelium ellipticum was isolated from the Trinidad Government Forest Reserves at Melajo and Arena, from the lowland forest along Moraballi Creek in Guyana, and more recently from the Amazon Basin of Colombia. The isolates were very small and delicate, barely visible on the isolation plates at $\times$ 30 magnification, and so considerably smaller than those of *A. leptosomum* on the same isolation plates. Cultivation was originally attempted on 0·1 % LP medium streaked with *Escherichia coli*. Germination and growth occurred with no further morphogenesis. Weak hay infusion medium (8 g. dried leached bluegrass/l.) with *E. coli* gave good growth and development. After several months cultivation the slime mould was able to complete its life-cycle on 0·1 % LP, but optimum growth and development occurred on a weak glucose–peptone medium (0·05 % glucose and 0·025 % peptone) at 20 to 25° when a suspension of *E. coli* and slime mould spores was spread in a broad band on the agar. *E. coli* and *Aerobacter aerogenes* were the best food sources. No development took place at 30°.

Growth and development

The elliptical spores measured 5·5 to 8·0 $\times$ 2·0 to 3·0 μm. (Pl. 5, fig. 31). After germination the myxamoebae formed a densely packed feeding front (Pl. 5, fig. 32). On DP medium inoculated with a suspension of spores and *Escherichia coli* the bacteria were consumed after 40 h. at 25°. The myxamoebae then clumped together (Pl. 5, fig. 32) in increasing numbers without streaming, to form small mounds (Pl. 5, fig. 35). Crude streams were observed only when a culture was cooled to 4° or on a medium of higher nutrient content (0·25 % DP) (Pl. 5, fig. 34) on which subsequent development was abnormal. The aggregation pattern therefore differs from *Acytostelium leptosomum* (Pl. 5, fig. 40), where radiate streaming occurs. Each mound-like aggregation produces one to several sorocarps (Pl. 5, fig. 38) depending upon its size: the highly gregarious fruiting of *A. leptosomum* (Pl. 5, fig. 40) does not occur. The developing sorogen is long and tapered (Pl. 5, fig. 33), and as the stalk lengthens it develops a bulbous posterior portion (Pl. 5, fig. 36); in contrast, the developing sorogens of *A. leptosomum* are naviculate (Pl. 5, fig. 40). The cells in the narrow anterior portion of the *A. ellipticum* sorogen probably secrete the acellular stalk tube: Raper & Quinlan (1958) found that in the sorogen of *A. leptosomum* the myxamoebae in the anterior end were arranged perpendicular to the stalk, indicating a secretory rather than a locomotive function. Following aggregation, secretion of the entire stalk (200 to 1000 μm. in length) took 2 to 3 h. Its delicate nature in respect to the mass of spores which it supported (Pl. 5, fig. 39) was an engineering wonder. A globular unpigmented sorus (18 to 40 μm. across) developed at the terminus of the stalk (Pl. 5, fig. 37). The average dimensions of both stalk length and sorus were less than for *A. leptosomum*.

DISCUSSION

Of the three new Acrasieae described here, perhaps *Dictyostelium dimigraformum* is of greatest interest because it possesses characteristics intermediate between those of *D. mucoroides* and *D. discoideum*. Its similarity to *D. discoideum* is most apparent in the migrating pseudoplasmodial phase of development when stalk formation does not occur. This is a more efficient means of migration than we find in *D. mucoroides* since cells are not needlessly expended. My observations concerning the response of the pseudoplasmodia to light essentially duplicate those of Newell *et al.* (1969) working with *D. discoideum*. The myxamoebae show a physiological similarity to those of *D. discoideum* since they are unable to digest the pigment prodigiosin. Myxamoebae of *D. mucoroides* digest this pigment. The sorocarps themselves as well as the stalked migrating pseudoplasmodia resemble those produced by *D. mucoroides* or *D. purpurem*. The organism provides good experimental material for the study of environmental factors which initiate stalk formation. The 17-fold variation in size of the spores of *D. dimigraformum* is of interest, for it has been argued that genetic heterogeneity brought about through para-sexual mechanisms is basic to cell differentiation in the Acrasieae (Ashworth & Sackin, 1969). The possible role which these large cells play in development is worthy of investigation.

Dictyostelium laterosorum has the beaded sorocarps of *D. rosarium* but differs from this species in pigmentation, spore shape, crampon base, and developmental pattern (see Raper & Cavender, 1968). *D. laterosorum* also resembles *D. lavandulum* in pigmentation of the sorocarps and the presence of a crampon base. The crampon-based Acrasieae are tropical in distribution. Hopefully, *Coenonia*, a crampon-based genus which is more highly differentiated than any cellular slime mould now in culture, will someday turn up in tropical soils.

Acytostelium ellipticum is one of the smallest Acrasieae. The sorocarps are more delicate than those of *Dictyostelium deminutivum* but the myxamoebae are larger. The pattern of aggregation and sorocarp distribution is closer to this species than to *A. leptosomum*.

Strains AR-5b, TBII-1 and AE-2 have been deposited with the American Type Culture Collection, Washington, D.C.

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The writer is indebted to John B. Thurston for aid in collecting the soils and in isolating Acrasieae from them.

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EXPLANATION OF PLATES

PLATE 1

Dictyostelium dimigraformum

Fig. 1. A germinating spore just before the splitting of the spore wall. × 1140.

Fig. 2. The same germinating spore. A myxamoeba is emerging from the split spore case. × 1140.

Fig. 3. A moving myxamoeba. × 1140.

Fig. 4. Spores, including a large one 17 μm. in length. × 1140.

Fig. 5. A very large spore, 26 μm in length. × 900.

Fig. 6. A pseudoplasmodium forming from a clump of spores which have germinated *en masse*. Some of the myxamoebae are wandering away. × 70.

Fig. 7. A typical wheel-like aggregation. × 29.

Fig. 8. A migrating pseudoplasmodium which is producing a stalk. Note the partially dried sorogen which developed from myxamoebae cast off in the slime track of a stalkless migrating slug. × 90.

PLATE 2

Dictyostelium dimigraformum

Fig. 9. An aggregation at a slightly later stage in development from that in Pl. 1, fig. 7. × 35.

Fig. 10. A pseudoplasmodium which has stopped migration after exposure to light and which is producing a stalk, visible in the interior. × 58.

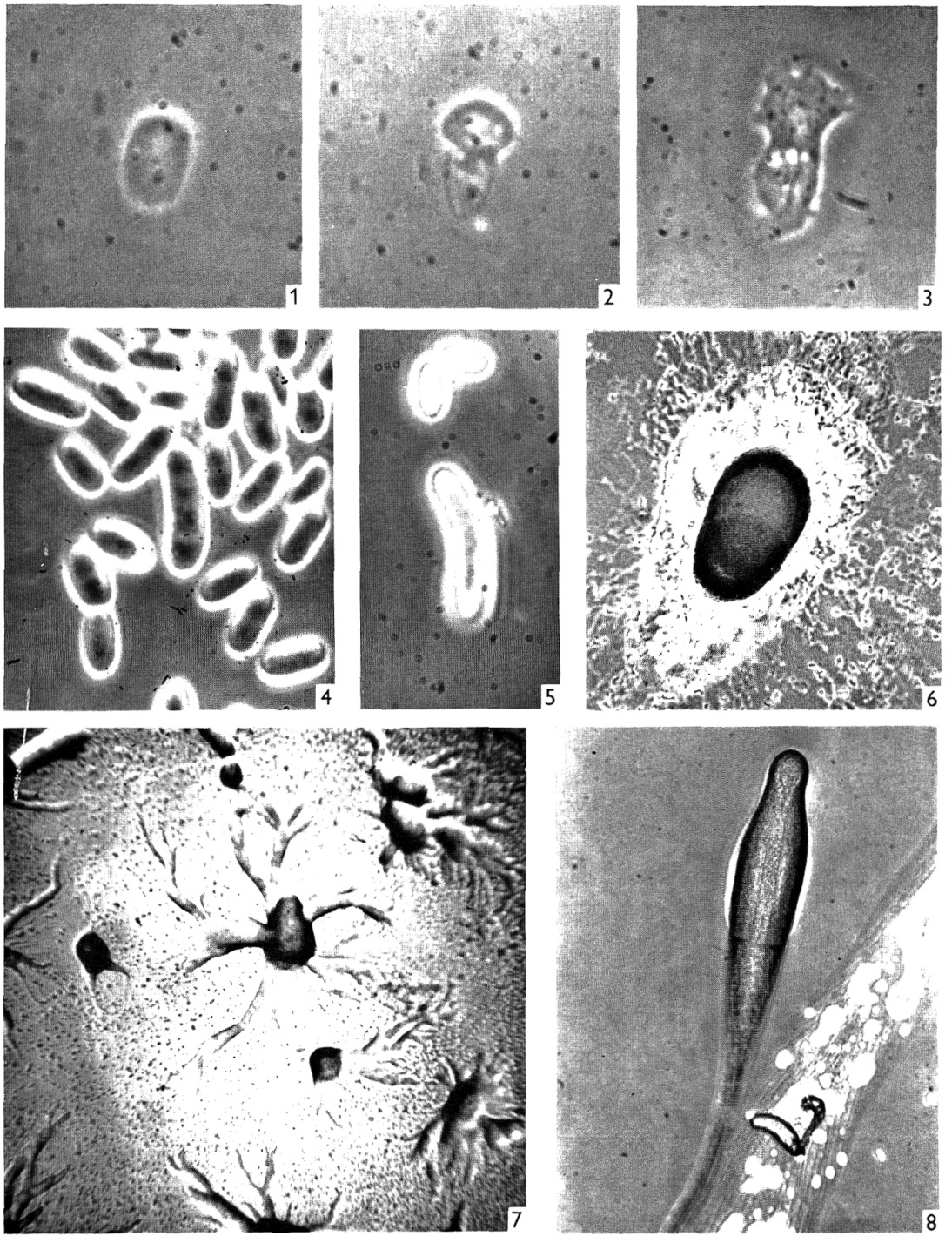
Fig. 11. A stalkless migrating pseudoplasmodium or slug. × 58.

Fig. 12. A pseudoplasmodium which has ceased migration and become orientated almost vertically. The pre-spore mass is about to be lifted off the agar surface. × 100.

Fig. 13. A mature sorocarp produced from a stalkless migrating pseudoplasmodium. Note the short horizontal portion surrounded by slime. × 44.

Fig. 14. A small rising sorogen. The stalk is composed of a single row of cells. × 35.

Fig. 15. Mature sorocarps, which have developed without migration, collapsed on the agar surface. × 5.



J. C. CAVENDER

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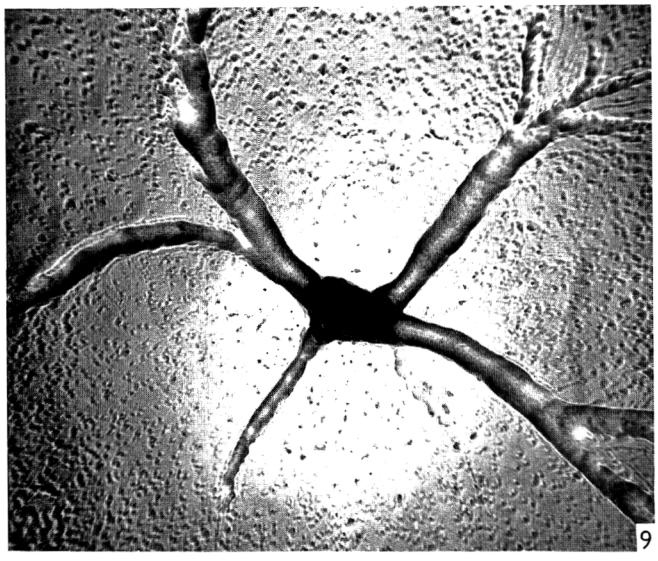
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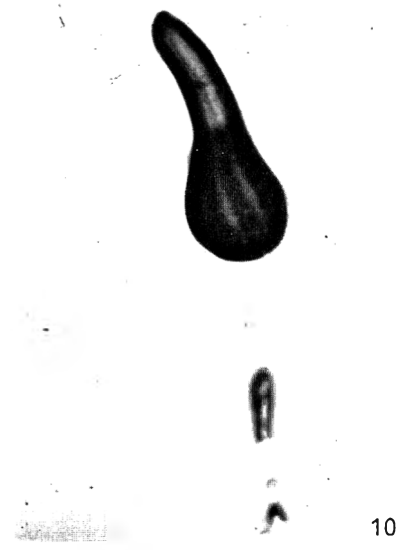
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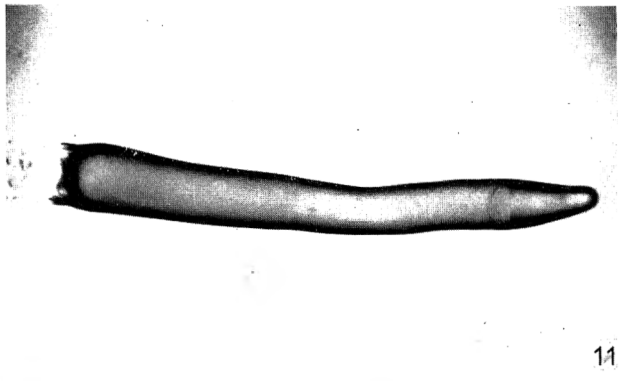
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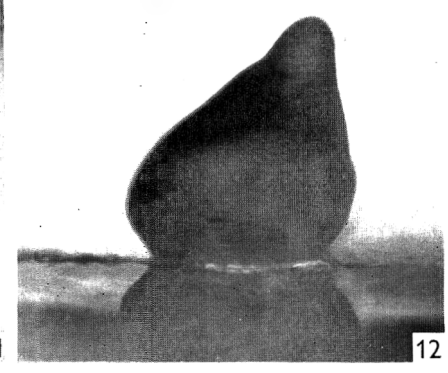
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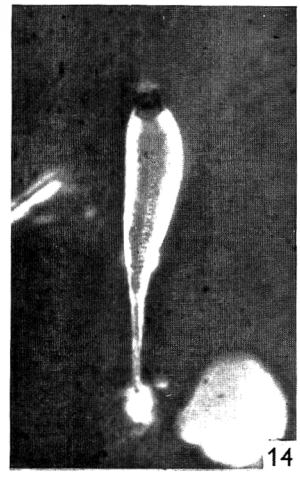
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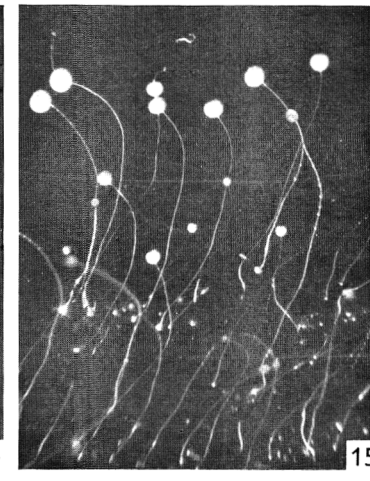
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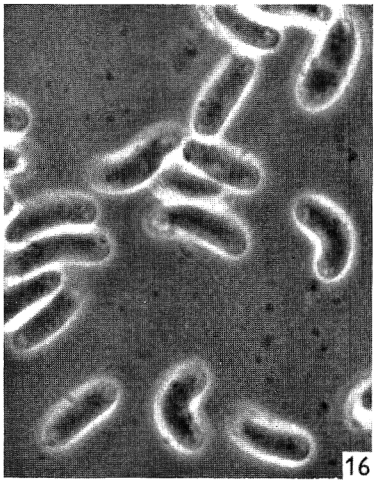
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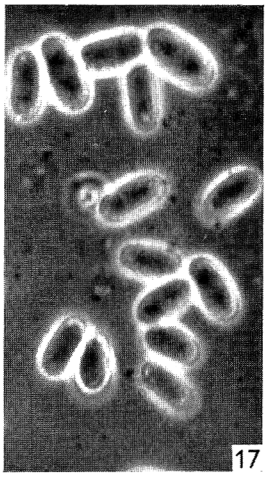
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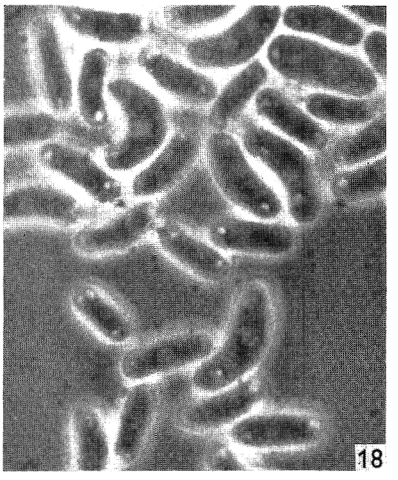
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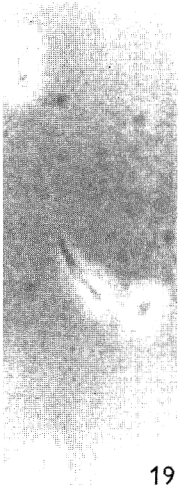
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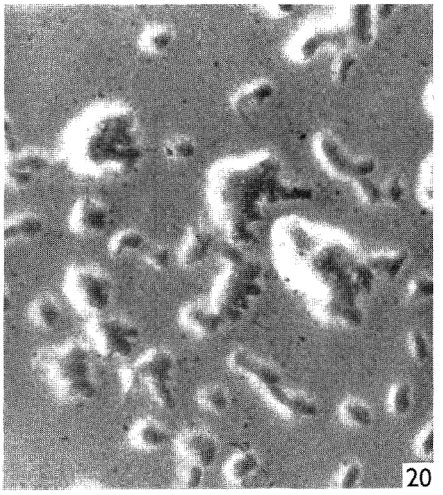
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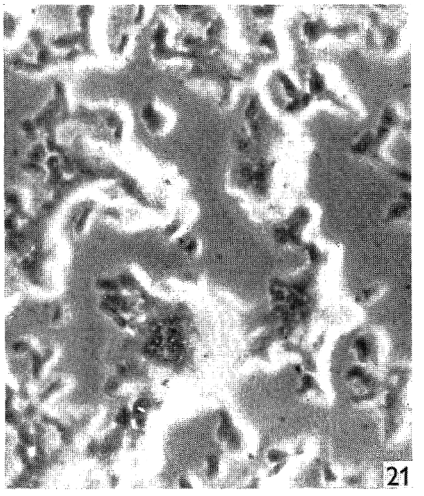
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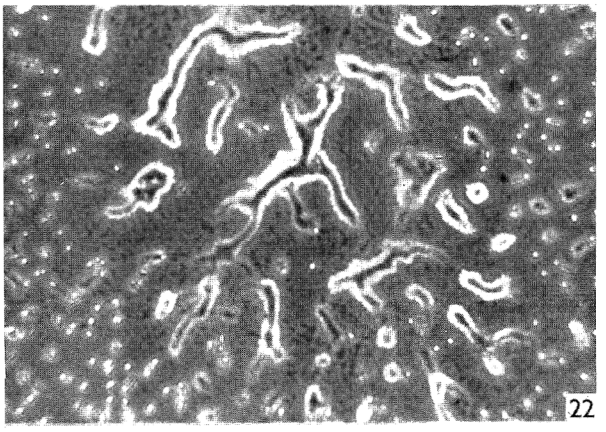
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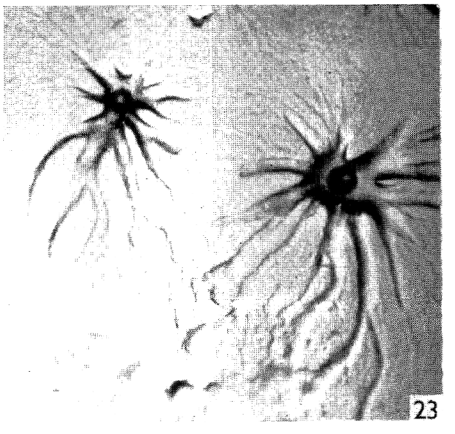
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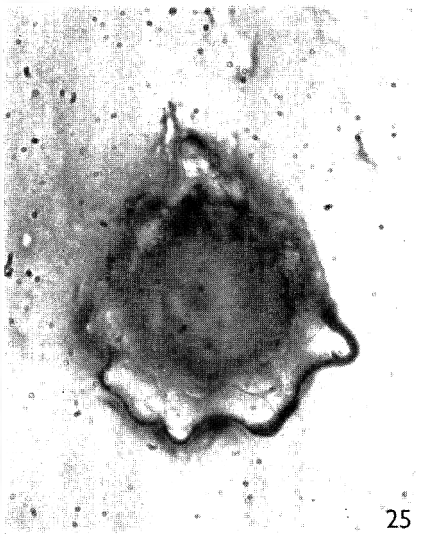
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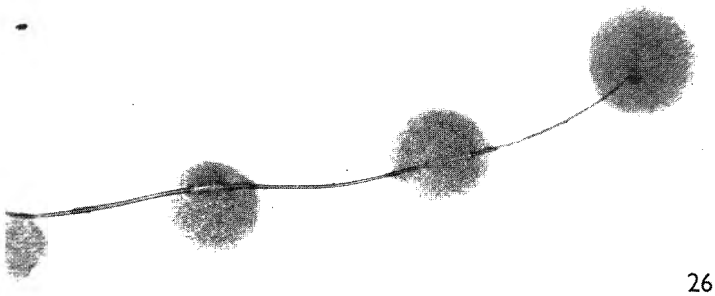
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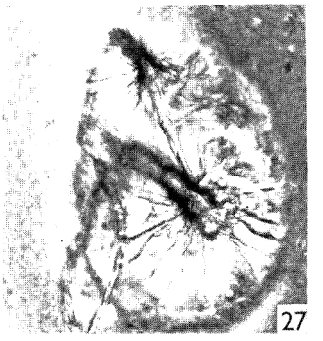
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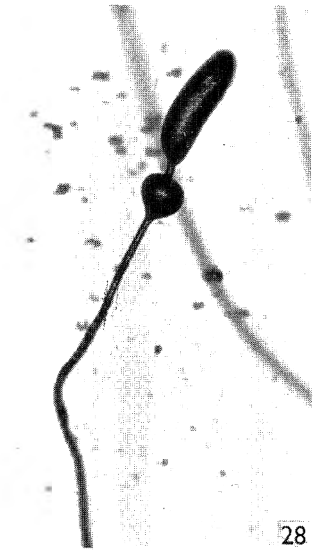
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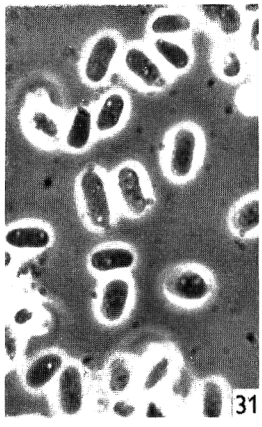
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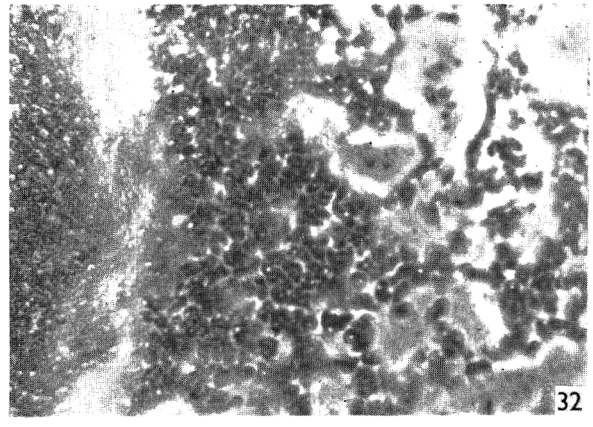
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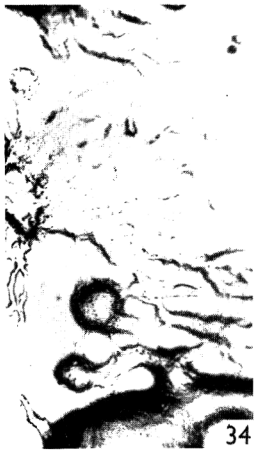
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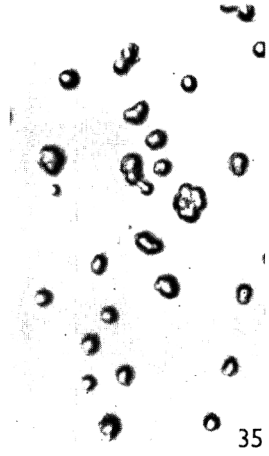
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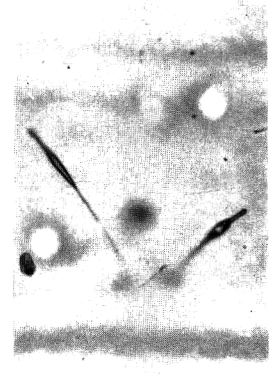
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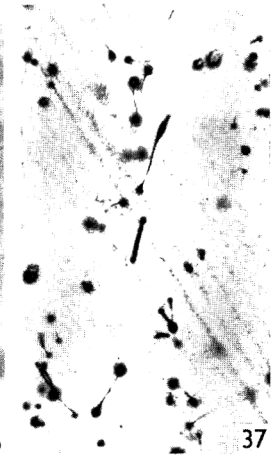
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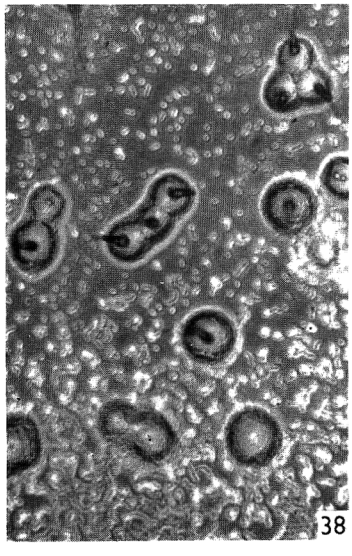
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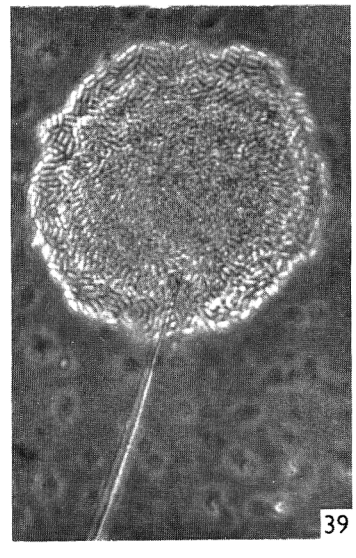
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PLATE 3

Dictyostelium laterosorum

Fig. 16. Spores of strain AE-4. Note the granules at each end. × 1140.

Fig. 17. Spores of strain TB II-1 before size change. × 1140.

Fig. 18. Spores of strain TB II-1 after size change. × 1140.

Fig. 19. The central myxamoeba is moving toward the lower right. Note the triangular shape and long tail. × 450.

Fig. 20. TB II-1. An early stage in aggregation. The cells have gathered into small clumps. × 340.

Fig. 21. TB II-1. A slightly later stage. The clumps have increased in size. × 290.

Fig. 22. TB II-1. An intermediate stage in aggregation. Dominant centres are forming which attract the myxamoebae from surrounding clumps. × 115.

Fig. 23. Wheel-like aggregates typical of strain AE-4. × 29.

PLATE 4

Dictyostelium laterosorum

Fig. 24. TB II-1. A late stage in aggregation. Strong centres have developed which attract myxamoebae from a relatively large area. × 9.

Fig. 25. A typical crampon-like base which supports the sorocarp. × 450.

Fig. 26. Terminal portion of the sorocarp showing the disposition of the sori. × 46.

Fig. 27. The expanded disc of slime material which surrounds the base of the stalk. × 115.

Fig. 28. A developing sorogen which has just abstricted a mass of myxamoebae from its posterior portion. The myxamoebae will differentiate into spores forming together a lateral sorus. × 53.

Fig. 29. Mature sorocarps showing the typical beaded appearance. × 23.

Fig. 30. A sorocarp on which a lateral branch has developed. × 23.

PLATE 5

Acytostelium ellipticum

Fig. 31. Spores showing the characteristic shape for the species. × 1140.

Fig. 32. Myxamoebae densely packed in a feeding front at the left. At the right aggregation is beginning to occur. × 290.

Fig. 33. Characteristic shape for the species of a young rising sorogen. × 230.

Fig. 34. Rudimentary stream formation by strain AE-2 on 0·25% dextrose–0·25% peptone agar. Subsequent development was abnormal. × 29.

Fig. 35. Typical aggregates formed on 0·25% dextrose–0·05% peptone agar. × 29.

Fig. 36. Rising sorogens. At this stage the sorogen has a relatively long narrow anterior portion and a more bulbous posterior. × 92.

Fig. 37. Sorocarps at various stages of development. × 46.

Fig. 38. Aggregates from which 1 to 3 sorocarps are developing. × 92.

Fig. 39. Terminal portion of a mature sorocarp collapsed on the agar surface. Note the dimension of the stalk tube in relation to the spore mass it supported. × 290.

Fig. 40. A typical aggregate of *A. leptosomum* showing stream formation, numerous clustered sorocarps and naviculate sorogens. × 23.

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SHORT COMMUNICATION

A Water-soluble Carotenoid-glycopeptide from *Sarcina morrhuae*

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(*Accepted for publication 16 March 1970*)

Free pigments have been isolated from *Sarcina morrhuae* by Nandy & Sen (1967), and their results have been confirmed. When no more pigment could be extracted, the bacterial remnants were still pigmented. The present work was undertaken to see how the remaining pigment was bound.

METHODS

Sarcina morrhuae was grown on 25 % (w/v) NaCl, 1 % (w/v) $MgSO_4.7H_2O$, 0·02 % (w/v) $CaCl_2.6H_2O$, 0·5 % (w/v) KCl, 1 % (w/v) yeast extract, 0·5 % (w/v) tryptone and 2·5 % agar no. 3 at 34° for 3 weeks. Pigment was removed by repeated ultrasonication in methanol and then repeated saponification with 10 % (w/v) methanolic KOH (at room temperature under an inert atmosphere and in the dark). When no further pigment was extracted, the residue was stirred at 40° in distilled water and a red-orange solution recovered.

The solution was desalted using Sephadex G75 followed by ultrafiltration in an Amicon high pressure cell. Homogeneity was tested by examining (*a*) sedimentation patterns in a Beckman analytical ultracentrifuge, (*b*) resolution on columns of (i) various grades of Sephadex, (ii) Biogel A15 (4 % agarose), and (*c*) resolution by disc-gel electrophoresis on 7·5 % cyanogum C40 gels at pH 4·7. An estimate of the molecular weight was obtained by (*a*) behaviour on dialysis (molecular weight cut-off 9000), (*b*) retention on columns of various Sephadexes and on 4 % agarose (standardized for both Stokes radius and molecular weight). An absorption spectrum was recorded from 210 to 600 nm. and the following were also determined: (*a*) dry weight and ash, (*b*) protein by the Folin–Lowry method using serum albumin as standard or by the quantitative ninhydrin method (Moore & Stein, 1954) with norleucine as standard, (*c*) carbohydrate (Whistler & Wolfrom, 1962), (*d*) amino acid analysis on a Technicon autoanalyser with norleucine as internal standard.

The hydrolysate prepared for carbohydrate estimation was chromatographed on Whatman no. 1 paper using either butanol + pyridine + water (6 + 4 + 3, by vol.) or ethyl acetate + pyridine + water (70 + 20 + 23, by vol.; Colombo *et al.* 1960) as solvents, with solutions of glucose, mannose, galactose and ribose as standards. Duplicate chromatograms were stained with 0·1 M-anisidine phthalate in ethanol (Pridham, 1956) or 2 % (w/v) ninhydrin in acetone.

RESULTS

The complex is a monodisperse system of molecules of low molecular weight giving a single symmetrical Schlieren pattern on the analytical ultracentrifuge. No resolution was apparent on Sephadex or on 4 % agarose. A single band was displayed on disc-gel electrophoresis with a speed of migration virtually the same as serum albumin in the system used. A molecular weight of 8000 to 9000 was suggested by (*a*) very slow diffusion through visking tubing, (*b*) no retention on Sephadex G25, but retention to an increasing degree on Sephadex G50, G75 and G100 and (*c*) behaviour on the 4 % agarose column, which indicated a Stokes radius of approximately 5 Å and a molecular weight of less than 10,000.

Absorption peaks were recorded at 237 and 276 nm. but their intensity was such that no fine spectrum was recorded in the visible range. The presence of carotenoid was deduced because (i) the bacterium produces red and orange free carotenoids and the complex was red-orange, and (ii) the complex gave a positive Carr–Price reaction (Morton, 1942). The ash content was 17·5 % (expressed as Cl^-, the main anion (Christian & Waltho, 1962)), the protein content was 59·7 %, the carbohydrate content was 7·7 % (as glucose) and the carotenoid content was 15·1 % (by difference). This gives molar ratios of protein (from average molecular weight of amino acids in Table 1): carbohydrate:carotenoid (as bacterioruberin) as 26·2:2·0:0·95. The amino acid composition of the complex is shown in Table 1. A small NH_3 peak was recorded and this suggests that little of the glutamic and/or aspartic acid is present as amide. Also, small unidentified peaks were located between Glu-Gly, Met-Ile, Phe-NH_3 and Lys-His.

Table 1. *Amino acid composition of the complex**

Aspartic acid	13·3	Isoleucine	5·6
Threonine	4·1	Leucine	8·9
Serine	2·7	Tyrosine	2·6
Glutamic acid	14·0	Phenylalanine	3·4
Glycine	13·0	Lysine	2·3
Alanine	11·1	Histidine	2·6
Valine	9·1	Ornithine+arginine	5·7
Methionine	1·6		

*Composition is expressed in moles per cent of amino acid.

When stained with anisidine–phthalate chromatograms of the hydrolysed complex showed one spot which co-chromatographed with glucose and another of low R_F value which was also ninhydrin-positive and probably a glycopeptide.

DISCUSSION

The extracted complex of carotenoid, glucose and peptide is a monodisperse system of molecules of approximately 9000 molecular weight. In the complex, glucose is bound to peptide and glycosidically to carotenoid since unhydrolysed material is anisidine phthalate negative. The amino acid analysis showed a wider variety of amino acids with less exact molar ratios compared to the carotenoid-glucopeptide of *Sarcina flava* (Thirkell & Hunter, 1969). This bound carotenoid represents one form in which pigment exists in the membrane where all such pigment is localized. Because

none of the chemical or enzymic methods applied (e.g. glycosidases or proteases) liberated material into organic solvents without degradation of the carotenoid, the latter was estimated by difference. An analogy with the carotenoid-glucopeptide from *S. flava* would suggest that bacterioruberin (the most polar free carotenoid) is involved in this complex. A high ash content for the desalted material was recorded. Since many enzymes in this bacterium are salt-dependent (Larsen, 1967), the membrane proteins may also have salt intimately associated with them, eg. as Na^+ and/or K^+ counterions associated with the aspartic and/or glutamic acid residues in the peptide.

D. T. is grateful to the Science Research Council for financial support, and M. I. S. H. is in receipt of an S.R.C. Research Studentship. We thank Dr Serafini-Fracassini for assistance with the amino acid analysis.

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CORRIGENDUM

In R. WHITTENBURY, K. C. PHILLIPS & J. F. WILKINSON (1970),
Journal of General Microbiology **61**, 205–218.

An error has been made in the description of the membrane systems on methane-utilizing bacteria. Type I membranes should have been referred to as type II membranes, and vice versa, to bring the nomenclature in line with that of the subsequent paper (S. L. DAVIES & R. WHITTENBURY, 1970, *Journal of General Microbiology* **61**, 227–232).

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