Volume 59, Part 3, December 1969

The Journal of General Microbiology

CAMBRIDGE UNIVERSITY PRESS
For the Society for General Microbiology

THE JOURNAL OF GENERAL MICROBIOLOGY

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THE JOURNAL OF GENERAL MICROBIOLOGY

Published monthly except in August, November, and December when semi-monthly.
Single parts 45*s*. net ($7.50 in U.S.A.), plus postage.
Three parts form a volume. The subscription price of a volume (which includes postage) is 100*s*. net ($17.00 in U.S.A.).
Second class postage paid at New York, N.Y.

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THE JOURNAL OF GENERAL MICROBIOLOGY

THE PREPARATION OF PAPERS

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

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

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(2) A paper should be written only when a piece of work is rounded off. Authors should not be seduced into writing a series of papers on the same subject as results come to hand. It is better to wait until a comprehensive paper can be written.

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

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

(5) Authors should remember that in preparing their typescript they are giving instructions to the printer (about layout, etc.) as well as attempting to convey their meaning to their readers.

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The following books may be found useful:

Bergey's Manual of Determinative Bacteriology, 7th ed. (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 ed. (1961). Kew: Commonwealth Mycological Institute.

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J. gen. Microbiol. (1969), 59, 289–301
Printed in Great Britain

The Genetics of Penicillinase Production in *Staphylococcus aureus* Strain PS 80

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(*Accepted for publication 3 September 1969*)

SUMMARY

PS80 is a naturally occurring, penicillinase-producing strain of *Staphylococcus aureus*, which differs from the majority of such strains in that the genetic determinants for penicillinase production are located on the chromosome. The strain is also resistant to various metal ions and the genes controlling these resistances are located on a plasmid—the π plasmid. When the strain is maintained on nutrient agar slopes at room temperature, a change occurs in its genetic constitution in that these unlinked genetic elements become closely linked and co-transducible with a high frequency. The results described in this paper are consistent with the hypothesis that the change is due to a duplication of the penicillinase genes with one copy of the genes being retained in its original chromosomal location and the other copy being incorporated in the π plasmid.

INTRODUCTION

In most penicillinase-producing strains of *Staphylococcus aureus* the genetic determinants for penicillinase production are on extrachromosomal particles or plasmids (Novick, 1963). In addition to the penicillinase genes these plasmids often carry genes controlling resistance to mercury (Richmond & John, 1964), arsenate, arsenite, cadmium, lead and zinc ions (Novick & Roth, 1968) and, in a few strains isolated in Japan, to erythromycin (Hashimoto, Kono & Mitsuhashi, 1964). Richmond (1968) has defined 11 different types of penicillinase plasmids based on the type of penicillinase produced (Richmond, 1965*a*), the degree of extracellularity of the enzyme, and the presence or absence of the genes controlling other resistance markers on the same plasmid. These plasmids have been divided into two compatibility groups (Com I and II) (Novick & Richmond, 1965; Richmond, 1965*b*). Plasmids belonging to the same compatibility group are incompatible with each other in that they are unable to co-exist in the same cell to form stable plasmid diploids. Stable plasmid diploids can only be constructed when the two plasmids belong to different compatibility groups (Richmond, 1965*b*). The reason for this lack of compatibility is not known but an attachment site hypothesis, similar to that proposed for replicons in *Escherichia coli* by Jacob, Brenner & Cuzin (1963), has been suggested. According to this, the plasmid has an attachment site, possibly on the cell membrane, to which the plasmid must become fixed in order to be stabilized and to ensure its equal distribution to progeny cells on division. Plasmids of Com I are assumed to have a Com I attachment site while plasmids of Com II have a second, equally specific attachment site. Some confirmation of this hypothesis has been provided by experiments of

Vol. 59, *No.* 2, *was issued* 23 *February* 1969

Novick (1967) and Richmond (1968) from which it appears that plasmids have a genetic locus, originally designated *mc* (for maintenance and compatibility) but now designated *mcr* (for maintenance, compatibility and replication) (Novick, 1969), which is responsible for recognizing the cellular attachment site for the particular plasmid and allowing its autonomous replication. In plasmids of Com I, the gene is known as *mcr-1* and in plasmids of Com II as *mcr-2*.

Although the genetic determinants for penicillinase production are on plasmids in most strains of staphylococci, a few strains have been described in which these genes probably have a chromosomal location (Asheshov, 1966*a*: Poston, 1966: Harmon, Baldwin, Tien & Critz, 1966: Miller & Harmon, 1967). One of these strains is PS80 (NCTC9789), the propagating strain for typing phage 80. Some of its characters were described previously (Asheshov, 1966*a*) and may be summarized as follows. The genes determining penicillinase production in this strain are unusually stable and attempts to isolate penicillinase-negative mutants have been unsuccessful. When these genes are introduced into a sensitive strain by transduction, the kinetics of transduction are those expected for chromosomal genes, i.e. the frequency of transduction is low but can be increased by giving the transducing phage small doses of ultraviolet light (Arber, 1960). Strain PS80 is resistant to arsenate, arsenite, cadmium and mercury ions and the genes controlling these resistances appear to be carried on a plasmid. Mutants sensitive to all of these metal ions arise spontaneously with a fairly high frequency but these mutants continue to produce penicillinase.

In the original transduction experiments in which PS80 was the donor of the penicillinase genes, co-transduction of resistance to the metal ions did not occur, which appeared to confirm the lack of linkage of these genes to the penicillinase genes. Further experiments have since been carried out, however, and it now appears that the genes controlling resistance to metal ions can become linked to the penicillinase genes, and under these conditions co-transduction of all these genetic elements occurs with a high frequency. The results to be described support the view that the plasmid in PS80 which carries the genes controlling metal ion resistances is able to incorporate penicillinase genes into its structure without causing a deletion of these genes from the chromosome. The strain thus appears to change from a penicillinase haploid strain to a penicillinase diploid strain.

METHODS

Strains of Staphylococcus aureus. Strain PS80. Wild-type PS80 is an inducible penicillinase producer and is resistant to arsenate, cadmium and mercury ions. Its genotype is defined as $i^{+}pen^{+}$ (π *asa-r*, *cad-r*, *mer-r*). In defining the genotype I have used the convention of Novick & Richmond (1965) with some of the modifications suggested by Novick (1969). The symbol pen^{+} refers to the structural gene for penicillinase and *i* the penicillinase inducibility locus, i^{+} representing wild-type inducible synthesis and i^{-} mutant constitutive synthesis; *asa-r*, *cad-r* and *mer-r* refer to the loci determining resistance to arsenate, cadmium and mercury respectively and their sensitive alleles are designated *asa-s*, *cad-s* and *mer-s*. Plasmid genes are enclosed in parenthesis and the Greek letter, designating the type of plasmid, precedes the markers carried by the plasmid.

Strain 17855. This strain was used as the recipient in all transduction experiments. It was kindly provided by Dr K. G. H. Dyke, who suggested its use. It is a

penicillinase-negative mutant of a strain which originally produced penicillinase and is resistant to arsenate and cadmium ions but sensitive to mercury ions. The genes controlling resistance to arsenate and cadmium are chromosomal (Dyke, personal communication).

Cultures were maintained on nutrient agar slopes at room temperature and were subcultured at 3-monthly intervals. At the time of subculture the strains were phage-typed and their antibiotic resistance pattern was determined.

Phages. Typing phage 80 (NCTC 9788) was used as the transducing phage in all experiments. It was propagated on the donor strains using the soft-agar layer method of Swanstrom & Adams (1951). Lysates were sterilized by filtration through sintered glass filters (a.p.d. 1·5 μ) and were stored at 4°.

The various preparations are designated by the phage number followed by the number of the strain on which propagation was carried out; for example, 80.80 indicates phage 80 propagated on strain PS 80. Batches of the various phages, propagated at different times, are further specified by a number in parenthesis.

Media. Nutrient broth and nutrient agar were prepared from Oxoid Nutrient Broth No. 2. Nutrient agar was solidified with 1·2 % (w/v) fibre agar. Peptone–water agar contained Oxoid peptone 20 g.; NaCl 5 g.; and Davis agar 10 g. (pH 7·4). Starch agar was prepared by adding soluble starch to a final concentration of 0·2 % (w/v) to either nutrient agar or peptone–water agar. It was used to detect penicillinase production.

Tests for resistance to penicillin and metal ions. Strains were tested for resistance either by the disc test or by plating on agar containing the different substances in suitable concentration. Discs contained the various substances in the following amounts: penicillin, 1·6 units of benzyl penicillin; arsenate ions, 0·32 mg. sodium arsenate; cadmium ions, 20 μg. cadmium sulphate; mercury ions, 0·13 μg. phenyl mercuric nitrate.

Agar plates containing the various inhibitory substances were used for testing individual colonies, for replica-plating, and for isolating transductants growing from single cells. A single concentration was adequate for all these purposes except when testing for resistance to mercury ions. However, the test for mercury resistance is influenced by inoculum size, and a concentration of mercuric chloride suitable for use with relatively large inocula—e.g. when streaking growth from a colony or for replica-plating—was too high to allow growth from single cells, as in transduction experiments. It was necessary to use two different concentrations of mercuric chloride. The final concentrations used were as follows: penicillin, 0·1 μg benzyl penicillin/ml.; cadmium ions, 10 μg. cadmium sulphate/ml.; mercury ions, 45 μg. $HgCl_2$/ml. (high concentration), 25 μg. $HgCl_2$/ml. (low concentration).

Either nutrient agar or peptone–water agar was used in testing for resistance to all of the substances except mercuric chloride. It was necessary to use peptone–water agar when testing for mercury resistance since the composition of the medium is critical (Moore, 1960; Green, 1962). Sodium citrate (10^{-2}M) was added to the agar when plates were used to isolate transductants, to inhibit lysis by free phage particles.

Tests for penicillinase production. Strains were recognized as penicillinase producers by their reaction on starch agar (Dyke, Jevons, & Parker, 1966). When penicillinase-negative mutants were being selected, methicillin (0·5 μg./ml.) was added as an inducer of penicillinase. In the absence of inducer the test allows one to distinguish between

inducible and constitutive penicillinase production. Quantitative estimations of penicillinase were carried out by the method of Richmond, Parker, Jevons & John (1964).

Loss of drug resistance during growth at 37° and 43·5°. The method used was essentially the same as that described previously (Asheshov, 1966*b*). The only modification was that, in some instances, penicillinase-negative mutants were sought by plating on starch-agar plates containing inducer rather than by replicating to penicillin agar. This allowed more clones to be screened in a single experiment.

Growth in the presence of acridine dyes. Log.-phase broth cultures, containing approximately 10^8 viable units/ml. were diluted 10^{-4} in nutrient broth (pH 7·6) containing concentrations of 5-amino acridine HCl ranging from 25 to 3·125 μg./ml. After incubation at 37° for 18 hr, the tube containing the highest concentration of dye in which visible growth occurred was suitably diluted and plated on nutrient agar. Colonies were tested for drug-resistance by replica-plating to drug-containing medium.

Transduction experiments. These were carried out as described previously (Asheshov, 1966*a*). In the majority of experiments the transducing phage was irradiated with u.v. light before being used to transduce. Five ml. of the phage suspension, diluted in nutrient broth, were placed in a sterile Petri dish at a distance of 23 cm. from the light source, a 15 W 'germicidal' lamp. After each dose, a sample was removed, titrated for survivors, and used to transduce. In some experiments, transduction of two different markers was measured by dividing the phage-treated suspension into two equal portions and screening each for a different marker. The frequency of transduction was calculated as the ratio of the number of transductants obtained to the number of plaque-forming units used in the experiment.

The use of ethyl methane sulphonate to produce mutations. The method used was the same as that described by Novick (1963). After treatment, the cultures were screened on starch agar, with or without inducer, depending on whether mutations in the structural gene or the inducibility locus were being sought.

RESULTS

Stability of the genes controlling penicillinase production and resistance to metal ions in PS80. Old laboratory cultures of PS80 were found to contain a small number of mutants that were sensitive to arsenate, cadmium and mercury ions but were still penicillinase producers. The frequency of loss of these markers could be increased, either by growth at 43·5° or in the presence of 5-amino acridine HCl (Table 1, 2). All of these mutants produced penicillinase. A more determined attempt was made to isolate penicillinase-negative mutants from PS80 after growth for 6 hr at 43·5°. However, out of about 84,000 colonies examined in a single experiment, no penicillinase-negative mutants were detected.

Transduction experiments. A transduction experiment in which PS80 was the donor of the genes controlling penicillin and mercury resistance to strain 17855 was done in 1965 using phage 80.80(1) as transducing phage. Frequency of transduction is plotted as a function of the u.v. dose given to the transducing phage (Fig. 1).

Transduction of the genes for penicillin resistance showed the curve expected for transduction of chromosomal genes in that there was a marked stimulation in frequency with small doses of u.v. light (Arber, 1960). The curve for transduction of mercury resistance was typical for transduction of extrachromosomal genes. Trans-

ductants were examined for co-transduction of the opposite unselected marker but no co-transduction of penicillin and mercury resistance was found.

In early 1966 a second preparation of phage was made—phage 80.80(2)—and the experiment was repeated. The kinetics of transduction of the two markers were similar to those obtained with the first phage preparation. However, when transductants, selected for penicillin resistance, were examined it was found that a minority (about 15 %) had been co-transduced to mercury resistance.

Table 1. *Effect of growth at 37° and 43·5° on the loss of resistance to cadmium ions in PS80*

Growth temperature					
37°			43·5°		
No. colonies screened	Cadmium-sensitive No.	Cadmium-sensitive %	No. colonies screened	Cadmium-sensitive No.	Cadmium-sensitive %
3050	1	0·03	1750	2	0·11
4275	1	0·02	5125	9	0·18

The results of two independent experiments are given.

Table 2. *Effect of growth in 5-amino acridine HCl on the loss of resistance to cadmium ions in PS80*

Acridine concentration					
0			6·25 μg./ml.		
No. colonies screened	Cadmium-sensitive No.	Cadmium-sensitive %	No. colonies screened	Cadmium-sensitive No.	Cadmium-sensitive %
2500	0	—	1390	2	0·14
3560	1	0·03	3832	5	0·13

The results of two independent experiments are given.

Table 3. *A comparison of the frequency of transduction of penicillin resistance and co-transduction of penicillin and mercury resistance with five different batches of phage 80/80 (recipient: strain 17855)*

Batch no.	Date of preparation	Frequency of transduction of: Penicillin-resistance	Frequency of transduction of: Penicillin and mercury resistance
1	1965	$1·5 \times 10^{-8}$	$<7·5 \times 10^{-9}$
2	Feb. 1966	$4·5 \times 10^{-9}$	$4·5 \times 10^{-9}$
3	Apr. 1966	$7·5 \times 10^{-9}$	$9·2 \times 10^{-8}$
4	Aug. 1966	$9·4 \times 10^{-9}$	$9·4 \times 10^{-8}$
5	Feb. 1967	$1·0 \times 10^{-8}$	$3·5 \times 10^{-6}$

Three more preparations of phage 80.80 were made subsequently and their ability to transduce penicillin resistance and to co-transduse penicillin and mercury resistance was determined (Table 3). Only frequencies with unirradiated phage are given. The frequency of transduction of penicillin resistance alone remained more or less the same

throughout these experiments. Frequency of co-transduction, however, increased strikingly during the 20 months elapsing between the preparation of the first and the fifth batch of phage.

Figure 2 shows the frequencies obtained for transduction of penicillin resistance alone, mercury resistance alone and the co-transduction frequency for both resistances obtained with the fifth batch of phage. The curve obtained for transduction of penicillin resistance showed the usual kinetics for chromosomal genes and resembled that obtained with the first batch of phage (cf. Fig. 1). The curve obtained for transduction of mercury resistance was also similar to that found with the first batch of phage. The curve for co-transduction of both resistance markers showed the exponential decrease expected for extrachromosomal genes with a slightly lower frequency than that found for transduction of mercury resistance.

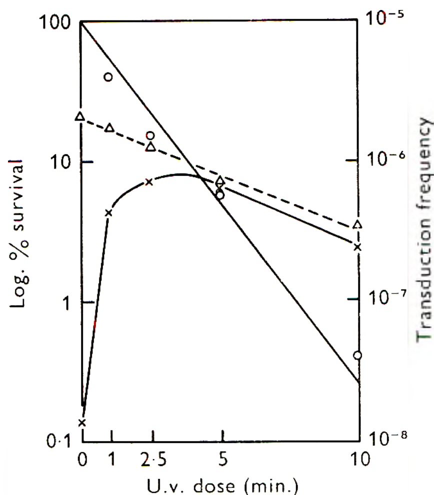

Fig. 1

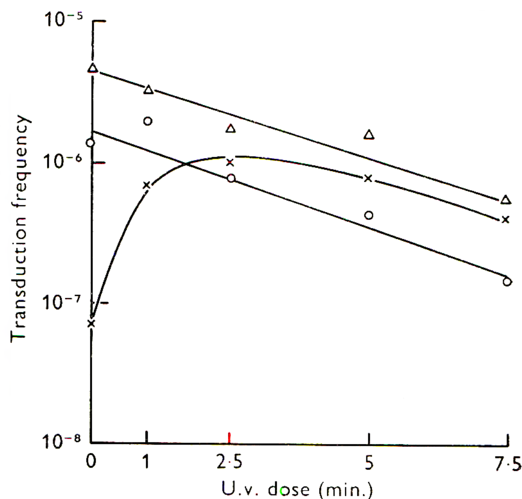

Fig. 2

Fig. 1. Frequency of transduction of penicillin and mercury resistance plotted as a function of the u.v. dose given to the transducing phage, phage 80/80 (1). Recipient: strain 17855. Donor: PS80. Phage inactivation curve: ○——○. Transduction of penicillin resistance: ×——×. Transduction of mercury resistance: △ - - - △.

Fig. 2. Frequency of transduction of penicillin resistance, mercury resistance and co-transduction of both resistances obtained with phage 80/80(5). Recipient: strain 17855. Frequencies are plotted as a function of the u.v. dose given to the transducing phage. Transduction of penicillin resistance: ×——×. Transduction of mercury resistance: △——△. Co-transduction of both resistances: ○——○.

The results suggested that the phage preparation used in this experiment contained three sorts of transducing particles: those carrying only the chromosomal penicillinase genes, those carrying the plasmid genes controlling resistance to the metal ions, and a third class carrying the genetic determinants for both penicillin and mercury resistance linked together. The simplest explanation to account for the presence of three different sorts of transducing particles would seem to be that the donor was heterogeneous and consisted of some cells in which these genes were linked and others in which they were unlinked.

This was confirmed by propagating phage 80 on ten well-isolated colonies of

PS80 and determining the ability of these ten preparations to transduce penicillin resistance and to co-transduce penicillin and mercury resistance (Table 4). Four of the phages co-transduced both resistances with a high frequency, four showed little or no co-transduction and the other two gave intermediate co-transduction frequencies.

This phenomenon was not confined to the particular culture of PS80 used in these experiments. A mutant of PS80 which produced penicillinase constitutively was isolated in 1965 after treatment of the wild-type strain with ethyl methane sulphonate (EMS). This mutant was used as the donor of the penicillinase genes soon after its isolation and, at that time, it failed to show any co-transduction of penicillin and mercury resistance. A second preparation of phage 80 was made in 1967 and this preparation co-transduced both resistances with a high frequency.

Table 4. *Frequency of transduction of penicillin resistance and co-transduction of penicillin and mercury resistance to strain 17855 with phage 80 propagated on 10 different clones of PS80*

Phage preparation	Frequency of transduction of: Penicillin resistance	Penicillin and mercury resistance
1	$1 \cdot 0 \times 10^{-7}$	$1 \cdot 1 \times 10^{-5}$
2	$9 \cdot 0 \times 10^{-8}$	$2 \cdot 9 \times 10^{-6}$
3	$2 \cdot 0 \times 10^{-7}$	$2 \cdot 0 \times 10^{-5}$
4	$2 \cdot 0 \times 10^{-8}$	$< 1 \cdot 0 \times 10^{-9}$
5	$8 \cdot 0 \times 10^{-8}$	$5 \cdot 0 \times 10^{-8}$
6	$3 \cdot 0 \times 10^{-8}$	$< 3 \cdot 0 \times 10^{-9}$
7	$4 \cdot 8 \times 10^{-8}$	$3 \cdot 2 \times 10^{-8}$
8	$9 \cdot 9 \times 10^{-8}$	$2 \cdot 5 \times 10^{-9}$
9	$7 \cdot 0 \times 10^{-8}$	$< 5 \cdot 0 \times 10^{-9}$
10	$9 \cdot 6 \times 10^{-8}$	$8 \cdot 6 \times 10^{-7}$

Linkage of the genetic determinants for penicillin and metal ion resistances in PS80. There appear to be two possible explanations of the change that has occuured in the genetic constitution of PS80 during the course of these experiments. The simplest one is that one or other of these originally unlinked genetic elements has changed its cellular location—either the penicillinase genes have been excised from the chromosome and incorporated into the structure of the π plasmid, or the π plasmid has integrated into the chromosome at a site sufficiently close to the penicillinase genetic region to allow co-transduction of all these genes with a high frequency. The second possibility is that duplication of the penicillinase genes has occurred with one copy being retained in its original chromosomal location and the second copy being integrated into the π plasmid.

If the first explanation is correct it should be reflected in a change in the stability of the genes involved. The stability of the penicillinase genes is presumably due to their chromosomal location. If they have been excised from the chromosome and become part of the π plasmid they should show the same instability as the π plasmid and it should be possible to isolate penicillinase-negative mutants with a reasonably high frequency. If, on the other hand, linkage has occurred through integration of the π plasmid into the chromosome this should have the effect of stabilizing these genes.

Two sub-lines of PS80 were chosen for study. These are isolates number 3 and 4 of Table 4. Isolate number 3 showed a high frequency of co-transduction of penicillin

and mercury resistance and should, therefore, be a strain in which most of the cells carry the genes controlling these resistances closely linked. It will be referred to as PS80HCD (high co-transducing donor). Isolate number 4 was a strain which showed no co-transduction of these resistances and, presumably in this strain these genes are unlinked. It will be referred to as PS80LCD (low co-transducing donor).

Table 5. *Loss of resistance to cadmium ions in PS80HCD and PS80LCD after growth at 43·5° and in the presence of 6·25 μg./ml. of 5-amino acridine HCl*

	Control culture			Treated culture		
	No. colonies	Cadmium-sensitive		No. colonies	Cadmium-sensitive	
Treatment	screened	No.	%	screened	No.	%
			PS80HCD			
43·5°	3015	3	0·10	945	7	0·74
43·5°	3210	18	0·56	2350	13	0·55
Acridine	4500	2	0·04	3110	11	0·35
Acridine	4400	14	0·31	4440	78	1·53
			PS80LCD			
43·5°	3530	0	—	750	2	0·26
43·5°	3050	1	0·03	1750	2	0·11
Acridine	2170	0	—	2000	0	—
Acridine	3560	1	0·03	3832	5	0·13

PS80HCD was first examined for stability of the penicillinase genes after growth at 43·5° for 6 hr. Approximately 10^6 clones were screened in a single experiment but no penicillinase-negative mutants were detected. It would seem, therefore, that there has been no change in the stability of the penicillinase genes in PS80HCD, which suggests that they are still chromosomal.

The stability of the genes controlling resistance to the metal ions was then examined in PS80HCD and PS80LCD after growth at 43·5° for 6 hr and after growth in 5-amino acridine HCl for 18 hr. The genes controlling metal ion resistances in PS80HCD showed a slightly increased instability compared with PS80LCD (Table 5). There was thus no evidence that either the penicillinase genes or the genes controlling resistance to the metal ions had changed their location in the cell.

The second possibility—that PS80 had changed from a penicillinase haploid to a diploid strain—was examined. It is possible to construct stable penicillinase diploids in PS80 in which one copy of the genes is on the chromosome and the other on a plasmid. The behaviour of two such diploids was described by Asheshov & Dyke (1968). These diploids were constructed by transducing the linked genes for penicillin and metal ion resistances, using a high co-transducing isolate of PS80 as the donor, into PS80 recipients which had lost the π plasmid after growth at 43·5°. Transductants were selected for cadmium resistance. The recipient and donor strains chosen for these experiments carried different alleles of the inducibility locus, i.e. when the recipient was i^+pen^+, the donor was i^-pen^+ and vice versa. It was found that when the i^+ gene was on the plasmid and the i^- allele on the chromosome the diploids were phenotypically inducible, judging both by their reaction on starch agar and by the level of enzyme produced in the absence of inducer. The plasmid i^+ gene was able to exert control *trans* on the chromosomal genes. However, when the location of these alleles

was reversed, the chromosomal i^+ gene exerted only partial control on the plasmid i^-pen^+ genes. These latter diploids appeared to be constitutive enzyme producers, judging by their reaction on starch agar. However, the level or penicillinase produced in the absence of inducer, though significantly higher than the basal level characteristic of normal inducible strains, was lower than that expected for constitutive strains. A gene dose hypothesis has been postulated to explain this positional effect (Asheshov & Dyke, 1968).

In order to show that PS80HCD carried two copies of the penicillinase genes it was necessary to introduce a mutation into one of the copies. It was argued that it should be possible to produce a mutation in the *i* gene of the plasmid which would cause the strain to appear constitutive on starch agar. Such a 'constitutive' should resemble the partial constitutive diploids constructed artificially in PS80 and should revert to an inducible phenotype on losing the genes controlling resistance to the metal ions. If PS80LCD is a penicillinase haploid strain, mutation to a constitutive phenotype would be expected to occur as the result of a mutation in the single chromosomal *i* gene. Such a mutant should be fully constitutive and should retain this character on losing resistance to the metal ions.

Table 6. *Transduction of penicillin resistance to strain 17855 from PS80HCD c-1 and PS80LCD c-1*

The transducing phages were given varying doses of u.v. irradiation before being used to transduce.

U.v. dose (min.)	No. of transductants of the phenotype:			
	i^+pen^+mer-s	i^-pen^+mer-r	i^+pen^+mer-r	i^-pen^+mer-s
	Donor: PS80LCD c-1			
0	0	0	0	20
1	0	0	0	393
2·5	0	0	0	818
5·0	0	0	0	707
	Donor: PS80HCD c-1			
0	6	1173	8	2
1	58	1193	12	2
2·5	125	854	11	3
5·0	247	666	1	3

These two strains were treated with EMS and the survivors plated to starch agar. Three apparently constitutive mutants were isolated from each strain in two separate experiments. These were designated HCDc-1, c-2, c-3 and LCDc-1, c-2, and c-3. These six mutants were grown at 43·5° and cadmium-sensitive segregants were isolated from each of them. The three HCD 'constitutives' all reverted to an inducible phenotype on losing resistance to the metal ions: the three LCD mutants retained their constitutive phenotype on losing resistance to the metal ions. These results strongly suggested that the HCD mutants were i^+/i^- diploids with the i^- allele on the π plasmid.

Transduction experiments were done in which each of the six mutants was used as the donor of penicillin resistance to strain 17855. Transductants were replica-plated to mercury agar containing starch, to allow determination of the penicillinase phenotype of any penicillin-resistant, mercury-resistant transductants. Mercury-sensitive trans-

ductants were purified and their penicillinase phenotype determined on starch agar. The results of an experiment in which PS80LCD C-I and HCD C-I were the donors are shown in Table 6.

All of the penicillin-resistant transductants obtained when the donor was PS80LCD C-I were constitutive and sensitive to mercury ions. The frequency of transduction was stimulated by u.v.-irradiation of the transducing phage. It would seem, therefore, that this donor carries a single copy of the penicillinase genes on the chromosome, unlinked to the metal ion resistance genes. When PS80HCD C-I was the donor, however, there were four phenotypically different classes of transductants: *i⁺pen⁺mer-s*, *i⁻pen⁺mer-r*, *i⁺pen⁺mer-r* and *i⁻pen⁺mer-s*. The majority belonged to the first two classes. The *i⁺pen⁺mer-s* linkage group showed the transduction kinetics expected for chromosomal genes while the *i⁻pen⁺mer-r* linkage group showed the kinetics expected for extrachromosomal genes. The results are consistent with the view that PS80HCD C-I is a penicillinase diploid with an *i⁺pen⁺* linkage group on the chromosome and an *i⁻pen⁺* linkage group on the π plasmid. The presence of a few recombinant types—*i⁺pen⁺mer-r* and *i⁻pen⁺mer-s*—indicates that there is a certain amount of recombination between the chromosomal and extrachromosomal penicillinase genes.

Table 7. *Quantitative determination of penicillinase produced by strains PS80HCD, PS80LCD, PS80HCD C-I, PS80LCD C-I, and transductants obtained from the latter two strains*

Strain	Penicillinase phenotype on starch agar	Presumed penicillinase genotype*	Units of penicillinase (mg. dry wt. bacteria) Uninduced	Induced	Induction ratio†
PS80HCD	Inducible	*i⁺pen⁺ (i⁺pen⁺)*	4·8	117	24·3
PS80LCD	Inducible	*i⁺pen⁺*	6·7	118	17·6
PS80HCD C-I	Constitutive	*i⁺pen⁺ (i⁻pen⁺)*	24·0	243	10·0
PS80LCD C-I	Constitutive	*i⁻pen⁺*	128·0	149	1·2
Transductants Donor, HCD C-I					
(*a*) *i⁺pen⁺mer-s*	Inducible	*i⁺pen⁺*	8·1	119	14·7
(*b*) *i⁻pen⁺mer-r*	Constitutive	*(i⁻pen⁺)*	110·0	268	2·4
(*c*) *i⁺pen⁺mer-r*	Inducible	*(i⁺pen⁺)*	3·5	181	51·6
(*d*) *i⁻pen⁺mer-s*	Constitutive	*i⁻pen⁺*	200·0	331	1·6
Donor, LCD C-I					
(*a*) *i⁻pen⁺mer-s*	Constitutive	*i⁻pen⁺*	116·7	142	1·2
(*b*) *i⁻pen⁺mer-s*	Constitutive	*i⁻pen⁺*	107·0	156	1·4

* Plasmid genes are in parentheses.

$$\dagger \text{ Induction ratio} = \frac{\text{units/mg. dry wt bacteria induced}}{\text{units/mg. dry wt bacteria uninduced}}.$$

All figures are the average of three separate determinations.

Quantitative estimations of penicillinase produced by PS80HCD C-I and the four types of transductants obtained when this strain was used as donor were carried out. Similar determinations were made on PS80LCD C-I and two of the transductants obtained with this donor. Enzyme levels produced by PS80HCD and LCD, the parent strains from which HCD C-I and LCD C-I were derived, are included for comparison (Table 7).

Strain LCD C-I is a magno-constitutive strain with an induction ratio of 1·2, and

the two transductants obtained from this strain show similar levels for uninduced and induced enzyme. Strain HCD C-1, however, is a partial constitutive, with an induction ratio of 10:1. The uninduced level of penicillinase produced by this strain, although significantly higher than that produced by the inducible parent, PS80HCD, is much lower than that expected for a fully constitutive strain. The presence of a chromosomal i^+ gene is apparently exerting only partial control on enzyme synthesis. Segregation of the two penicillinase linkage groups carried by HCD C-1 is apparent in the transductants obtained from this strain. The two phenotypically i^+pen^+ transductants show enzyme levels and induction ratios characteristic of wild-type inducible strains, while the two i^-pen^+ transductants have enzyme levels and induction ratios characteristic of magno-constitutive strains. It is interesting that PS80HCD, a penicillinase diploid strain, and PS80LCD, a penicillinase haploid strain, produce the same amount of enzyme on induction, as though only one structural gene in PS80HCD is being expressed. This is similar to results obtained by Richmond (1965*b*) for i^+/i^- plasmid diploids.

DISCUSSION

The conclusion that PS80 carries a copy of the penicillinase genes on the chromosome is based on several lines of evidence. First, the genes are unusually stable and attempts to isolate penicillinase-negative mutants have failed. Secondly, the kinetics of transduction of these genes are those expected for chromosomal genes. Finally, the fact that PS80 is able to form stable penicillinase diploids, not only with the α and γ plasmid (unpublished results) but also with the homologous genes when they are incorporated into the π plasmid, is consistent with this conclusion.

The same sort of evidence supports the conclusion that the genetic determinants for resistance to the various metal ions are carried on a plasmid in PS80. These genes are unstable and are lost on storage, during growth at 43·5°, and in the presence of 5-amino acridine HCl. Moreover, the transduction kinetics for these genes are those expected for extrachromosomal genes. The plasmid carrying these genes—the π plasmid—has at least one characteristic in common with certain wild-type penicillinase plasmids in that it is unable to coexist with the γ plasmid (unpublished results). It has, accordingly, been assigned to compatibility group I and is assumed to carry an *mcr-1* locus in addition to its resistance markers.

Although the penicillinase genes are chromosomal in PS80 and the genes controlling resistance to metal ions are extra-chromosomal, these separate genetic elements can become closely linked when the strain is maintained on nutrient agar at room temperature. The experimental evidence indicates that this occurs through a duplication of the penicillinase genes, with one copy being retained on the chromosome and the other being integrated into the π plasmid. A similar phenomenon was described by Ames, Hartman & Jacob (1963) in a Salmonella strain in which the genes controlling histidine biosynthesis were duplicated, with the second copy being incorporated into a cryptic episome. Sweeney & Cohen (1968) have recently described a wild-type strain of *Staphylococcus aureus* which resembles PS80HCD both in the number and location of the penicillinase genes.

The ability of the π plasmid to incorporate chromosomal genes suggests that, at some time, the plasmid becomes intimately associated with the chromosome. It may be that the π plasmid has some regions genetically homologous with the chromosomal

penicillinase genetic region. However, there is no evidence for this at the present time. The π plasmid does not carry a functional *pen* gene. Neither does it appear to carry an intact i^+ gene since its introduction into an i^-pen^+ mutant of PS80, in which the penicillinase genes were chromosomal, failed to change the uninduced enzyme level. If the π plasmid does associate with the chromosome, the association appears to be a transient one. Any permanent association would be expected to increase the stability of the genes carried by the plasmid. However, in PS80HCD these genes were, if anything, more unstable than in PS80LCD.

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J. gen. Microbiol. (1969), **59**, 303–316
Printed in Great Britain

Heritable Mass Conversion of a Mutant Penicillinase-negative Culture of *Bacillus cereus* to a Positive Fully De-repressed State

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(*Accepted for publication 4 September 1969*)

SUMMARY

A small proportion of spontaneous penicillinase-negative mutants of the penicillinase-magno-constitutive *Bacillus cereus* strain 569/H prove to be highly unstable and revert to the relatively stable magno-constitutive parent phenotype at a high rate spontaneously. If incubated at 45° or at the normal growth temperature of 35° with chloramphenicol (20 μg./ml.) the organisms of a broth culture of this meta-stable negative strain can be induced to undergo 100 % conversion to the fully de-repressed magno-constitutive state within 90 to 120 min. The conversion event appears to occur at random amongst the bacilli, whether single or joined in pairs; there is little or no phenotypic lag between committal to reproduction in the derepressed state and full expression of gene potential in the form of maximal rate of penicillinase production and a 1000-fold increase in penicillin resistance. Induced conversion is inhibited by nalidixic acid (10 μg./ml.).

It is concluded that the conversion event, for which some possible mechanisms are discussed, is controlled by a thermo-labile protein inhibitor.

INTRODUCTION

The penicillinase-magno-constitutive *Bacillus cereus* mutant (strain 569/H) of the wild-type inducible strain 569 is known to be, under certain circumstances, slightly unstable and will spontaneously give rise, on prolonged subculture, to a range of variants, most of which show little or no penicillinase activity. The overwhelming majority of the penicillinase-loss mutants are genetically stable. A small proportion, however, revert to their parent (magno-constitutive) phenotype spontaneously at a very high rate.

On further analysis of one (strain 569/H/14) of these meta-stable 'negatives', it was found that conversion of the positive state could be achieved, without any significant loss in viability, in up to 100 % of a culture within an hour or two by incubation either at a raised temperature of 45° or at the usual growth temperature of 35° in the presence of chloramphenicol.

This conversion shows similarities to phase variation in Salmonella (see Hayes, 1964) and seems possibly to be analogous to 'paramutational' events in maize (see reviews by McClintock, 1961, 1965; and by Brink, Styles & Axtell, 1968) and at the *Pal* locus in Antirrhinum (Harrison & Fincham, 1964) as well as to certain other unstable mutational phenomena in Salmonella (Smith-Keary & Dawson, 1964;

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Dawson & Smith-Keary, 1963) and in *Escherichia coli* (Morse, 1967) and other types of variations reported in higher organisms (see Brink, 1964).

We here report the results of some studies indicating that this conversion in *Bacillus cereus* is under the control of a protein repressor.

METHODS

Bacterial strains. The magno-constitutive mutant 569/H was obtained spontaneously from the wild-type inducible *Bacillus cereus* NRRL 569 (Kogut, Pollock & Tridgell, 1956). Four other constitutive mutant strains (569/K, 569/M, 569/L and 569/A) were also obtained spontaneously from strain 569 by a similar technique.

The unstable strain 569/H/14 was isolated as a penicillinase-negative mutant colony on the indicator plates described below, from a spore suspension of strain 569/H treated with ethylmethane sulphonate (EMS) (Dubnau & Pollock, 1965).

The classifying terminology used for describing these enzyme mutants is that suggested by Collins, Mandelstam, Pollock, Richmond & Sneath (1965); but bacilli or colonies of strain 569/H/14 in the negative or positive state (i.e. before or after conversion) are referred to respectively simply as 'micros' and 'magnos'.

Media. The liquid medium used was a 1 % peptone broth ('S' broth, Pollock, 1953*a*) and solid indicator medium (AA) was made by incorporating 1·5 % agar in the above and adding Andrade indicator (Kogut *et al.* 1956). During conversion experiments in liquid media, gelatine was added to a final concn. of 1 % (w/v) when turbidity reached the equivalent of between 0·02 and 0·05 mg. dry bacterial wt/ml.

Culture conditions. Unless otherwise stated, inocula were derived from standard (3×10^8 viable cells/ml.) spore suspensions (Pollock, 1953*a*) first plated out on AA to produce single colonies (either overnight at 30°, or 24 hr at 25°) which were subcultured at 35° in 'S' broth or inoculated on the surface of, or as deep layers in, AA plates.

For conversion experiments a spore inoculum from strain 569/H/14 was spread on the surface of AA in a Petri dish so as to produce single colonies. After incubation overnight at 30°, or for 24 hr at 25°, portions from each of ten single, marked colonies were subcultured into 2·0 ml. 'S' broth and shaken at 35° for approx. 2 hr. The original plate was developed (see below) with penicillin to identify, and exclude, any spontaneous magnos (convertants) which might be present. One of the cultures derived from a negative colony was chosen and maintained in the logarithmic phase of growth, if necessary by serial dilutions in broth, at a concn. corresponding to about 0·05 mg. dry wt/ml., at which level (unless otherwise stated) the experiment was started.

Penicillin sensitivity of individual organisms is expressed as the lowest concentration of benzylpenicillin in AA that caused a reduction of at least 50 % in the viable count from an inoculum into layered agar (Pollock, 1957).

β-Lactamase activity. Penicillinase (E.C. 3.5.2.6) was assayed quantitatively by the Perret (1954) iodine method with benzylpenicillin as substrate and the activity expressed as units (defined by Pollock & Torriani, 1953). Samples (5 ml.) were kept at 0° with chloramphenicol (50 μg./ml.) until assayed. Cephalosporinase activities were assayed by the same method, using cephalosporin C as substrate, after the addition of 1·67 mM zinc sulphate (final concentration) to allow full activation of the enzyme (Kuwabara & Abraham, 1967).

Analysis of populations. The proportions of micros and magnos in a suspension were determined by inoculating an appropriate broth dilution either on the surface of AA plates or into 20 ml. of molten AA at 45° poured into Petri dishes and subsequently covered with 3·0 ml. of AA. The plates were then incubated overnight at 25° or 30°.

Development. The colonies were scored by flooding the surface of the plates with approx. 5 ml. of a 10 % (w/v) aqueous benzylpenicillin solution and counting the proportion of colonies becoming red (magnos) due to recolourization of indicator by penicilloic acid. The remaining white colonies (micros) could be clearly differentiated: surface colonies within a minute and deep colonies within about 30 min., at room temperature.

Special materials. Chloramphenicol (Parke-Davis Ltd.), mitomycin and caffeine, anhydrous (Sigma London Chemical Co. Ltd.), Streptomycin sulphate B.P. (Glaxo Ltd.) and nalidixic acid (Winthrop Laboratories) were purchased as commercial preparations. Cephalosporin C (as sodium salt) was a gift from Glaxo Research Ltd.; actinomycin D was a gift from Merck, Sharp and Dohme Ltd. and 5-fluorodeoxyuridine (FUDR) a gift from Roche Products Ltd. We acknowledge these gifts with gratitude.

Viable counts. Viable organisms were counted by deep inoculation of appropriate broth dilutions into AA. Mean counts from two dilutions (differing by 2- or 2½-fold) were taken when absolute counts were required. In some experiments only the proportion of magno or micro colonies developing after surface inoculation was scored, counting a total of 100 colonies within one area of the plate.

In experiments with the addition of 20 μg. chloramphenicol per ml. to the incubation medium, samples for viable counts were taken without any attempt to remove the drug because it appeared to be purely bacteriostatic (rather than bactericidal). It was found that the dilutions necessary were sufficient to decrease the concentration of the drug below that causing reduction in viability, although the colonies were slightly smaller in size.

In some experiments the number of bacilli per 'unit' (chain of bacilli) was recorded microscopically as the number of individual bacilli (recognized by the formation of complete cell-wall septa) in each of 100 separate units.

Optical density. The absorption of bacterial suspensions was measured at 675 mμ and expressed as mg. dry bacterial wt/ml. by reference to a standard dry wt/turbidity curve for *Bacillus cereus* 569.

RESULTS

Properties of the unstable micros

Origin. Although the original unstable micro mutant strain, 569/H/14 was isolated after treatment of its parent strain with EMS (see methods) similar mutants have been obtained spontaneously at apparently very high rates from strain 569/H after repeated subculture in liquid media.

Indeed, penicillinase-loss mutants of various kinds have been repeatedly isolated from cultures of strain 569/H during prolonged growth (e.g. six or more consecutive subcultures entailing inoculation of one loopful into 7·5 ml. of broth, each incubated for 24 hr or more at 35° with shaking). Table 1 summarizes the numbers in the various categories isolated in a series of three separate experiments. Most of those

isolated (including the unstable micros) showed no measurable penicillinase activity (i.e. less than 0·5 units/mg. dry bacterial wt, which is the lower limit of the Perret assay method) and all (also including the unstable micros), except four out of the 59 tested, retained the strict constitutivity of the parent strain.

Table 1. *Numbers and types of spontaneous penicillinase-'loss' mutants from the magno constitutive Bacillus cereus mutant strain 569/H*

Class of 'loss' mutant	Differential rate of enzyme formation (units/mg. dry bact.wt)	No. of isolates
Magno-inducible (wild-type revertant)		4
Meso-constitutive	> 3·0 units/mg.	8
Micro-constitutive	0·5–3·0 units/mg.	8
Negative (stable)	< 0·5 units/mg.	37
Negative (unstable) (569/H/14 micro types)	< 0·5 units/mg.	2
	Total	59

Table 2. *Phenotypic properties of Bacillus cereus strains 569/H, 569/H/14 (micro and magno)*

		569/H/14	
	569/H	Micro	Magno
Lecithinase	+++	+++	+++
Amylase	+	+	+
Proteinase	++	++	++
Nitrate reduction (to nitrite)	++	++	++
Alkaline phosphatase (repressed by inorganic phosphate)	+	+	+
Urease	−	−	−
α-glucosidase (inducible by maltose)	+	+	+
Voges–Proskauer test	+	+	+
Sugar fermentations			
Salicin	+	+	+
Glucose	+	+	+
Arabinose	−	−	−
Lactose	−	−	−
Sucrose	+	+	+
Maltose	−	−	−
Xylose	−	−	−
Penicillin sensitivity (units/ml.)	10	0·0075	7·5
Differential rate of penicillinase formation (units/mg. dry bact. wt)	2500	< 0·5	1800

All the above tests, except where indicated otherwise, were carried out according to methods described by Cruickshank (1965). Alkaline phosphatase and α-glucosidase assayed under conditions described by Torriani (1960) and Pollock (1961) respectively: proteinase as described by Smith, Gordon & Clark (1952) using 'Marvel' as sourc eprotein and amylase by observing decolorized zones around colonies growing on nutrient agar (AA) containing 0·5 % starch, subsequently 'developed' with 1 % iodine solution.

Two further, separately-isolated magno-constitutive mutant strains, 569/K and 569/M, showed the same tendency to throw off loss mutants on subculture, whereas the semi-constitutive mutant (569/A, see Pollock, 1957) and a meso-constitutive (569/L) appeared completely stable.

Cultures of strain 569/H/14 produced no detectable penicillinase activity before magno-type convertants began to appear. Apart from the absence of penicillinase there were no demonstrable biochemical morphological or specific growth character differences between it and the parent strain 569/H; nor was the magno (convertant) from 569/H/14 distinguishable phenotypically from the original parent 569/H (see Table 2). However, the similarity did not extend to the genotypic character determining the types of penicillinase-loss variants appearing on continuous subculture. At 35°, subcultures of 569/H and 569/H/14-magno showed no differences, most of the loss mutants being stable. At 25°, however, cultures of 569/H/14-magno yielded a much higher proportion of unstable micro variants (in one experiment, 100 %) than was obtained from 569/H, which behaved as at 35°. It seems likely therefore that the failure to demonstrate the greater tendency of 569/H/14 convertants to produce unstable micros at 35° was due to much higher spontaneous rate of conversion from unstable micros to relatively stable magnos at the higher temperature, leading to selection in favour of stable micros. This emphasizes the truly reversible, albeit polarized, nature of the micro to magno conversion.

Penicillin sensitivity. The penicillin resistance of individual bacilli from a 569/H/14 micro culture was, like that of stable micro-constitutives, very low (approx. 0·01 units/ml.). By contrast, bacilli converted to the magno state had the same high resistance as the parent strain 569/H (over 10·0 units/ml., i.e. more than 1000 times greater).

During conversion, resistance rapidly increased towards the 569/H level, along with an ultimate comparable increase of at least 3000-fold: 0·5 to 1800 units/mg. dry wt, in the differential rate of penicillinase formation.

Co-ordinate production of the two β-lactamases. Cephalosporinase ('β-lactamase II') activity has recently (Kuwabara & Abraham, 1967) been shown in this strain to be due to a species of protein quite distinct from penicillinase (β-lactamase I). Nevertheless both activities are induced co-ordinately in the wild type (Crompton, Jago, Crawford, Newton & Abraham, 1962; M. R. Pollock, unpublished observations). In addition we have found a co-ordinate increase in both activities following conversion of 569/H/14 from the micro to the magno state: the cephalosporinase activity of the 569/H parent strain, and of 569/H/14 during and after the conversion, was always from 5 % to 7 % of the benzylpenicillinase activity.

Effect of sporulation. Sporulation appeared not to have any effect on the properties of 569/H/14. The degree of genetic instability (i.e. speed and conditions of conversion from the micro to the magno state) and the actual percentage magnos present in the population at any given time were not significantly altered after preparation of a spore suspension by heating a 48 hr old broth culture for 60 min. at 60° and subsequent washing of the organisms as previously described (Pollock, 1953*a*).

All-or-none effect. The difference between the micro and magno states of 569/H/14 was generally clear cut and of the 'all-or-none' type. Colonies derived from 569/H/14 grown in or on AA overnight at 25° or 30° and developed with penicillin at room temperature (see Methods) were either white (unchanged) or bright red (due to penicilloic acid production).

Colonies derived from a culture of 569/H/14 in the micro state were shown to be pure: they yielded 100 % white colonies after re-spreading on AA plates and analysis (see Methods) at 25°. However, if the same original culture were grown in AA at 35°, a significant proportion of developing colonies were found on analysis to be mixed (usually with reds very much in the minority). This suggests that spontaneous conversion from micro to magno had occurred in these colonies during growth. The effect was more marked at 40°, when nearly all colonies (whether scored as white or red) proved to be mixed. Population analyses of colonies indicated that they would be likely to be scored as red if their populations consisted of more than 15 % bacilli already in the magno state (i.e. giving rise to red colonies even at 25°).

These tests show that the danger of *spontaneous* intra-colonial conversion during growth in AA vitiating the results of a population analysis could be avoided by not employing an incubation temperature above 30°.

Broth subcultures from white colonies (identified through velvet pad replica plates in order to avoid contact with penicillin) incubated at 35° had detectable penicillinase activity only to the extent expected from the small (but variable) proportion of magno type cells they sometimes contained (as indicated by the proportion of red colonies appearing on further subculture and analysis on AA plates).

Broth subcultures from red colonies (similarly isolated in the absence of penicillin) generally consisted of 100 % magno-type cells and produced enzyme at the full differential rate expected of the 569/H parent type. An important exception to this general rule will be discussed later.

Characteristics of the conversion reaction

Kinetics

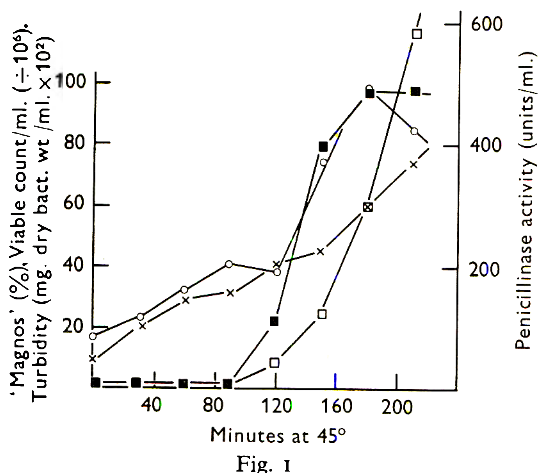
At 45°. Environmentally stimulated appearance of magno varieties from 569/H/14 micros was first observed by noticing the increased number of red colonies (on AA after developing with penicillin) appearing after incubation at 42° instead of 35° and the almost complete stability of the micro variety during growth at 25°. It was then found that incubation of a broth culture of 569/H/14 micros at 45° provoked up to 100 % conversion to the magno state in less than 2 hr (Fig. 1).

Although not shown in Fig. 1, incubation of an aliquot culture from the same micro-type colony at 35° involved no conversion to the magno state during the 3 hr of the experiment. Neither the wild-type strain 569 nor other micro-constitutive strains derived from 569/H showed any tendency to give rise to magnos during growth at 45°.

Although the total viable count and turbidity increased steadily throughout the experiment, the switch to the magno state occurred suddenly, after an absolute lag of 80 to 90 min. and was practically complete after a further hour. The 50 % conversion point (the time to reach 50 % of the maximum number of convertants) usually occurred between 90 and 120 min. after transfer to 45°. The amount of growth at 45°, measured either as increase in optical density or total count, was found to vary considerably from one experiment to another—largely no doubt because this temperature is critical, being at the extreme upper limit for growth of the *Bacillus cereus* 569 family. However, the kinetics and degree of conversion seemed to be quite unrelated to the extent of growth, even in certain experiments where there was a net drop in viable count.

Figure 1 also shows that detectable penicillinase activity appeared at the same time as the increase in the magno count and thereafter rapidly increased to a final level (approx. 1800 units/mg. dry bact. wt, not shown in Fig. 1) not much below that (2500 units/mg.) expected of a typical culture of 569/H.

With chloramphenicol. Addition of 20 μg. chloramphenicol/ml. to the culture during incubation at 35° was unexpectedly found also to induce up to 100 % conversion of a 569/H/14 micro culture with similar kinetics (Fig. 2). However, results with chloramphenicol were not so quantitatively reproducible as those involving conversion at 45°.



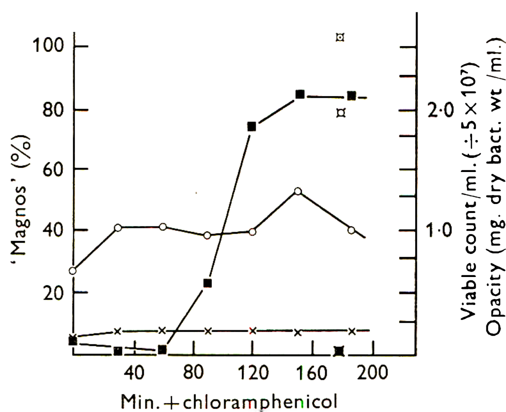


Fig. 1. Complete genotypic and phenotypic conversion of a population of *Bacillus cereus* 569/H/14 from the non-penicillinase producing micro state into the fully derepressed penicillinase-producing magno state, by incubation at 45°. ×——×, Optical density of culture; ○——○, total viable count; ■——■, magnos: as % of total count; □——□, penicillinase activity units/ml.

Fig. 2. Genotypic conversion of a population of *Bacillus cereus* 569/H/14 from the micro to the magno state by incubation at 35° in the presence of chloramphenicol (20 μg./ml.). ×——×, optical density; ○——○, total viable count; ■——■, magnos: as % of total count; ⌧, ⊡, ■ = points respectively indicating optical density, total viable count and magnos as % of total count of a control culture incubated similarly without chloramphenicol.

Variations in the magno colony count according to the temperature of incubation of the inoculated AA plates suggested that residual chloramphenicol, perhaps bound to the cells, was continuing to act in stimulating conversion after plating out samples. Washing the bacilli free of the drug caused no significant differences; and it was not possible immediately to reverse its inhibitory effect on growth by such treatment (nor could enzyme formation by an apparently converted population be demonstrated during the first 2 hr after treatment), although the viable count was unaffected. It is clear therefore that there is considerable growth lag after plating out chloramphenicol-treated organisms, and during this time a critical event, essential for conversion, may take place.

Phenotypic lag and development of penicillin resistance. A comparison between enzyme production and the appearance of magno-type colonies shown in Fig. 1 indicate that there can be little, if any, lag between the time when a bacillus is committed to reproduce in the magno state and when it begins to produce enzyme at the full, de-repressed rate.

Penicillin-resistance of individual cell units can also be measured. This has been done using final concentrations of 0·1 and 1·0 units of benzylpenicillin per ml. in the agar plates. These levels can be shown, in reconstruction experiments with mixtures from cultures of stable micro and magno strains, to inhibit completely the growth of micro-type cells into colonies, whilst allowing normal growth of 569/H and other magno-types.

Table 3. *Development of penicillin resistance in Bacillus cereus 569/H/14 (micro-type) during conversion to the magno-type by incubation in broth at 45°*

Plating-out medium		Viable counts ($\div 10^7$) per ml. at the following times (min.) after transfer from 35° to 45°					
		0	45	80	105	140	200
Plain nutrient agar (AA)							
Total		2·4	5·0	13·0	19	24	35
Magno-type		—	—	0·6	4·9	24·5	34
AA+0·1 unit benzylpenicillin/ml.	Total*	0·0026	0·004	0·75	9·0	24	35
AA+1·0 unit benzylpenicillin/ml.	Total*	0·00032	0·004	1·0	9·0	22	34

* All colonies developing on penicillin agar were of the magno-type.

Table 3 shows the comparative micro and magno counts on plain AA and on penicillin AA during a conversion experiment at 45°. On penicillin plates the total numbers of colonies developing (all of which were of the magno type) corresponded to the numbers of magno type colonies appearing on the plain agar plates. After 140 min. these represented practically 100 % of the total viable count.

This experiment confirms the rapid phenotypic expression of the conversion and shows that all the converted cells must be immediately capable of forming the enzyme approximately equally and at maximal rate.

Significance of mixed colonies

It was discovered that during the early stages of induced conversion a proportion of the colonies scored as red proved, on subsequent analysis, to be mixed (defined as containing not more than 95 % of the predominant type). Most of these mixed colonies contained roughly equal proportions of reds and whites. At least 100 colonies were analysed from each sample except towards the end of the conversion experiment when the proportion of reds had passed 50 % and only 50 were tested.

The proportion of mixed colonies in general declined as conversion of the population proceeded. Figure 3 shows the relationship between the number of such mixed reds as a proportion of total reds and the total reds as a proportion of the total colony count in a series of four experiments on conversion induced at 45°.

It could, of course, be argued that these mixed reds result from conversions occurring *after* plating out into AA and that, for this reason, the kinetics of induced conversion as followed in this work may be very inaccurate. It has already been argued that this could be true for conversions induced by chloramphenicol and, for this reason, these have been excluded from the results summarized in Fig. 3. But this argument is unlikely to apply to conversion at 45° because in this case there is no significant phenotypic lag in the expression of conversion (measured either in the form

of penicillinase production or as development of penicillin-resistance)—as indicated in Fig. 1 and Table 3.

One explanation of the decreasing proportion of mixed red colonies found during conversion would be that they reflect the diminishing proportion of multi-cell units containing unconverted cells in a population where the conversion event occurred randomly in individual cells. Although there was considerable variation between experiments and during the conversion process, Table 4, summarizing the results of the four experiments illustrated in Fig. 3, shows that a mean of only a little under

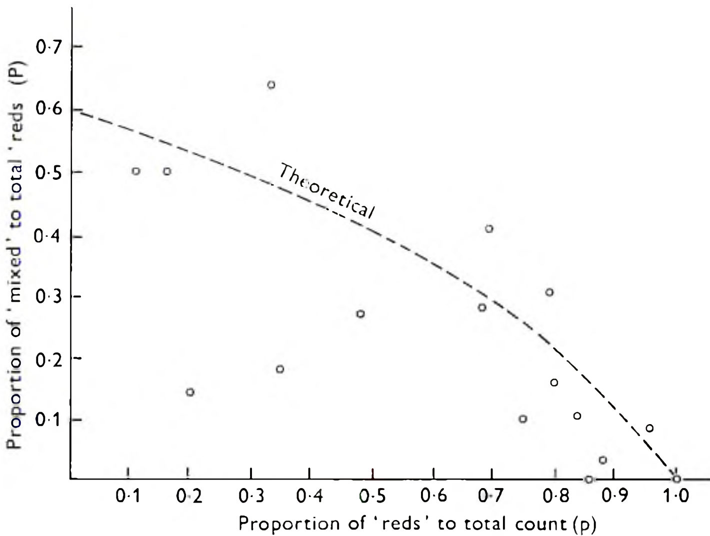


Fig. 3. The proportion of mixed colonies (i.e. appearing on plates as 'reds' (magnos) but subsequently proving on analysis to consist of micro and magno cells), P, occurring during conversion of populations of *Bacillus cereus* 569/H/14 from the micro to the magno state induced by incubation at 45° (in a total of four separate experiments), expressed in relation to the proportion of recorded 'reds' in the total viable count (p). – – –, Theoretical curve expected on the basis of conversion occurring at random in each cell and the observed mean proportion of double cell units in the population (see text).

Table 4. *Mean numbers of bacilli per chain ('unit') in cultures of Bacillus cereus 569/H/14 during conversion at 45°*

Expt. no.	Units in following classes (%): One bacillus	Two bacilli	More than two bacilli
1	58	33	9
2	64	33	3
3	54	41	5
4	51	43	6
Over-all range	51–64	33–43	3–9
Over-all mean	57	37	6

Mean numbers of bacilli per 'unit', in 100 unselected units in up to seven samples taken sequentially from each culture during 180 min. incubation at 45° were recorded and results expressed as percentages in each class.

50 % of units contained more than one cell and that over 85 % of these multi-cell units contained only two cells. Suppose that the proportions of two-cell and one-cell units were equal and three- (or more) cell units negligible, two-thirds of the conversion events, if randomly distributed amongst individual cells, would yield mixed red colonies at the very beginning of the conversion process.

The actual proportions of mixed red colonies to be expected on this hypothesis can be calculated accurately from the following formula (for which we are much indebted to Dr J. F. Collins), making only the simplifying approximation of including the few more-than-two-cell units within the two-cell unit group.

$$P = \frac{2a-2+2x}{a+1+x},$$

where $x = \pm\sqrt{[(1+a)^2-4ap]}$, a being the proportion of units containing two cells, p the proportion of all colonies that are 'red' and P the proportion of red colonies that prove to be mixed.

The theoretical curve in Fig. 3, giving expected values for P, has been calculated thus from the data summarized in Table 4 (giving a value of 0·43 for a) and the progress of conversion (P) during the same four experiments.

The scatter of points is high but the general trend suggests that the hypothesis of random conversion at the cell level is not unreasonable.

The metabolic basis of conversion

Phase and extent of growth. It was found that if treatment with chloramphenicol or at 45° was delayed until growth level had reached 0·05 mg. dry bacterial wt/ml., conversion was significantly retarded. After a level of 0·2 mg./ml., no conversion was detected for up to 3 hr.

If bacilli were removed from the growth medium and resuspended in phosphate alone, conversion—either with chloramphenicol or at 45°—was greatly reduced.

Although growth—measured either by viable count or opacity increase—is clearly not essential for conversion (as demonstrated by the effect of chloramphenicol and by those experiments at 45° where little growth took place) it must be concluded that fairly complex metabolic events underlie the phenomenon.

Inhibitors and other promoters of conversion. Other inhibitors of protein synthesis were tested under conditions identical to those used with chloramphenicol, but gave relatively slight and somewhat variable stimulation as follows:

Streptomycin (10 μg./ml.) gave 7·5 % conversion, puromycin (24 and 94 μg./ml.) up to 15 %, and actinomycin D (3·3 μg./ml.) up to 40 %, all against a control value of not more than 1 % and conversion with chloramphenicol of up to 100 %. None of these compounds had any effect on the extent or kinetics of conversion in the presence of chloramphenicol. Known inhibitors of DNA synthesis, mitomycin C (0·01 μg./ml.), caffeine (4·8 and 9·7 mg./ml.) FUDR (25 μg./ml.) and nalidixic acid (10·0 and 100 μg./ml.) had no effect by themselves (except that both mitomycin and FUDR were very toxic and caused a significant reduction in the viable count). But nalidixic acid (10 μg./ml.), if added during treatment at 45°, caused inhibitions of conversion from 70 % to 98 % after 3 hr treatment, usually without causing significant loss of viability.

In whatever manner the possible role of DNA synthesis may be assessed, results with chloramphenicol and treatment at 45° strongly support the idea that some

protein repressor substance must control the rate of conversion in normal cultures of 569/H/14.

The conversion-promoting effect of incubation at 45° could be explained if it is supposed that in 569/H/14 this protein repressor of conversion is somewhat thermolabile.

The two types of treatment that promote conversion—incubation at 45° and incubation with chloramphenicol at 35°—would, on this hypothesis, operate towards the same end (elimination of the same protein) by very different means: the former by inactivating the protein and the latter by preventing its formation. The pre-conversion lag could, in most instances, be due to the time necessary to allow the concentration of this protein repressor to fall below a critical, effective level.

Unlike the effect of combined addition of two different inhibitors of protein syn-

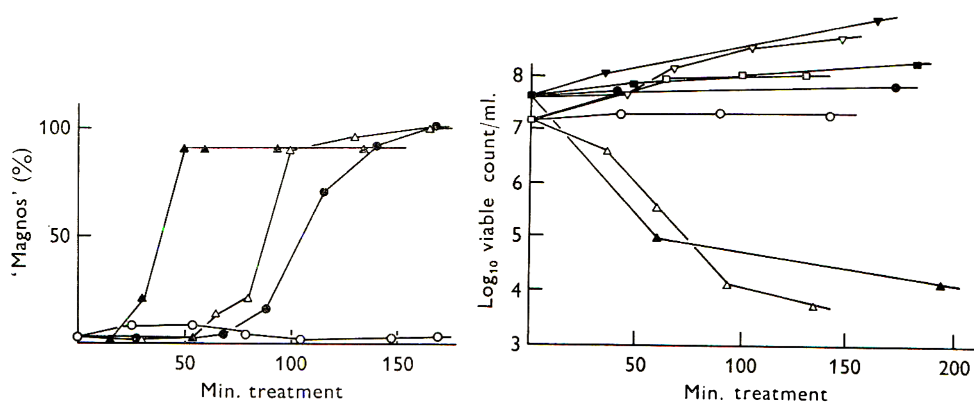


Fig. 4. Synergistic effect of addition of chloramphenicol and incubation at 45° in shortening the lag for the genotypic conversion of 569/H/14 from the micro to the magno state. All results expressed as % magnos in total viable count. ○——○, Control (incubation at 35° without chloramphenicol); ●——●, 35°+chloramphenicol (20 μg./ml.); △——△, 45°, no chloramphenicol; ▲——▲, 45°+chloramphenicol (20 μg./ml.).

Fig. 5. Comparative effects of incubation at 35° and 45°, with and without chloramphenicol, on the viable counts of the micro and magno varieties of *Bacillus cereus* 569/H/14. Open symbols: micro type; filled symbols: magno type. ▽, 35° (no chloramphenicol); □, 45° (no chloramphenicol); ○, 35°+chloramphenicol (20 μg./ml.); △, 45°+chloramphenicol (20 μg./ml.).

thesis, treatment at 45° and treatment with chloramphenicol should be powerfully synergistic in their effect and lead to a shortening of the pre-conversion lag. Figure 4 gives the results of an experiment showing that the predicted synergism did indeed occur in a very marked manner. The lag was reduced by over 70%—from around 75 min. to 20 min.—and the 50% conversion time was more than halved.

Unfortunately, whereas neither 45° nor chloramphenicol by themselves generally caused decrease in viability, the combined effect of both was markedly bactericidal, the counts falling to one-tenth the original level by the time conversion was completed. Although there was still a significant absolute increase in magno count during the earlier stages of the experiment, thereafter there was a decrease and it is possible formally to argue that some selection of magnos could have occurred.

That this is not indeed the case is shown in Fig. 5 where the comparative counts of

micros (during conversion) and of mature magnos (after conversion) treated under the same conditions do not differ significantly. This shows that the two varieties are equally sensitive to the traumatic effects of incubation with chloramphenicol at 45°.

DISCUSSION

A satisfactory interpretation of this phenomenon must await the development of a more precise system for the study of genetics in *Bacillus cereus*. Until very recently the many attempts made with this species have not borne fruit and claims to have demonstrated transformation (Felkner & Wyss, 1964) have not been substantiated (Goldberg & Gwinn, 1968). However, now that a generalized transducing phage for *B. cereus* has been discovered and successfully applied by Thorne (1968), it may be hoped some progress may at last be made in the genetics of this recalcitrant organism.

Until then it must be accepted that it is not even possible to characterize the genetic event underlying the original mutation from the wild-type inducible strain 569 to the magno-constitutive 569/H, let alone the basis of the loss mutation which gave rise to the unstable 569/H/14 micro strain or the conversion phenomenon itself.

However, even now it may be supposed that the reversibility of the micro to magno conversion makes it rather unlikely that the switch simply involves a loss (deletion) of a *unique* genetic element (e.g. an extrachromosomal plasmid) forming a repressor of the penicillinase structural gene. Beyond that, possible hypotheses—none of which can yet be excluded—group themselves into three broad classes as follows:

(1) Metabolic switches of the Delbrück (1949) model type or cycles *not* involving heritable modification of DNA (Pollock, 1953*b*; Monod & Jacob, 1961). Such possibilities probably cannot be formally excluded until the micro or magno states can be separately transferred from one organism to another, though they are made unlikely by the persistence of these distinct states through the sporulation cycle.

(2) Consistent errors or alterations occurring at a specifically relevant locus during chromosome replication. Since the individual cells of most two-cell units (as recorded in Table 4) are presumably derived from a common mother cell and therefore synchronized for cell division, it might be argued that a truly random occurrence of conversion amongst organisms joined in pairs would be evidence against the possibility of conversion being associated with the replication of a specific locus on the chromosome. However, although the kinetics of the appearance of mixed colonies during conversion hints at the possibility of a random occurrence at the cell level, it is no more than suggestive and in any case the above argument holds only if it can be shown either that *Bacillus cereus* cells have but a single chromosome or that, if more than one is present, their replication is synchronous.

(3) Position effects, involving translocations or duplications of genetic regions whereby controlling elements are separated from, or brought into contiguity with, the structural gene they regulate, causing a switching on or off of production of the relevant enzyme: in this case penicillinase. In so far as such a recombination may involve synthesis of DNA, the antagonistic effect of nalidixic acid, a known inhibitor of DNA formation (Goss, Dietz & Cook, 1964; Cook, Brown, Boyle & Goss, 1966; Barbour, 1967), on conversion gives some support to the above interpretation. This type of hypothesis may be difficult to prove (or exclude) even with sophisticated genetic techniques. But it is one—in various forms—which seems to be naturally well favoured at the moment as a basis for the interpretation of unstable genetic states in Aspergillus

studied by Bainbridge & Roper (1966) and Nga & Roper (1968), in Antirrhinum by Fincham (1967) and of those involving the Gal_3 locus in *Escherichia coli* as reported by Morse (1967) and of both the *pro* −401 locus (Smith-Keary & Dawson, 1964) and antigenic phase variation in Salmonella (B. A. D. Stocker, private communication), as well of course as the complicated variations of control of characters in maize (particularly anthocyanin production) worked out by McClintock (1961). All these phenomena may eventually be found to share some common molecular mechanism. And their possible relevance to mechanisms for tissue differentiation and antibody formation in higher organisms cannot be ignored.

What could in fact be a special case of the above hypothesis might be considered in the light of the possibility, suggested recently by the work of Fraser, Baird & Kleeman (1969), that *Bacillus cereus* may, under certain circumstances, be diploid. An unstable situation might arise if only one of the chromosomes in a pair was actively expressed at any one moment, as for the *X* chromosomes in female mammals and other instances in higher organisms (see Lyon, 1968). Conversion could then be a reflexion of a switch over of the active state from one chromosome to the other in a pair heterozygous for penicillinase formation. An explanation based on simple segregation to the haploid state would be excluded because conversion involves 100 % of the cells and is anyway not completely irreversible.

Whatever may be the nature of the event underlying this conversion phenomenon it would appear to be under the control of a protein repressor which, at least in the case of strain 569/H/14 (micro), is thermo-labile. Unless the reverse change from magno to micro is due to some quite different type of event, it must be supposed that this thermolability is lost in the conversion process. Restoration of a stable repressor might therefore be coupled with restoration of activity to the penicillinase gene and be part of the same phenomenon.

We are grateful to Miss Kathleen O'Hare and Miss Alexis Smith for technical assistance during summer vacations, and to Dr J. F. Collins for helpful discussions and for calculating the formula on which the theoretical curve illustrated in Fig. 3 is based.

The senior author (M.R.P.) is Honorary Director of a Research Group on 'Bacterial Enzyme Variation' supported by the Medical Research Council.

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J. gen. Microbiol. (1969), **59**, 317–328
Printed in Great Britain

The Effect of Long Chain Fatty Acid Isomers on Growth, Fatty Acid Composition and Osmotic Fragility of *Mycoplasma laidlawii* A

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(*Accepted for publication 8 September 1969*)

SUMMARY

Growth of *Mycoplasma laidlawii* A in a tryptose medium, with lipid pre-extracted, containing charcoal-treated bovine plasma albumin, occurred only after addition of long-chain fatty acids. Of the long-chain fatty acids tested, positional as well as geometrical isomers of octadecenoic acids were effective in growth promotion; *trans* isomers were more effective in promoting growth and in lowering osmotic fragility than their corresponding *cis* isomers. Tetradecenoic or hexadecenoic acids did not replace the growth requirement for an octadecenoic acid. Although fatty acids containing cyclopropane-rings promoted growth, a greater toxicity and lower growth response was observed. No fatty acid isomerization by this mycoplasma was detected and the octadecenoic acids added to the growth medium were recovered from membrane polar lipids unchanged. Fatty acid analyses revealed that myristic and palmitic acids were the major saturated fatty acid components of the membrane polar lipids. No differences were found in the fatty acid content or composition of membrane polar lipids from mycoplasmas grown at 25° and 37°. Major differences in the fatty acid composition between glycolipids and phospholipids were not apparent.

INTRODUCTION

The mycoplasma cell is bound by a lipoprotein membrane containing virtually all of the cellular lipids (Razin, 1967). While mycoplasmas not requiring sterols are capable of synthesizing saturated fatty acids from acetate (Rottem & Razin, 1967*a*; Pollack & Tourtellotte, 1967) some require an exogeneous octadecenoic acid for growth (Razin & Rottem, 1963; Razin, Tourtellotte, McElhaney & Pollack, 1966) and others do not (Henrikson & Panos, 1969; Panos & Henrikson, 1969). Thus far, all have been found capable of incorporating both saturated and unsaturated fatty acids into their membrane polar lipids (Razin *et al.* 1966; Rottem & Razin, 1967*a*).

Practically nothing is known about the possibility of replacing the unsaturated fatty acid requirement for growth of a non-sterol-requiring mycoplasma with monoenoic positional or geometrical isomers or by fatty acids containing a cyclopropane ring. The ability to alter the fatty acid composition of mycoplasmal membranes by changing the lipid composition of the growth medium and the resulting effect on osmotic fragility of intact cells has been reported (Razin *et al.* 1966). An incorporation of elaidic acid into membrane lipids of the sterol-requiring *Mycoplasma* sp. Y, was reported by Rodwell (1968). This acid replaced both the saturated and un-

saturated fatty acid growth requirements and was incorporated, unchanged, into the membrane lipids of this organism. The incorporation of *trans* monoenoic acid isomers as well as cyclopropane ring containing and methyl-branched fatty acids into membrane lipids has also been reported for certain non-sterol-requiring mycoplasmas (Panos & Henrikson, 1969; McElhaney & Tourtellotte, 1969).

The present work examines the effect of various positional and geometrical monoenoic acids and of the cyclopropane-ring containing acids on growth and osmotic fragility of *Mycoplasma laidlawii* A, an octadecenoic acid-requiring organism. The fatty acid composition of the membrane polar lipids of this organism when grown in the presence of these exogeneously supplied fatty acids is also discussed.

METHODS

Organism and growth conditions. Mycoplasma laidlawii (oral strain) was kindly provided by Dr S. Razin (The Hebrew University—Hadassah Medical School, Jerusalem, Israel). This strain is related to *M. laidlawii* A (Rottem & Razin, 1967*b*) and requires an octadecenoic acid for growth. For growth experiments, mycoplasmas were grown in 5 ml. medium consisting of 2 % (w/v) tryptose (Difco Laboratories Inc., Detroit, Michigan, U.S.A.) from which lipid had been extracted (Henrikson & Panos, 1969), 0·5 % (w/v) glucose, 0·5 % (w/v) sodium acetate, 500 units penicillin G/ml. and 0·25 % (w/v) bovine plasma albumin fraction V (Armour Pharmaceutical Co., Kankekee, Illinois, U.S.A.) which had been treated with charcoal to remove fatty acids according to Chen (1967). Each tube received 0·05 ml. of an ethanolic solution (70 %, v/v in water) containing various amounts of the fatty acid to be tested and 0·05 ml. of inoculum grown in the medium described above with 2 μg. oleic acid/ml. The initial inoculum contained about 10^7 colony-forming units (c.f.u.)/ml. medium as determined by the colony-count technique of Butler & Knight (1960). Inoculated tubes were incubated statically at 37°. Growth was estimated after 24 to 36 hr by viable count (Butler & Knight, 1960) and/or extinction at 400 nm. For the analyses of fatty acids from membrane polar lipids, organisms were grown in 4 l. volumes of medium as described above, supplemented with 2 to 4 μg./ml. of a particular monoenoic fatty acid. For isotope experiments, [1-^{14}C]oleic acid (57·2 mc/m-mole) or [1-^{14}C]elaidic acid (52 mc/m-mole) was added to the growth medium (1 μc/l.). For osmotic fragility experiments, 100 ml. of medium supplemented with 10 to 20 μg./ml. of a monoenoic or cyclopropane-ring containing fatty acid was used. Organisms were routinely harvested after 24 to 36 hr at 37° (17,000 *g* for 20 min.), washed once with 0·25 M-NaCl and the amount of cell protein determined according to Lowry, Rosebrough, Farr & Randall (1951).

Assessment of osmotic fragility. A portion of washed mycoplasmas was suspended in a small volume of 0·25 M-NaCl and 0·05 ml. of suspension added to test-tubes containing 2 ml. 0·25 M-NaCl or 2 ml. o 0·05 M-NaCl. The change in turbidity (from 0·500 extinction) was measured at 500 nm. with a Zeiss PMQ II spectrophotometer after incubation at 37° for 15 min. and the results expressed as % lysis in 0·05 M-NaCl.

Lipid extraction and chromatography. Lyophilized membranes isolated by osmotic lysis of washed organisms as described by Razin *et al.* (1966), were extracted with 20 volumes of chloroform + methanol (2 + 1, v/v) according to Folch, Lees & Sloane-Stanley (1957). The lipid extract was dried under a stream of nitrogen, dissolved in

0·5 ml. chloroform and applied to a 1·6 × 10 cm. column of activated silicic acid (Unisil, 100 to 200 mesh, Clarkson Chemical Co. Inc., Williamsport, Penna., U.S.A.) that had been washed with chloroform. Neutral lipids were eluted with 200 ml. chloroform followed by 200 ml. methanol which eluted the polar lipids (Ansell & Hawthorne, 1964). After removal of neutral lipids, separation of phospholipids from glycolipids was achieved by successive elution with 200 ml. acetone and 150 ml. chloroform + methanol (1 + 1, v/v) (Shaw, Smith & Kostra, 1968). Total phosphorus in lipid fractions was determined according to Fiske & SubbaRow (1925) after digestion of samples with 70 % perchloric acid at 120° for 24 hr.

Analyses of fatty acids. The alkaline hydrolysis–fatty acid extraction methods of Hofmann, Henis & Panos (1957), modified by the omission of bicarbonate washings, were used throughout. Methylation of the free fatty acids, hydrogenation and infrared analyses were performed as described elsewhere (Panos, Cohen & Fagan, 1966); Weinbaum & Panos, 1966). Separation of methylated fatty acid geometrical isomers was achieved by thin-layer chromatography on silica gel G plates containing 15 % (w/v) $AgNO_3$ and 0·01 % (w/v) Rhodamine 6G. Methylated fatty acid mixtures were dissolved in benzene and applied on plates as bands with each plate containing approximately 200 μg. of material. The developing solvent was a mixture of light petroleum (b.p. 38° to 46°) + diethyl ether (9 + 1, v/v). Fatty acid zones were located under ultraviolet radiation. For recovery of labelled acids in radioactive experiments, bands were scraped off plates, transferred to tubes and the silica gel extracted (3 times) with multiple portions (5 ml.) of light petroleum. After transfer to scintillation vials and subsequent removal of solvent under a stream of nitrogen, 10 ml. of scintillation fluid (3 g. 2,5-diphenyloxazol and 0·1 g. 1,4-bis-(5 phenyl:oxazolyl-2)-benzene in 1 l. toluene) was added and samples counted in a Packard Tricarb Scintillation counter. Esterified positional monoenoic acid isomers, together with the other components of such mixtures, were resolved by capillary column gas chromatography using a 150 foot (40·6 m.) × 0·01 inch (0·25 mm.) column coated with Carbowax K-20 M + V-93 (99 + 1) (Perkin-Elmer Corp., Norwalk, Conn., U.S.A.) as described previously (Panos *et al.* 1966; Panos & Henrikson, 1968). The designated abbreviations (C13T?, C14T?) of the tentatively identified branched methyl fatty acids are based upon their total carbon atom content.

Chemicals. Oleic, *cis*-vaccenic, elaidic and *trans*-vaccenic acids were purchased from the Hormel Institute (Austin, Minn., U.S.A.) and were greater than 99 % pure. [1-^{14}C]Oleic acid (57·2 mc/m-mole) and [1-^{14}C]elaidic acid (52 mc-/m-mole) were the products of Applied Science Laboratories (State College, Penna., U.S.A.) and were checked for purity before use. Dihydrosterculic (*cis*-9,10-methyleneoctadecanoic) acid had been prepared by the hydrogenation of *Sterculia foetida* kernel oil according to the method of Tausig (1955) following low-temperature crystallization of the free acid and purification of the methyl ester by thin-layer chromatography as described above. Lactobacillic (*cis*-11,12-methyleneoctadecanoic) acid was obtained by vacuum frational distillation of methylated fatty acids derived from *Escherichia coli* E-26 grown in a defined medium (Hofmann, Lucas & Sax, 1952; Weinbaum & Panos, 1966).

RESULTS

The fatty acid composition of the tryptose+albumin ingredients of the growth medium is given in Table 1. The intensive extraction of dried tryptose and bovine serum albumin resulted in an almost insignificant combined fatty acid content remaining, giving approx. 0·5 μg. unsaturated fatty acid/ml. medium when reconstituted. Charcoal treatment of bovine serum albumin decreased its fatty acid content from 0·147 % to 0·008 % without changing the relative amount of each of its residual acids. The dominant fatty acids in charcoal-treated albumin preparations were oleic (29 %), palmitic (25 %) and stearic acids (22 %). Almost no growth of *Mycoplasma laidlawii* A occurred in this extracted medium without the addition of an octadecenoic acid.

Table 1. *Residual fatty acid composition and content of the lipid-extracted tryptose + albumin ingredients of the growth medium**

Fatty acids	Composition (%)
Lauric	0·33
C_{13} sat'd	0·04
Tridecanoic	0·04
C_{14} sat'd	0·04
Myristic	2·87
C_{14} unsat'd	0·10
Pentadecanoic	0·31
Palmitic	33·22
Margaric	0·30
Stearic	1·80
Oleic	38·27
Linoleic	0·67
Total fatty acids = 0·0059%	

* Tryptose extracted with chloroform+methanol and ethyl ether, albumin by charcoal treatment

The absolute requirement of *Mycoplasma laidlawii* A for an unsaturated fatty acid has been described (Razin *et al.* 1966). This requirement can be fulfilled by oleic acid and to a lesser degree by linoleic or linolenic acids (Razin & Rottem, 1963). Figure 1 shows that this mycoplasma can be grown in a tryptose pre-extracted medium containing 0·25 % (w/v) charcoal-treated albumin with other fatty acids as well. Either positional or geometrical isomers of octadecenoic acids served as effective growth promoters. Furthermore, a *trans* isomer, elaidic (*trans*-9-octadecenoic) acid, was more active in promoting growth than its corresponding *cis* isomer (oleic acid). Likewise, two cyclopropane-ring containing acids, lactobacillic (*cis*-11,12-methyleneoctadecanoic) and dihydrosterculic (*cis*-9,10-methyleneoctadecanoic) acids were able to replace this need for an octadecenoic acid for growth. Although shorter-chain monoenoic fatty acids such as lauroleic, *cis*-5-tetradecenoic, myristoleic or palmitoleic were devoid of growth-promoting abilities, they were none the less incorporated into complex lipids and some elongated. This aspect will be dealt with in a subsequent publication. The optimal concentration of the various octadecenoic fatty acids for growth was 2·5 to 5·0 μg./ml. medium, higher concentrations began to exert a toxic effect. The lowest growth response was found to occur with the cyclopropane ring-

containing fatty acids (Fig. 1), with about 87% inhibition of growth at 15 µg./ml. medium (Table 2). A similar toxic effect was not observed with any of the other positional or geometrical octadecenoic acid isomers tested. Also, marked lysis occurred when mycoplasmas were grown with 4 µg./ml. of either of these ring-containing fatty acids upon reaching the stationary phase of growth.

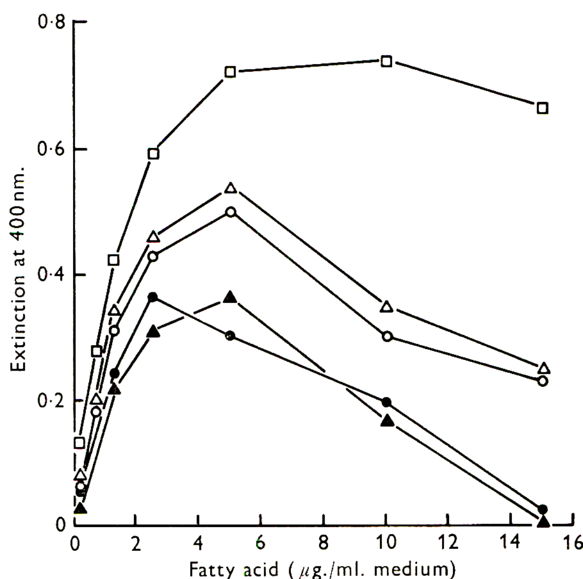

Fig. 1. The effect of long-chain fatty acid isomers on growth of *Mycoplasma laidlawii* A in a lipid pre-extracted tryptose medium. △, Oleic (*cis*-9-octadecenoic) acid; ○, *cis*-vaccenic (*cis*-11-octadecenoic) acid; □, elaidic (*trans*-9-octadecenoic) acid; ●, dihydrosterculic (*cis*-9,10-methyleneoctadecanoic) acid; ▲, lactobacillic (*cis*-11,12-methyleneoctadecanoic) acid. Growth estimated after 36 hr.

Table 2. *Growth inhibition of Mycoplasma laidlawii A by long-chain fatty acids*

Organisms grown in a lipid pre-extracted medium containing 0·25% charcoal-treated albumin. Fatty acids added as ethanolic solutions.

Fatty acid	Concentration (µg./ml.)	Colony-forming units/ml.	Inhibition* (%)
Elaidic	5	$8·3 \times 10^9$	
	15	$6·1 \times 10^9$	26·5
Oleic	5	$6·1 \times 10^9$	
	15	$2·4 \times 10^9$	60·7
Dihydrosterculic	5	$2·4 \times 10^9$	
	15	$3·1 \times 10^8$	87·1

* As compared to mycoplasmas grown with fatty acid, 5 µg./ml.

Quantitative results from capillary column gas chromatograms are given in Table 3 and illustrate the intensive incorporation of *cis*-vaccenic acid into membrane polar lipids as well as their high myristic acid content as compared with that of the growth medium (Table 1). The total fatty acid content of these membranes was 18·6%. No changes in fatty acid content or in the relative amount of the various fatty acids of the membrane polar lipids was noticed when *Mycoplasma laidlawii* A was grown at 25°.

A comparison of the fatty acid content of membrane phospho- and glyco-lipids is shown in Table 3, which also shows a small but consistently greater *cis*-vaccenic acid content of the phospholipids always apparent during these studies. Glycolipid fractions were eluted from silicic acid columns with acetone and contained approximately 40 % of the membrane polar lipids. Membrane phospholipids were similarly eluted with chloroform + methanol (1 + 1, v/v). These fractions contained over 43 % of the total polar lipids and all of the organic phosphorus of the membrane lipids.

Table 3. *Fatty acid composition (%) and distribution of Mycoplasma laidlawii A membrane polar lipids**

Organisms grown in a lipid pre-extracted medium containing 0·25% charcoal-treated albumin. *cis*-Vaccenic acid added as ethanolic solution.

Fatty acid†	Polar lipids	Phospholipids	Glycolipids
Capric	0·01	0·01	0·01
Lauric	7·04	6·72	7·96
C_{13} sat'd (C13T?)	0·49	0·40	0·39
Tridecanoic	1·02	0·92	0·98
C_{14} sat'd (C14T?)	0·02	0·01	0·02
Myristic	30·12	28·62	31·40
C_{14} unsat'd	0·40	0·38	0·25
C_{14} unsat'd	0·93	0·91	0·93
Pentadecanoic	1·48	1·47	1·43
Palmitic	16·61	15·01	17·12
Margaric	0·01	0·01	0·01
Stearic	1·27	1·32	1·06
cis-Vaccenic	39·09	41·98	38·01
Linoleic	1·30	1·15	1·20
Unsat'd/sat'd ratio	0·72	0·80	0·70

* Mycoplasmas grown with *cis*-vaccenic acid, 2 μg./ml., average of three experiments.
† Unsat'd = unsaturated, sat'd = saturated.

When *Mycoplasma laidlawii* A was grown with elaidic acid the fatty acid profile and composition of its membrane polar lipids was the same as that for organisms grown with oleic acid. However, the characteristic configurational *trans* band (10·3 μ) appeared in the infrared spectra of those methylated fatty acid mixtures from membrane polar lipids of mycoplasmas grown with elaidic acid (Fig. 2). The possibility that part of the elaidic acid was isomerized to the corresponding *cis* isomer was excluded by the use of labelled fatty acids in the growth medium and the subsequent separation of geometrical octadecenoic acid isomers by thin-layer chromatography. Table 4 shows that this mycoplasma was incapable of isomerizing an octadecenoic acid either from or to the *trans* configuration. All of the [1-^{14}C]elaidic or [1-^{14}C]oleic acid added to the growth medium was recovered from membrane polar lipids without redistribution of radioactivity. The low amount of radioactivity appearing in the *cis* and trans *areas* of the respective *trans* and *cis* thin-layer chromatographic fractions (Table 4) was due to impurities in the isotopic reagents as supplied by the vendor.

An increase in the concentration of oleic or elaidic acid in the growth medium up to 12 μg./ml. resulted in an increase in the total amount of each acid incorporated into the mycoplasmas (Fig. 3). However, further increases in the external fatty acid concentration did not increase incorporation. The maximal amount of octadecenoic

acid/mg. cell protein under these conditions was approx. 150 μg. Although no differences were noted in the amount of oleic or elaidic acid incorporated into *Mycoplasma laidlawii* A, a marked change in its osmotic fragility was observed. This difference was most pronounced when the concentration of such acids in the growth medium increased above 5 μg./ml. The differences in cell yields, viable counts and osmotic fragility of *M. laidlawii* A grown with relatively high concentrations of various fatty acids are summarized in Table 5. The *trans* isomer, elaidic and *trans*-vaccenic acids improved growth and increased the resistance of the organisms to osmotic lysis more than did their corresponding *cis* isomers, oleic and *cis*-vaccenic acids or their homologous *cis*-cyclopropane-ring containing fatty acids, dihydrosterculic and lactobacillic acids.

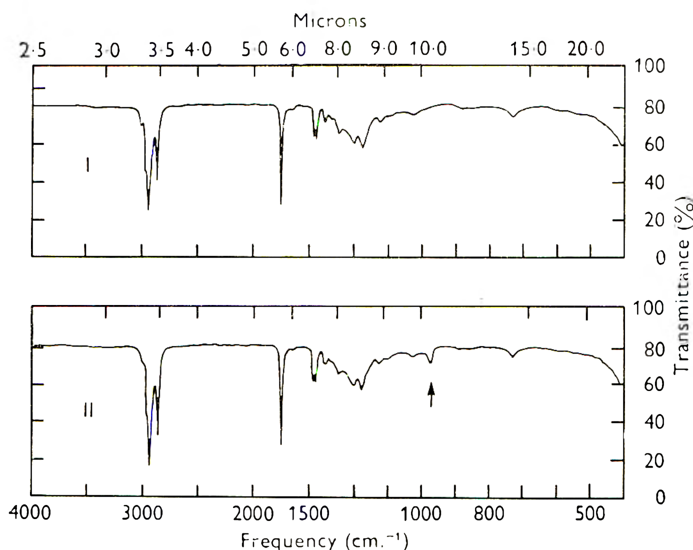

Fig. 2. Infrared spectra of methylated fatty acids from membrane polar lipids of *Mycoplasma laidlawii* A. I, Grown with oleic acid, 2 μg./ml. II, Grown with elaidic acid, 2 μg./ml. Arrow indicates *trans* band.

Table 4. *Incorporation of geometrical octadecenoic acid isomers into Mycoplasma laidlawii A membrane polar lipids*

Organisms grown in a lipid pre-extracted medium containing 0·25 % charcoal-treated albumin and labelled fatty acid indicated. Fatty acids added as ethanolic solutions. Methylated fatty acids separated into fractions by thin layer ($AgNO_3$) chromatography

		Radioactivity (counts/min.)	
Fractions	R_F	[1-^{14}C]Oleic acid	[1-^{14}C]Elaidic acid
Saturated acids	0·85	42	33
Trans acids	0·66	120	6218
Cis acids	0·45	4843	270

DISCUSSION

A growth requirement for long-chain fatty acids has been established for certain mycoplasmas: *Mycoplasma* var. *mycoides mycoides* and *Mycoplasma* sp. Y require both an unsaturated and a saturated fatty acid for growth (Rodwell & Abbot, 1961;

Table 5. *Effect of octadecenoic and cyclopropane ring containing fatty acid isomers on growth and osmotic fragility of Mycoplasma laidlawii* A

Organisms grown in lipid pre-extracted tryptose medium containing 0·25 % charcoal-treated bovine serum albumin with fatty acids indicated.

Fatty acid added to growth medium	Concentration (μg./ml.)	Cellular yields (mg. protein/100 ml.)	No. of c.f.u.* (c.f.u./ml.)	Osmotic fragility†
Oleic	20	1·72	$6 \cdot 20 \times 10^{8}$	44·5
	10	2·94	$2 \cdot 60 \times 10^{9}$	47·0
Elaidic	20	3·86	$1 \cdot 65 \times 10^{10}$	26·4
Dihydrosterculic	10	2·00	$7 \cdot 00 \times 10^{8}$	58·6
cis-Vaccenic	20	1·61	$4 \cdot 35 \times 10^{8}$	53·6
	10	3·10	$2 \cdot 30 \times 10^{9}$	55·0
trans-Vaccenic	20	3·45	$4 \cdot 65 \times 10^{9}$	32·1
Lactobacillic	10	1·90	$8 \cdot 25 \times 10^{8}$	52·6

* C.f.u. = colony-forming units.
† Percentage lysis in 0·05 M-NaCl (0·25 M-NaCl as control).

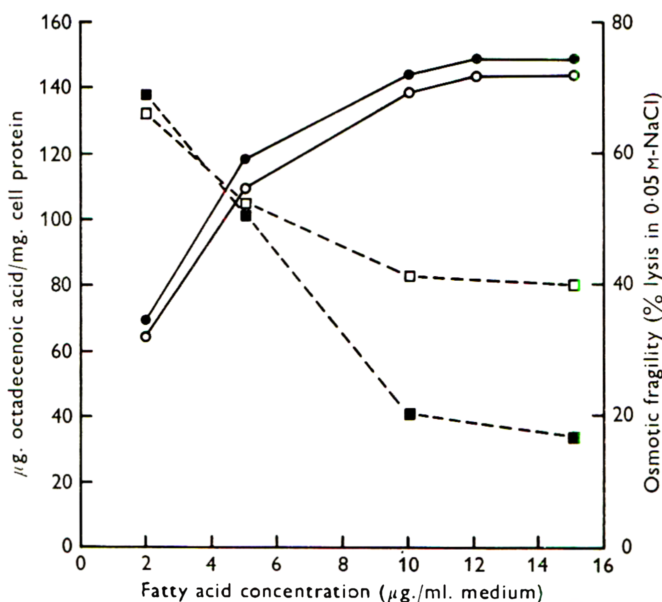

Fig. 3. Incorporation of octadecenoic acid isomers into *Mycoplasma laidlawii* A and their effect on osmotic fragility. Incorporation: oleic acid (○) and elaidic acid (●). Osmotic fragility: organisms grown with oleic (□) or elaidic acids (■). Fragility determined after 24 hr.

Rodwell, 1967). However, while some non-sterol-requiring strains require an unsaturated fatty acid for growth (Razin & Rottem, 1963; Razin *et al.* 1966), others do not (Henrikson & Panos, 1969; Panos & Henrikson, 1969). In the present studies, the requirement for an unsaturated fatty acid by *M. laidlawii* A was fulfilled by either positional or geometrical octadecenoic acid isomers, but not by shorter-chain mono-

enoic fatty acids. The inability of shorter-chain fatty acids to be elongated beyond their respective hexadecenoic acid isomers by this mycoplasma has been discussed by Panos & Rottem (1969). Growth of *M. laidlawii* A was greatest when elaidic acid replaced oleic acid as the octadecenoic acid requirement. Elaidic acid provided a greater growth response and was less toxic at higher concentrations than its corresponding *cis* isomer under otherwise identical growth conditions. Rodwell (1968) reported that both the saturated and unsaturated fatty acid needs of *Mycoplasma* sp. Y could be fulfilled by elaidic acid. This greater response of *M. laidlawii* A with elaidic acid may be due to a better packing of its membrane polar lipids necessary for greater growth and cellular activity. Surprisingly, cyclopropane-ring-containing fatty acids (lactobacillic and dihydrosterculic acids) were able to substitute for oleic acid in the growth medium. While these acids are known to replace the octadecenoic acid requirement for certain bacteria (Hofmann & Panos, 1954), their presence in the mycoplasmas has been only speculative (O'Leary, 1962; Pollack & Tourtellotte, 1967). Although nothing is known about the physical effects of ring-containing fatty acids on, for example, lipoidal membrane packing sequences, it is known that 'the cyclopropane-ring appears to restrict the number of "economical" modes of packing the long chains into a crystal lattice' (Hofmann, 1963). Thus the greater toxicity and lowest growth response of mycoplasmas grown with lactobacillic or dihydrosterculic acids and the lysis of *M. laidlawii* A, once the stationary phase of growth was reached, may conceivably be related to an indiscriminate incorporation resulting in an alteration of the spatial requirements for membrane stability, with eventual lysis.

The fatty acid composition of mycoplasma lipids has been a subject of previous publications (Razin *et al.* 1966; Rodwell, 1968; Panos & Henrikson, 1969). A comparison of the fatty acid profile of mycoplasma lipids with that of the growth medium has also been reported (Smith, Kostra & Henrikson 1965; Razin *et al.* 1966). Using high-resolving capillary gas chromatography, Henrikson & Panos (1969) showed that when *Mycoplasma* sp. KHS or *M. laidlawii* B were grown in a medium almost devoid of fatty acids, myristic acid comprised almost 50% of their total fatty acids. The presence of significantly greater amounts of myristic acid than palmitic acid in the membrane polar lipids from *M. laidlawii*, together with the reverse observed in uninoculated growth-medium controls, reflected a net synthesis of myristic acid and confirmed the earlier results of others concerning the *de novo* synthesis of saturated fatty acids from acetate (Rottem & Razin, 1967*a*; Pollack & Tourtellotte, 1967). Contrary to these whole-cell growth experiments, stearic acid was the major saturated fatty acid synthesized by a cell-free system from this organism (Rottem & Panos, 1969), indicating the possibility of an altered regulatory mechanism once cellular integrity had been destroyed. The fatty acid content of the complex lipids from *M. laidlawii* A revealed minute but consistent differences in the saturated fatty acid content of the glycolipid and phospholipid fractions. However, their over-all similarity in fatty acid composition indicated the use of a possible common precursor (2,3-diglycerides?) for the syntheses of gly co- and phospho-lipids (Kates, 1966).

Elaidic acid was incorporated into the membrane polar lipids to the same extent as its corresponding *cis* isomer, indicating that the enzymes catalysing the esterification of the fatty acids do not discriminate between *cis* and *trans* isomers in *Mycoplasma laidlawii* A. An equal incorporation of *cis* and *trans* isomers into the 2 position of phosphatidylcholine was described by Lands (1965) for mammalian cells. The fact

that most of a *trans* isomer added to the growth medium could be recovered unchanged from *Mycoplasma* sp. Y and *Mycoplasma* sp. KHS had been mentioned previously (Rodwell, 1968; Panos & Henrikson, 1969). Our findings that no isomerization of a *trans* octadecenoic acid occurred in *M. laidlawii* A confirms these earlier results. The use of radioactive geometrical octadecenoic acid isomers made the investigation of the lack of isomerization possible by a method with greater sensitivity than that of capillary gas chromatography.

Mycoplasmas are relatively resistant to osmotic lysis as compared to bacterial protoplasts (Razin & Argaman, 1963). Since almost all of the polar lipids reside in the mycoplasmal membrane (Smith, 1967), it is reasonable to assume that changes in membrane lipid composition may result in changes in the elasticity of this cellular component. The stability of myelin has been assumed to be due to its high saturated fatty acid content, allowing for a closer packing of its phospholipids (O'Brien, 1965). Likewise, Chapman, Owens & Walker (1966), using an artificial system, showed that phospholipids containing a *trans* acid formed a more condensed monolayer than those containing the corresponding *cis* derivative. Among the mycoplasmas it has been found that unsaturated long-chain fatty acids increase the resistance of *Mycoplasma laidlawii* B to osmotic lysis (Razin *et al.* 1966), presumably because of increased membrane elasticity. These authors inferred that unsaturated fatty acids may allow for a looser packing of the mycoplasmal membrane lipids resulting in greater elasticity. However, our studies showed that although osmotic fragility decreased with increasing concentrations of oleic acid in the growth medium, elaidic acid lowered fragility still further. The need for an optimal ratio of saturated:unsaturated fatty acids for both growth and osmotic stability of the mycoplasmal membrane has been suggested (Razin *et al.* 1966). The ability of elaidic acid to serve both the unsaturated and saturated fatty acid requirement for growth of a sterol-requiring mycoplasma (Rodwell, 1968) seemingly supports this assumption. Although no change in the saturated or unsaturated fatty acid content of the membrane polar lipids was noted when *M. laidlawii* A was grown with elaidic acid, as compared with oleate grown cells, the possibility still remains that this *trans* acid may be more suitable in maintaining an optimal lipoidal membrane orientation for better growth and decreased osmotic fragility.

We are grateful to Mrs O. Leon for technical assistance. Dr S. Rottem was a postdoctoral fellow in these laboratories from the Hebrew University—Hadassah Medical School, Jerusalem, Israel. These investigations were supported by grants AI-04543 and AI-04495 from the National Institute of Allergy and Infectious Diseases, U.S. Public Health Service, and a contract (NR 136-756) from the Office of Naval Research. One of us (C.P.) is a Senior Career Development Awardee (U.S.P.H. 5-K3-GM 15,531).

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J. gen. Microbiol. (1969), **59**, 329–336
Printed in Great Britain

Enhancement of Damage to *Escherichia coli* Strain B/R after Ultraviolet and γ Irradiation

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(*Accepted for publication 10 September 1969*)

SUMMARY

Death of *Escherichia coli* after exposure to ultraviolet (u.v.) radiation or to γ-rays was markedly increased when the bacteria were incubated on media containing chloramphenicol or puromycin. A similar effect occurred when an irradiated histidine-requiring strain of *E. coli* B/R was deprived of its requirement but not when several other amino acid auxotrophic mutants of *E. coli* B/R were similarly deprived. The extra killing may be associated with the synthesis of RNA rather than be a direct consequence of inhibition of protein synthesis.

INTRODUCTION

Death (i.e. the inability to form a visible colony) in *Escherichia coli* strain B/R after exposure to ultraviolet (u.v.) or ionizing radiation depends partly on the conditions in which the bacteria are treated after irradiation. For example, a marked decrease in survival occurs when the bacteria are incubated on a nutrient medium containing chloramphenicol for a short period after irradiation (Okagaki, 1960; Alper, 1963; Gillies & Brown, 1967). This effect is apparently caused by inhibition of protein synthesis in the irradiated bacteria; there is some evidence that it may result from loss of substances which are not resynthesized (Gillies & Brown, 1967). In the present paper data are presented on the effect on survival of irradiated *E. coli* B/R of puromycin, which inhibits protein synthesis (Yarmolinsky & de la Haba, 1959), of laevorphanol, which inhibits the uptake of [^{14}C]uracil into bacterial RNA (Simon, 1963), and of withholding a required amino acid from auxotrophic strains.

METHODS

Strains. The strain *Escherichia coli* B/R was that used previously by Alper & Gillies (1960). Mutants auxotrophic for amino acids were isolated from this strain by using methods described by Gillies (1961):

Mutant	Requirement	No. of mutants
E. coli B/R his$^-$	Histidine	3
E. coli B/R 7	Proline	1
E. coli B/R met$^-$	Methionine	1
E. coli B/R trp$^-$	Tryptophan	1

Stock cultures were maintained at 4° on slopes of Oxoid Blood Agar Base.

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Media. All strains were grown to stationary phase in Oxoid nutrient broth at 37°. For colony counting after irradiation *Escherichia coli* B/R was incubated at 37° on Oxoid Blood Agar Base or on a defined nutrient medium known as M 19 (Forage & Gillies, 1964). The irradiated auxotrophic mutants were incubated on medium M 19, from which the required amino acid was omitted as desired.

Various substances were added to the post-irradiation growth medium where indicated. Chloramphenicol was heat-sterilized and added at 5 μg./ml. Puromycin was sterilized by filtration through an Oxoid membrane filter (pore size not greater than 1 μ) and added at 40 μg./ml. Laevorphanol was heat-sterilized and used at 1·2 mg./ml.

Extraction and measurement of protein containing [^{14}C]leucine and of RNA containing [^{14}C]uracil. The rates of protein synthesis and of RNA synthesis were determined by measuring the incorporation of [^{14}C]leucine (purity, 99 %; specific activity, 6·6 mc/m-mole) and [^{14}C]uracil (purity, 99 %; specific activity, 40·6 mc/m-mole) into protein and RNA respectively. Both labelled compounds were obtained from the Radiochemical Centre, Amersham, Bucks. Radioactive samples were counted in a Tri-Carb liquid Scintillation Counting System, Model 314 Ex, set at 3·3 high voltage.

Bacteria were grown to stationary phase, centrifuged and resuspended in about 8 ml. M/15, pH 7, Na/K phosphate buffer at 10^8 organisms/ml., and 5 ml. of the irradiated suspension were inoculated into 50 ml. medium M 19 containing 0·2 μc of [^{14}C]leucine or 0·02 μc/ml. of [^{14}C]uracil. At intervals, 4 ml. samples were removed and pipetted into 0·5 ml. 50 % ice-cold trichloroacetic acid (TCA) and filtered through a Millipore membrane filter (GSWP 02500, pore size 0·22 μ, diameter 25 mm.). The precipitate was washed four times with 4 ml. 5 % ice-cold TCA, and the filter dried at 37° for 1 hr. When completely dry, the filter was placed in the bottom of a scintillation bottle and 4 ml. of scintillator (5 g. 2,5 diphenyloxazole and 0·3 g. 1,4 bis-2-(4-methyl-5-phenyl-oxazoly)-benzene dissolved in 1 l. scintillation-grade toluene) were added.

Irradiation experiments. Bacteria were washed three times and resuspended in phosphate buffer for irradiation.

Ultraviolet irradiation. Ten ml. suspension were pipetted into a 9 cm. glass Petri dish and placed under a water-jacketed Hanovia 15 W u.v. germicidal lamp emitting more than 90 % of its energy at 2537 Å. The energy emitted from the lamp was 286 ergs/mm.^{2}/min. at 42 cm. The dish was gently shaken during irradiation, after which the suspension was diluted and plated away from direct light.

γ-Irradiation. Bacteria were exposed to γ-irradiation from a ^{60}Co source (Vickers-Armstrong Mk IV Hotspot Irradiation Unit) in the vessel described by Forage & Gillies (1964), with oxygen-free nitrogen bubbled through the suspension. The dose rate in the ^{60}Co unit, as measured by ferrous sulphate dosimetry (Miller & Wilkinson, 1952), was 2·2 krads/min.

Dilutions of unirradiated and irradiated bacterial suspensions were dispensed with an Agla microsyringe (Burroughs Wellcome Co., London) on to cellophan carriers (2 × 1 in.; 5 × 2·5 cm.) lying on the surface of the growth medium previously warmed to 37°. Bacteria could thus be transferred rapidly from one medium to another (Alper & Gillies, 1958).

Although in most of the experiments the cellophan technique was used, the investigations with laevorphanol were made in liquid media. Unirradiated and irradiated bacteria were inoculated into bottles containing 19 ml. nutrient broth containing

laevorphanol or chloramphenicol, incubated and at intervals samples for the determination of the viable count were removed, diluted and plated on to nutrient agar. In both procedures the plates were incubated overnight at 37° and macro-colonies scored.

RESULTS

Relationship between protein synthesis and survival in Escherichia coli B/R

Puromycin and chloramphenicol. After exposure of the bacteria to u.v. radiation the two antibiotics caused additional killing to the same extent (Fig. 1). After exposure to a dose of γ-rays, which caused the same killing as u.v. radiation, both antibiotics decreased survival to about one-third. These results suggest that the decrease in survival may be caused by general inhibition of protein synthesis, with which puromycin and chloramphenicol interfere (Morris & Schweet, 1961; Jardetzky & Julian, 1964).

A difficulty in the use of supposedly specific inhibitors is that they may cause other unknown effects in the organism. To overcome this problem we have studied the effect on survival of withholding the required amino acid from irradiated auxotrophic mutants of *Escherichia coli* B/R. Under these conditions protein synthesis would be expected not to occur or to be strictly limited.

Histidine-requiring auxotrophs. When *Escherichia coli* B/R his⁻ was incubated on a defined nutrient medium lacking histidine immediately following γ-irradiation under anaerobic conditions, survival was decreased to about the same as by chloramphenicol (Fig. 2). A series of experiments in which different doses of γ-rays were used are summarized in the survival curves shown in Fig. 3. Qualitatively similar results were obtained for u.v.-irradiated bacteria although the enhancement of death was only half as much as that found when they were treated with chloramphenicol. Two other histidine-requiring mutants, isolated on different occasions, responded in similar fashion.

Proline-requiring auxotroph. After γ-irradiation under anaerobic conditions no decrease in survival was observed when *Escherichia coli* B/R 7 was incubated for a period on medium M 19 lacking proline. After u.v.-irradiation, however, there was an increase in colony-forming ability following incubation without proline (Fig. 4).

Methionine-requiring and tryptophan-requiring auxotrophs. There was a small but insignificant decrease in the survival when cells of *Escherichia coli* B/R met⁻ and *E. coli* B/R trp⁻ were incubated on media lacking their requirement after exposure to relatively large doses of γ-rays. There was no additional killing when the bacteria were similarly treated after u.v.-irradiation.

Protein synthesis in Escherichia coli B/R. It is difficult to account for the difference in survival of the histidine-requiring strain compared with the other amino acid auxotrophs. In each strain a decrease in survival might have been expected since the results with chloramphenicol and puromycin suggested that, when protein synthesis is inhibited in *E. coli* B/R, survival is decreased. However, because of possible variation in the sizes of amino acid pools and the rates at which the amino acids are utilized, cessation of protein synthesis may occur more rapidly in some auxotrophs than in others. Strains of *E.coli* B/R which had been exposed to u.v.-irradiation incorporated [^{14}C]leucine as shown in Fig. 5. Some uptake of [14C]leucine into protein took place in all the auxotrophic strains and also in bacteria treated with puromycin. Only in bacteria treated with chloramphenicol did synthesis of protein not occur. Qualitatively

similar results were found for γ-irradiated bacteria. Thus protein synthesis occurred in bacteria treated with puromycin or in *E. coli* B/R his⁻ under conditions which caused additional death, whilst no protein synthesis occurred in bacteria treated with chloramphenicol, a condition which also caused additional death. Protein was synthesized to a similar extent in all the auxotrophs deprived of their requirement, yet only in the histidine-requiring strain did an appreciable decrease in survival occur.

Since these results showed no obvious correlation between survival and protein

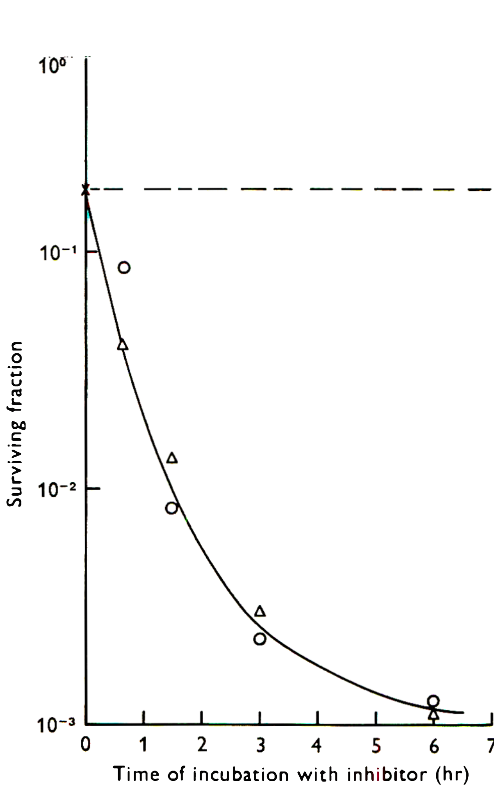

Fig. 1

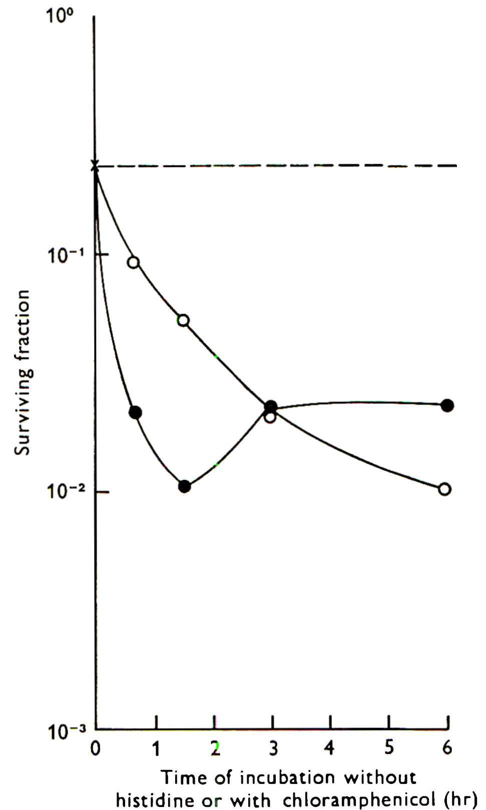

Fig. 2

Fig. 1. Survival of stationary phase *Escherichia coli* B/R after exposure to u.v. radiation (180 ergs/mm.^{2}). ×, Survival of bacteria incubated throughout on Oxoid Blood Agar Base. Survival of bacteria incubated on first medium for the intervals indicated before transfer to second medium:

	1st medium		2nd medium
△	Oxoid Blood Agar Base+puromycin	→	Oxoid Blood Agar Base
○	Oxoid Blood Agar Base+chloramphenicol	→	Oxoid Blood Agar Base

Fig. 2. Survival of stationary phase *Escherichia coli* strain B/R his⁻ after exposure to γ-rays (99 krads) under anaerobic conditions. ×, Survival of bacteria incubated throughout on medium M 19+histidine. Survival of bacteria incubated on first medium for the intervals indicated before transfer to second medium:

	1st medium		2nd medium
○	M 19	→	M 19+histidine
●	M 19+histidine+chloramphenicol	→	M 19+histidine

synthesis in *Escherichia coli* B/R, we examined the effect of these post-irradiation conditions on RNA synthesis in bacteria. Both puromycin and chloramphenicol, whilst inhibiting protein synthesis, lead to the accumulation of RNA in bacteria (Yarmolinsky & de la Haba, 1959; Nomura & Watson, 1959). It is possible, therefore, that additional death caused by post-irradiation treatments might be due to the accumulation of RNA in the irradiated bacteria.

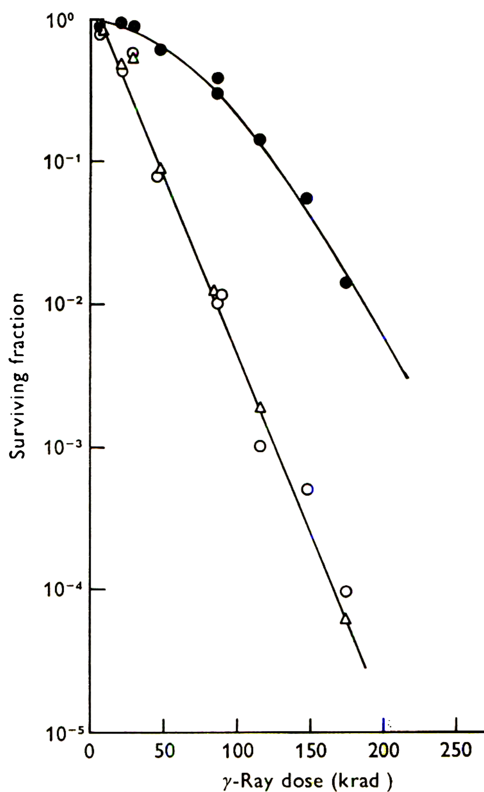

Fig. 3

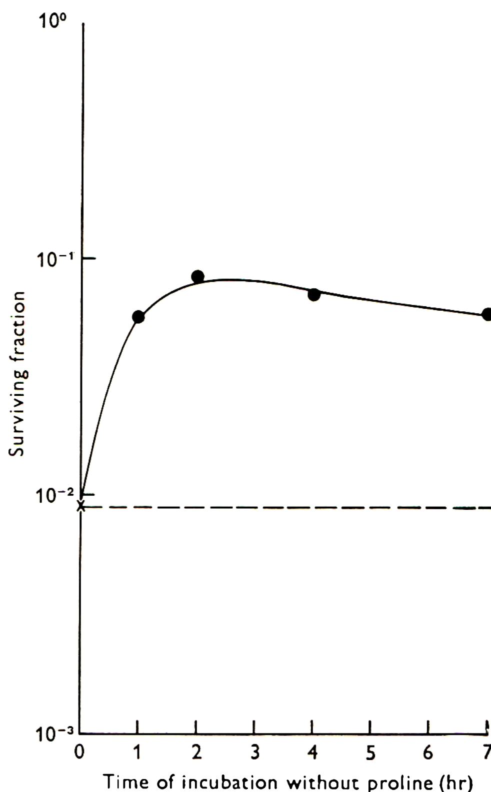

Fig. 4

Fig. 3. Survival of stationary phase *Escherichia coli* strain B/R his⁻ after exposure to γ-rays under anaerobic conditions. ●, Survival of bacteria incubated throughout on medium M 19 + histidine. Minimum degree of survival of bacteria incubated on first medium before transfer to second medium:

	1st medium	2nd medium
○	M 19	→ M 19 + histidine
△	M 19 + histidine + chloramphenicol	→ M 19 + histidine

Fig. 4. Survival of stationary phase *Escherichia coli* strain B/R 7 (proline-requiring) after exposure to u.v. radiation (215 ergs/mm.^{2}). ×, Survival of bacteria incubated throughout on medium M 19 + proline; ●, Survival of bacteria incubated on medium M 19 for the intervals indicated before transfer to medium M 19 + proline.

Relationship between RNA synthesis and survival in irradiated Escherichia coli B/R

Laevorphanol. This drug had no effect on the survival of irradiated *Escherichia coli* B/R nor did it alter the extent of chloramphenicol-induced death. Very limited uptake of [^{14}C]uracil into RNA took place initially in the u.v.-irradiated bacteria treated with

laevorphanol (Fig. 6). The incorporation of [^{14}C]leucine was commensurate with the uptake of labelled uracil into RNA. Thus in the presence of laevorphanol or chloramphenicol some RNA but no protein was synthesized.

Puromycin, chloramphenicol and amino-acid requiring auxotrophs. The uptake of [^{14}C]uracil into u.v.-irradiated *Escherichia coli* B/R in which protein synthesis was

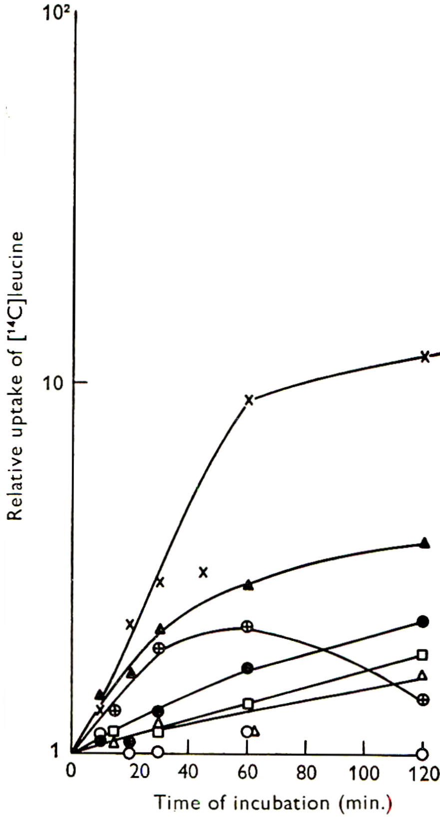

Fig. 5

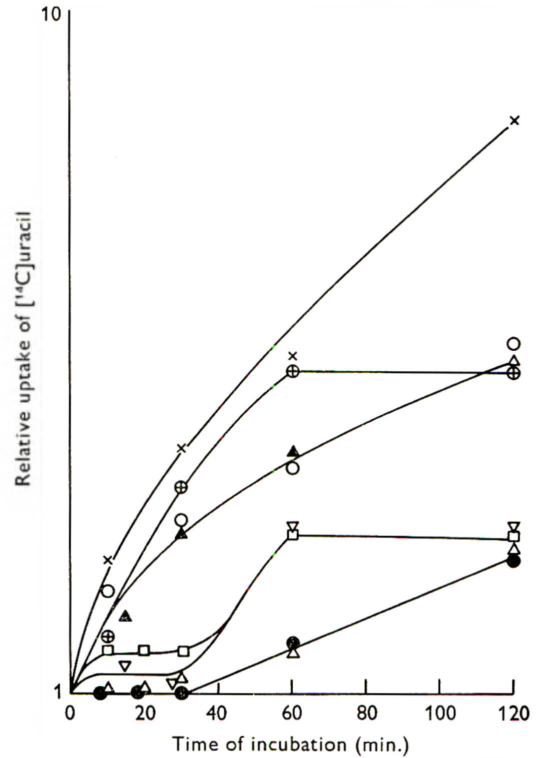

Fig. 6

Fig. 5. Incorporation of [^{14}C]leucine into the acid-insoluble fraction of *Escherichia coli* strain B/R after exposure to u.v. radiation (190 ergs/mm.^{2}). ×, *E. coli* B/R incubated in medium M 19; ○, *E. coli* B/R incubated in M 19+chloramphenicol; ⊕, *E. coli* B/R incubated in M 19+puromycin; ▲, *E. coli* B/R his$^-$ incubated in M 19 lacking histidine; ●, *E. coli* B/R 7 incubated in M 19 lacking proline; △, *E. coli* B/R trp$^-$ incubated in M 19 lacking tryptophan; □, *E. coli* B/R met$^-$ incubated in M 19 lacking methionine.

Fig. 6. Incorporation of [^{14}C]uracil into the acid-insoluble fraction of *Escherichia coli* strain B/R after exposure to u.v. radiation (190 ergs/mm.^{2}). ×, *E. coli* B/R incubated in medium M 19; ○, *E. coli* B/R incubated in M 19+chloramphenicol; ⊕, *E. coli* B/R incubated in M 19+puromycin; ▽, *E. coli* B/R incubated in M 19+laevorphanol; ▲, *E. coli* B/R his$^-$ incubated in M 19 lacking histidine; ●, *E. coli* B/R 7 incubated in M 19 lacking proline; △, *E. coli* B/R trp$^-$ incubated in M 19 lacking tryptophan; □, *E. coli* B/R met$^-$ incubated in M 19 lacking methionine.

inhibited or limited is also illustrated in Fig. 6. No uptake of radioactivity into RNA occurred in *E. coli* B/R 7 or *E. coli* B/R trp$^-$ during the first 30 min. of incubation in medium lacking proline or tryptophan respectively, and only a very small amount of

incorporation took place into *E. coli* B/R met⁻ during the same period in medium lacking methionine.

In contrast, when bacteria were treated with chloramphenicol or puromycin, or when histidine was withheld from the histidine-requiring auxotroph, RNA synthesis proceeded at a comparatively Although rapid rate. the rate and extent of synthesis was not so great as that in uninhibited cells, there was a 3- to 4-fold increase in the amount of RNA accumulating after 120 min. incubation under these conditions. Qualitatively similar results were found for γ-irradiated cells.

DISCUSSION

Incubation of *Escherichia coli* B/R with puromycin or chloramphenicol or incubation of *E. coli* B/R his⁻ without histidine all enhanced radiation killing. As some protein synthesis did occur in bacteria treated with puromycin or lacking histidine it would appear that the additional killing is not due entirely to abolition of protein synthesis immediately after irradiation. It is unlikely to result from insufficient synthesis of protein, perhaps required in an unknown repair process, since approximately the same limited amount of protein was synthesized in mutants requiring tryptophan, methionine or proline with no increase in killing. There is thus no direct correlation between the amount of protein synthesized and the survival of *E. coli* B/R after irradiation.

There was, however, a definite correlation between conditions which allowed RNA synthesis to begin immediately after irradiation and those which enhanced death. Incubation with puromycin or chloramphenicol, or incubation of *E. coli* B/R his⁻ in the absence of added histidine, all allowed some RNA synthesis while the viable count was decreasing. Incubation of the tryptophan, methionine and proline mutants without their requirement, or of *E. coli* B/R with laevorphanol, allowed little, if any, synthesis of RNA for at least 30 min. and also had no effect on survival.

In conditions where no RNA synthesis took place, protein was still synthesized but at less than normal rate. When synthesis of both protein and RNA was stopped, during incubation of the bacteria with chloramphenicol + laevorphanol, enhanced killing occurred. Thus, even in the absence of RNA synthesis increased killing did occur when protein synthesis was inhibited at the same time, indicating a requirement for new protein to prevent further death among organisms unable to synthesize RNA.

Although we are unable to suggest a specific mechanism to account for the enhanced killing of irradiated *Escherichia coli* B/R, it is tempting to speculate that the phenomenon may be associated with the synthesis of an RNA component which is toxic to the bacteria. It seems reasonable to suggest that an abnormal messenger RNA could be synthesized on a DNA template which had been altered by exposure to u.v. radiation or to ionizing radiation and that this damage would be expressed if the abnormal RNA were involved in the synthesis of new protein.

One of us (A.J.F.) is indebted to the Medical Research Council for a studentship.

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J. gen. Microbiol. (1969), **59**, 337–349
Printed in Great Britain

Incorporation of Thymine into Prototrophic and Thymine-dependent Mutants of *Bacillus anthracis*

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(*Accepted for publication 10 September 1969*)

SUMMARY

Mutants of *Bacillus anthracis* which were prototrophic in a defined medium below 32° required either thymidine (mutant *thy*) or low concentration of thymine (mutant *thytlr*) for growth at 37°. No thymidylate synthetase was produced by these mutants at 37°. Normal enzyme activity was found in bacteria grown at 25°. Absence of thymidylate synthetase activity in bacteria grown at 37° could not be accounted for either by a heat-sensitive enzyme protein or by an inhibitor of this enzyme activity.

The incorporation of thymine by prototrophic bacteria of *Bacillus anthracis* was negligible at either temperature. Mutant *thytlr* and its prototrophic revertant both readily took up thymine at 37° but not at 25°. Thymine uptake by both prototrophic and *thy* bacteria was markedly enhanced by the presence of any of the deoxyribonucleosides except thymidine. Moreover, thymidine antagonized thymine uptake. No thymidine phosphorylase activity was detected in *B. anthracis*, but phosphorolytic decomposition of deoxyuridine was found and that of deoxyadenosine seemed likely. The phosphorolytic reaction of deoxyuridine resulted in an accumulation of deoxyribose by *thytlr* bacteria, whilst both wild-type and *thy* bacteria catabolized this sugar. The secondary mutation in *thy* bacteria presumably resulted in a defect in synthesis of 1,5-phosphodeoxyribo mutase or deoxyribose 5-phosphate aldolase (E.C. 4.1.2.4). Although no thymidine phosphorylase was detected in *B. anthracis*, resting suspensions of this bacterium transferred the deoxyribosyl moiety from thymidine to thymine. This indicates the presence of the enzyme nucleoside purine (pyrimidine) deoxyribosyl transferase in *B. anthracis*. This particular enzyme may also be involved in the increased thymine uptake in the presence of different deoxyribonucleosides.

INTRODUCTION

The isolation of mutants from *Bacillus anthracis* unable to synthesize thymidine nucleotide has been reported (Ivánovics, 1964). These mutants required thymine for growth only when they were cultivated in a defined medium for *B. anthracis* at temperatures above 32°. To approach the uniform nomenclature in bacterial genetics recommended by Demerec *et al.* (1966), mutant VC⁻TdR⁻ isolated from prototrophic bacteria will be referred to as strain *thy*; one of its variants, a secondary mutant, isolate VC⁻T⁻, as strain *thytlr* (thymine low requirement). Strain *thy*, as noted previously, had about a 50-fold greater thymine requirement than did *thytlr*. Strain *thy* grew normally in the presence of thymidine (TdR), but this nucleoside inhibited the multiplaction of *thytlr* bacteria (Ivánovics, 1964). The mutants grown in the absence of pyrimidines at low termperature showed a normal replication of macromolecules and normal cell morphology. A shift up of the cultivation temperature to

37° in the absence of thymine resulted in an unbalanced synthesis of macromolecules followed by the death of both *thy* and *thytlr* bacteria (Ivánovics, 1963, 1964). Our earlier study of the thymine auxotrophs was restricted to nutritional experiments. The purpose of the present work was to elucidate the site and nature of the block in the thymine pathway, and to gain some insight into thymine incorporation by tracer experiments and enzymic studies.

METHODS

Bacteria. The original mutants of *Bacillus anthracis* produced an extracellular proteolytic enzyme during growth. Since a contamination of cell extracts with proteolytic enzyme would affect the enzymic activity under investigation, non-proteolytic mutants of *thy* and *thytlr* were isolated. Bacteria of these strains were treated with ethylmethanesulphonate and the surviving organisms cultivated until marked sporulation occurred. Spores thus obtained were spread on milk agar plates and incubated. Colonies which developed without a proteolytic halo were purified by streaking. Stable non-proteolytic derivatives of strains *thy* and *thytlr* were used in all experiments. A prototrophic revertant obtained from *thytlr*, referred to as strain *thytlrrev*, and a non-proteolytic wild-type strain NPA which came from the collection of Professor H. Smith (Birmingham) were also used in these studies. A non-capsulogenic derivative of NPA which will be referred to as strain P, was used throughout. Spores were prepared as described previously (Ivánovics, 1962), and a single batch of spores from each strain was used to provide inocula.

The strain *Escherichia coli* B was from the collection of this laboratory.

Media. A basal casein hydrolysate medium (BCM) was used for cultivating bacteria under defined conditions; it contained 1 μg. thiamine/ml. and was prepared as previously described (Ivánovics, Varga & Marjai, 1963). Milk agar plates were made from yeast-extract peptone agar by adding 40% (v/v) centrifuged milk.

Chemicals. DL-L-tetrahydrofolic acid was prepared as recommended by Kisliuk (1957). All other chemicals used were reagent grade purchased from different commercial firms.

TPB buffer. 0·05 M-tris+HCl buffer (pH 7·2) containing 0·1 mM-sodium phosphate.

Extinctions. The extinctions of the bacterial suspension was measured with a Spectromom 20, at 600 mμ. A spectrophotometer, MOM 201 (Budapest), served to measure extinction at different wavelengths.

Growth of organisms for enzyme assays. Bacteria of strains *thy* and *thytlr* were grown in BCM either in the absence of pyrimidine at 24° or in the presence of thymidine and thymine, each at 5 to 10 μg./ml. at 37°. Except where otherwise stated, no pyrimidine was present in the medium when strains P, *thytlrrev* or *E. coli* were grown.

To obtain a washed suspension, a culture in the exponential phase was centrifuged, the deposit washed with TPB, and suspended in TPB to give an extinction value of 0·7; this corresponded to 0·6 mg. dry bacteria/ml.

Crude bacterial extracts were made from 200 ml. cultures harvested in the middle log. phase as follows. The culture was rapidly cooled, centrifuged and the deposit washed with TPB. The packed bacteria were taken up in 5 ml. TPB and treated in a MSE Ultrasonic Power unit at 2° to 4° for 4 min. The homogenate was sharply centrifuged, its supernatant fluid removed and 50 mg./streptomycin sulphate added to it; the bulky precipitate caused by the streptomycin was removed by centrifugation.

Assay for thymidylate synthetase activity. A fraction, obtained from the crude bacterial extracts by precipitation with between 26% and 35% (w/v) ammonium sulphate, as recommended by Wahba & Friedkin (1962), was assayed for its activity by the Method III of these authors. The assay system was incubated at 25° for 60 min., and enzymic activity calculated from the values measured between 2 and 12 min. One enzyme unit is defined as the amount of enzyme catalysing the formation of 1 μmole thymidylate/hr/mg. protein.

Thymidine:orthophosphate deoxyribosyl transferase. (Thymidine phosphorylase, E.C. 2.4.2.4.) Three tests were used to demonstrate this enzyme activity of bacteria. (1) The crude bacterial extract was mixed with an equal volume of 0·04 M-cysteine hydrochloride solution and 0·1 M-potassium phosphate buffer (pH 7·2) and the activity measured by the method of Razzel & Khorana (1958). (2) A washed bacterial suspension containing 0·6 mg. dry bacteria/ml. was treated with toluene and assayed as recommended by Fangman & Novick (1966). (3) Either strain P or *thy* was grown in BCM containing 0·1 to 1·0 mM of thymidine at 37° overnight; a 1/20 dilution was made from this inoculum with the same medium and incubated until the extinction reached to 0·7 to 0·8. The supernatant fluid of the culture was sucked through a membrane filter (no. 4 Göttigen), the filtrate mixed with the same volume of 0·3 N-NaOH, and its extinction measured at 300 mμ. The conversion of thymidine to thymine was calculated from the increase of extinction as recommended by Hotchkiss (1948).

Tests for enzymic degradation of uridine deoxyriboside (UdR) and adenine deoxyriboside (AdR). To assay the enzyme capable of catalysing the phosphorolysis of UdR washed bacteria were made up in TPB with 1 mM-UdR. Assay systems were prepared in duplicate, with and without 1 mM sodium arsenate, both were incubated for 60 min. at 37°. When 1 μmole UdR is converted to uracil, there is an increase of 4·1 in extinction value at 290 mμ in the presence of 0·1 N-NaOH. This served to estimate the enzyme activity from the amount of uracil formed. When the enzyme activity was measured by the formation of sugar from the nucleoside, a sample from the assay system was deproteinized with $HClO_4$ (0·5 M), and the clear supernatant fluid heated in boiling water for 3 min. The amount of deoxyribose, both free and phosphorylated, was determined by the diphenylamine test (Burton, 1956). A sample was also taken from the assay system without arsenate, deproteinized by $Ba(OH)_2 + ZnSO_4$ (Manson & Lampen, 1951) to determine the free sugar.

The degradation of AdR could not be followed in a way similar to that for UdR. Therefore, a qualitative test was devised inolving thin-layer chromatography; this gave an indication of the decomposition of AdR by washed bacteria. The technique of chromatography was as described in the next paragraph.

Assay for nucleoside:purine (pyrimidine) deoxyribosyl-transferase (E.C. 2.4.2.6.). Different unlabelled deoxyribonucleosides were used as donors and tritiated thymine as acceptor for the deoxyribose moiety. The reagent to detect the enzyme activity contained 8 m-mole of the deoxynucleoside and 3 m-mole of tritiated thymine (2 μC/ml. activity) dissolved in TPB diluted 1/10 with water. Two ml. of the reagent was mixed with same volume of either washed bacterial suspension or the uncentrifuged ultrasonic fraction and incubated for 60 min. at 37°. The assay system was rapidly cooled and centrifuged; 12 mg. charcoal were added to 3 ml. of the supernatant fluid, and shaken for 15 min. The charcoal was sedimented, and eluted by a mixture of 8 vol. ethanol + 1 vol. water + 1 vol. ammonium hydroxide solution (0·88 sp.gr).

Elution done 5 times with 5 ml. portions of the mixture. Recovery of the radioactivity in the pooled eluate amounted to 95 %. With the aid of an intensive cool air stream, the volume of the pooled eluate was evaporated to about 1 ml. The exact volume if the concentrate was measured by weight on an analytical balance. Samples (20 to 40 μl.) of eluate were put on a thin layer of cellulose (MN 300) containing 3 % of $Al(OH)_3$, and run with a solvent of 15 ml. *n*-butanol + 60 ml. acetone + 20 ml. water + 5 ml. concentrated ammonium hydroxide. A very good resolution of thymine and thymidine was achieved in this way. R_F values: thymine = 0·55; thymidine = 0·66; UdR = 0·43; uracil = 0·32.

Material scraped off the spots of thymine and thymidine was suspended in scintillation liquid and its activity counted. Scintillation liquid was 0·5 g. POPOP and 5 g. PPO in 1 l. toluene. Radioactivity was counted in a TRI Carb Packard, Model 2101, scintillation spectrometer. The amount of radioactive thymine converted to thymidine was calculated from the percentage distribution of the label between the base and its nucleoside. Neglecting the loss of activity which occurred during isolation of products by charcoal treatment, enzyme activity was expressed by the amount of thymidine formed μmole/hr/ml. bacterial suspension.

Determination of bacterial multiplication. Bacillus anthracis is a chain former, therefore the bacterial protein content of the culture was determined instead of colony count. A 0·5 ml. sample was mixed with 0·5 ml. cold 10 % (w/v) TCA and centrifuged; the bacteria were washed twice with 5 % TCA, finally suspended in 0·5 ml. of 10 % NaOH, and kept overnight in cold. The protein content of the extract was determined by the method of Lowry *et al.* (1951). The number of generations was calculated by the formula ($\log A_t - \log A_0$) divided by 0·301. A is the amount of protein per ml. at 0 and t times, respectively.

Incorporation of thymine into bacteria. Tritiated thymine ($-CH_3$-^3H) was obtained from the Hungarian State Isotopic Laboratory. The stock solution contained 1·173 mg. thymine/ml. with an activity of 1·077 mC/ml. Labelled thymine was diluted with cold thymine as appropriate.

Tritiated thymine, 5 μg./ml. (1 μC/ml. activity), was added to bacteria growing exponentially in BCM. Samples of the bacterial culture (0·1 ml.) were taken at intervals and added to 5 ml. cold 5 % TCA containing 10 μg. thymine/ml., sucked through a membrane filter, followed by several washings of the filter with 2 ml. cold TCA and cold saline. Radioactivity of the dried filter was measured; counts given by the zero time sample was considered background activity and subtracted from the timed sample.

RESULTS

Heat-activated block in the de novo pathway of the chromosome replication

Previous studies (Ivánovics, 1964) suggested the existence of a block in thymidylate-5-phosphate synthesis which exerts its effect on the chromosome replication in the mutants only at an elevated temperature. Table 1 shows that no thymidylate synthetase activity was detected in preparations obtained from mutants grown at 37° with exogenous pyrimidine. However, this enzyme activity was found in preparations from the mutants cultivated at 25° and in the preparations obtained from prototrophic wild-type and revertant bacteria (strain *thytlrrev*) grown at 37°. The same enzyme activity was recovered from *Escherichia coli* cultivated at 37°.

Table 2 shows that there was no difference in the heat sensitivity of the enzymic activity of preparations obtained either from prototrophic or from thymine-dependent bacteria.

The lack of thymidylate synthetase activity in preparations of *thytlr* bacteria cultivated with exogenous thymine at 37° was not due to the presence of an inhibitor of this enzyme since, if this were the case, the inactive preparation of *thytlr* would inhibit the enzyme activity of a preparation from the prototrophic bacteria. No such an effect was observed.

Table 1. *Specific thymidylate synthetase activity of individual preparation obtained from bacteria grown at low and elevated temperature*

For growing strains *thy* and *thyltr* at 37°, casein hydrolysate medium BCM contained 5 μg./ml. thymidine and thymine, respectively. Zero value indicates that less than 0·01 enzyme unit was found in each of 2 to 4 individual preparations investigated

Bacterium	Specific enzyme activity: μmole/hr/mg. protein from bacteria grown at 25°	37°
B. anthracis, P	N.I.*	0·078
B. anthracis, *thy*	0·065 0·078	0 .
B. anthracis, *thyltr*	0·075 0·077 0·081	0 . .
B. anthracis, *thyltrrev*	N.I.	0·088
E. coli B	N.I.	0·079

* Not investigated.

Table 2. *Inactivation of thymidylate synthetase activity of preparations by heating at 50° in tris buffer-Na phosphate at pH 7·2*

Time of heating (min.)	Specific enzyme activity of preparation from strain *thyltr*	*P*
0	0·075	0·070
5	0·070	0·065
10	0·052	0·047
15	0·032	0·032
20	0·023	0·019
30	0·009	0·013

Incorporation of thymine by strains of Bacillus anthracis

A summary of results is shown in Table 3. Only an insignificant incorporation of label was detected in prototrophic bacteria at either temperature and in the mutants at 25°. On the other hand, a marked uptake of thymine by strains *thytlr* and *thytlrrev* was observed at 37°, although the revertant was a thymine-independent derivative of *thytlr* with thymidylate synthetase activity at 37°. A striking feature of the thymine

incorporation was its dependence on temperature. Apparently the intactness of the pathway leading to thymidylate synthetase was not the only factor which regulated thymine uptake. Strain *thy* could not be examined under identical condition since this strain does not grow at such a low concentration of thymine.

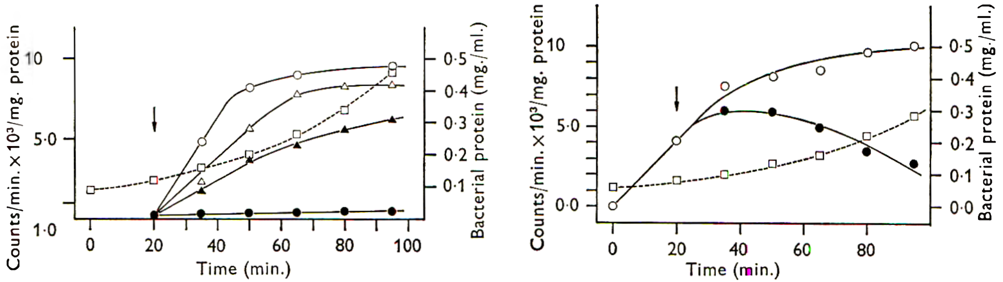

Fig. 1 Fig. 2

Fig. 1. The effect of different deoxyribonucleosides on the incorporation of tritiated thymine into prototrophic strain P of *Bacillus anthracis*. A culture growing exponentially in casein hydrolysate medium BCM containing 5 μg. labelled thymine/ml. with 1 μc activity/ml. was divided into four samples at 20 min.; they were supplemented with different nucleosides; 20 μg./ml. each. ●, Thymine only (T); ○, T+AdR; △, T+UdR; ▲, T+CdR; □, bacterial protein in culture (mg./ml.). The growth rates of the bacteria were identical in all cases; the generation time was 45 min.

Fig. 2. The incorporation of tritiated thymine into prototrophic *Bacillus anthracis* in the presence of AdR and TdR. 20 μg. AdR/ml. and 5 μg. tritiated thymine/ml. with 1 μc activity/ml. was added to an exponentially growing culture. The culture was divided in two parts at 20 min.; one of them was reincubated while the other was supplemented with 5 μg. thymidine/ml. before reincubation. Incorporation of thymine in systems: ○, T+AdR; ●, T+AdR+TdR; □, weight of bacterial protein.

Table 3. *Thymine incorporation into Bacillus anthracis at 25° and 37°*

Bacteria were grown overnight at 25° in casein hydrolysate medium BCM containing unlabelled thymine 5 μg./ml. The cultures were diluted 1/10 with the same medium and reincubated at 37° until their extinction reached to 0·2; this was considered 0 time when tritiated thymine 1 μc/ml. was added to the cultures. Reincubation was either 25° or 37°.

Time of cultivation (min.)	Strain *P*		Strain *thy*		Strain *thy/tr*		Strain *thy/tr rev*	
	25°	37°	25°	37°	25°	37°	25°	37°
	C.p.m./mg. bacterial protein							
25	350	351	350	N.I.*	290	3970	351	3840
75	480	490	720	N.I.	485	8542	515	10344

* Not investigated.

Exogenous thymine utilization by strains of *Bacillus anthracis* was increased in the presence of various deoxyribonucleosides. Figure 1 shows that prototrophic bacteria readily incorporated thymine in the presence of deoxyribonucleosides; AdR was most effective in this respect, UdR is next, whilst CdR exhibited only a moderate effect. By contrast, adenosine, deoxyribose and thymidine were unable to enhance the effect of label-incorporation into the acid-insoluble material of prototrophic bacteria. Moreover, thymidine inhibited thymine uptake into prototrophic bacteria even in the presence of AdR (Fig. 2).

The addition of thymidine to the culture stopped thymine uptake by prototrophic bacteria, although this did not affect the growth rate of the bacteria. This particular effect of thymidine was also valid for *thy* and *thytlr* bacteria when they were grown at 37°. Multiplication of *thy* bacteria was not supported by a low concentration of thymine (5 μg./ml.), therefore no label was taken up under this condition. Thymidine itself, on the other hand, gave a good growth of this organism. Deoxyribonucleosides (e.g. AdR) augmented the thymine uptake by *thy* bacteria, resulting in label incorporation and growth. Label incorporation, however, was blocked when thymidine was present in the culture (see Fig. 3).

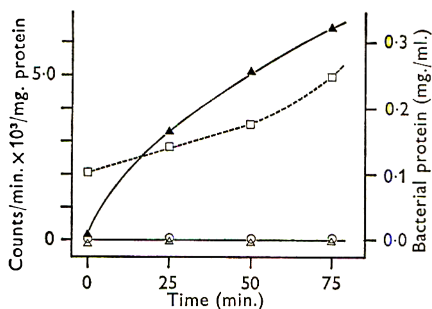

Fig. 3

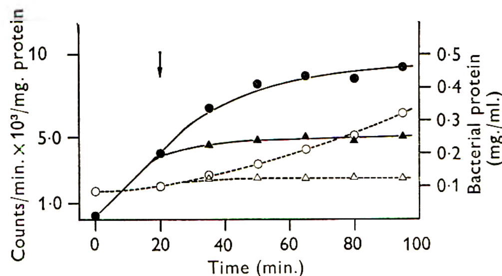

Fig. 4

Fig. 3. The effect of different deoxyribonucleosides on the incorporation of tritiated thymine into *Bacillus anthracis thy*. Bacteria were grown in casein hydrolysate medium BCM containing 5 μg. thymidine/ml. until an extinction value of 0·3. The bacteria were centrifuged down, washed with BCM medium and suspended in 40 ml. BCM medium which contained 5 μg. thymine/ml. with 1 μc activity/ml. The suspension was distributed in 10 ml. samples and the samples supplemented with nucleosides. The growths of individual cultures were identical. □, Protein content of the cultures; generation time of bacteria, 60 min. Incorporation of thymine: ▲, 20 μg. AdR/ml. + thymine; ○, 5 μg. thymidine/ml. + thymine; △, AdR + thymidine + thymine.

Fig. 4. The growth of strain *thyltr* of *Bacillus anthracis* and the incorporation of thymine in the presence and absence of thymidine. Bacteria were grown in casein hydrolysate medium BCM with 5 μg. unlabelled thymine/ml.; 1 μc tritiated thymine/ml. was added at 0 time and the culture reincubated. The culture was divided into equal volumes; at 20 min. thymidine 1 μg./ml. was added to one of the samples; the other served as control. Incorporation of thymine: ●, in absence of thymidine; ▲, in presence of thymidine; ○, bacterial protein without thymidine, and, △, with thymidine.

The inhibitory effect of thymidine on thymine uptake by *Bacillus anthracis* was most notably exhibited by mutant *thytlr*; addition of thymidine to the culture growing with thymine at 37° stopped both label-incorporation and growth (Fig. 4).

Examination of enzymes involved in metabolism of pyrimidine nucleosides

Deoxynucleoside phosphorylases. Phosphorolytic reactions are known to participate both in the degradation of nucleosides in the presence of orthophosphate yielding deoxyribose 1-phosphate and base, and in the formation of deoxyribonucleoside from sugar-phosphate with any of the nucleic acid bases. As far as we are aware, nothing is known of these enzymic reactions in *Bacillus anthracis*; therefore it appeared rewarding to extend our study in this direction. Crude extracts from bacteria grown in the presence of 10 to 20 μg. thymidine/ml. were assayed for thymidine phosphorylase

activity. Material from mutant strain *thytlr* could, of course, only be obtained from bacteria cultivated without this nucleoside. Values were found to be very low in all cases; specific enzyme activities of preparations varied between 0·2 and 0·3, whilst those from *Escherichia coli*, for comparison, showed a value of 5·2 to 5·8. When enzyme assays were made with washed bacterial suspensions, no thymidine phosphorylase activity was detected in the strains of *B. anthracis*. On the other hand, the *E. coli.* suspension was effective in degrading thymidine by phosphorolysis.

Growth of mutant *thy* was not sustained by a low concentration of thymine, although it grew well on thymidine concentrations as low as 2 to 5 μg./ml. Should this organism

Table 4. *Degradation of thymidine into thymine by exponentially growing bacteria*

Flasks with casein hydrolysate medium BCM containing either 0·1 or 1·0 μM of thymidine were inoculated and incubated overnight; dilutions of cultures were made with same medium and reincubated at 37° with shaking. The cultures were harvested at an extinction of 0·7 in Expt. 1, and at 0·5 in Expt. 2; they were membrane-filtered and mixed with an equal volume of 0·2 N-NaOH. Their extinctions were measured at 300 mμ; a non-inoculated medium served as a blank.

	TdR concentration in medium (μM)			
	Expt. 1:0·1		Expt. 2:1·0	
Bacterium	Change of extinction*	Thymine formed (μmole/ml.)	Change of extinction	Thymine formed (μmole/ml.)
B. anthracis, P	+0.008	0·004	+0·006	0·003
B. anthracis, *thy*	−0·040	Zero value	−0·003	Zero value
E. coli, B	+0·192	0·106	+0·260	0·144

* Multiplied by 2 when the amount of thymine was calculated. Minus values may derive from an exogenous product released by bacteria during growth.

have an effective thymidine phosphorylase activity, the bacteria would be expected soon to exhaust the low concentration of thymidine in the medium by splitting it to thymine which would not be utilizable. To test this, *Bacillus anthracis* and *Escherichia coli* were cultivated in the presence of thymidine and the conversion of thymidine to thymine determined. Thymidine phosphorylase is known to be an inducible enzyme in *E. coli*, therefore bacteria used as the inoculum were also grown in the presence of high concentrations of thymidine. Table 4 shows the results. *Escherichia coli* split thymidine considerably during its growth, whereas the strains of *B. anthracis* did not exhibit a significant degradation of it. The very low rate of thymidine degradation either with crude extracts of *B. anthracis* or with living bacteria did not furnish evidence for the existence of thymidine phosphorylase in this organism.

In contrast to these observations, a marked phosphorolysis of UdR was shown by *Bacillus anthracis*. As shown by Table 5, a resting bacterial suspension of prototrophic *B. anthracis* decomposed UdR, releasing free base but no sugar; however, both base and sugar accumulated in equimolar quantity in the assay system with arsenate. Mutant *thytlr* cleaved UdR by releasing both base and sugar in the absence of arsenate, which indicates that deoxyribose was not catabolysed by this mutant. An evidence for phosphorolytic cleavage of UdR was furnished by the identification of sugar-phosphate in the assay system of *thytlr* bacteria when a sample was taken from

it, treated with Zn + Ba reagent (Manson & Lampen, 1951), and the sugar determined in the supernatant fluid. In Expt. 1 (see Table 5) only 0·04 μmole diphenylamine-positive substance/hr/ml. was left in the supernatant fluid, the bulk of deoxyribose was removed by the Zn + Ba treatment which indicated its phosphorylated form.

Table 5. *Release of uracil and deoxyribose from UdR by resting bacterial suspensions of Bacillus anthracis*

Prototrophic bacteria were grown in the presence of UdR 10 μg./ml.; the concentration of UdR in assay system was 1 μM, incubation at 37° for 30 min. The growth of strain *thyltr* was sustained by thymine 5 μg./ml.

B. anthracis strain	Expt. no.	Presence of arsenate	Products in suspension (μmole/hr/ml.): Free uracil	Deoxyribose
Prototrophic	1	−	0·28	0·02
	2	−	0·29	0·02
	2	+	0·28	0·28
thyltr	1	−	0·31	0·28
	2	−	0·25	0·21

Table 6. *Deoxyribosyl transfer by resting bacteria in the presence of various deoxyribonucleosides as donors*

Assay system contained tritiated thymine 3 μmole/ml., various deoxyribonucleosides as donors, and the washed bacterial suspension. More than one experiment was made with each assay system; the results show the mean values.

	Amount of radioactive thymidine formed by washed bacterial suspension of:			
		B. anthracis strain:		
	E. coli	P	*thy*	*thyltr*
Donor	Thymidine formed (μmole/hr/ml.)			
UdR	1·73	1·09	1·23	1·36
TdR	0·78	0·50	0·81	0·64
CdR	1·23	1·05	1·14	1·41
AdR	< 0·03	< 0·03	< 0·03	< 0·03
GdR	< 0·03	< 0·03	< 0·03	< 0·03

The degradation of AdR by the prototrophic and mutant strains of *Bacillus anthracis* was also demonstrated. The presence of both adenine and hypoxanthine in the assay system indicated the cleavage of AdR and partial deamination of the base. No attempt was, however, made to show the presence of phosphate-sugar in the reaction mixture. The phosphorolysis of AdR by *B. anthracis* was therefore only presumable.

Nucleoside:purine (pyrimidine) deoxyribosyl transferase. In this enzymic reaction tritiated thymine served as an acceptor of the deoxyribose moiety which came from different purine or pyrimidine deoxyribonucleosides. The results are summarized in Table 6. In some experiments, bacterial suspensions were homogenized by ultrasonic treatment and used as an enzyme preparation in the presence of UdR as donor. None of the homogenates of the strains of *B. anthracis* was capable of catalysing the transfer reaction, in contrast to the test made with the *Escherichia coli* homogenate

where tritiated thymidine was formed; however, it was somewhat less (1·47 μmole/hr/ml.) than that formed by intact bacteria. In summary, an enzyme activity catalysing the transfer of the deoxyribosyl radical from pyrimidine deoxyribosides by the whole organisms of *B. anthracis* was found; however, neither AdR nor GdR were capable of taking part in this reaction.

DISCUSSION

With different species of Enterobacteriaceae, variable phenotypes of thymine mutants, depending on the quantitative requirements of thymine, have been described (Alikhanian *et al.* 1966; Brietman & Bradford, 1967*a*; Roepke, 1967). It has been considered that thymine mutants with low base requirements are actually double mutants (Harrison, 1965; Stacey & Simpson, 1965; Alikhanian *et al.* 1966; Breitman & Bradford, 1967*b*; Eisenstark, Eisenstark & Cunningham, 1968), just as it was concluded in the case of thymine-dependent mutants of *Bacillus anthracis* (Ivánovics, 1964). It has been pointed out by Breitman & Bradford (1967*b*) that the second mutation results in lack of deoxyribose aldolase activity which is associated with a low thymine requirement of the bacteria. The ability to incorporate thymine into DNA at a low exogenous concentration also may be caused by a mutation involving the loss of deoxyribomutase (Munch-Petersen, 1968; Barth, Beacham, Ahmad & Pritchard, 1967). The consequence of the second mutation is an intracellular accumulation of deoxyribose-1-phosphate which increases the utilization of exogenous thymine (Kammen, 1967; Breitman & Bradford, 1967*b*). It has been suggested that genes controlling the catabolism of deoxyribonucleosides constitute an operon (Barth *et al.* 1968). This operon controls the production of thymidine phosphorylase, purine nucleoside phosphorylase (E.C. 2.4.2.1), deoxyribomutase and deoxyriboaldolase (E.C. 4.1.2.4) which are capable of catalysing either the biosynthesis or breakdown of deoxyribonucleosides. The operon conception concerning deoxyribose catabolism has been advanced and the *deo* designation for this operon was suggested to indicate the genes determining enzymes necessary for growth on deoxythymidine or deoxyuridine as sole carbon source (Lomax & Greenberg, 1968). Any of the mutations leading to a low thymine requirement in strains lacking thymidylate synthetase are also associated with a phenotype characterized by a sensitivity of bacterial growth to thymidine or to other deoxyribonucleosides (Alikhanian *et al.* 1966; Eisenstark *et al.* 1968). As shown by Barth *et al.* (1967), thymine mutants of *Salmonella typhimurium* highly sensitive to thymidine are lacking in deoxyriboaldolase, and the less sensitive mutants lack deoxyribomutase.

In the present work the first mutation in *Bacillus anthracis* affected the capacity of bacteria to produce thymidylate synthetase at 37°. Similarly, this enzyme was detected in the extracts of *thy tlr B. anthracis thy tlr* bacteria grown with exogenous thymine at 37°; however, effective enzyme activity was found in extracts obtained from these mutants when grown at 25°. Apparently the heat sensitivity of DNA replication in the mutants could not be accounted for either by an alteration in the structure of enzyme protein which renders the enzyme extremely heat labile, or by an inhibitor of the thymidylate synthetase activity. It seems that the synthesis of this enzyme itself is a heat-labile process. Heat-sensitive thymine mutants are not uncommon among bacteria. Alikhanian *et al.* (1966) found that half their thymine mutants isolated from *Escherichia coli* K 12 mapping at the site of *thy* locus were temperature-

sensitive. We would stress that the majority of thymine auxotrophs of *B. anthracis* isolated by using ethylmethanesulphonate as mutagen, needed thymidine only at temperatures higher than 32° (Ivánovics *et al.* 1963), like the strain *thy* used in this study.

With the exception of thymidine, all the deoxyribonucleosides tested enhanced the incorporation of tritiated thymine into acid-insoluble material of *Bacillus anthracis*. Moreover, thymidine totally blocked thymine utilization by prototrophic bacteria and thymine auxotrophs. The rate of bacterial multiplication, however, appeared to be independent of exogenous thymine uptake except when thymidine was added to cultures of mutant *thy tlr* growing in the presence of thymine; in this case both thymine incorporation and growth were arrested.

A striking feature of the enzymic make-up of *Bacillus anthracis* is the lack of thymidine phosphorylase activity. On the other hand, definite evidence was found that UdR was decomposed by phosphorolysis by protrophic and by *thy tlr* bacteria. The absence of deoxyribose from the assay system of prototrophic bacteria indicated that deoxyribose-1-phosphate formed by the phosphorolytic enzyme was metabolized. Mutant *thy tlr* was found, in contrast, to be incapable of metabolizing the sugar-phosphate formed by the phosphorolysis of UdR, indicating that either deoxyribose mutase or aldolase is missing in this strain. Although no detailed studies were made to analyse the decomposition of AdR, preliminary observations pointed to a probable phosphorolysis by *B. anthracis*.

Cultivation experiments with mutant *thy* suggested the presence of trans-*N*-deoxyribosylase in *Bacillus anthracis* (Ivánovics, 1964). Since no phosphorylitic breakdown of thymidine could be detected in *B. anthracis*, the conversion of thymine to thymidine in the presence of deoxyribonucleosides could only be related to a deoxyribosyl transfer reaction. Indeed, tritiated thymine was converted into thymidine by washed bacterial suspensions in presence of unlabelled pyrimidine deoxyribonucleosides, whereas purine deoxyribonucleosides did not function as substrates in a transfer reaction. The deoxyribosyl transfer reaction appears to be specific as it is known under certain experimental conditions (Roush & Betz, 1958) and assay systems (Zimmerman & Seidenberg, 1964). Although evidence was secured for the existence of deoxyribosyl transfer reaction in *B. anthracis*, further studies are needed to identify the presence of nucleoside: purine pyrimidine deoxyribosyltransferase in the organism.

It is hard to give a satisfactory explanation of increased thymine uptake by *Bacillus anthracis* in the presence of UdR and AdR, although phosphorolytic breakdown of these deoxyribonucleosides was either proved or rendered presumable. Secondary mutation in *B. anthracis* resulting in a block in the catabolism of deoxyribose which in turn increased the availability of intracellular deoxyribose donors rendered the bacteria capable of utilizing low concentrations of exogenous thymine as is known for some Enterobacteriaceae. Nevertheless, there are several points which do not allow a similar conclusion which is valid for Enterobacteriaceae as to the incorporation of thymine. These contradictory facts are as follows. (1) The lack of thymidine phosphorylase in *B. anthracis* does not allow the conversion of the accumulated deoxyribose-1-phosphate plus thymine to thymidine. (2) Exogenous thymidine prevents thymine uptake by either prototrophic or mutant bacteria, although thymidine itself is a deoxyribose donor in transfer reaction in resting bacteria. (3) In spite of that AdR did not serve as a deoxyribosyl donor in transfer reaction in resting bacteria, AdR

highly increased the thymine incorporation in growing bacteria. The facts listed above obscure the mechanism of thymine incorporation in *B. anthracis*, therefore further data are needed for its complete explanation.

The authors are indebted to Dr J. McGeachie (Royal Infirmary, Glasgow) for help in preparing the manuscript.

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J. gen. Microbiol. (1969), **59**, 351–358
Printed in Great Britain

The Respiratory Metabolism of *Acanthamoeba rhysodes* During Encystation

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(*Accepted for publication 12 September 1969*)

SUMMARY

The endogenous respiration of vegetative amoebae, harvested during active growth, was similar in both the culture medium and in the high salt medium used to induce the encystation of starved amoebae. The respiration of starved amoebae and early stages of encystation was suppressed in high salt medium and stimulated when returned to the culture medium. A transient increase in respiration was observed early in encystation, while the respiration of later encystation stages decreased to the low level observed in mature cysts.

The respiratory metabolism of mitochondria isolated from encysting amoebae was similar to that of mitochondria from vegetative amoebae while cyst mitochondria exhibited little respiration. The catalase and acid phosphatase activities of amoebae increased during starvation and encystation.

INTRODUCTION

Hartmannellid amoebae, currently classified in the genus *Acanthamoeba* (Page, 1967), are obligate aerobes during vegetative growth (Band, 1959) and during encystation (Band, 1963). The mature cyst, however, exhibits little respiratory activity (Reich, 1948) and survives under anaerobic conditions (Band, 1963). Electron microscope observations of the cyst (Bauer, 1967; Bowers & Korn, 1968, 1969; Vickerman, 1962) reveal the appearance of dense areas in the mitochondria and a possible alteration in the structure of the mitochondrial cristae. These observations imply that reduced respiratory activity may be due to changes in the metabolism of the mitochondria.

This paper reports observations on the respiration of whole amoebae and isolated mitochondria during encystation. Acid phosphatase and catalase were used to estimate the activity of lysosomes and peroxisomes in amoebae and in mitochondrial fractions during encystation.

METHODS

Encystation. The methods of cultivation and the induction of encystation have been described previously (Band, 1959, 1963) for *Acanthamoeba* (*Hartmannella*) *rhysodes*. For the purpose of clarity, some of the observations from Band (1963) and related papers will be given below.

Amoebae were starved for 48 hr in a filtered peptone medium which contained 10 times less glucose than would support growth (PPGF). This procedure was a modification of the original method (Band, 1963) where the PPGF contained as much glucose as the culture medium (0·1 M). After 48 hr in PPGF, starved amoebae appeared

normal but growth did not occur. Incubation for 48 hr in PPGF, or in a defined medium (Band, 1962) with 10 times less glucose, was required for optimum encystation of amoebae when they were placed in a high salt medium (HSM, 0·25 M-NaCl, 3·2 mM-$MgCl_2 . H_2O$, 0·36 mM-$CaCl_2$). A misinterpretation by Griffiths & Hughes (1968) indicated that the original method (Band, 1963) gave a very low per cent encystation. Although incubation of vegetative amoebae in HSM caused approximately 50 % encystation (Band, 1963), incubation of PPGF-starved amoebae in HSM was reported to induce approximately 100 % encystation.

Before 24 hr, amoebae in the HSM resembled vegetative amoebae, but did not adhere to a glass surface and exhibited a net surface charge density similar to that of mature cysts rather than vegetative amoebae (Band & Irvine, 1965). During this time addition of a carbon source inhibited encystation (Band, 1963). After 24 hr the amoebae appeared round, exhibited an Alcian blue-positive surface coat and addition of a carbon source did not inhibit encystation. Cysts started to appear after 32 hr in HSM, although the process was not completed until later. Aeration was required throughout encystation.

Respiration. Amoebae were washed once and suspended in fresh medium when the respiration medium was the same as the incubation medium, or washed twice when the respiration medium was different from the incubation medium. Respiration was determined at 30° using a Clark electrode (Estabrook, 1967) with 1·5 to 1·75 × 10^6 amoebae/ml. respiration medium, or 1·5 to 2 mg. mitochondrial protein.

Fractionation. Although vegetative amoebae could be ruptured by relatively gentle means (i.e. a Teflon pestle of the Potter–Elvehjem type homogenizer) to obtain mitochondria, encysting amoebae became progressively more resistant to various disruption methods. The Teflon pestle did not rupture encysting amoebae or cysts in the HSM. Brief ultrasonic treatment or passage through a hand-operated mill (A. H. Thomas Co.) ruptured vegetative amoebae and encysting amoebae but not cysts. Cysts were ruptured with a ball mill, prolonged ultrasonic treatment or the French press (American Instrument Co.); the French press was used in the present study at 20,000 lbs./in^2. Disruption of vegetative amoebae with the French press yielded metabolically active mitochondria at the same pressure so homogenization with the French press was not deleterious to cyst mitochondria. The differential response to methods of homogenization made it possible to remove defective forms at various stages of encystation; vegetative and dead amoebae could be removed from encysting amoebae with the Teflon pestle and encysting amoebae could be removed from cysts with the hand-operated mill.

The mitochondrial fraction was isolated at 4°, in general, according to the method of Schneider & Hogeboom (1950), except that the isolation and wash medium was 0·25 M-sucrose containing 0·001 M-ethylenediaminetetraacetic acid and 0·5 % (w/v) bovine serum albumin (crystallized and lyophilized, Sigma Chemical Co.), pH 7·4. Respirometric measurements were made after adding 1·19 μmole Na adenosine-5′-diphosphate (ADP) (Sigman Chemical Co., Grade I) to 4 ml. of a respiration medium which contained 0·225 M-sucrose, 10 mM-KH_2PO_4, 5 mM-$MgCl_2$, 20 mM-KCl, pH 7·4 at 30°; 13 μmole Na succinate or K α-ketoglutarate or 80 μmole each of Na pyruvate and Na malate.

Acid phosphatase determinations were based on the hydrolysis of *p*-nitrophenyl phosphate (Sigma Chemical Co.) in the presence of 0·1 % (v/v) Triton X-100

(Wattiaux & de Duve, 1956) and 0·022 M-Na citrate, pH 4·3, expressed as μmole *p*-nitrophenol liberated/mg. protein/min. at 37°. Catalase activity was determined by the change in absorption at 240 mμ (Beers & Sizer, 1952) of a 0·02 M-H_2O_2 solution containing 0·017 M-$KH_2PO_4 + Na_2HPO_4$ buffer, pH 7·0, and the homogenate or mitochondrial fraction and expressed as μmole H_2O_2 destroyed/mg. protein/min. at 24°.

Protein concentration was determined by the Folin–Ciocalteau method modified by Miller (1959).

RESULTS

Respiration of whole amoebae. The respiration of starved amoebae declined during encystation in HSM (Table 1); HSM had no effect on the respiration of vegetative amoebae. The first effect of HSM on respiration was observed after the 48 hr starvation period in PPGF which preceded incubation in HSM to induce encystation.

Table 1. *Respiration of Acanthamoeba rhysodes*

The average of *n* determinations (± S.D.) in nmoles O_2/min./10^6 amoebae, with an oxygen cathode at 30°.

Respiration medium	Source of amoebae						
			Amoebae during encystation (hr)†				
	Vegetative amoebae	Starved amoebae*	1 to 2	3 to 4*	5 to 6	24	Cysts
Culture	12·3 ± 1·5 *n* = 4	12 ± 2 *n* = 7	13·7 ± 3·5 *n* = 12	15·5 ± 3·6 *n* = 16	13·1 ± 3·2 *n* = 8	5·9 ± 2 *n* = 9	2·5 ± 1·2 *n* = 4
HSM	12 ± 1·8 *n* = 4	6·9 ± 1 *n* = 10	7 ± 2·5 *n* = 10	8·9 ± 2·7 *n* = 10	8·3 ± 3 *n* = 6	5 ± 1·3 *n* = 6	1·8 ± 0·5 *n* = 4
LSM‡	10 ± 1·2 *n* = 3	10 ± 2 *n* = 3	—	—	—	—	—
Culture§	9·1	14	—	18	—	4	2
Culture§ + KCN, 0 time	9·8	22	—	28	—	7	2·8
Culture§ + KCN, 30 min.	9·1	15	—	24	—	6	1·8

* Analysis of variance for starved amoebae and 3 to 4 hr encysting amoebae indicated significant differences in respiration between these at the 5 % level:

	Culture d.f.	Culture Mean squares	HSM d.f.	HSM Mean squares
Between	1	60	1	20
Within	21	10·5	18	4·2

† In high salt medium (HSM).

‡ Respiration in low salt medium (0·05 M-NaCl with the divalent ions of HSM).

§ Matched sets of single observations in which respiration was determined before, immediately after and 30 min. after the addition of 8 μmole KCN to 4 ml. of amoebae.

During the first few hours in HSM before round forms appeared (24 hr), respiration was stimulated again if encysting amoebae were returned to the culture medium (PPG); this effect diminished later. The effect of HSM on respiration and its reversal in PPG was osmotic in origin. Incubation in a low salt medium (HSM with NaCl lowered to 0·05 M) did not suppress the respiration of vegetative amoebae from PPG and had no effect on the respiration of starved amoebae from PPGF (Table 1).

Table 2. *Respiratory activity of mitochondria isolated from Acanthamoeba rhysodes*

Oxygen uptake (Rate), measured with an oxygen cathode, was expressed as an average of n determinations in mμmole O_2/min./mg. mitochondrial protein, after the addition of adenosine-5′-diphosphate (ADP). The ADP:O ratio was the ratio of O_2 utilized to the amount of ADP phosphorylated; the respiratory control ratio (RCR) was the respiratory rate with ADP relative to the respiratory rate following the expenditure of ADP.

	Source of mitochondria									
	Vegetative amoebae			Starved amoebae			Encysting amoebae (24 hr in HSM)			Cysts*
Substrate	Rate	ADP/O	RCR	Rate	ADP/O	RCR	Rate	ADP/O	RCR	Rate
Succinate	136 ± 8·5† n = 2	2·0 ± 0·1	2·0 ± 0·3	—	—	—	—	—	—	—
	60·8 ± 7·4 n = 9	1·5 ± 0·2	1·4 ± 0·2	68 ± 24 n = 4‡	1·6 ± 0·4	1·5 ± 0·2	63 ± 5·6 n = 7	1·8 ± 0·2	1·8 ± 0·4	6·9 ± 3·4 n = 3
α-keto-glutarate	51 ± 17† n = 5	2·7 ± 0·2	1·9 ± 0·8	—	—	—	—	—	—	—
	24·6 ± 8 n = 3	2·2 ± 0·2	1·9 ± 0·2	45 n = 1	3·6	3·1	41 ± 7 n = 4	3·1 ± 0·3	2·1 ± 0·2	8·1 ± 5·0 n = 2
Pyruvate and malate	51·4 ± 4 n = 5	2·5 ± 0·5	1·8 ± 0·4	68 ± 24 n = 3	2·6 ± 0·4	2·2 ± 0·4	54·5 ± 7 n = 11	3·2 ± 0·3	2·3 ± 0·7	8·7 ± 0·68 n = 3

* ADP did not stimulate the respiration of mitochondria from cysts.

† Homogenized with a Teflon pestle; other data from French press homogenization.

‡ ADP did not stimulate the respiration of mitochondria from five additional isolations.

The respiration of early encystation stages increased during the first 1 to 6 hr in HSM and then decreased to the low level exhibited by cysts (Table 1). This transient increase was observed with either HSM or PPG as the respiration medium and was statistically significant at the 95 % level when the respiration of the PPGF-starved amoebae was compared with that of encysting amoebae after 3 to 4 hr in HSM.

Cyanide (8 μmole KCN/4 ml. of amoebae) caused an initial increase in respiration; after 30 min. respiration returned nearly to the original rate (Table 1). This was true for vegetative amoebae, starved amoebae and the various stages of encystation and cysts.

Activity of mitochondria. Vegetative mitochondria obtained using the French press were not as active as vegetative mitochondria isolated by Teflon homogenization. This difference was more apparent for the rate of respiration than for phosphorylation. The respiratory activity of mitochondria isolated from PPGF-starved amoebae was very erratic (Table 2). Many preparations of mitochondria from PPGF-starved amoebae did not exhibit respiratory control in that ADP did not stimulate respiration. The respiration rate of mitochondria from PPGF-starved amoebae was greater than that from either vegetative mitochondria or encysting amoebae.

Table 3. *Acid phosphatase and catalase activity of Acanthamoeba rhysodes*

Acid phosphatase activity expressed as μmole of *p*-nitrophenol liberated/min./mg. protein at 37°; catalase activity expressed as μmole H_2O_2 destroyed/min./mg. protein at 24°. Assay procedures in Methods section; data given as an average of *n* determinations.

Acid phosphatase	Vegetative amoebae	Starved amoebae	Encysting amoebae*	Cysts
Whole amoeba homogenate	0·6 ± 0·08 *n* = 4	0·81 ± 0·17 *n* = 2	1·0 ± 0·1 *n* = 2	0·98 ± 0·23 *n* = 3
Mitochondrial fraction	0·28 ± 0·04 *n* = 2	0·145 ± 0·01 *n* = 2	0·3 ± 0·09 *n* = 2	0·38 ± 0·11 *n* = 2
Catalase				
Whole amoeba homogenate	9·1 ± 4 *n* = 4	28·8 ± 8 *n* = 3	17·5 ± 4 *n* = 2	18·3 ± 1 *n* = 2
Mitochondrial fraction	2·7 ± 1·9 *n* = 2	17·6 ± 1·6 *n* = 2	6 ± 1·4 *n* = 2	4·3 ± 4·2 *n* = 2

* In HSM for 24 hr.

Mitochondria from encysting amoebae, after 24 hr in HSM, had respiration rates, ADP/O ratios and respiratory control similar to or greater than those of mitochondria from vegetative amoebae homogenized with the French press. Mitochondria isolated from cysts were almost inactive.

Respiratory metabolism was not changed when cytochrome c (0·04 μmole) was added to mitochondria oxidizing succinate. When cytochrome c (0·04 μmole) and nicotinamide adenine dinucleotide (4 μmole) were added together to mitochondria from PPGF-starved amoebae oxidizing pyruvate and malate, respiration was stimulated and respiratory control was absent. These cofactors had no effect on mitochondria isolated from vegetative amoebae, encysting amoebae or cysts.

Acid phosphatase and catalase. Acid phosphatase activity in whole amoeba homogenates increased during starvation and encystation but the specific activity of acid phosphatase in the mitochondrial fractions was less than the activity in the whole

amoeba homogenates (Table 3). Catalase activity in whole amoeba homogenates from PPGF-starved amoebae, encysting amoebae and cysts was greater than in homogenates of vegetative amoebae (Table 3); PPGF-starved amoebae were the most active. The specific activity of catalase in the mitochondrial fractions was less than the activity in whole amoeba homogenates. The mitochondrial fraction from PPGF-starved amoebae contained much greater catalase activity, however, in proportion to the activity of the whole amoeba homogenate, than did the other mitochondrial fractions.

DISCUSSION

Endogenous respiration. The observations presented in this study on the respiration of vegetative amoebae from growing cultures were similar to those reported by Reich (1948) on *Acanthamoeba* (*Mayorella*) *palestinensis* and by Neff, Neff & Taylor (1958) on Neff's *Acanthamoeba* sp. However, *A. rhysodes* amoebae from actively growing cultures respired at a similar rate over a wide range of osmotic pressure (0·15 to 0·5 osmolar in culture medium vs. HSM) while Neff *et al.* (1958) reported decreased respiration at elevated salt concentrations.

The sensitivity of endogenous respiration to osmotic pressure following starvation may have been the basis for using starvation to induce optimum encystation in HSM (modification from Band, 1963). The endogenous respiration of starved amoebae and early stages of encystation was reversibly suppressed in HSM. The osmotically sensitive respiratory pathway appeared to be progressively inactivated later in encystation.

The transient increase in respiration, observed during the early stages of encystation, did not appear to be osmotically sensitive since the increase was similar in HSM and in culture medium. The total endogenous respiration of mature cysts was comparable to this increase alone and was not osmotically sensitive. Thus, the transient increase in respiration may be due to the appearance of a new respiratory pathway which is responsible for the respiration of mature cysts.

Mitochondrial respiration. Mitochondria isolated from encysting amoebae did not reflect in their metabolism the changes in endogenous respiration of whole, encysting amoebae. Although the respiration of encysting amoebae after 24 hr in HSM was not stimulated on return to a lower osmotic pressure, mitochondria isolated from them behaved similarly to vegetative mitochondria.

Electron microscope observations on the cysts of related hartmannellid amoebae (Bauer, 1967; Bowers & Korn, 1969; Vickerman, 1962) indicated that mitochondria were retained by the cyst in a modified form. This is consistent with the present observation that mitochondria from mature cysts respired poorly and failed to exhibit respiratory control. Electron microscope observations also revealed autolysosomes and pockets of cytoplasmic debris trapped inside the cyst walls. If inert mitochondria were present in the debris, these could have been present in the mitochondrial fraction from cysts and would have distorted the data on mitochondrial metabolism. Estimates of the number of mitochondria present in the cyst's cytoplasm in a related hartmannellid amoeba (Bowers & Korn, 1969) suggested little change in mitochondrial number from vegetative amoebae. This would favour the possibility that most of the mitochondria from the cyst in the present work came directly from the cytoplasm.

The differential centrifugation methods used to isolate mitochondria might also harvest a portion of the amoeba's lysosomes and peroxisomes. These organelles have

been detected in hartmannellid amoebae by electron microscopy (Bowers & Korn, 1968, 1969), by histochemistry (Müller & Møller, 1967) and their presence suggested by chemical analysis of the culture medium (Lasman, 1967). Their presence in the mitochondrial fraction could be deleterious to the activity of isolated mitochondria and invalidate comparisons of mitochondrial activity at various stages of encystation. The activities of acid phosphatase and catalase, present in lysosomes and peroxisomes respectively (e.g. Müller, Hogg & de Duve, 1968), were similar in all fractions except the catalase activity of the mitochondrial fraction from starved amoebae. Since phosphorylation by this fraction was variable, it might have been contaminated by peroxisomes.

Effect of cyanide on respiration. The failure to inhibit respiration of vegetative, starved and encysting amoebae with cyanide did not correspond to the reports of others. At similar cyanide concentrations, vegetative amoebae of both *Acanthamoeba palestinensis* (Reich, 1955) and Neff's *Acanthamoeba* sp. (Neff *et al.* 1958) were inhibited in their respiration. A strict comparison of these two previous reports with observations presented in this paper was not possible as both authors used different methods and different specific amoebae. Since Reich (1955) observed a slight increase in respiration with cyanide in the presence of glucose over control amoebae without cyanide, he proposed that cyanide stimulated a respiratory pathway that utilized glucose. Since the culture conditions used by Reich (1955) and those used in the present paper were not the same, a difference in endogenous reserves may have existed. If this were true, then the amoebae in the present paper may have reacted to cyanide in a manner comparable to Reich's amoebae in the presence of glucose.

This investigation was supported by a U.S. Public Health Service Research Grant (AI-06117-06) from the National Institute of Allergy and Infectious Diseases.

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J. gen. Microbiol. (1969), **59**, 359–367
With 1 *plate*
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Genetic Evidence for Hybridization in an Interspecific Cross in the Genus *Sordaria*

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(*Accepted for publication 15 September 1969*)

SUMMARY

When the two heterothallic species *Sordaria heterothallis* and *S. thermophila* are crossed, two types of viable ascospores are recovered. The first, the mature black ascospore, is produced with a mature eight-spored ascus formed from an intraspecific fusion of nuclei of the same mating type. The second, the non-pigmented germinable ascospore, is produced by the interspecific fusion of nuclei of opposite mating types within an ascus in which all other ascospores are aborted. The non-pigmented ascospore is the product of the mixed genomes of the two species. It is a recombinant spore from which either species may be obtained by the partial substitution of its genome through successive backcrosses. Its inability to develop pigmentation, the irregular growth patterns of some of the strains which it produces and their inability to react in complementation tests, appear to be the result of a lack of affinity between the two species.

INTRODUCTION

Two new heterothallic species, *Sordaria heterothallis* (Fields & Maniotis, 1963) and *S. thermophila* (Fields, 1968), have recently been described. Previously reported studies on the hybridization experiments between these species (Fields, 1963; Lewis, 1968*a*, 1969) have indicated that the primary result was the formation of perithecia in which most of the asci were aborted at varying stages of maturation, and in which an occasional mature eight-spored ascus was recovered. These asci (recovered at a frequency of 1 per 10,000 asci examined) were found to have been homozygous for the mating type factor. Lewis (1968*b* and 1969) described evidence which suggests that they were formed by the intraspecific fusion of nuclei of the same mating type. Although most of the spores formed in these asci were normal in colour and shape and showed reasonable viability, these studies also indicated that aneuploid ascospores were formed during meiosis and that the resultant loss of chromosomes contributed to the inviability observed in some ascospores.

Another type of viable ascospore produced from the hybridization attempts was the non-pigmented germinable ascospore. The aim of this study was to determine whether this ascospore was the product of intra- or interspecific nuclear fusions. Proof of the latter would determine whether hybridization had occurred between the two species.

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MATERIALS

Wild-type strains of *Sordaria heterothallis* and *S. thermophila* were supplied by Dr W. G. Fields. The wild-type strains of *S. heterothallis* were designated as WT *5-A* and WT *16-a*, and those of *S. thermophila* as WT *7-A* and WT *17-a*. In each case *A* and *a* refer to the two mating types. Differences between the two species, and descriptions of the media, the cultural conditions employed and the techniques used in the isolation, characterization and mapping of mutants have been previously reported (Lewis, 1969). The *S. heterothallis* mutants used in this study were stu_1, stu_4, stu_5, stu_6 (strains with stunted growth) and ol_3 (olive-green ascospores). Those of *S. thermophila* were col_1, col_2, col_3 (strains with a colonial type of growth) and *t* (tan coloured ascospores). In *S. heterothallis*, stu_1 and stu_6 were 0·1 units apart and 1·0 unit from the mating type locus. Stu_4 and stu_5 were 2·0 units apart on another chromosome.

Several types of interspecific crosses were made: (1) Between the two wild-type strains. (2) $Stu_6 \times t$ (cross no. 4, Table 1). (3) Heterocaryons forced between *stu* strains of *Sordaria heterothallis* were crossed with wild-type strains of *S. thermophila* (crosses 1 to 3, Table 1). From these crosses it was hoped to determine whether the f_1 progeny (1) segregated only as parentals and whether strains recombinant for the markers in the constituent strains of the heterocaryon could be recovered; (2) were the products of interspecific fusions. Progeny were analysed for recombination between the *stu* markers and the mating type factors in order to determine whether the non-pigmented germinable ascospores were derived from interspecific nuclear fusions. f_1 strains produced by non-pigmented germinable ascospores were classified to species by back-crossing to wild-type strains of both species. The genotypes of stunted progeny from interspecific crosses were determined by complementation tests with $stu_{1,4,5}$ and stu_6 of *S. heterothallis* and with the restricted colonial mutants of *S. thermophila*.

RESULTS

Fields (1963) carried out the initial interspecific matings between *Sordaria heterothallis* and *S. thermophila*. He found that there was an abundant production of perithecia in which almost all the asci contained aborted protoplasts or delimited ascospores which did not mature. He also observed (personal communication) that these 'immature' ascospores occasionally germinated.

A closer study of these non-pigmented germinable ascospores showed that they were produced in asci which contain no black spores, and in which only one spore germinated. The other seven ascospores in the asci showed various degrees of abortion and no germination (Pl. 1, fig. 1). These asci were produced at the rate of 20 to 30 per perithecium, and the delimited ascospores ranged in colour from hyaline to beige. Most asci contained eight aborted ascospores and made up the bulk of the cluster in interspecific matings (Pl. 1, fig. 2). The germinable ascospores germinated without any of the pre-germination activation that was mandatory for *Sordaria heterothallis* and *S. thermophila*. The ascospores of *S. heterothallis* are only known to germinate when incubated on a medium containing 7 % sodium acetate (Fields & Maniotis, 1963), and those of *S. thermophila* require a 2-hr heat shock treatment and incubation on a medium containing 0·55 % ammonium acetate (Lewis, 1969).

Non-pigmented germinable spores isolated from wild-type interspecific matings

Table 1. *Analysis of non-pigmented germinable ascospores isolated from Sordaria heterothallis × S. thermophila crosses*

Cross no.	*S. heterothallis* × *S. thermophila*	Spore no.	Phenotypes		Complementation results*	MT	f_1 backcross reactions with		Recombinant spores
			Colour	Growth			*S. thermophila*	*S. heterothallis*	
1	$(stu_5+stu_6)a$ × WT 7,*A*	1,4,7,10,11	Hyaline	*WT*	—	*A*	Inter†	Inter	—
		2	Hyaline	*WT*	—	*a*	Inter†	Inter	—
		3,12	Hyaline	*stu*	Not tested	*a*	Inter	Inter	+
		5,6,8	Hyaline	Sub *WT*	Not tested	*A*	Inter	Inter	—
		9	Light brown	*WT*	—	*A*	Inter	Inter	—
2	$(stu_1stu_4ol_3+stu_5)a$ × WT *A*	1	Hyaline	Sub *WT*	Negative	Not tested	—	—	—
		2,5							
		7–12,14,15,	Hyaline	*stu*	Negative	Not tested	—	—	—
		17–20							
		6,13,16	Beige	*WT*	—	Not tested	—	—	—
		21–22							
		24–25	Hyaline	*WT*	—	*a*	Inter	Inter	+
		23	Hyaline	*stu*	Negative	*A*	Inter	Inter	+
3	$(stu_1stu_4ol_3+stu_5stu_6)a$ × *WT* 7,*A*	1,§3,4,16,17	Hyaline	*stu*	Negative	*A*	Inter	Inter	+
		2,10,12,15	Hyaline	*stu*	Negative	Not tested	Inter	Inter	—
		5,9,14	Hyaline	*stu*	Negative	*a*	Inter	Inter	—
		8,13	Hyaline	Sub *WT*	—	Not tested	Inter	Inter	—
		11	Hyaline	*WT*	—	*A*	Inter	Inter	+**
		6	Beige	*stu*	Negative	Not tested	Inter	Inter	—
4	stu_6 *a* × t_1 *A*	1–3							
		5–7	Hyaline	*WT*	—	*A*	Inter	Inter	—
		4	Hyaline	*stu*	Not tested	*a*	Inter	Inter	—

* Complementation tests were carried out with stu_1, stu_2, stu_4 and stu_6 of *S. heterothallis* and col_1 of *S. thermophila*. A positive complementation test is scored when pairs of *stu* cultures of the same mating type produce fans of wild type growth, when the control, *stu* cultures of opposite mating types remain stunted. In a negative test only stunted growth is observed.

† Interspecific.

‡ This strain produced an intraspecific reaction with *S. thermophila* after three subculturings.

§These strains produced a few perithecia in which some asci had 4 to 8 black spores.

** See text: formation of strains phenotypically resembling *S. heterothallis* from a non-pigmented ascospore.

showed two basic types of growth, normal wild type growth and a sub-wild type growth. The sub-wild type growth microscopically had an abnormal mycelial development, with much spiralling and more branching than the wild type. The growth rate was slower than the wild type, but the colony eventually grew to the edge of the Petri dish. In some sub-wild type strains, the colony gave rise to numerous sectors. These observations and the inability of the spores to develop normal pigmentation suggest that factors inhibiting the phenotypic expression of wild type were present in these non-pigmented ascospores, although some had become sufficiently mature physiologically to germinate and grow. When strains from the non-pigmented germinable ascospores were crossed among themselves, the asci produced showed an even greater degree of ascospore abortion than that produced in interspecific crosses. The non-pigmented germinable ascospores produced showed no phenotypic differences from their f_1 counterparts, and their mycelial strains had similar characteristics.

Progeny from the four matings shown in Table 1 are described phenotypically. f_1 cultures showed the phenotypes of either the mutant parental strains used in these matings, the sub-wild type mycelium, with its characteristic irregular growth and sectoring, or wild type growth with the normal phenotype. Thirty-five slow growing isolates were tested for complementation with stunted and colonial strains of both species. The results in Table 1 show that no complementation took place. It was assumed that failure of complementation might be due to the recombinant nature of the non-pigmented ascospores.

Twenty-nine of the isolated non-pigmented germinable ascospores were back-crossed to the wild type strains of both species. All strains showed an interspecific reaction with both species. This result led to the conclusion that the genomes of these strains were interspecifically recombinant, and that both parents contributed to their genotypes. Genetic proof of this is described in the following sections.

Twelve of the f_1 non-pigmented ascospores showed apparent recombination between the *stu* markers and the mating type factors (Table 1). The apparent recombinant spores with stunted growths, no. 23 of cross no. 2, and nos. 1, 3, 4, 16 and 17 of cross no. 3, could not be classified as true recombinants without further study, since the *stu* markers could not be identified through complementation, and a 1:1 segregation could not be determined from the aborted f_2 asci and ascospores. Proof of their recombinant nature is provided in the crosses discussed below.

(1) Cross no. 3, ascospore no. 3 (Table 1). This spore, an apparent recombinant, produced a culture which had a stunted growth and carried the *A* mating type allele. The backcross reactions with both *Sordaria heterothallis* and *S. thermophila* were interspecific. The *S. thermophila* backcross produced aborted asci with no germinable ascospores. In the *S. heterothallis* backcross, a few aborted asci with 1 to 2 mature spores were produced, and one mature eight-spored ascus was recovered. The eight black spores which germinated were wild type in growth and *a* in mating type. Three non-pigmented germinable spores—two of stunted and one of wild type growth—were recovered and analysed. The stunted strains were determined to be stu_5a through complementation tests. The recovery of the stu_5 marker in the backcross progeny proves that the original non-pigmented ascospore must have been recombinant, with the stu_5 marker derived from *S. heterothallis* and the *A* mating type locus from *S. thermophila*. This ascospore could only have resulted from an interspecific nuclear fusion.

(2) Cross no. 3, ascospore no. 14 (Table 1). This spore produced a mycelium with stunted growth, which was *a* in mating type and gave a negative complementation test. The backcross with *Sordaria heterothallis* produced aborted asci with 1 to 2 mature ascospores; several non-pigmented germinable ascospores were isolated. Of these, two were wild type, one sub-wild type and four stunted in phenotype. One of these stunted strains was determined to be $stu_1 stu_4$ *a*, through complementation tests. Thus the f_1 non-pigmented ascospore which was stunted in phenotype must have carried the *stu* markers. That these f_2 non-pigmented ascospores could take part in complementation reactions with *stu* strains of *S. heterothallis* reflects the fact that they contained a larger part of the *S. heterothallis* genome than the f_1 ascospores. That they were non-pigmented reflects the fact that they were the products of mixed genomes. From this result it may be concluded that ascospores no. 1, 4, 16 and 17 from cross no. 3, which were phenotypically stunted, carried the *stu* markers and were recombinant. Ascospore no. 11 was also shown to be recombinant for the stu_5 marker and mating type factor, as is described in the section in which strains phenotypically resembling *S. heterothallis* are obtained through backcrossing.

Ascospore no. 2, cross no. 1 (Table 1), and ascospores 21, 22, 24 and 25 of cross no. 2 were also interspecific recombinants. The initial backcross reactions of spore no. 2, cross no. 1 with *Sordaria heterothallis* and *S. thermophila* were interspecific. This strain was however repeatedly subcultured over 3 months while other experiments were in progress. On further testing, the backcross was found to produce an interspecific reaction with *S. heterothallis* but an intraspecific reaction with *S. thermophila*. This f_1 segregant, *a* MT, was clearly an interspecific recombinant.

These results demonstrated that the non-pigmented germinable ascospores contained a mixture of the genomes of *Sordaria heterothallis* and *S. thermophila*. Such mixtures appeared to impair some of the physiological functions of the cells; spore pigmentation is prevented, and *stu* strains failed to show complementation either among themselves or when mixed with *stu* strains of *S. heterothallis*. Proof that these strains were the products of mixed genomes was also shown by the recovery of both of the original species from them as is demonstrated below.

Formation of strains phenotypically resembling S. thermophila from a non-pigmented ascospore

Strains phenotypically resembling *Sordaria thermophila* were obtained from f_1 non-pigmented ascospore cultures by successive backcrossing to the *S. thermophila* wild type strain. One such series of backcrosses (Fig. 1) was started with the culture from ascospore no. 1 of cross no. 3 (Table 1). The mycelium from this ascospore had a stunted growth and was *A* in mating type. Only non-pigmented ascospores were recovered from the interspecific backcross with *S. heterothallis* but none could be induced to germinate. The backcross with *S. thermophila* produced two types of perithecia. (1) Several perithecia contained asci with 1 to 8 black spores (Pl. 1, fig. 3). Six asci with eight black spores were isolated; the ascospores germinated on ammonium only after the 2-hr heat treatment at 50°. All germinating ascospores were wild type in growth, although one parent appeared to carry the *stu* marker. This and the fact that this culture gave rise to two types of perithecia in this cross suggests that strain 1 of cross no. 3 was a heterocaryotic culture. Figure 1 shows the results of the analysis of ascus no. 4 of the f_2 backcross. Both mating type factors were recovered.

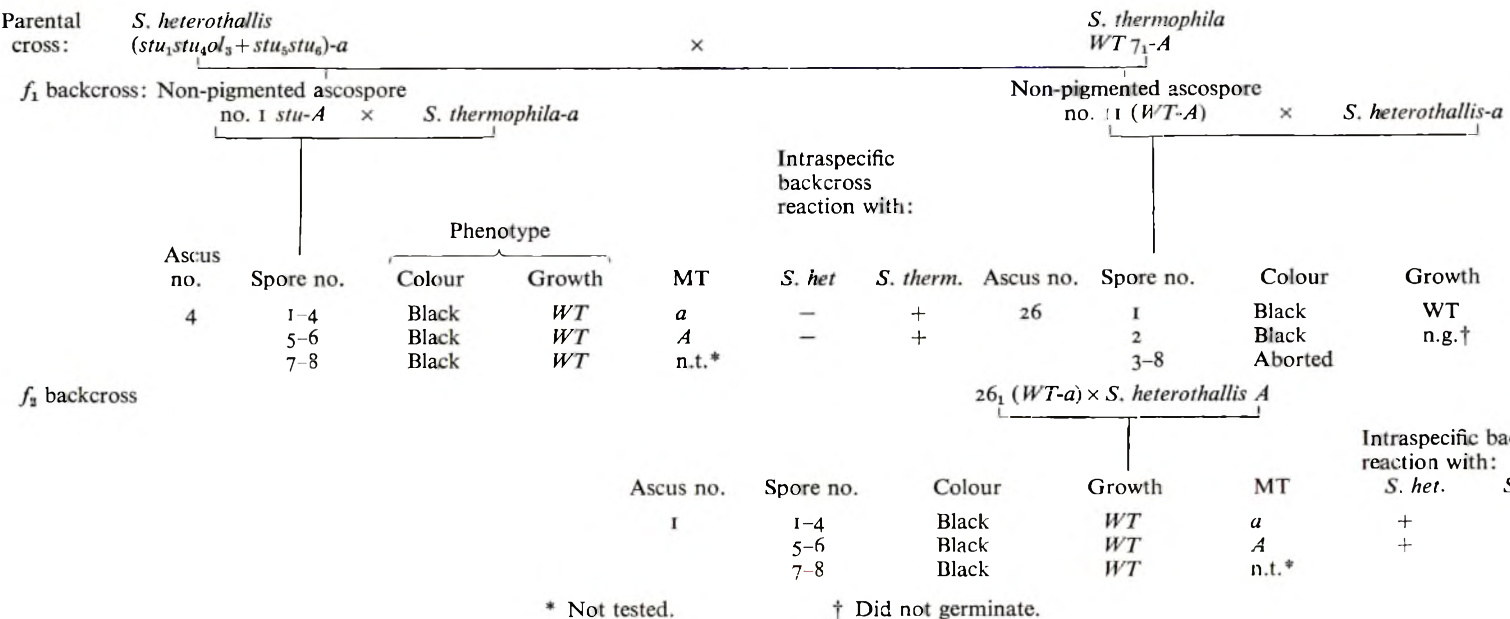


Fig. 1. Formation of strains phenotypically resembling *S. heterothallis* and *S. thermophila* from non-pigmented germinable ascospores.

Mycelia from each of three pairs of ascospores of this ascus were backcrossed to the wild type strains of *S. heterothallis* and *S. thermophila* respectively. Normal segregation of the mating type alleles was observed in all crosses with *S. thermophila*, with asci and ascospores showing the typical shape and size of this species (Pl. 1, fig. 5). Strains with the *S. thermophila* phenotype were thus isolated among the f_3 progeny. (2) The majority of f_1 perithecia in this backcross contained asci which showed an interspecific reaction, with some asci containing 1 to 2 black ascospores (Pl. 1, fig. 4). One mature eight-spored ascus which was recovered from these perithecia was homozygous for the wild type growth and *a* mating type of *S. thermophila*. This confirms the results described previously (Lewis, 1969) that the formation of the mature eight-spored ascus was exclusively an intraspecific event.

Formation of strains phenotypically resembling S. heterothallis from a non-pigmented ascospore

Strains phenotypically resembling *Sordaria heterothallis* were obtained from recombinant f_1 non-pigmented germinable ascospores through successive backcrosses to the *S. heterothallis* wild type. Figure 1 shows a series of backcrosses which were started with ascospore culture no. 11 of cross no. 3 (Table 1). This spore produced a wild type mycelium of *A* mating type. Backcrosses to *S. heterothallis* and *S. thermophila* were both interspecific, but perithecia from the former produced numerous aborted asci with 1 to 4 black ascospores. Cultures from eight of the 16 isolated black spores were stu_5a in genotype. Thus the original f_1 ascospore was interspecifically recombinant. One of the wild type f_2 ascospores of *a* mating type was backcrossed to *S. heterothallis A*. The f_3 asci contained mostly 4 to 8 black spores (Pl. 1, fig. 6). Six asci with eight black spores were dissected, and all ascospores showed wild type growth. Figure 1 shows the analysis of ascus no. 1. A mycelium from each of three spore pairs was backcrossed to both *S. heterothallis* and *S. thermophila*; all produced intraspecific reactions with *S. heterothallis* (Pl. 1, fig. 7) and interspecific reactions with *S. thermophila*. Strains with the *S. heterothallis* phenotype were thus isolated among the f_4 progeny. The replacement of parts of the genomes of the non-pigmented germinable ascospores with parts of the genomes of *S. heterothallis* or *S. thermophila*, the demonstration of interspecific recombination and the fact that these spores react inter-specifically with both species, is evidence that these non-pigmented spores are inter-specific recombinants.

DISCUSSION

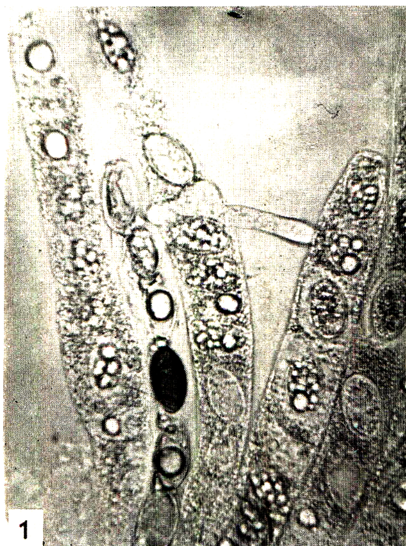
The non-pigmented germinable ascospore is a cell containing parts of the genomes of the two species *Sordaria heterothallis* and *S. thermophila*. The mixture of chromosomes appears to impair the growth rate in some strains and inhibits spore pigmentation in all cases. It is probable that the synthesis of the spore pigment is controlled by several loci on a number of chromosomes, and that the necessary complementation between the products of these loci which is needed for pigment synthesis cannot or can only partially take place, as in the production of beige coloured germinable ascospores, when the chromosomes are from different species. This may reflect the evolutionary differences between the chromosomes of these two species. A hypothesis of this nature can be formulated on the basis of the results obtained from attempts to force heterocaryons between strains carrying the *stu* markers. If one or both of these strains is

derived from a non-pigmented germinable ascospore no complementation takes place, although the *stu* markers were originally derived from the same species. It cannot be assumed that the differences in cytoplasms may be responsible for this result; for example, the inability of *S. heterothallis* proteins to be formed on *S. thermophila* polysomes. Lewis (1969) has shown that the genome of *S. heterothallis* can express itself normally in the cytoplasm of *S. thermophila* and vice versa. Another explanation for these irregular phenotypic expressions may be that the nuclei in these ascospores are aneuploid. This appears to be the explanation for the ascospore culture no. 2 of cross no. 1, which may have lost its extra chromosomes of *S. heterothallis* during successive subculturing. In this case, however, the *a* mating type allele from *S. heterothallis* appears to have been incorporated into the mating type chromosome of *S. thermophila* resulting in a recombinant genome. In the genus *Neurospora* Dodge (1931) was able to introduce certain factors from *N. sitophila* into *N. crassa* and show their normal segregation in eight-spored asci from the $f_1 \times$ *N. crassa* backcross. More detailed work along these lines in *N. crassa* has been done by Fincham (1951), and Scott-Emuakpor (1965), who have compared the recombination frequencies of markers first in one species and then after transfer into the genome of the other. One result of the presence of aneuploid nuclei would be the eventual formation of heterocaryotic strains (Pittenger, 1954) as in ascospore culture no. 11 of cross no. 3. This phenotypically wild type culture was shown to contain nuclei containing the stu_5 marker. On the other hand the phenotypically stunted culture of ascospore no. 1 cross no. 3 when crossed with the wild type strain of *S. thermophila* gave rise to asci with eight spores which formed cultures with wild type growth. This suggested that only nuclei without the *S. heterothallis stu* marker took part in the formation of these asci.

The recovery of *Sordaria heterothallis* and *S. thermophila* from recombinant ascospores by backcrossing is similar in some respects to the work done with Neurospora by Dodge (1928, 1931, 1936).

The hybrid asci obtained from crosses between *Neurospora stiophila* and *N. tetrasperma* (Dodge, 1928) showed less abortion than did those in the hybridization of our Sordaria species. There was better delimitation of ascospores, and a large number of mature ascospores were obtained, although the number of perithecia produced seems to have been very small. Dodge was able to recover *N. tetrasperma* after two backcrosses, as has been the case with *Sordaria thermophila* in the present study. Dodge recovered the *N. sitophila* genome by intercrossing certain f_1 isolates. When f_1 from interspecific crosses in Sordaria were crossed among themselves the asci contained more rather than fewer aborted ascospores.

Hybridization studies in Sordaria suggest that these two species are less closely related than the two species, *Neurospora sitophila* and *N. crassa*. A large number of f_1 ascospores with mixed genomes which were recovered from the Neurospora hybridization appeared morphologically and physiologically normal, and markers introduced from both species were expressed normally (Dodge, 1931). Very similar results were obtained in crosses involving *N. sitophila* and *N. tetrasperma* (Dodge, 1931, 1936). The fact that strains from the f_1 non-pigmented ascospores reacted interspecifically with both species of Sordaria, although they were composed of parts of the genomes of both species, as has been shown through the results of genetic analysis, suggests that there must be very little affinity between the two species.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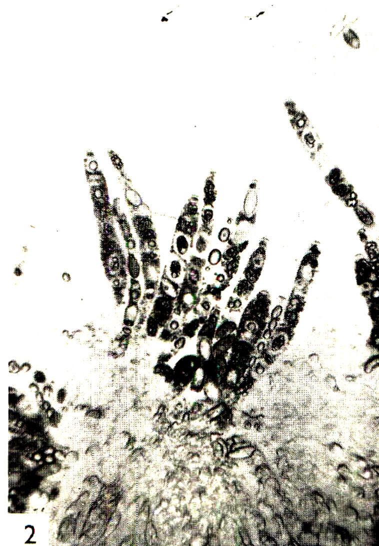
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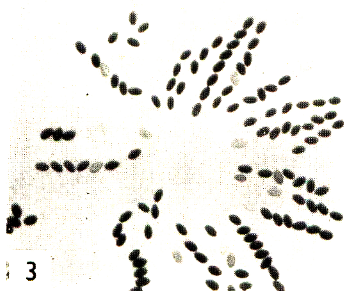
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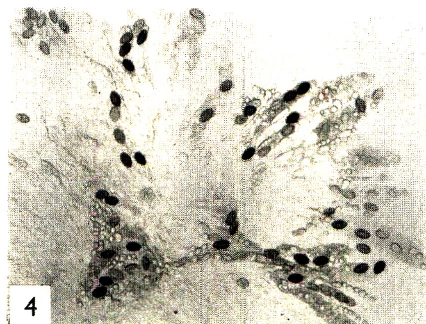
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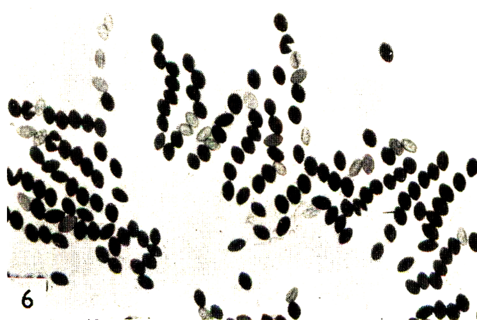
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The author wishes to express his appreciation to Professor Lindsay S. Olive for his guidance and support, and for his many helpful suggestions during the course of these studies, and to Dr W. G. Fields for his advice and suggestions. The willingness of Dr Fields to supply the cultures used in this study is gratefully acknowledged.

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EXPLANATION OF PLATE

PLATE 1

Fig. 1. Portion of Fig. 2 enlarged to show non-pigmented ascospore germinating in an ascus plated in corn meal agar. ×2500.

Fig. 2. Interspecific cluster from *Sordaria heterothallis* × *S. thermophila* cross, showing asci with aborted ascospores in all stages of development. Note ascus with germinating ascospore. ×550.

Fig. 3 to 4. Magnifications, ×350.

Fig. 3. Clusters of asci with 1 to 8 mature ascospores from the following cross: Mycelium from non-pigmented f_1 ascospore no. 1 of cross no. 3 (Table 1) *A* × *S. thermophila a*.

Fig. 4. Cross similar to that in Fig. 3. Asci from perithecia which produced interspecific clusters.

Fig. 5 to 7. Magnifications, ×350.

Fig. 5. f_3 *S. thermophila* ascospores from the f_2 back-cross of mycelium derived from spore of cluster shown in Fig. 3, to *S. thermophila* wild type.

Fig. 6. f_3 asci mostly with 6 to 8 mature ascospores from the following cross: f_2 ascospore progeny *a* (Fig. 3 ascospore 26_1) × *S. heterothallis A*. Most of these f_3 ascospores show a resemblance to *S. heterothallis* wild type ascospores.

Fig. 7. f_4 *S. heterothallis* ascospores obtained by crossing progeny from clusters like those in Fig. 6 with *S. heterothallis* wild type.

J. gen. Microbiol. (1969), **59**, 369–382
With 6 plates
Printed in Great Britain

An Albino Mutant of *Blastocladiella emersonii*: Comparative Studies of Zoospore Behaviour and Fine Structure

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(*Accepted for publication 16 September 1969*)

SUMMARY

A stable, ultraviolet-induced, albino mutant of *Blastocladiella emersonii* Cantino and Hyatt is described. It differs from wild type in: (*a*), capacity to form brown resistant sporangia (RS), both as 2nd and 3rd generation RS in clones derived from 1st generation ordinary colourless (OC) plants, and as 1st generation RS produced directly in response to exogenous bicarbonate; (*b*) the inhibition of growth by low concentrations of bicarbonate; (*c*) the longer generation time, stemming from a combination of decreased growth rate and delay in spore release until terminal sporangia have become unusually large; (*d*) the increased incapacity to discharge *in situ* on solid media with increasing temperatures above 23°; (*e*), reduced motility of zoospores and increased tendency to become amoeboid and encyst prematurely; (*f*), certain changes in the fine structure and organization of mitochondria and their associated lipid sacs, γ particles and the double membranes that surround nuclear caps and nuclei of spores chilled to low temperatures.

INTRODUCTION

Reports of mutations and genetic analysis of the aquatic Phycomycetes are rare [Emerson & Wilson (1954), Emerson (1950)]. Although some aquatic Phycomycetes are sensitive to radiation (Deering, 1968) stable mutations in aquatic Phycomycetes have been observed infrequently. Among the Chytridiomycetes, *Allomyces macrogynus* displays clearly circumscribed haploid and diploid phases; yet, even in such haploid thalli, where recessive mutations should not be masked, mutations have apparently been rare. Thus, while female-less (Stumm, 1958) and albino (Foley, 1958) variants have been produced, as well as mutants with decreased capacity to form RS (De Long, 1965), the latter (according to De Long) was but the third example in the literature of a well established single-gene mutation in Allomyces, and the first example of a single-gene lesion displayed by the diploid sporophyte. In biflagellate fungi (often assumed to be haploid, although morphological and cytological evidence for Achlya, Dictyuchus, Pythium and Phytophthora; Mullins & Raper, 1965; Sansome, 1965, now suggests they may be diploid) the situation is much the same.

The biochemistry of RS morphogenesis and the changes in fine structure associated with zoosporogenesis in *Blastocladiella emersonii* have been studied in some detail (Cantino & Lovett, 1964; Lessie & Lovett, 1968); as yet genetic studies have not been

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possible. Because the fungus reproduces asexually by motile spores released from colourless thin-walled OC plants, it has been tempting to think that (as in some species of its close relative, Allomyces) meiosis might be taking place in RS plants when sporogenesis occurs. However, RS spores look and behave much like OC spores (Cantino & Hyatt, 1953*a*; Cantino, 1969*b*) and true sexual fusions between any kinds of spores have never been observed. Spores from OC and RS plants can give rise to a few O (orange) plants that release orange swarmers in which fewer cytoplasmic γ particles occur (Cantino & Horenstein, 1956). Most orange swarmers are non-viable, and none has ever been seen to exhibit conventional sexual activity (Cantino & Hyatt, 1953*a*). During some 20 years of almost daily culturing of *B. emersonii*, variant clones displaying altered colours and/or gross texture and morphology have appeared spontaneously only about six times (E. C. Cantino, unpublished data); from only two of these were isolates obtained that could be propagated as stable mutant strains for a number of years; both of these were orange phenotypes. Possible relationships among the orange cell types produced by the Blastocladiales are considered more fully elsewhere (Cantino, 1966).

In the present study our original aim was to produce nuclear markers with which we could begin a genetic analysis of *Blastocladiella emersonii*, define its ploidy, detect and establish the nature of recombination if it occurred and eventually study the bicarbonate trigger mechanism for RS differentiation with incompetent mutants. Repeated attempts were made to produce and isolate such markers, yet only one stable variant (strain 9) incompetent to form RS was ever produced. The following report concerns differences between strain 9 and its wild type parent.

METHODS

Nature of the wild-type stock culture

Following the first studies (Cantino, 1951) and subsequent description (Cantino & Hyatt, 1953*a*) of *Blastocladiella emersonii* as a new species, day-to-day working cultures of the fungus have generally been maintained by successive cultivation using spores derived from clones of OC plants. But to ensure stability of the strain, RS spores from aged RS clones were used every few months to initiate new stocks of OC plants. As far as we know, cultures maintained in this way for the past 18 years do not differ from the original isolate. We refer to such strains as 'wild type'.

For some 4 years we have also kept one strain going exclusively via OC plants and OC spores. This strain now differs in some respects from wild type. On PYG agar (Difco Laboratories, Detroit, Mich., U.S.A.) its clones do not become pigmented as deeply brown as do those of wild type because fewer of the thalli develop into brown pitted RS types; furthermore, among those that do, many do not lay down the usual amount of pigment in the thickened wall. Thus, altered capacity for morphogenesis and melanogenesis may stem in part from a decreased capacity to respond to bicarbonate. Also, when OC spores are inoculated to PYG agar containing $NaHCO_3$ at concentrations which induce RS in the wild type, not all the growing thalli respond to form individual RS plants. Instead, variable numbers of plants arise that possess the usual distinctive shape of an RS thallus grown under these conditions but do not discharge spores *in situ* as would an OC cell; yet these plants bear larger thinner-walled sporangia than their RS counterparts in the populations. In fact, they strikingly

resemble the RS-like plants induced many years ago (Cantino, 1951) with exogenous pyrophosphate. The proportion of our 'pseudo RS' types (cf. Domnas, 1968), in a population of bicarbonate-induced RS cells can range from nil to very high levels depending upon a variety of factors among them are population density and media autoclaving time. The differences between this variant and its parent have not yet been fully analysed. To distinguish between the two, we have designated the variant as 'wild type var. 1'. In the present report wild type var. 1 was the only wild type used.

Production of zoospore suspensions

About 5×10^3 zoospores were spread out on freshly poured PYG agar in 150 mm. diam. plastic Petri plates at about 32 spores/cm^2 and incubated in the dark at 20° (wild type var. 1) or 24° (mutant strain 9). After about 24 hr, 75 to 90 % of the OC cells had discharged spores into the residual surface film of fluid remaining from the original inoculum. Populations of spores produced under these conditions consisted predominantly if not exclusively of elongate amoeboid swarmers. Plates were then flooded with 9 ml. water; after 5 min., the resulting spore suspension (10^6 to 5×10^6 spores/ml.) was removed, placed in an ice bath, and samples fixed immediately for electron microscopy and/or measured in a model B Coulter counter. When used as inocula for synchronized liquid cultures, spores were kept at 1 to 2° for 2 hr to improve synchrony (Cantino, Truesdell & Shaw, 1968).

Synchronized single generation cultures

Spores pre-chilled for 2 hr were inoculated into water-jacketed, 500 ml. spinner flasks (Bellco Glass Inc., Vineland, New Jersey) containing 200 ml. PYG + phosphate + citrate (PYG-PC; Cantino & Goldstein, 1967) at 24°. Population densities were 10^5 and 5×10^5 spores/ml. in various experiments; aeration, at 2 l./min., and magnetic stirring were started 2 hr after inoculation. Thalli were induced to differentiate sporangia and discharge spores by replacing spent media with the '1/2 DS' solution of inorganic salts of Murphy & Lovett (1966).

Ultraviolet irradiation

About 5×10^5 wild type var. 1 spores were spread out on about 57 cm^2 surface of wettable cellulose film placed on top of PYG agar, incubated until most thalli had reached the 8-nucleate stage (*c.* 7 hr), irradiated with a General Electric no. G3028 30 W germicidal lamp for 160 sec., and then incubated in the dark for an additional 14 hr. The film with organisms attached was then transferred to 10 ml. of $\frac{1}{2}$ DS solution; zoospores released therefrom were collected after 4 hr and served as the source of mutants. Unless zoospore formation was induced this way, maturation of OC organisms and zoospore discharge was extremely asynchronous as compared with non-irradiated controls.

Electron Microscopy

Spore suspensions were derived either from plate cultures or from OC plants (about 10^5/ml.) grown in spinner flasks as described above; the spent media in flask cultures were replaced by $\frac{1}{2}$ DS solution after 18 hr, and samples removed and fixed at various times before and during spore cleavage. Spore suspensions were centrifuged at 1800*g* for 1 to 2 min.; the loose pellet thus produced was fixed as follows.

Fixation. Stock solutions of glutaraldehyde (50 %), OsO_4 (2 %) and $KMnO_4$ (4 %)

were diluted with $1 \cdot 8 \times 10^{-2}$ M-Na veronal + Na acetate buffer (pH 7·8) to yield 2 %, 1 % and 2 % solutions, respectively (Lessie & Lovett, 1968). Spores fixed 1 hr in glutaraldehyde were postfixed 1 hr in either OsO_4 or $KMnO_4$; the latter was also used alone (2 hr). Equal volumes, usually 3 ml., of spore suspension and cold fixative were mixed, loosely pelleted immediately at low speeds, suspended in fresh cold fixative and kept on ice for the desired times.

Dehydration. Pellets were dehydrated successively in 25, 50, 75 and 95 % ethanol in water, containing 1 % $MgCl_2$ for 10 min. each, followed by a final dehydration in absolute ethanol.

Embedding. Pellets were treated successively with ethanol solutions containing increasing proportions (3 + 1, 1 + 1, 1 + 3) of propylene oxide for 10 min. each, and with pure propylene oxide for 30 min., transferred to propylene oxide + Epon 812 (1 + 1) overnight and finally embedded in gelatin capsules with Epon 812 (7 + 3).

Sectioning. Sections were cut with a Porter-Blum MT-2 ultramicrotome with a diamond knife, mounted on 400-mesh copper grids and examined with a Phillips 100B electron microscope.

RESULTS

Isolations and characterization of the albino mutant

Spores from irradiated OC organisms were dispersed on 40 plates of PYG agar, half of them with about 1·3 spores/cm^2 and the other half about 2·6 spores/cm^2. Viability, in terms of the capacity to form first generation OC plants, was roughly comparable to that of non-irradiated spores. After 5 days almost all clones derived from irradiated spores, like those on control plates, contained many brown RS cells. However, 10 white clones were found in which RS were absent or only rarely detected. We determined, therefore, whether the progeny from such clones were competent to differentiate RS organisms when exogenous $NaHCO_3$ was provided. Random samples of zoospores from each clone were transferred at about 0·9 to 1·8 spores/cm^2 to PYG media containing bicarbonate at several concentrations between zero and 5×10^{-2} M. After 3 days populations were scored for plants that released spores to form clones and for brown RS among those that did not discharge. Figure 1 A shows the cloning competence of progeny derived from nine variants and from three wild type var. 1 controls. Although all isolates differed from the wild type in their response to $NaHCO_3$, strain 9 was outstanding in that (*a*), no detectable melanin was produced by plants grown on any of the media; and (*b*), its spores displayed very low viability on PYG containing 5×10^{-3} M-$NaHCO_3$, while those that did germinate did not develop into mature cells.

Additional tests with successive generations derived from most of these strains showed that capacity for cloning on bicarbonate media and for melanin production was variable, whereas strain 9—after partially regaining some cloning competence during successive sub-culturing—became particularly stable in both of these respects. The response of progeny derived from three such clones of the stabilized strain 9 is compared in Fig. 1 B with that from three clones of wild type. Cloning by strain 9 was completely inhibited by 5×10^{-3} M-$NaHCO_3$, a concentration whereon most wild-type thalli developed into OC plants which then discharged spores and formed clones. Instead, strain 9 produced uniformly large colourless cells without thickened walls which neither discharged nor developed further in any obvious way. Strain 9 did form

clones on lower concentrations of $NaHCO_3$, but such colonies did not contain the pitted brown RS plants which typified wild-type clones. The colonies did produce regularly, but at low frequency, thin-walled yellow-orange cells which resembled the O-cells of Cantino & Hyatt (1953*a*). Periodic tests have shown that lack of cloning competence on bicarbonate media has remained a stable feature of strain 9 for 3 years.

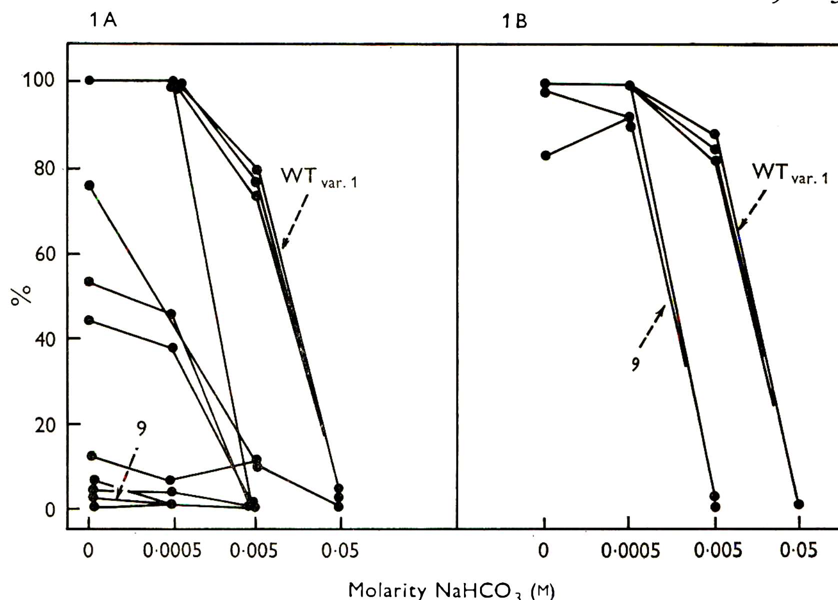

Fig. 1 A. Cloning competence of populations of individual plants (% discharging to yield clones) derived from zoospores of three wild type var. 1 and nine mutant albino strains (including strain 9) on PYG-agar media containing up to 5×10^{-2} M-$NaHCO_3$.
Fig. 1 B. Cloning competence of three clones from subsequent progeny (several generations later) of albino strain 9, and three clones of wild type var. 1; conditions as for Fig. 1 A. (Mechanism by which cloning competence was regained during subculturing is not understood.)

Individual plants of strain 9 on PYG agar also matured less rapidly than those of wild type var. 1. Rates at which populations of the wild type and mutant plants discharged spores *in situ* on PYG agar were compared at temperatures between 17 and 32° (Fig. 2). At all temperatures the interval between the time a spore was placed upon the agar and the time the OC cell derived from it released its spores onto the medium (the generation time) was longer for the mutant. Furthermore, with temperatures above 23°, the populations of strain 9 plants discharged their spores with decreased synchrony (note decreasing slopes in Fig. 2, top); also, more and more of them lost the capacity to produce and/or discharge spores. The temperature which yielded the highest proportion of normally discharging cells in the shortest time was 26° for the wild type and 23° for strain 9.

Synchronized single-generation liquid cultures of the mutant and wild type were also started with spores ($1{\cdot}6 \times 10^4$/ml.) in medium PYG-PC at 24° agitated with a stream of air; plant sizes were recorded during development (Fig. 3). The rate of increase in cell volume was higher for the wild type; however, the mutant thalli continued growing at their slower rate for a longer time, and achieved a larger size before they formed papillae and differentiated spores.

Comparative behaviour of zoospores

Mutant cells were grown on PYG agar (about 6·5 plants/cm^2) until a few had begun to discharge spores and were then flooded with water. As zoospores emerged from sporangia, they were seen to differ from the wild type in their behaviour. They lacked the intense activity of wild-type spores under similar conditions; they swam sluggishly for brief periods and then settled quickly on the agar surface, where they exhibited amoeboid movements and then either encysted or swam slowly again.

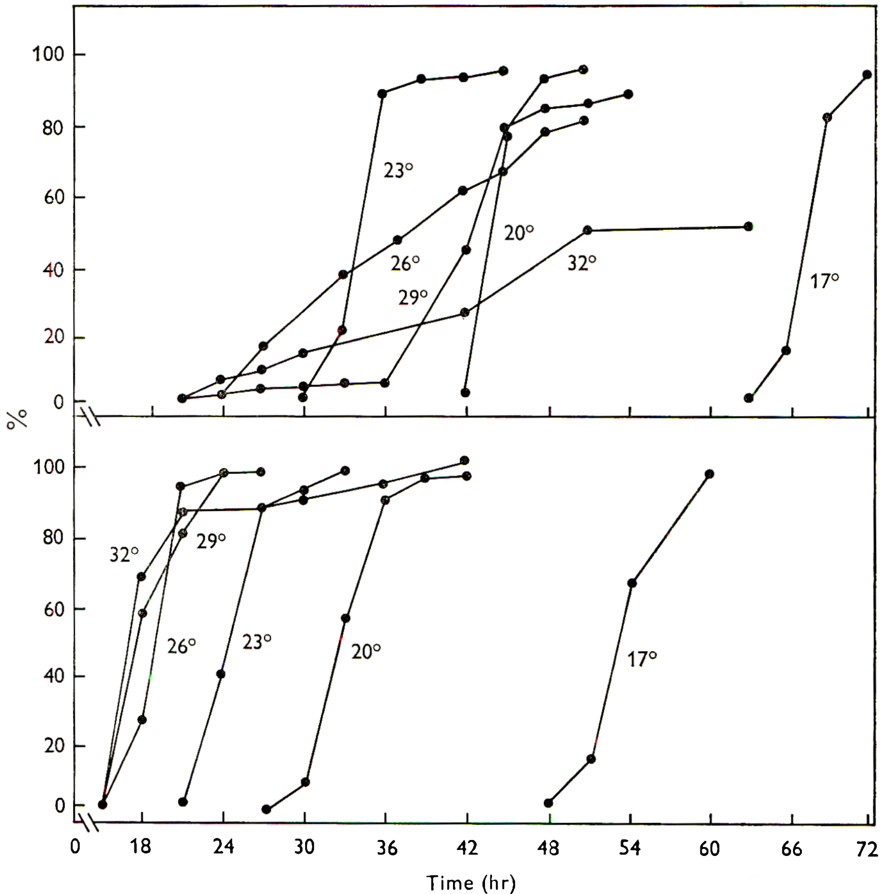

Fig. 2. Rate of maturation of populations of individual plants (as % discharging spores *vs.* time) of wild type var. 1 (bottom) and albino mutant strain 9 (top) on PYG-agar at different temperatures.

Fine structure of wild-type and mutant zoospores

Actively swimming wild-type zoospores of *Blastocladiella emersonii* are subglobose to ellipsoid. Numerous aspects of the fine structure of such spores have already been described (Cantino, Lovett, Leak & Lythgoe, 1963; Fuller, 1966; Lessie & Lovett, 1968). When spores were first released from dense populations of OC plants into a surface film of fluid on solid media, they were irregularly elongated rather than

smoothly ovoid and crawling amoeboids rather than active swimmers. With the harvesting method used here, we examined the structure of these amoeboid spores, compared it with that of similar spores stored over ice for 2 hr, and contrasted this with known facts about swimming spores (cf. Cantino *et al.* 1968).

The amoeboid and chilled zoospores of wild type var. 1. The fine structure of amoeboid spores differed little from previously published pictures. They displayed more or less elongate shapes with pseudopodial protrusions. The single nucleus was bounded

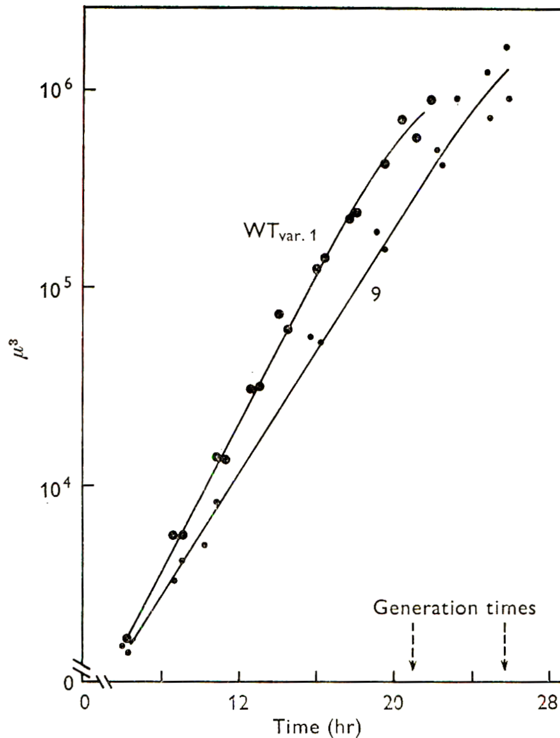

Fig. 3. Rate of increase in average cell volume of wild type var. 1 and albino mutant strain 9 inoculated with $1 \cdot 6 \times 10^4$ spores/ml. and grown in aerated liquid medium PYG-PC at 24°. (Representative results from one experiment.)

by a partially porous membrane and was almost completely surrounded on its posterior side by ribosomal particles enclosed within a continuous double membrane that delimited the spore's nuclear cap, now chemically characterized (Lovett, 1963).

An unexpected feature of wild-type amoeboid spores was the persistent occurrence of one to several satellite ribosome packages (Cantino, 1969*a*) apparently identical in texture and osmiophilic properties with the particles in the nuclear cap. These ribosome packages were delimited by their own continuous double membranes, and were situated in the cytoplasm *outside* the nuclear cap, and sometimes even in locules almost completely enclosed by the mitochondrion. The membrane around these ribosome packages is now known to be connected solely to the outer unit membrane of the nuclear cap (Cantino & Mack, 1969).

The spore's single mitochondrion was asymmetrically disposed as usual about the nucleus at the posterior end of the spore, where it was associated with flagellar fibres

and membrane-covered banded rootlets as in swimming spores. In view of the presently unresolved nature of the attachment between rootlets and kinetosome, it is noteworthy that one of our pictures (Pl. 4, fig. 7) of this association is very similar to one (Fig. 55) published by Lessie & Lovett (1968) which prompted their comment that '...in some sections two of the rootlets actually seem to be arms of a single continuous structure that passes close to (or is attached to) the basal body fibrils...' Finally while we have frequently seen sections (of both the wild type and wild type var. 1) showing three mitochondrial canals, only two of them ever contained banded rootlets, the third containing only ill-defined material. We have yet to see unmistakable evidence, either among our own sections or those published by others, for the conclusion (Fuller, 1966; Reichle & Fuller, 1967) that the zoospore of *Blastocladiella emersonii* possesses three banded rootlets.

The significance of the differences among the granules situated in the double-membraned lipid sac (Cantino *et al.* 1963, Lessie & Lovett, 1968; Reichle & Fuller, 1967) along the outer edge of the mitochondrion is still uncertain. In amoeboid as in swimming spores, these granules always appeared in two forms: one, an often elongated membrane-bound organelle (possibly a continuous matrix) of even electron density; the other, situated among the former, a somewhat polyhedral body with an electron-transparent centre and an electron-opaque limiting region. Originally, these two types of bodies were simply lumped together and termed 'lipid granules' (Cantino *et al.* 1963); later, they were distinguished as spherical lipid granules and 'sb' granules by Lessie & Lovett (1968), who re-interpreted earlier views (Reichle & Fuller, 1967) of these granules.

Other structures in amoeboid spores, seen previously *in vivo* by phase microscopy (Cantino *et al.* 1968), are the large transparent vacuoles found most often in the cytoplasm at the amoeboid's anterior end. Sometimes they were so disposed as to suggest that they were in the process of breaking through to the outside. And finally, the structure of the γ particles appeared to be normal. In contrast, the wild-type zoospore chilled for 2 hr exhibited some marked structural changes. The cell was now globose or sub-globose (Pl. 1, fig. 1). Its nuclear apparatus (i.e. its assemblage of nucleus, nucleolus and nuclear cap) was displaced far to one side probably due to the change in shape of the mitochondrion. The latter had become almost completely spherical but remained secured to the basal region of the flagellum by a small loop, all that remained of it on one side of the still anchored flagellum (Pl. 4, fig. 7, arrow). Simultaneously, the volume of the spore increased, and its cytoplasm became more electron transparent. The membranes of the lipid sac were frequently fragmented and lipid bodies often lay scattered in the cytoplasm. Other details about the structure and metabolic activity of such chilled wild-type spores have been reported elsewhere (Cantino, Suberkropp & Truesdell, 1969).

The amoeboid and chilled zoospores of mutant strain 9. Unchilled amoeboid spores of the mutant also contained one or more satellite ribosome packages bound by double membranes and located outside the nuclear cap. Other organelles apparently did not differ from those in unchilled wild-type amoeboid spores.

However, additional differences became apparent after mutant spores were chilled. They were generally larger than chilled wild type spores and in most the double membrane around the nuclear cap was incomplete (arrows in Pl. 1, fig. 2; Pl. 2, fig. 3 and 4). Such holes—in a membrane very rarely imperfect in wild-type spores—

almost always occurred along the sides of the nuclear cap near the normal point of continuity of its membrane with that of the nucleus; this normal joining point is illustrated in Pl. 5, fig. 9 (arrow).

The double membrane of the nuclear cap was sometimes seen (Pl. 2, fig. 4, dotted arrow) to be continuous with the membrane of the lipid sac, yet separate from the nuclear membranes. This, however, is a different kind of breach of the cap's intactness that also occurs in wild-type spores (Cantino & Mack, 1969, fig. 11; Lessie & Lovett, 1968).

The smaller of the two gaps in the nuclear cap membrane pictured in Pl. 2, fig. 3 (dotted arrow) clearly demonstrated both the double nature of the membrane and the fact that such breaks were not due to artifacts of section preparation. Angular vacuoles were sometimes seen (Pl. 1, fig. 2; Pl. 2, fig. 3) near the gaps in the nuclear caps.

In addition to their imperfect nuclear caps, chilled mutant zoospores also displayed imperfect nuclear membranes (Pl. 3, fig. 5, arrow). Such spores appeared normal in other respects. Sections of sporangia undergoing sporogenesis also revealed (Pl. 3, fig. 6, arrow) that discontinuities in nuclear membranes were detectable even before zoospores had been fully differentiated and released. Examples of the sort depicted in this figure were commonly seen in sporangia.

Chilled mutant spores had globose shapes resembling those of chilled wild-type spores; yet, their mitochondria (although they did become compacted, Pl. 1, fig. 2; Pl. 2,; Pl. 6, fig. 11) never took on the nearly spherical shape so typical of mitochondria in chilled wild-type spores (compare Pl. 6, fig. 10, with Pl. 6, fig. 11). The associated lipid sacs did not become as disrupted as those in the wild type. The γ particles, entirely normal in unchilled mutant spores, took on seemingly distorted and sometimes unusual shapes when spores were chilled.

The banded rootlets in the mutant seemed to be normally placed in mitochondrial canals and associated with the kinetosome. Striations were seen in longitudinal sections of rootlet axes; little is known about their nature or significance. When fixed with $KMnO_4$ alone (Pl. 4, fig. 7), the bands in chilled spores appeared to be of two types, one alternating with the other. An unusual view of a banded rootlet (Pl. 4, fig. 8)—in this case, in an unchilled mutant spore stained with lead citrate and uranyl acetate—suggested that these striations were enclosed within a distinct sleeve only vaguely visible in Pl. 4, fig. 7.

DISCUSSION

Ultraviolet irradiation of *Blastocladiella emersonii* led to formation of some visibly altered clones; one such phenotype, the albino strain 9, described here, differed structurally and functionally from its parental wild type var. 1 in that: (*a*), the fine structure of its zoospores, particularly the membrane system delimiting the nuclear apparatus, was especially sensitive to modification by cold treatment; (*b*), its zoospores displayed decreased motility; (*c*), the growth rate of the thalli derived from these spores was less, and the size of plants, at generation time, greater than those from wild type strains; (*d*), spore germination and/or subsequent growth were inhibited by concentrations of exogenous $NaHCO_3$ that induced RS formation in wild type strains; (*e*), it did not produce RS plants in response to those concentrations of exogenous $NaHCO_3$ that did permit growth.

Deering (1968) reported some pronounced changes in the u.v. sensitivity of germ-

lings of *Blastocladiella emersonii* when they were irradiated at different developmental stages, and he discussed possible reasons for these results in terms of nuclear division cycles and other factors. Apparently, no stable albino mutant such as ours arose or was established in the course of Deering's studies. However he worked with one- to four-nucleate germlings while we irradiated eight-nucleate plantlets.

Table 1. *Comparative properties of Blastocladiella emersonii and some strains derived from it**

	Wild type var. 1	Mutant strain BEM	Mutant strain 9
Comparison of zoospore characteristics (vs. wild type)			
Size (wild type = about 7 × 9 μm. for colourless spores)	Similar	Similar	Similar
Motility	Similar	Altered ('zig-zag')	Altered (sluggish)
Viability	Similar†	Greatly decreased (to about 1 %)	Somewhat decreased
γ particles/cell (wild type = about 13 for colourless spores)	?	8	?
Changes in fine structure?	No	?	Yes
Nature of populations of first generation individual thin walled plants derived from zoospores‡			
Generation time (as % of wild-type gen. time)	About 100 %	About 120 %	About 120 %
Colour at maturity (vs. wild type = 99 % colourless, 1 % orange)	Similar	100 % orange	% orange erratic, variable; mostly colourless
Change in capacity to grow like wild type in 10^{-2}M to 5×10^{-3}M bicarbonate?	No	Yes (total loss)	Yes (total loss)
Change in capacity to form RS plants directly in response to bicarbonate like wild type?	Yes (reduced)	Yes (total loss)	Yes (total loss)
Change in capacity to form clones bearing RS plants on non-bicarbonate media like wild type?	Yes (reduced)	Yes (total loss)	Yes (total loss)
Changes in enzyme activity characteristic of wild type?	?	Yes	?

* For references to the original papers upon which this summary is based, see Cantino, 1966.

‡ It is noteworthy that in *Blastocladiella britannica* (Horenstein & Cantino, 1961), an albino variant (strain TW) incapable of producing RS plants in clones was also characterized by: (*a*) lack of capacity to produce RS in response to bicarbonate; (*b*), inability to grow at concentrations of bicarbonate between 10^{-2}M and 5×10^{-3}M; (*c*), an extended generation time as high as three times that of the parental type.

† Soll, Bromberg & Sonneborn (1969) reported that the zoospores of wild type var. 1 behave identically with the wild type in their germination assays.

In any case, the altered properties (Table 1) of our albino strain 9 are reminiscent of the features displayed previously by spontaneous orange mutants (e.g. strain BEM; Cantino & Hyatt, 1953*b*) of *Blastocladiella emersonii*. In view of the number and kinds of changes involved, perhaps albino strain 9 also arose (as suggested earlier for orange mutants; Cantino & Lovett, 1964) by mutation in a single pleomorphic nuclear

gene. Without more direct evidence, however, the changes which gave these two new phenotypes remain obscure.

Aside from questions about the origin of Blastocladiella mutants, there emerged some interesting details about zoospore fine structure, namely, the cold-induced breaks in the membrane around the nuclear apparatus. The enclosure of a zoospore's ribosomes in a membranous structure such as a nuclear cap apparently is not vital for all uniflagellate aquatic fungi; the motile spores of some Chytridiomycetes, for example, bear naked aggregates of ribosome-like particles either randomly distributed or localized around or near their nuclei, while others may enclose them only partially within loose networks of imperfect double membranes (Fuller, 1966; Chambers, Markus & Willoughby, 1967). And in the spores of Monoblepharella (Fuller & Reichle, 1968), ribosome-like bodies are found in two major areas: scattered at the cell's posterior, where they are not delimited by membranes; and closely packed around the nucleus, where they are partially delimited by flattened cisternae but otherwise in contact with cytoplasm. At the other extreme is the situation found in *Blastocladiella emersonii* and its derivatives: the regular appearance in unchilled zoospores of a double-membraned nuclear cap that encloses essentially all its ribosomes, and (at least in some spores) additional small packets of ribosome-like particles, also delimited by double membranes but situated in the cytoplasm. Such satellite ribosome packages had not been seen (Cantino *et al.* 1963)—or seen only very rarely (Reichle & Fuller, 1967; Lovett, personal communication)—by previous workers in spores from wild-type stocks of *B. emersonii*, and it had not been determined whether they were connected to the cap or enclosed by separate membranes of their own. It is now known (Cantino & Mack, 1969) that they connect solely to the outer unit membrane of the nuclear cap's double membrane, and that they can be released as separate entities. The high frequency with which they have been found in the present study and in later work (Cantino, 1969*b*; Cantino & Mack, 1969) suggests that they may be especially prevalent in, if not a special feature of, amoeboid spores as compared to swimming spores. At present the functional significance of these satellite ribosome packages is unknown.

With the nuclear cap itself, cold shock induces it to fracture in the albino mutant but not in the wild type. Rupture of this cap and release of its ribosomes to the cytoplasm is apparently an obligatory event associated with germination of wild-type zoospores (Lovett, 1968). It is conceivable, therefore, that an increase in germinability (or improvement in the synchrony of germination) of mutant spores might result from a premature induction of such a membrane lesion by cold shock; unpublished data do suggest that the percentage germination for a population of cold-shocked mutant spores is increased. However, the potential significance of this correlation is weakened by the fact that cold shock also affects the behaviour of wild-type spores (Cantino *et al.* 1968; Cantino *et al.* 1969), yet their nuclear caps remain intact at these lowered temperatures.

In contrast to these changes in the mutant's nuclear cap, the shape of the single mitochondrion in the albino's spore is much less affected by cold shock than is the wild type's. It would seem as if the membranes around these two types of organelles respond quite differently to a decrease in temperature, but in reciprocal fashion. If lowered temperatures should cause physical modification of the lipid structures in the spore membranes so as to change their permeability, as suggested by Schramm,

Eisenkraft & Barkai (1967) for other biological systems, perhaps cold-induction of different degrees of 'leakiness' among the organelles in wild-type spores and mutant spores is involved in the phenomenon. Perhaps microtubules might also be involved, considering (*a*), reports that in *Blastocladiella emersonii* they extend upward (as an array of nine sets of three) from the kinetosome region to ensheath the nuclear cap (Lessie & Lovett, 1968; Fuller & Calhoun, 1968), and (*b*), that low temperatures cause microtubules to disappear in other organisms (Roth, 1967; Tilney & Porter, 1967). At the moment, however, we have no direct evidence to support these possibilities. Although work has begun (Cantino *et al.* 1969) to analyse in physiological terms the effect of cold shock upon wild-type spores, this has not been done with the albino mutant.

The work reported herein was supported by a NATO Post-doctoral Fellowship to D.S.S., and by research grants from the National Institutes of Health and the National Science Foundations to E.C.C.

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EXPLANATION OF PLATES

PLATE 1

Fig. 1 and 2. Zoospores harvested from PYG agar cultures of wild type var. 1 and strain 9, respectively; chilled 2 hr before permanganate fixation. Arrows point to breaks in nuclear cap. × 13,300 and × 13,600.

PLATE 2

Fig. 3 and 4. Zoospores harvested from PYG agar cultures of strain 9; chilled 2 hr before permanganate fixation. Solid arrows point to breaks in nuclear cap; for dotted arrow, see text. × 1400 and × 14,300.

PLATE 3

Fig. 5 and 6. Zoospore harvested from PYG agar culture, and a sporangium undergoing sporogenesis harvested from PYG-PC spinner flask cultures, respectively, of strain 9; the spore was chilled 2 hr before permanganate fixation. Arrows point to breaks in nuclear membrane. × 16,800 and × 17,100.

PLATE 4

Fig. 7. Zoospore harvested from PYG agar culture of wild type var. 1; chilled 2 hr before permanganate fixation. Arrow points to anchoring loop of mitochondrion; see text. × 34,600.

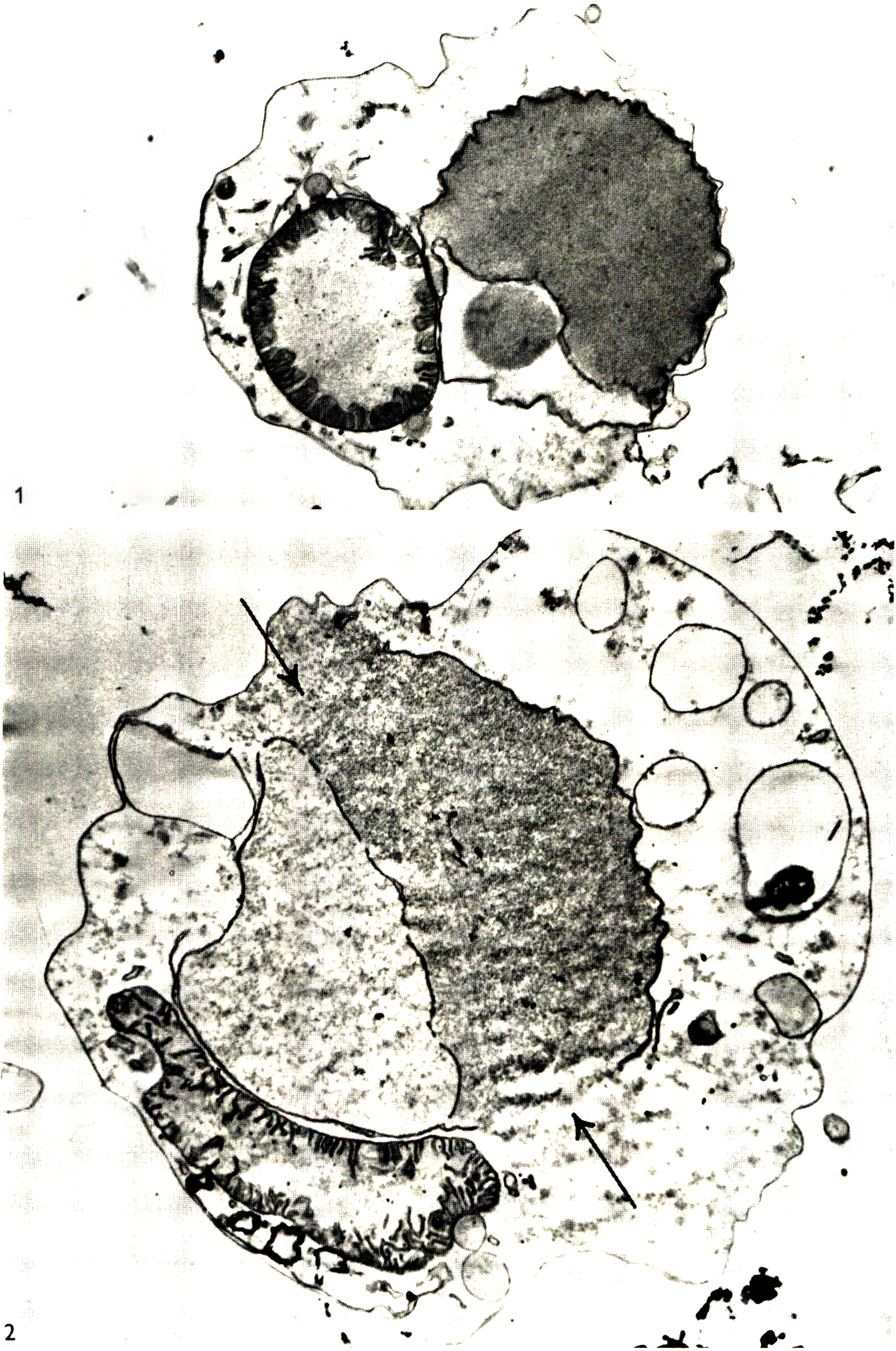
Fig. 8. Zoospore harvested from PYG-PC spinner flask culture of albino mutant strain 9; unchilled, fixed with glutaraldehyde-osmic acid, and stained with uranyl acetate and lead citrate. × 37,900.

PLATE 5

Fig. 9. Zoospore harvested from PYG agar culture of strain 9; chilled 2 hr before permanganate fixation. Arrow points to connection of nuclear cap and nuclear membranes. × 31,000.

PLATE 6

Fig. 10 and Fig. 11. Zoospores harvested from PYG agar cultures of wild type var. 1 and strain 9, respectively; chilled 2 hr before permanganate fixation. × 31,500 and × 31,600.



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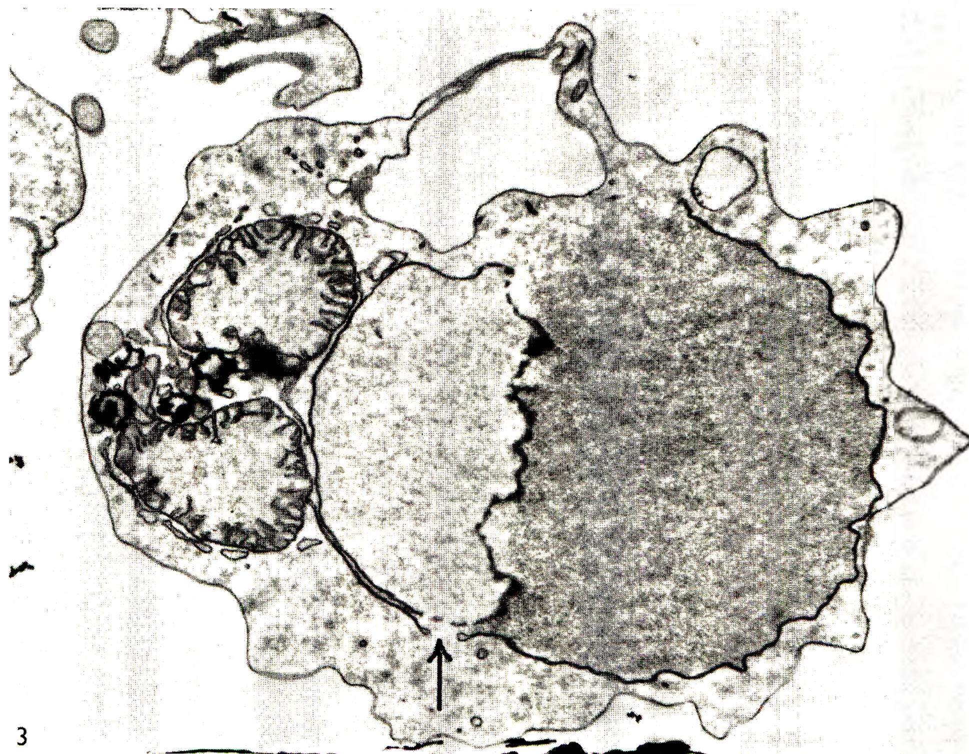

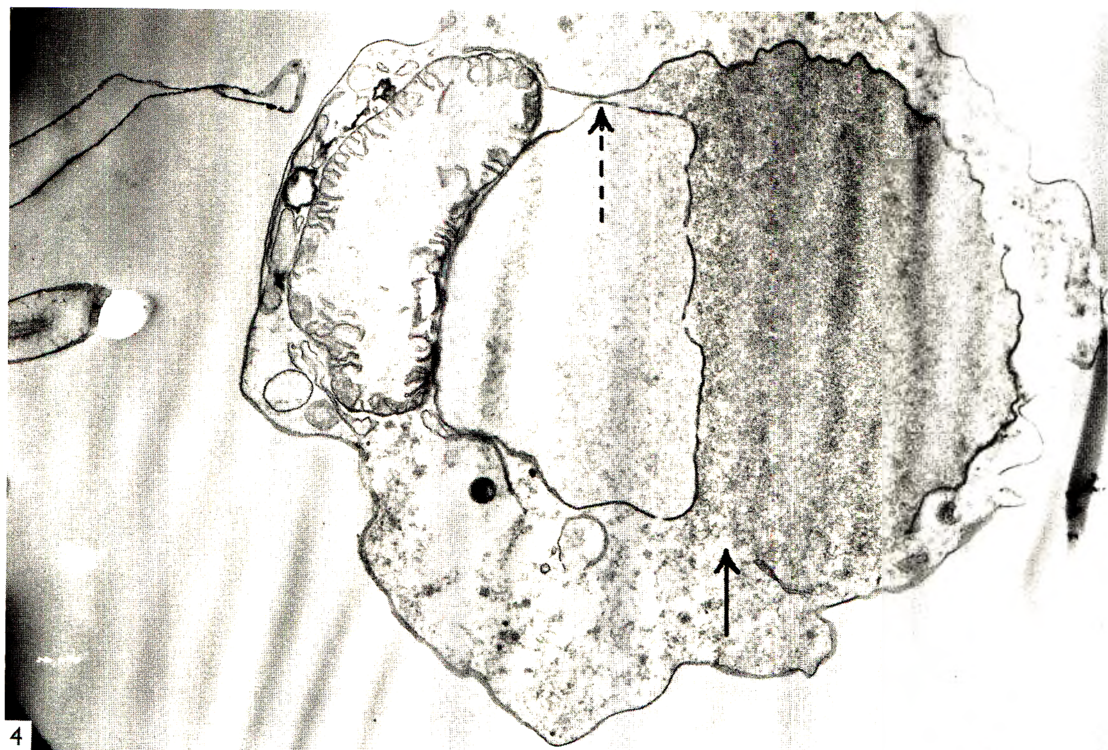

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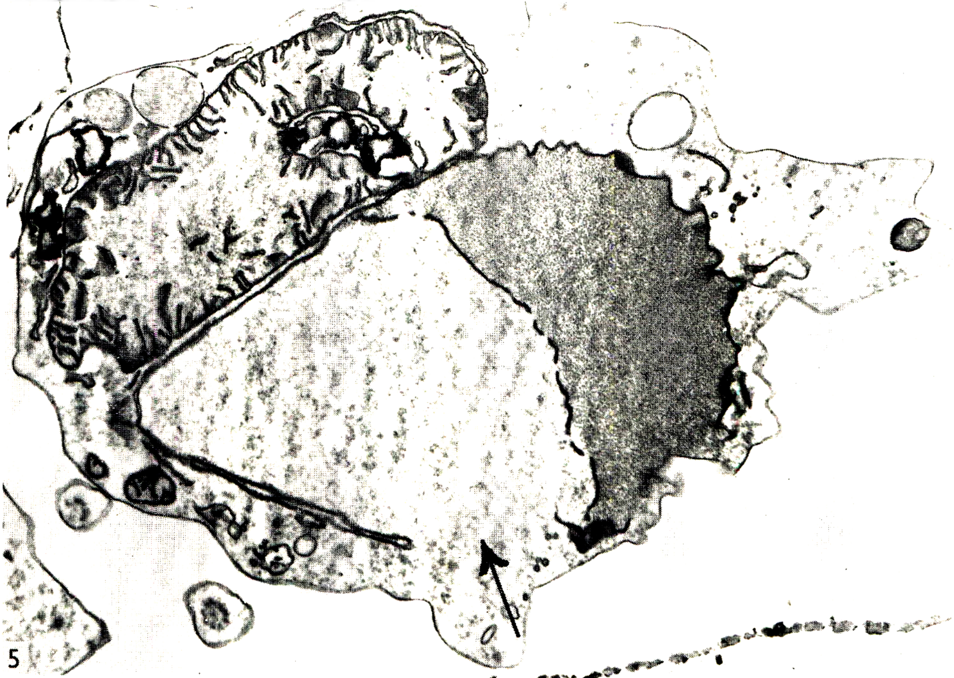

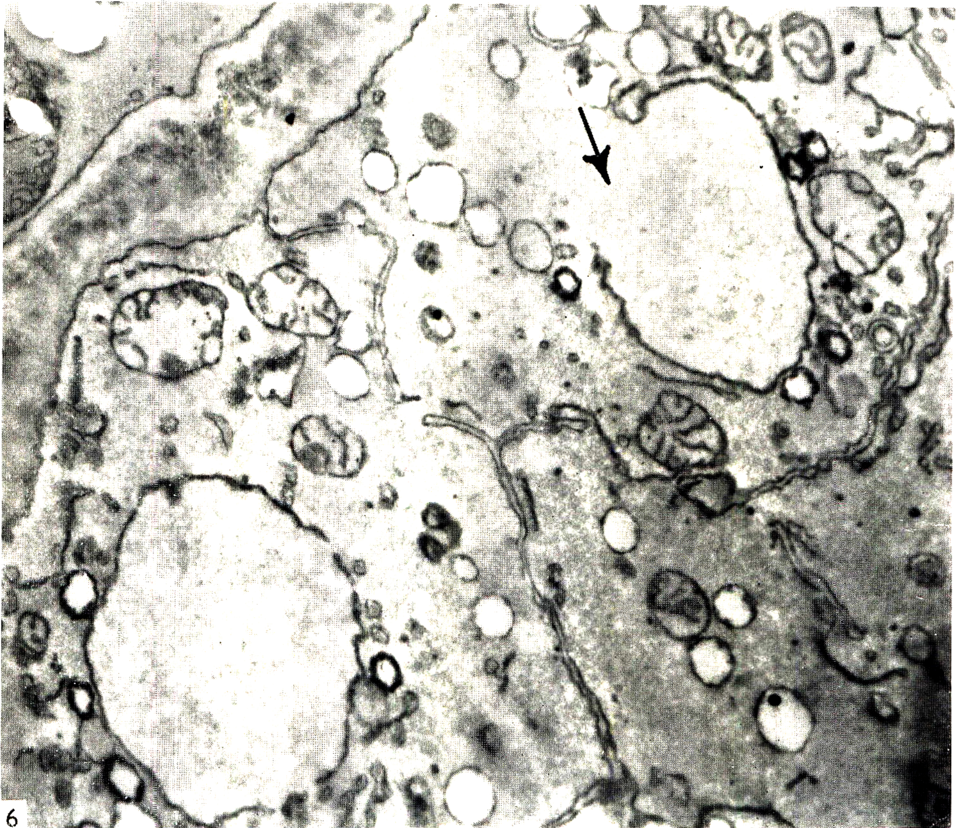

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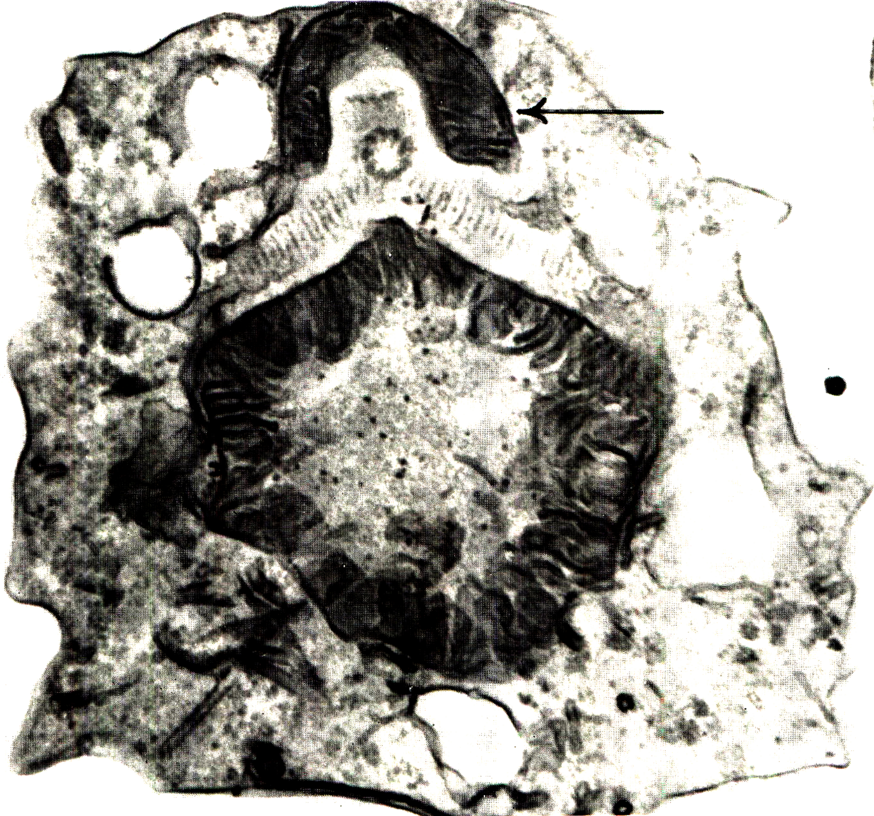

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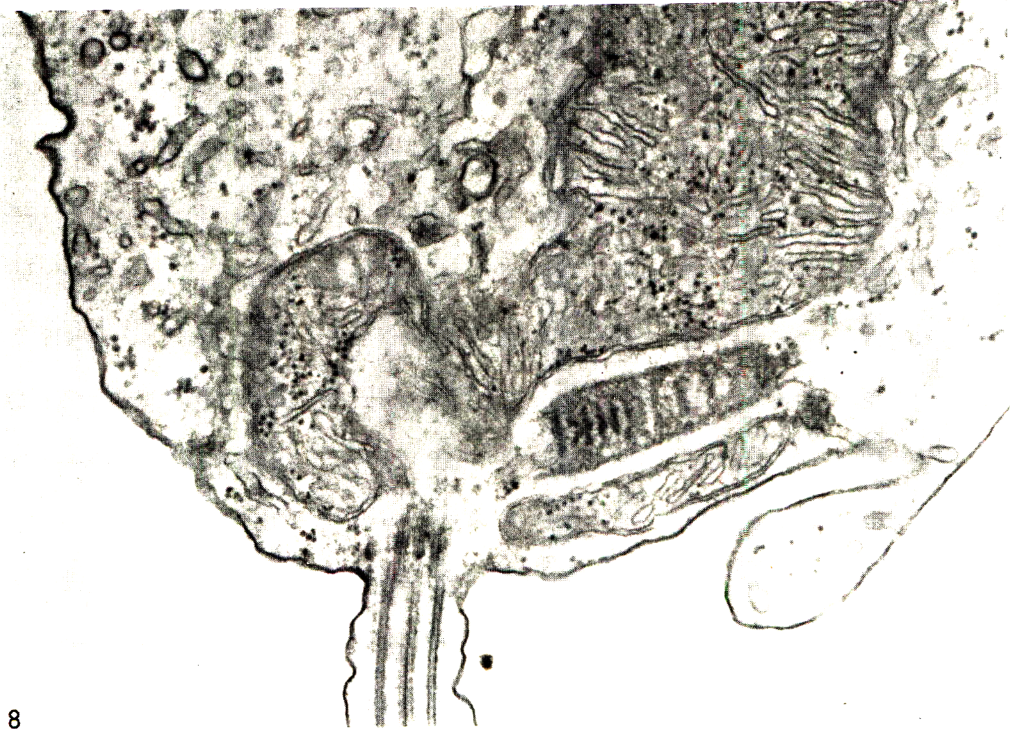

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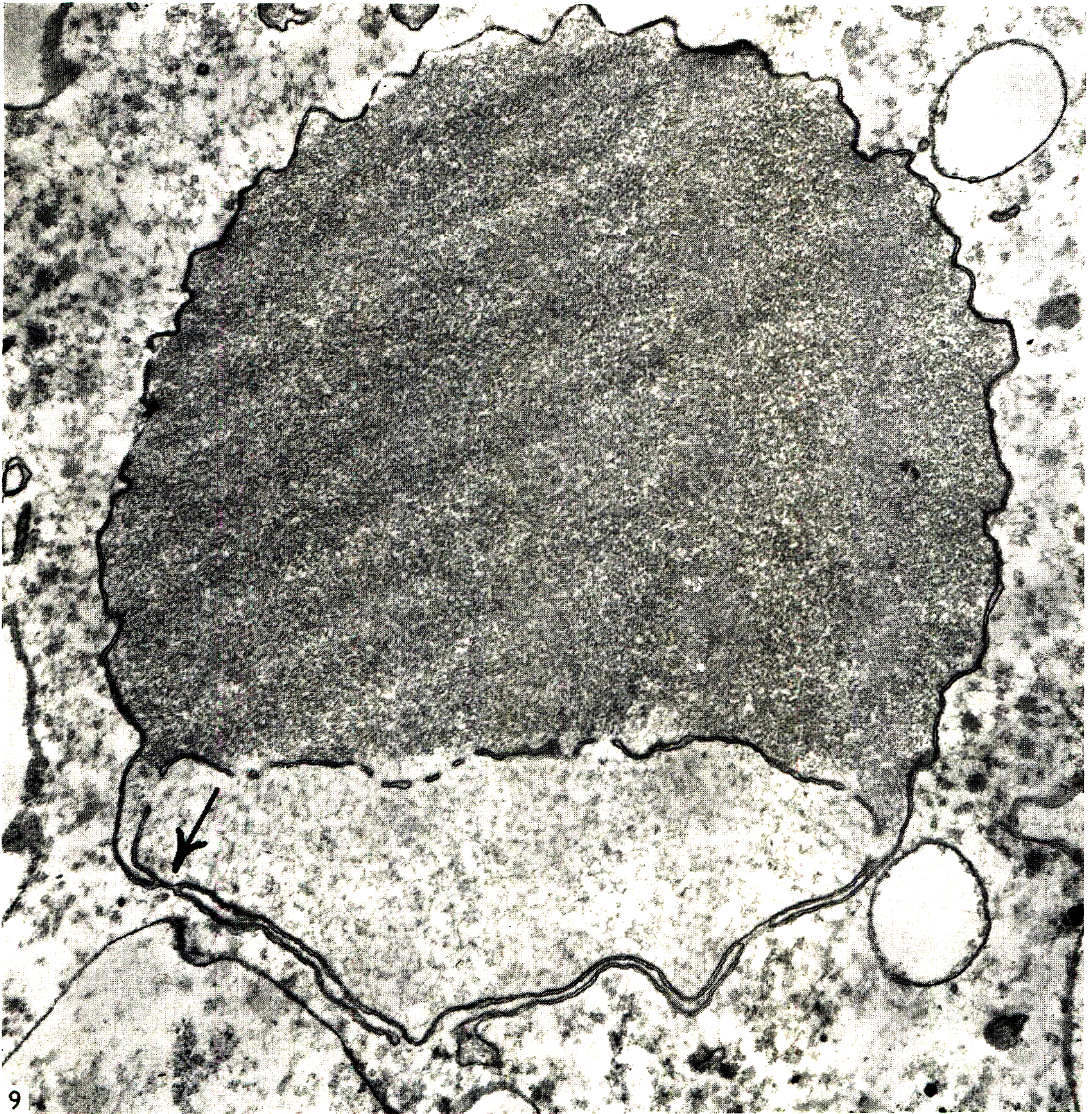

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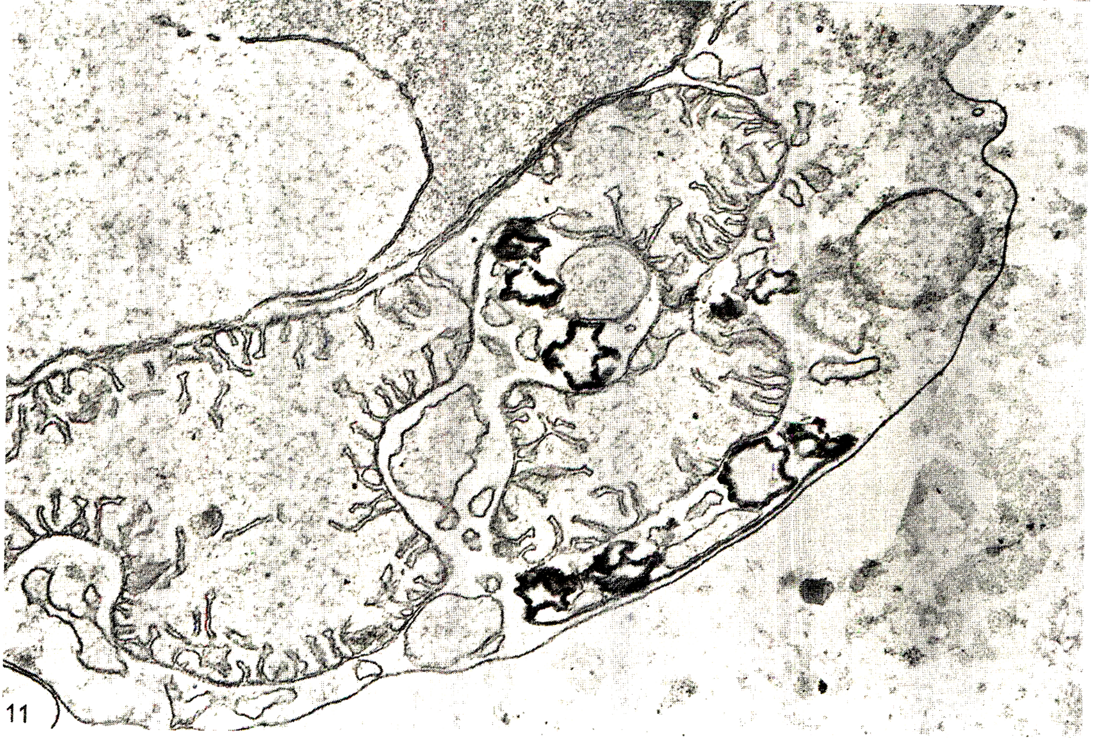

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J. gen. Microbiol. (1969), 59, 383–391
Printed in Great Britain

The Immunodepressive Effect of a Murine Plasmodium and its Interaction with Murine Oncogenic Viruses

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(*Accepted for publication 17 September 1969*)

SUMMARY

Plasmodium berghei yoelii (p.b.y.) was found to cause an acute self-limiting infection in Balb/c mice, lasting for 14 to 18 days. A sharp fall in the primary response to sheep erythrocytes, as measured by the number of haemolytic plaque-forming cells in the spleen, and by the appearance of antibodies in the serum, coincided with high levels of parasitaemia between the 8th and 11th days of p.b.y. infection. A secondary response to sheep erythrocytes was similarly affected when animals were infected with p.b.y. 9 days before the second antigen injection. Mice were resistant to reinfection with p.b.y., which produced either transient or no parasitaemia, and no immunodepression.

In mice carrying an immunodepressive leukaemogenic virus by vertical transmission, infection with a similar dose of p.b.y. was usually fatal.

Murine sarcoma virus (Harvey: m.s.v./H) produces tumours and splenomegaly in newborn mice but very rarely in adults. When injected into adult Balb/c mice at the height of p.b.y. infection, m.s.v./H produced a high incidence of splenomegaly 4 weeks later, although the splenomegaly induced by the plasmodium alone had by then subsided.

These results are discussed in relation to Burkitt's (1969) hypothesis of a causal connection between chronic malarial infection and development of Burkitt lymphoma in children.

INTRODUCTION

The peculiar pattern of incidence of African lymphoma of childhood, first described by Burkitt (1959) and frequently confirmed (e.g. Haddow, 1963; Edington & Maclean, 1964; Ten Seldam, Cooke & Atkinson, 1966), has led to an intensive search for an insect-vectored virus as its cause. Although several viruses have been found associated with Burkitt's lymphoma (B.l.), and its distribution has been shown to correspond to that of certain mosquitoes, neither the hypothesis of viral causation, nor that of insect transmission, has been proved or disproved (see Wright, 1967; Harris, 1967; Henle, 1968, for reviews).

Recently it has been suggested that the significance of B.l. distribution may not be its correspondence with the habitat of certain vectors, but with the prevalence of another vectored disease, malaria. O'Conor (1961) considered the possibility that parasitic infection might be a factor in the development of B.l., as had others for other lymphomas (Higginson, 1963). It was Dalldorf and his colleagues (1964) who first

noted that high incidence of B.l. occurred in areas of holo-endemic malaria, i.e. where splenomegaly and parasitaemia are found in over 75 % of children of 2 to 9 years, adult splenomegaly rate is low, and tolerance of infection in adults high. The association was found both in Kenya and in New Guinea. They suggested that the 'primary cause' of B.l. is a widely distributed agent, but that lymphoma develops only when the reticuloendothelial system (r.e.s.) is stimulated by chronic malarial infection. Since then others have noted the correspondence between B.l. and malarial incidence (Edington & Maclean, 1964; Henle, 1968). Burkitt (1969) has recently collected evidence for the association, and speculated upon its meaning.

Significant correlations are notoriously difficult to establish where standards of diagnosis and record differ widely, and where many variables cannot be controlled. Further surveys will be awaited with interest, but in the meantime it is worth considering how chronic malarial infection might influence the development of a lymphoma.

The authors quoted confine themselves to supposing, with Dalldorf and his colleagues (1964), that the r.e.s. may react 'differently' to the 'cause' of B.l. (presumably a virus) after malarial infection. What kind of difference might this be?

There are several possibilities, e.g. (i) malaria causes great hyperplasia of the r.e.s., and this may provide more cells able to support proliferation of an oncogenic virus, and thus increase the likelihood of malignant transformation; (ii) prolonged stimulation by plasmodial antigens may eventually lead to malignant change among immune-reacting cells; or (iii) the parasite may produce an altered immune reactivity, leading to an abnormal response to an oncogenic virus or to the cellular antigens which it induces. For the first there is no factual evidence. The second has been suggested in other cases (Metcalf, 1961; Schwartz & Beldotti, 1965; Walford, 1966; East & de Sousa, 1966) but certainly not proved. The third is considered in the present work.

The strong cellular antigenicity of many strains of B.l. (Henle & Henle, 1966; O'Conor, 1961), the unusually high cure rate after relatively small doses of drugs (Ziegler *et al.* 1967; Burkitt, 1967), and the occurrence of spontaneous cures (D. P. Burkitt, personal communication), all suggest that the disease is strongly influenced by the patient's immune reaction. Some have postulated that the difference between B.l. and acute leukaemia is due to a difference in immune reaction to a causative agent (Burchenal, 1966; Clifford, 1966). Leukaemia, it is supposed, may follow an early (prenatal or neonatal) transmission of an oncogenic agent which induces tolerance, while B.l. may result from a later transmission (by contact or vector) of the agent which induces an immune reaction. Alternatively, a higher immune reactivity in the African B.l. patient may be due to intense exposure to a variety of other infectious agents (Henle & Henle, 1966), such as several species of plasmodia. In both cases the assumption is that the B.l. patient is making a stronger immunological response than the leukaemic to an oncogenic virus or to the cellular antigens which it induces.

If both conditions were caused by the same virus, antigenic cross-reactivity between the cells of B.l. and of leukaemias would be expected; none has been found so far (Henle & Henle, 1966). If B.l. were merely leukaemia confined to certain sites by a strong immune response, we might expect it to arise in the presumptive sites of origin of leukaemic cells: lymphoid organs or bone marrow. B.l., at any rate in its early stages, conspiciously spares these sites. Moreover there is no evidence that B.l. patients react particularly strongly to other antigenic stimuli.

Although immunoglobulins IgM and IgG increase earlier in life and reach a higher

adult level in Africans than in black or white residents in other countries, there is no evidence that the former have more active immune responses. In malarial and other parasitic diseases raised IgM and IgG levels are common, though only partially accounted for by a parallel increase of protective antibody against the causative organism (Smithers, 1967; Curtain, Kidson, Champness & Gorman, 1964).

There is much evidence that procedures or agents which damage or functionally exhaust the r.e.s. affect the course of malarial and other parasitic infections (Goble & Singer, 1960). Surprisingly little is known about the response of a host infected with malaria to other antigens. The only relevant report we have found is that of McGregor & Barr (1962), who found a higher incidence of non-reactors to tetanus toxoid among malarious than non-malarious children in the Gambia. Intercurrent infections in malaria have been little investigated, and we do not know whether bacteria or viruses evoke a greater or a lesser antibody response from a host with an acute or long-term malarial infection. The effect of protozoan infections on the general capacity of an organism to produce immune responses to other antigens has been little explored.

Depression of immune responses by certain viruses and bacteria is well established, and has been recently reviewed (Salaman, 1969; Floersheim, 1969). In the present work, methods used in the study of immunodepression by murine leukaemogenic viruses (Salaman & Wedderburn, 1966; Wedderburn & Salaman, 1968) have been employed to examine the effect of a murine plasmodium on immune responses in mice.

METHODS

Mice

Balb/c mice, maintained by brother-sister mating, were fed a pellet diet (FFG, made by F. G. Dixon Ltd., Ware, Herts) and water *ad lib.*

Virus preparations

Murine Sarcoma Virus (*Harvey*) (*m.s.v./H*). A 10 % extract of livers and spleens from animals infected with this agent was received from Dr J. J. Harvey. It produced splenomegaly and multiple sarcomas in 100 % of animals injected when newborn.

Urethane Leukaemia Virus (*u.l.*). This leukaemogenic virus was isolated by one of us from urethane-induced leukaemia (Salaman, 1963), and was found to pass by vertical transmission (v.t.). Balb/c mice carrying the virus (u.l./v.t.) develop gross splenomegaly with or without lymph node enlargement at 6 to 9 months.

Plasmodial infection in mice

In preliminary experiments several murine plasmodia were tested with the object of finding one which would produce, in mice of a strain previously used for immunological tests, an acute self-limiting disease of sufficient duration for the performance of these tests. The infection produced in 5- to 14-week-old Balb/c mice by intraperitoneal (i.p.) injection of approximately 10^6 organisms of *P. berghei yoelii* (p.b.y.) was found to be suitable.

A group of mice were injected with 70,000 infective sporozoites per mouse. Eight days later the mice, which had 10 to 15 % parasitized erythrocytes were bled, and the blood, diluted 1+1 with Ringer containing 15 % glycerin and 20 units heparin/ml., was stored at −70°. For experimental purposes two mice received i.p. 0·1 ml. of

the stored infected blood. Six days later they were bled, and the blood was diluted so that 10^6 parasitized erythrocytes were contained in a volume suitable for i.p. injection into test mice.

In the infection caused by this dose, parasitaemia, first detectable on the 3rd day, rose, sometimes showing a small peak on the 4th day, until on the 9th to 11th day mice had 15 to 35 % parasitized erythrocytes The percentage then declined until the 15th to 17th day, when less than 1 % parasitized erythrocytes were found. Splenomegaly, detectable by palpation on the 3rd day, reached a peak about the time of the peak of parasitaemia, when spleens weighed 1 to 2 g. (the normal weight for adult mice of this strain is 0·1 to 0·15 g.), and persisted for some time after parasitaemia was no longer detectable. The mice looked fluffed and hunched and were anaemic (haematocrit 25 to 35 %) between the 8th and 13th days, and then recovered. No deaths occurred among mice aged five weeks or more, but of a batch of three-week-old mice which received a smaller dose (10^5 parasitized erythrocytes) half died.

Immunological tests

Serum haemagglutinin (ha) and haemolysin (hl) titres and also the number of haemolytic plaque-forming cells (p.f.c.) in the spleen were determined after the intravenous (i.v.) injection of $2{\cdot}5 \times 10^8$ sheep erythrocytes. The number of p.f.c. were determined by the method of Jerne, Nordin & Henry (1963) with minor modifications. Details of the immunological tests used have been described elsewhere (Salaman & Wedderburn, 1966; Wedderburn & Salaman, 1968).

RESULTS

Immune response to sheep erythrocytes in mice infected with malaria

Eight-week-old Balb/c mice were infected with plasmodia, and equal groups were then given $2{\cdot}5 \times 10^8$ sheep erythrocytes (s.e.) i.v. 4, 7 and 9 days later. Seven days after antigen injection ha and hl titres of the first two groups showed a three- to fourfold depression compared with uninfected controls; however, the titres then began to rise, and had reached control levels 3 weeks after s.e. injection. Animals which received s.e. 9 days after p.b.y. had no detectable ha or hl 7 days later, and antibody was still barely detectable 3 weeks after antigen injection.

In unimmunized Balb/c mice there are 40 to 140 p.f.c. per spleen against s.e. In mice infected with p.b.y. a gradual rise in this 'background' number of p.f.c. began 4 days after infection, and reached 500 to 1000 p.f.c. by the 7th day. The count remained at about this level till the 13th day, after which it declined slowly, but was still significantly raised 2 months after infection.

The number of p.f.c. per spleen 4 days after a dose of $2{\cdot}5 \times 10^8$ s.e. was then determined for groups of four mice which had received the antigen at different intervals after infection with the plasmodium. The results are shown in Fig. 1, together with the degree of parasitaemia at the time of s.e. injection. Notwithstanding the rise in 'background' p.f.c. in infected mice there was a short but severe impairment of the immune response to the antigen when given during a period which roughly coincided with the period of maximum parasitaemia.

The number of p.f.c. per spleen in mice which had received s.e. 10 days after p.b.y. was followed for some time, to ascertain whether the reduction in the p.f.c. response at

4 days was merely a delay. Results showed that there was no significant delay, but a true reduction. The number of p.f.c. in immunized infected mice never rose higher than 1600 per spleen. Uninfected immunized mice, on the other hand, showed a peak response of 250,000 to 300,000 p.f.c. per spleen on day 4.

The primary p.f.c. response after repeated injections of plasmodia was next examined. When mice received a second injection of p.b.y., or a series of four injections at fortnightly intervals, no recurrence of parasitaemia was observed, and when a second injection of p.b.y. was followed by s.e. there was no depression of the p.f.c. response.

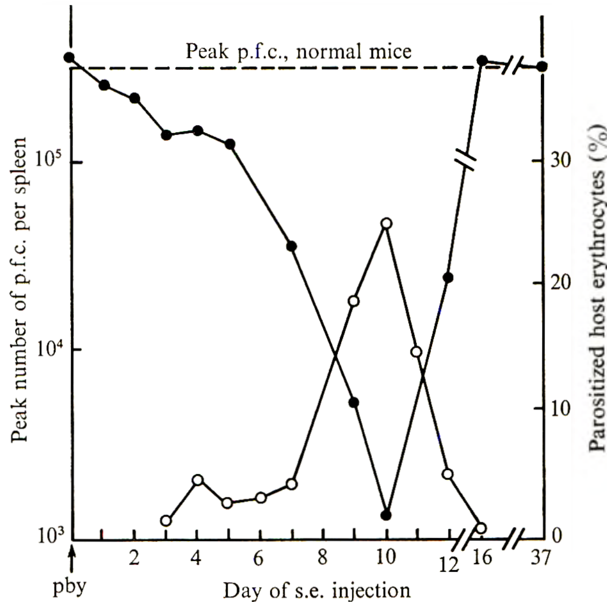

Fig. 1. Effect of *Plasmodium berghei yoelii* (p.b.y.), injected at various times before a standard dose of sheep erythrocytes (s.e.) on the peak number of haemolytic plaque-forming cells (p.f.c.) per spleen. Four mice in each group. Mice received 10^6 parasitized erythrocytes (p.b.y.) intraperitoneally. $2{\cdot}5 \times 10^8$ sheep erythrocytes were injected i.v. on the day indicated. Mean peak number of p.f.c. per spleen (measured 4 days after s.e. injection): —●—●—; mean percentage of erythrocytes parasitized: —○—○—.

The effect of plasmodial infection on the secondary response was examined briefly. When mice were infected 9 days before a primary injection of s.e., to which they showed an extremely depressed response, as described above, they nevertheless responded vigorously to a second dose of s.e. given 5 weeks after the first: they were obviously not tolerant to the antigen. However, further work is necessary to determine whether this response has the characteristics of a primary or of a secondary reaction. On the other hand mice which received p.b.y. 9 days before the second of two injections of s.e. separated by 4 to 5 weeks showed a 10-fold rise in the residual p.f.c. count usually found 4 to 5 weeks after a primary s.e. injection and by a 20-fold depression of the response to the second s.e. injection. This result is qualitatively similar to that observed when p.b.y. was given before a primary s.e. injection.

Effect of malarial infection on the pathogenicity of an oncogenic virus

Since murine plasmodial infection depressed the immune response to s.e., it was of interest to discover whether it altered the response to infection by oncogenic viruses. Murine sarcoma virus (Harvey) (m.s.v./H) (Harvey, 1964) was chosen as an example, because it causes tumours, splenomegaly, and early death when injected into newborn animals, but is much less pathogenic in adults. If the reason for this difference, in this case and in those of other viruses with similar age-dependent pathogenicity, were the relative incompetence of the neonatal immune system, then it would be expected that the effects of m.s.v./H in plasmodium-infected adults would approximate to those in normal newborn mice.

Two groups of eight Balb/c mice aged 10 weeks received 10^6 p.b.y. per mouse. Ten days later one group received i.p. 0·1 ml. of m.s.v.-infected plasma and a third untreated group also received m.s.v.

Four and a half weeks after the injection of m.s.v. the mice were killed. The group which had received p.b.y. followed by m.s.v. had spleens weighing from 0·25 to 1·5 g. (average 0·71 g.), with numerous superficial nodules. Histologically they presented the usual appearance of m.s.v. spleens, but contained in addition a considerable amount of malarial pigment (F. C. Chesterman, personal communication). One mouse which had received m.s.v. only appeared sick at this time. It had a ruptured spleen weighing 0·47 g., and was the only mouse in this group in which any evidence of m.s.v. infection was seen; the others all had spleens weighing less than 0·25 g., and showed no gross pathology. No tumours were found in either of the m.s.v.-infected groups. Animals which had received p.b.y. only had spleens weighing less than 0·25 g., containing much malarial pigment.

It is clear therefore that the pathological effects of m.s.v. in the spleens of adult mice were greatly increased by prior infection with p.b.y., while the production of solid tumours was not increased, at least over the time-span used.

Malarial infection in mice carrying a vertically transmitted virus derived from urethane-induced leukaemia

Balb/c mice carrying the virus u.l. by vertical transmission (u.l./v.t.) develop gross splenomegaly, with or without lymph node enlargement, at 6 to 9 months. Gradually increasing splenomegaly, and a slight degree of immune depression, are found before this, i.e. from about the 8th week. U.l./v.t. mice of 6 to 10 weeks of age were infected with p.b.y. in the usual way, in the hope that recovered mice might show a changed incidence or time of development of overt leukaemia.

The malarial infection progressed in the usual way until the 12th day. After that, instead of recovering like normal Balb/c, the u.l./v.t. mice continued to deteriorate, and out of 19 mice 16 died between the 15th and 21st days, with 50 % or more parasitized erythrocytes and gross anaemia.

The severe but short-lived immune depression which occurs during a primary infection of Balb/c mice with p.b.y. does not prevent the development of resistance which terminates the infection and prevents reinfection. However, the above experiment shows that development of effective resistance is prevented by the presence of a virus which exerts a more prolonged immunodepressive effect than the parasite itself.

DISCUSSION

The question originally asked, whether malarial infection can predispose to infection by an oncogenic virus, has not been fully answered. It has been shown that a murine plasmodium severely depresses immune reactivity for a short period at the height of parasitaemia, and that the pathogenicity of a murine sarcoma virus injected during that period is enhanced. Conversely it has been shown that mice carrying a leukaemogenic and immunodepressive virus succumb to a dose of *Plasmodium berghei yoelli* which is followed by only temporary illness in normal mice.

An incidental finding was that the number of spleen cells capable of lysing sheep erythrocytes in unimmunized mice is significantly raised in plasmodial infection. This recalls the suggestion that increased titres of haemolysins and haemagglutinins to foreign erythrocytes in Africans may be related to infectious processes, especially malaria (Zuckerman, 1966; Kano, McGregor & Milgrom, 1968).

There is little evidence to indicate the mechanism of immunodepression in this case. The infected mouse is carrying a large burden of foreign antigen at the time of maximum immune depression, and the possibility of antigenic competition is not excluded. This possibility, in relation to immune depression by a virus, was discussed in a previous paper, and suggestive though not conclusive evidence against it was presented (Wedderburn & Salaman, 1968). Further work is needed to determine what part, if any, antigenic competition plays in immunodepression by plasmodial infection.

The best animal model of the situation which Burkitt has suggested in man would be chronic plasmodial infection in a mouse carrying a leukaemogenic virus. Our attempt to examine the immune status of mice chronically infected with the plasmodium has been frustrated by solid resistance to reinfection. Repeatedly injected mice show no illness and no immunodepression. However, Jerusalem (1968) repeatedly infected Swiss mice with *P. berghei berghei*, usually lethal in these mice, by cutting short the initial infection with a *p*-aminobenzoic acid-free diet. At 11 days these mice failed to reject both homo- and allogeneic skin grafts, and after 6 months of repeated injections there was some evidence of increased incidence of malignant lymphoma (Jerusalem, 1968, and personal communication).

The significance of these findings in the relation of plasmodial infection to neoplasia is a matter for speculation until further evidence accumulates. A range of immune reactions, cell-associated as well as humoral, against a variety of antigens, should be examined at various stages of plasmodial infection.

The early immunodepression in acute plasmodial infection of mice, and the increased incidence of malignant lymphoma in chronically infected mice, suggests, by analogy, a reason for the correlation which Burkitt has noted between the incidence of Burkitt lymphoma and malaria in man.

In planning this work we received valuable advice from Professor P. C. Garnham. We are indebted to Dr F. C. Chesterman for histological diagnosis, to Mr R. S. Killick Kendrick, Mrs B. Adkins and Mr R. Saldanha for technical, and to Miss C. Cowen for secretarial, assistance.

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J. gen. Microbiol. (1969), 59, 393–400
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Isolation and Characterization of Substance in Yeast Extract which Inhibits Growth of Thymine-less Strains of *Escherichia coli*

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(*Accepted for publication 19 September 1969*)

SUMMARY

Yeast extract which promotes the growth of wild-type strains of *Escherichia coli* had a potent bactericidal action on thymine-less mutants. The active principle in the yeast extract was found to be adenosine. All of the other nucleosides and their bases tested except guanine, hypoxanthine and inosine also showed various degrees of bactericidal activity. The activity of adenosine was competitively annulled by the addition of excess thymidine to the medium, but thymine showed practically no anti-adenosine effect.

INTRODUCTION

During a study of thymine metabolism by thymine-less mutants of *Escherichia coli* and *Klebsiella* (*Aerobacter*) *aerogenes*, Harrison (1965) observed inhibition of growth by yeast extract, a nutrient frequently used in bacterial culture media. From the previous findings of Cohen & Barner (1956, 1957) and Zamenhof & Giovanni (1956) that the growth of thymine-less strains of *E. coli* was inhibited by some nucleosides, Harrison (1965) suggested that the active principle in the yeast extract might be nucleosides, although he did not isolate and identify the principle.

We also observed a similar phenomenon to that of Harrison (1965), that is, the growth of thymine-less strains of *Escherichia coli* was inhibited in a polypeptone agar medium containing yeast extract whereas the growth of wild-type strains was greatly enhanced in the same medium. Accordingly we examined the active principle in yeast extract and found it to be adenosine. We then made a systematic survey of the effects of naturally occurring nucleosides and their bases on the growth of thymine-less bacteria; the results of these experiments are reported in this paper.

METHODS

Bacteria. Three strains of bacteria and their thymine-less mutants were used. *Escherichia coli*, B3 (S. Brenner), 15T$^-$ (S. S. Cohen) and W3110-22 (T. Okada) required 1 μg., 1 to 2 μg. and 7 to 10 μg. thymidine/ml., respectively, to sustain full growth. *Escherichia coli*, strains BN, 15WT and W3110 were prototrophic.

Media and buffer. In the following formulae quantities of components are given in g./l. of distilled water. λ-Broth medium: polypeptone (Daigo Nutritive Chemicals Co., Ltd.), 10; NaCl, 5, adjusted to pH 7·2 with N-NaOH. GSC medium: $(NH_4)_2HPO_4$, 2·5; KH_2PO_4, 1·5; NaCl, 5; Na glutamate, 3; adjusted to pH 7·2 with N-NaOH. After

sterilization of the mixture, 10 ml. of 30 % (w/v) glucose, 10 ml. of 10 % casamino acids, 0·5 ml. of 20 % $MgSO_4$ and 5 ml. of 0·1 M-$CaCl_2$, each sterilized separately were added to 1 l. of the mixture. λ-Agar and λ-top agar: agar (Nihon Eiyokagaku Co., Ltd.) 15 and 7, respectively added to 1 l. λ-broth. Supplements of yeast extract, thymidine and the other compounds related to nucleic acids were added to each medium as indicated in the results. Dilution buffer contained: KH_2PO_4, 1·902; Na_2HPO_4, 5·76; and NaCl, 15 (pH 7·2): after sterilization, 1 l. was mixed with 1 ml. of sterilized 0·5 M-$MgSO_4$.

Assay of bactericidal activity. Two assay methods were used. The first method was done on λ-agar containing various concentrations of test compounds. Test bacteria were suspended in λ-broth to an extinction of 0·1 at 660 nm. and the suspension streaked on the λ-agar with a standard wire loop of 3 mm. diameter. Plates were incubated at 37° overnight. The bactericidal activity of the test compounds was defined as the greatest dilution which did not give any bacterial growth on the agar. In the second method bacteria were incubated with shaking with various concentrations of the test compounds in GSC medium at 37° and after incubation colony counts were made by the agar layer technique. Bacterial growth (net mass increase) was also measured in terms of extinction at 660 nm.

Fractionation and purification of the active principle from yeast extract. The various steps used in the isolation of the bactericidal principle from yeast extract are outlined in Fig. 1; further purification was achieved by chromatography on a silica gel column. Fraction D (see Fig. 1) was dissolved in a small quantity of elution solvent, the lower layer of a mixture of $CHCl_3$ + MeOH + water (70 + 30 + 10, by vol.) and applied to a column (25 × 250 mm.) of silica gel (Kanto Chemical Co.; for chromatographic use) by the dry system method. The column was eluted with 900 ml. of the above solvent and 100 ml. fractions collected. The elution was completed by using 100 ml. MeOH. Fractions were evaporated to dryness. Small needle-shaped crystals were obtained from fractions no. 5 and 6, which were re-chromatographed repeatedly and re-crystallized from water. Measurements were made of the melting point and ultraviolet spectra of the small white needle crystals thus obtained and they were subjected to elementary analysis. Their activity was assayed by the first method described above.

Thin layer chromatography (t.l.c.). All fractions obtained by column chromatography and the purified preparation of active principle were subjected to t.l.c. on plates coated with silica gel G (Merck) and the same solvent system as that used for column chromatography. Substances were applied in a line 1·5 cm. from the end of a plate 20 cm. long. Solvent was allowed to ascend 15 cm. from the origin. The spots of material were located by spraying the plate with 10 % (w/v) H_2SO_4 and then heating it.

Chemicals. All the nucleosides and bases used were purchased from Sigma Chemical Company.

Apparatus. A Hitachi Ltd. photoelectric photometer, type EPO-B, a Shimazu recording spectrometer, type SV-50A, and a Yanagimoto C.H.N. Coder, type MT-1, were used.

RESULTS

Effect of yeast extract in solid medium on bacterial growth

The capacities for growth of the three thymine-less mutants and their original wild parents were tested on λ-agar containing 0, 0·037, 0·063, 0·125, 0·5, 1·0 and 2·0 %

(w/v) yeast extract, respectively. Plate 1 shows that, with 0 % yeast extract, both of the auxotrophs and the wild type bacteria grew uniformly, but growth of strain W3110-22 was inhibited completely with 0·037 % yeast extract, $15T^-$ strain with 0·125 % and B3 strain with 0·25 %. This order of sensitivities for yeast extract seemed to be correlated with the thymine requirements of the three bacteria. The growth of all three wild bacteria was enhanced by increase in the yeast extract concentration. With 2 % yeast extract, the wild strains yielded rather thick lawns of growth.

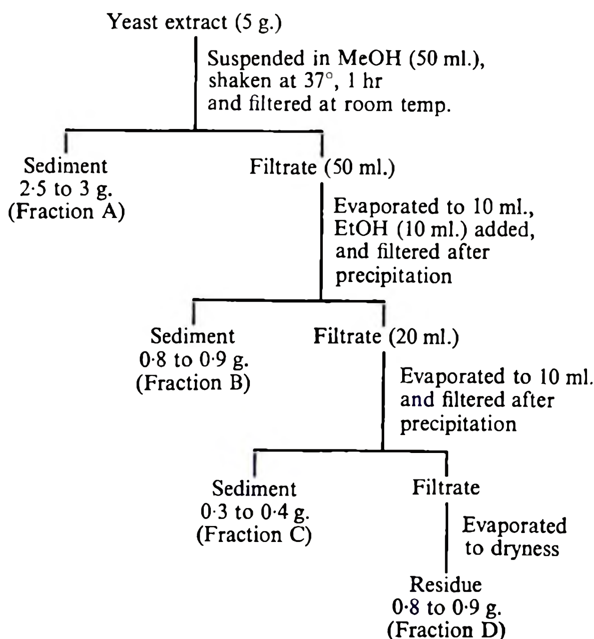


Fig. 1. Fractionation of the active principle of yeast extract.

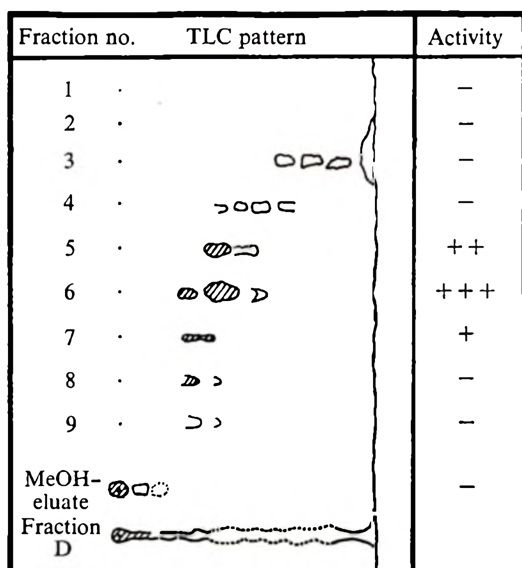
Extraction and identification of the active principle from yeast extract

Methanol and ethanol were used for the extraction. The procedure is outlined in Fig. 1. Fractions A and B had no activity. There was a trace of activity in fraction C but almost all the activity was recovered in fraction D which formed an orange resinous residue on evaporation of the solvent.

Silica gel chromatography of fraction D. To purify the active principle further, it was subjected to column and thin layer chromatography. Figure 2 shows the patterns on t.l.c. and the activity of each of the residues of the fractions obtained by column chromatography. Small needle-shaped crystals were obtained from fractions no. 5 and 6, which contained most of the activity. In Fig. 2 spots with oblique shading are those which became black on heating, suggesting that the active principle might contain a sugar.

The crystals from fractions no. 5 and 6 were combined, re-chromatographed and crystallized from a minimum amount of water. Small white needle-shaped crystals were obtained.

Identification of the active principle. The R_F value of the recrystallized active principle was the same as that of adenosine but not of other nucleosides and adenine (Fig. 3). The ultraviolet spectra of the purified crystals were measured in acidic, neutral and alkaline solutions. The $\lambda_{max.}$ and $\lambda_{min.}$ of the substance almost coincided with those of adenosine (Table 1). The melting points of crystals of the purified material and its picrate were 229° and 195 to 198°, respectively, and the former agreed with that of adenosine and was not lowered by admixture with authentic adenosine. Analysis on the unknown crystals gave the following: C, 44·40; H, 4·76; N, 25·6 %; and authentic adenosine C, 44·90, H, 4·76; N, 26·21 %. These results indicated that the active principle in yeast extract was definitely adenosine.



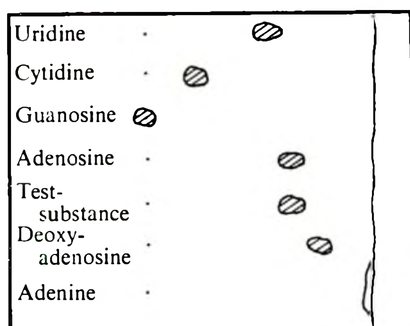


Fig. 2

Fig. 3

Fig. 2. Activity and pattern produced by thin layer chromatography of each subfraction obtained from column chromatogram. Fraction D was subjected to silica gel chromatography and the various sub-fractions thus obtained were tested for their anti-bacterial activity and their behaviour on thin layer chromatography. Spots with oblique shading scorched deep black and other spots became yellow, orange or brown. Bactericidal activity is expressed as + + +, strong; + +, medium; +, weak and −, negative.

Fig. 3. Identification of the active principle by thin layer chromatography. All spots except adenine scorched black.

Table 1. *$\lambda_{max.}$ and $\lambda_{min.}$ in ultraviolet spectra of the active principle and adenosine*

Solution	Active principle $\lambda_{max.}$ (mμ.)	Active principle $\lambda_{min.}$ (mμ.)	Adenosine $\lambda_{max.}$ (mμ.)	Adenosine $\lambda_{min.}$ (mμ.)
0·1 N-HCl	258·5	229·8	258·5	230·4
In water	260·5	227·3	260·0	226·5
0·1 N-NaOH	262·5	233·7	262·0	235·0

Inhibition of growth of thymine-less mutants on λ-agar by natural bases and nucleosides

The bactericidal activities of a wide variety of natural bases, ribonucleosides and deoxyribonucleosides were tested at the same time on agar plates (Table 2). All these natural metabolites except guanine and its nucleosides had some activity. Growth of the three wild-type bacteria, used as controls, tended to be stimulated by these substances as by yeast extract.

Table 2. *Bactericidal activities of bases or their nucleosides in λ agar*

	Strain					
	E. coli W3110–22		*E. coli* 15T⁻		*E. coli* B3	
	Inhibition					
Agent	complete	partial	complete	partial	complete	partial
	mM	mM	mM	mM	mM	mM
Uracil	0·25	0·125	0·5	0·25	.	2·0
Cytosine	.	2·0	.	2·0	.	–
Adenine	.	2·0	.	2·0	.	–
Guanine	.	–	.	–	.	–
Uridine	0·5	0·25	1·0	0·5	2·0	1·0
Cytidine	0·5	0·25	1·0	0·5	2·0	1·0
Adenosine	0·5	0·25	1·0	0·5	2·0	1·0
Guanosine	.	–	.	–	.	–
Deoxyuridine	0·25	0·125	0·5	0·25	0·5	0·25
Deoxycytidine	1·0	0·5	2·0	1·0	2·0	1·0
Deoxyadenosine	.	×	.	×	.	×
Deoxyguanidine	.	.	.	×	.	×

–, No inhibition with 2 mM; ×, no inhibition with 4 mM.

The order of sensitivities was *Escherichia coli* W3110-22 > *E. coli* 15T⁻ > *E. coli* B3 and that of bactericidal activities of the agents was deoxyuridine, uracil > adenosine, uridine, cytidine > deoxycytidine > adenine > cytosine > deoxyadenosine.

Growth inhibition of a thymine-less mutant by natural bases and nucleosides in liquid medium

The inhibitory effects of natural bases and nucleosides on the growth of thymine-less mutants were also studied in liquid medium using *Escherichia coli* B3. The use of 15T⁻ or W3110-22 was avoided, since the former is known to be colicinogenic (Ryan, Fried & Mukai, 1955) or lysogenic (Sandoval, Reilly & Tandler, 1965; Endo *et al.* 1965; Mennigmann, 1965; Frampton & Brinkley, 1965) and the properties of the latter are not as well known as those of B3. In this experiment even when the bacteria were washed with GSC after only brief treatment with the active agent, they did not survive. Microscopic examination showed that about 90 % of the bacteria formed filaments within 90 min. after treatment with the agent and did not divide during further incubation. These results can be summarized as follows. The effects of deoxyribonucleosides differed as between liquid medium and solid medium. Thus deoxyribonucleosides stimulated the growth of *E. coli* B3 in liquid medium (Fig. 4). However, when

the concentration of bacteria was very low, deoxyribonucleosides inhibited growth (Fig. 5). Accordingly, it seems that the action of deoxyribonucleosides depends on the bacterial concentration. On the contrary, the bactericidal effect of adenosine did not seem to depend on the concentration of bacteria. Of the bases, uracil had the strongest activity while as in solid medium neither guanine nor hypoxanthine had any activity (Fig. 6). The growths of all wild-type strains were stimulated by all the bases

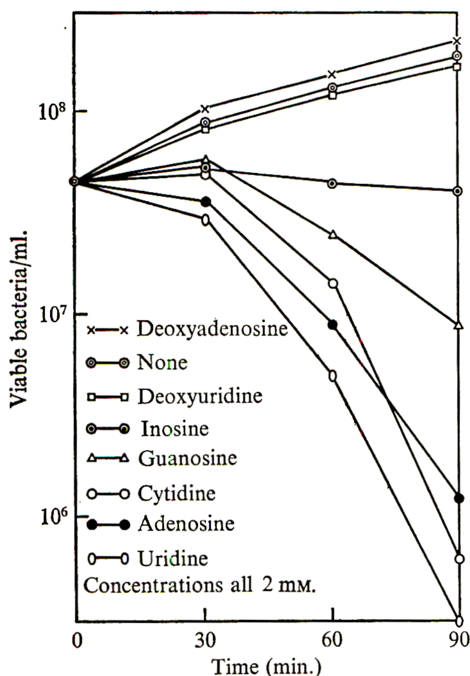


Fig. 4

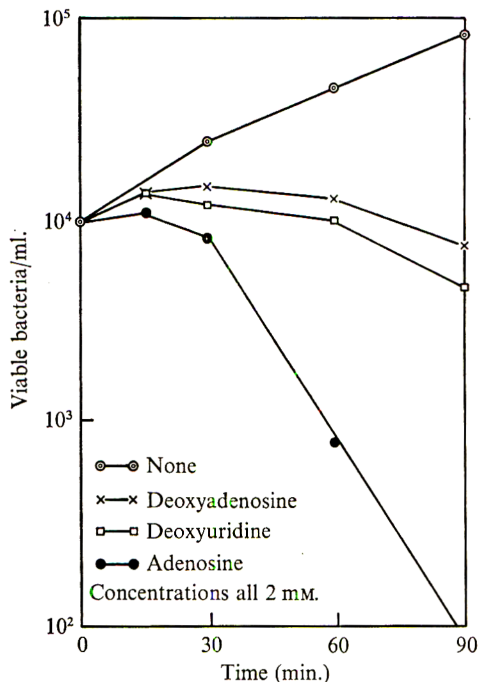


Fig. 5

Fig. 4. Effect of various nucleosides on growth in GSC medium. *Escherichia coli* B3 growing exponentially in GSC medium with 1 μg. thymidine/ml. was used. Effects were tested in GSC medium containing 1 μg. thymidine/ml. and the required supplement.

Fig. 5. Effect of deoxyribonucleosides when bacterial concentration of *Escherichia coli* B3 was small. The conditions of bacterial growth and medium were the same as for Fig. 4.

and nucleosides tested. When sufficient thymidine was added in the presence of a bactericidal concentration of adenosine, the bactericidal activity was annulled competitively in proportion to the thymidine concentration (Fig. 7). On the other hand, when thymine was used as the nutritional requirement, the growth rate of the bacteria was decreased to about half that in thymidine and also the antagonistic effects on the bactericidal action of adenosine were considerably decreased as compared with those obtained with thymidine (Fig. 7).

DISCUSSION

Harrison (1965) first reported a difference in the sensitivities to yeast extract, uridine and cytidine of mutants of *Aerobacter aerogenes* and *Escherichia coli* requiring 25 μg. thymine and those requiring 1 to 2 μg./ml. He also reported that the strains which

needed a relatively high concentration of thymine were more sensitive to these agents. Our results also showed the same tendency as his. In general, uracil or its nucleosides produced a greater bactericidal activity than the other bases or their nucleosides. Regarding the effects of deoxyribonucleosides, previous workers have reported only their stimulatory effect on the growth of thymine-less bacteria. However, in the present work deoxyuridine was the most potent inhibitor of the thymine-less mutants on solid

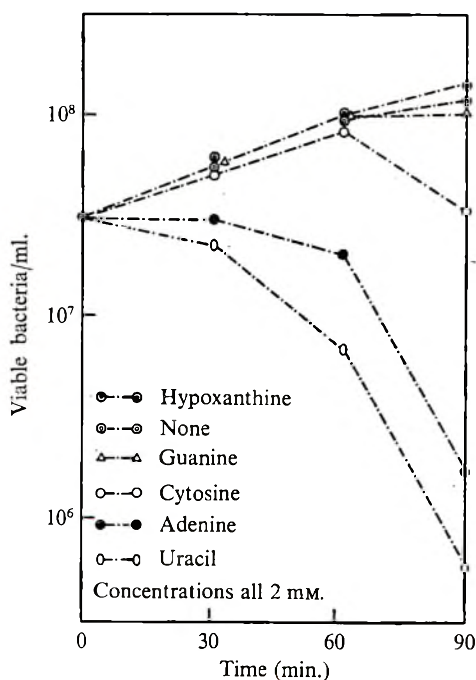


Fig. 6

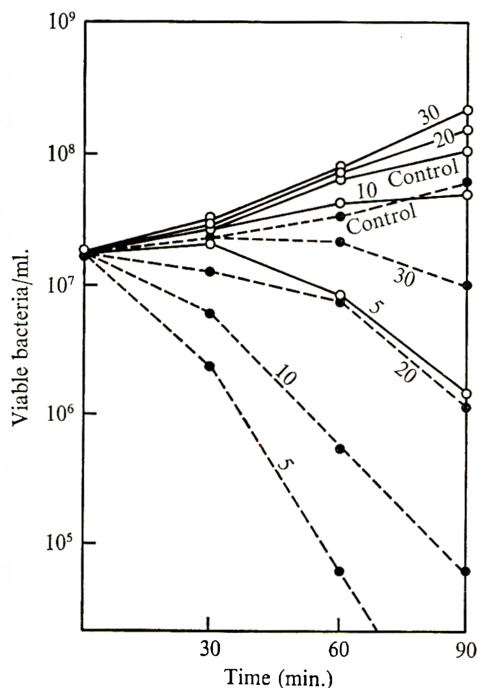


Fig. 7

Fig. 6. Effect of various bases on growth in GSC medium. Except for the agents added the conditions were as for Fig. 4.

Fig. 7. Effect of thymidine or thymine concentration on inhibition by adenosine. Numbers represent the final concentration (μM.) of thymidine (—O—O—) or thymine (--●--●--) in the medium which, except for the control, contained 2 mM adenosine. The two control media respectively contained only 5 μM. thymidine or thymine.

medium. An inhibitory effect of deoxyuridine was also seen in liquid medium when the bacterial concentration was very low.

The present findings may be related to thymine-less death (Cohen & Barner, 1954) as well as to the regulation of DNA synthesis in thymine-less bacteria. It is interesting that, in spite of the presence of enough thymine to sustain growth of these mutants, natural metabolites related to nucleic acid have a strong bactericidal action on such mutants but not on the parent wild-type strains. Recently, we have found that a thymine-less strain produced thymine riboside from thymidine *in vivo* when the strain was incubated in the medium with a high concentration of adenosine (in preparation). It seems that this phenomenon could be related to the results of Fig. 7, since thymine which possesses no deoxyribose could be easily converted to its riboside by a reaction

involving adenosine. Since thymine and uracil are of similar molecular structure, the bactericidal action of uracil and its nucleosides may be due to competition effects in enzyme systems involving thymine.

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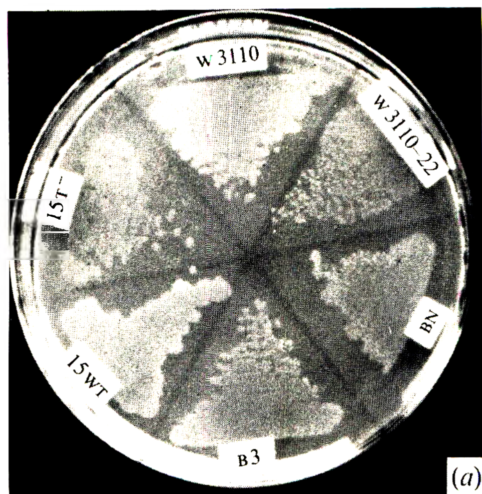
EXPLANATION OF PLATE

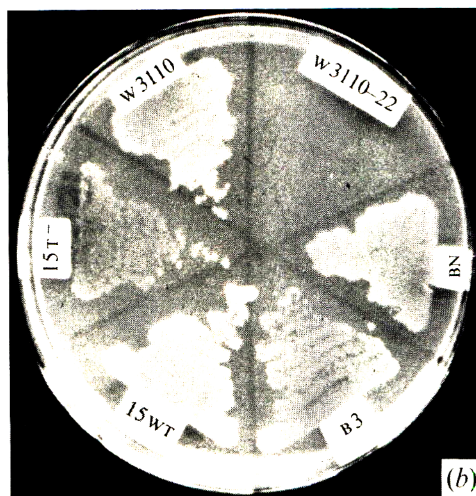
PLATE 1 (*a*, *b*, *c*, *d*)

Growth of *Escherichia coli* mutants on λ agar containing yeast extract. The nutritional properties of the bacteria are described in the Methods.



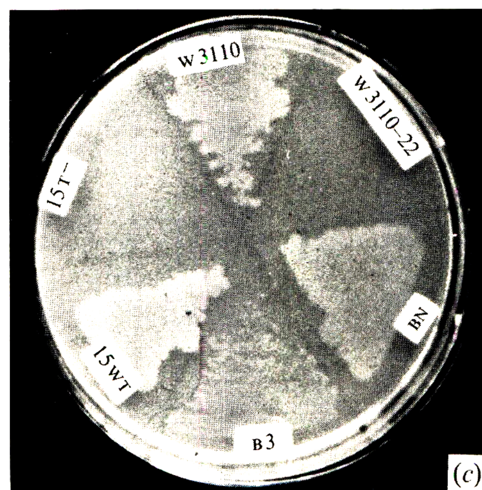
(*a*)

Yeast extract 0%



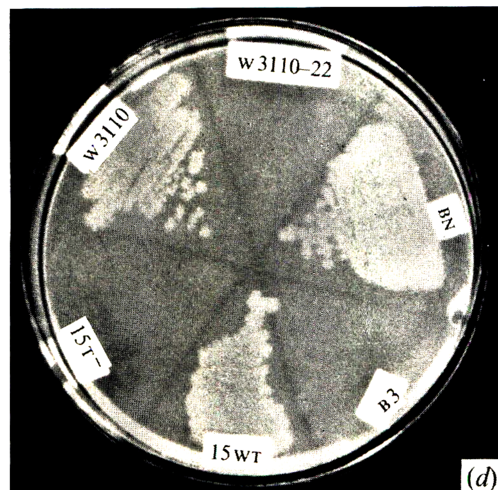
(*b*)

Yeast extract 0·037%



(*c*)

Yeast extract 0·125%



(*d*)

Yeast extract 0·25%

N. KINOSHITA, H. AKIYOSHI AND H. ENDO (*Facing p.* 400)

J. gen. Microbiol. (1969), 59, 401–410
With 2 plates
Printed in Great Britain

Host-independent Growth of *Bdellovibrio bacteriovorus* in Microbial Extracts

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(*Accepted for publication 23 September 1969*)

SUMMARY

Three *Bdellovibrio bacteriovorus* wild-type strains tested grew into long forms, up to 80 times their original lengths, when incubated in cell-free microbial extracts. This growth occurred both in extracts of organisms permissive and non-permissive for Bdellovibrio parasitic growth, but not in any other medium tested. Under certain osmotic conditions the long forms segmented into chains of cells. Some division of chains occurred. Only approximately 5 % of the bdellovibrios grew into long forms. ^{3}H-thymidine was incorporated into the long forms but not into short forms. A component of *Pseudomonas aeruginosa* extracts which supported growth of Bdellovibrios was macromolecular, heat stable and resistant to pronase, DNAse and RNAse.

INTRODUCTION

Bdellovibrio bacteriovorus is a bacterial parasite which multiplies inside susceptible bacterial host organisms. After growing inside the host, it segments into daughter cells which are released from the ghosted host (Scherf, DeVay & Carol, 1966; Starr & Baigent, 1966; Shilo, 1969). The bdellovibrio-host system provides an experimentally accessible model for studying certain aspects of intracellular parasitism which are often more difficult to approach with parasites of animals and higher plants (Shilo, 1969). A problem encountered with all such systems is the difficulty of varying the environment in which the parasite grows. In an attempt to minimize this difficulty with bdellovibrios we have developed a new system that permits limited extracellular growth and division.

METHODS

Bacterial strains and growth. The *Bdellovibrio bacteriovorus* strain routinely used was a streptomycin-resistant isolate (spontaneous mutation) of strain 109. Bdellovibrios were grown in liquid culture using *Escherichia coli* K12 strain P567 as host, according to the conditions of Varon & Shilo (1968). *Escherichia coli*, *Pseudomonas aeruginosa* and *Bacillus subtilis* strain 68A were grown on rotary shakers at 37° in nutrient broth; *B. cereus* T, at 30° in nutrient broth; *Micrococcus lysodeikticus*, at 37° on brain heart infusion; and *Saccharomyces cerevisiae*, at 30° in nutrient broth + 2 % (w/v) glucose, adjusted to pH 6·0. *Pseudomonas aeruginosa* was isolated from a patient at Hadassah Hospital, Jerusalem. Other strains were from the collections of the Departments of Microbiological Chemistry and Bacteriology, Hebrew University, Hadassah Medical

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School. Bdellovibrio plaques were assayed on *E. coli* P567, by the method of Varon & Shilo (1968).

Preparation of cell extracts. Cultures were harvested in late log phase (or for bdellovibrios, soon after host lysis was complete). Organisms were sedimented in a centrifuge, washed once and resuspended in a solution containing 0·3 % (w/v) Difco-Casamino acids (vitamin free), pH 7·5 (hereafter called 'amino acid buffer'). Bacteria were broken by sonic treatment for 5 to 10 min. at 4 to 6 amps in 10 to 30 ml. on a Bronson sonifier model no. 5110. For some experiments *Pseudomonas aeruginosa* organisms were lysed by incubation at 30° (pH 7·8) for 20 min. in a solution containing 30 μg. lysozyme/ml. and 4 mM-EDTA, after which they were incubated for 10 min. at 20° in a solution containing 20 μg. DNAse/ml. and 10 mM-$MgSO_4$. Debris and most remaining intact cells were removed by centrifugation at 20,000 *g* for 15 min. Extracts were then filtered through HA filters (Millipore Corp., Bedford, Mass.) to remove any remaining intact organisms. For some experiments extracts were separated into supernatant and pellet fractions by centrifugation at 120,000 *g* for 2 hr at 4°. In these cases the pellet was washed in amino acid buffer, recentrifuged as above and resuspended in amino acid buffer. Supernatant and pellet fractions were refiltered through HA filters. Extracts or fractions were then dialysed in tubing (No. 18/32, Scientific Instrument Centre, London, England) which had been sterilized by being autoclaved suspended from a capped glass tube into a flask containing the liquid to be dialysed against. Unless stated otherwise, 'bacterial extract' refers to an extract of *P. aerugenosa* prepared by sonication, filtered and dialysed overnight (1:200) against amino acid buffer. These extracts retained 50 to 90 % of their 'activity' (see below) after storage up to 1 month at 4°.

Incubation mixture for induction of long forms. The standard incubation mixture contained $1·5 \times 10^9$ bdellovibrios/ml., 5 mg. Difco-Casamino acids (vitamin free), pH 7·5/ml. and bacterial extract or other material being tested for its ability to induce growth. Incubation was at 30°, with shaking, for the time stated. Bdellovibrios were prepared from cultures cooled to 4° when lysis of *Escherichia coli* appeared by phase contrast microscopy to be complete. After 0 to 8 hr, cultures were centrifuged at 750 *g* for 2 min. to remove any precipitate present in the growth medium, and then at 27,000 *g* for 20 min. to pellet the bdellovibrios. These organisms were then resuspended in modified TM buffer (0·01 M-tris (pH 7·5) + metals; Varon & Shilo, 1968), the concentration measured spectrophotometrically and a portion added to the incubation mixture as $\frac{1}{3}$ to $\frac{1}{2}$ the final volume. Bacterial extract was added to a final concentration of 1·0 mg. protein/ml., unless otherwise stated. Protein determinations were by the Biuret method.

Thymidine-incorporation experiments. To measure thymidine incorporation into acid-precipitable material, thymidine-methyl-^{3}H (2 c/mM, Radiochemical Centre, Amersham, England) was added at the designated time to the standard incubation mixture just described, normally to final concentration 3 μc./ml. At the same time, non-labelled thymidine was added to final concentration $0·5 \times 10^{-4}$ M. At appropriate times, 50 μl. samples were removed and applied to paper discs (Whatman 3 MM). Discs were passed successively through three beakers containing cold 10 % TCA and two beakers containing ethanol, and were dried. Radioactivity was measured in a scintillation counter. For the standard rate-of-incorporation assay, ^{3}H-thymidine was added after the standard incubation mixture had shaken for 12 to 13 hr. A 50 μl. sample was removed 2 hr later and the acid precipitable counts determined as above.

Microscopy. For determining the fraction of long forms in a Bdellovibrio population, cultures after 13 hr incubation were suspended in 0·05 M-Na azide plus 0·1 M-Na acetate (pH 6·0) to stop motility and then examined by phase contrast in a Petroff–Hausser counter. Each examination included 450 to 500 organisms. Those organisms which were both noticeably fatter than, and at least 4 × the length of, the normal small bdellovibrios present, were counted as long forms.

Materials. Nutrient broth, casein amino acids, yeast extract, tryptone and brain heart infusion were from Difco Laboratories, Detroit, Mich. RNAse, RNAse-free DNAse and alcohol dehydrogenase were from Worthington Biochemicals, Freehold, N.J. Pronase (grade B) was from Calbiochem, Los Angeles, Cal. Yeast RNA and calf thymus DNA were from Sigma Chemical Co., St. Louis, Mo.

RESULTS

Bdellovibrio growth in host-free cell extracts

When Bdellovibrio strain 109 was aerated at 30° in the presence of dilute dialysed cell-free extracts of *Pseudomonas aeruginosa*, approximately 5 % of the organisms grew into long forms. Morphological changes were first observed by phase contrast microscopy after 4 to 5 hr, at which time some of the bdellovibrios were fatter and slightly longer and had lost their motility. Within an hour, some fatter organisms were 3 to 4 times the length of most of the visible bdellovibrios, and were characteristically limp, bending and flopping on their shorter axis in response to random disturbances of the liquid. During the next 5 to 10 hr, progressively longer forms were visible. Over 80 % of these were clearly segmented into units approximately 0·4 μ × 0·9 μ (Pl. 1). Chains as long as 100 units appeared.

Partial division of chains less than 15 units long was observed occasionally. In these instances the intact chains appeared to be quivering. Single breaks occurred after some minutes, seemingly at random along the chain. Usually, additional breaks were observed at intervals of several minutes, and single units released swum out of the field. Plate 1, fig. 3–4 shows chains as they appeared during division.

Growth of long forms also occurred in dialysed cell-free extracts of *Escherichia coli*, *Micrococcus lysodeikticus*, *Saccharomyces cerevisiae* and *Bdellovibrio bacteriovorus* strain 109 itself (final protein concentrations of 1 mg./ml.). These forms were indistinguishable by phase contrast microscopy from those of Plate 1. We verified that *M. lysodeikticus* and *S. cerevisiae* are not hosts for Bdellovibrio strain 109. Little or no attachment of bdellovibrios to these cells was observed by phase contrast microscopy. No plaques were formed on these strains, even when loops of bdellovibrios at 10^9/ml. were spotted on cell lawns. Extracellular growth of bdellovibrios did not occur in any of a variety of complex media tested (see below).

In addition to Bdellovibrio 109, two strains independently isolated in Jerusalem from sewage were tested for extracellular growth (strains GB and T28, isolated by M. Varon). In *Pseudomonas aeruginosa* extract, each grew into long forms which closely resembled those of Plate 1.

In each of the above instances, only a fraction of the Bdellovibrios grew into long forms. Standard incubation mixtures prepared on separate days were estimated to be 6·2, 3·9, 5·3 and 7·0 % long forms (for standard conditions and estimation procedure, see Methods). We were unable to increase this fraction by varying extract con-

centrations, Bdellovibrio concentrations, the species source of the extract or the Bdellovibrio strain. The length of time after host cell lysis that the bdellovibrios were harvested had little effect. Other instances of phenotypic variation in a genetically homogeneous Bdellovibrio population, involving attachment to hosts, have been reported (Varon & Shilo, 1968, 1969*a*).

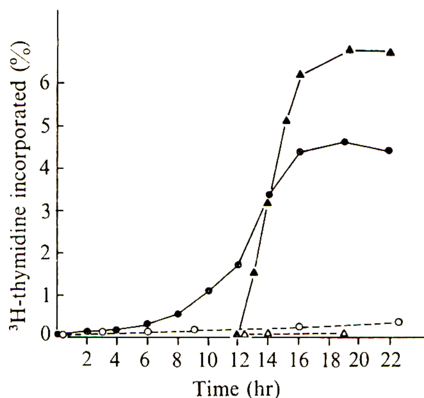

Fig. 1

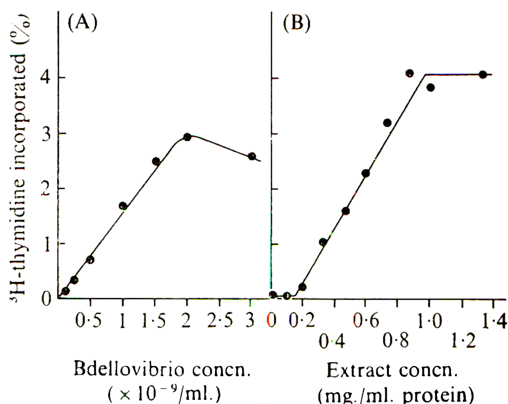

Fig. 2

Fig. 1. Incorporation of ^{3}H-thymidine during growth of long forms. Bdellovibrios were incubated with bacterial extract under standard conditions (see Methods) and 50 μl. samples were removed at various times for determination of acid-precipitable counts. ●, ^{3}H-thymidine added at $t = 0$; ▲, ^{3}H-thymidine added at $t = 12$ hr; open symbols, extract replaced by amino acid buffer in the incubation mixtures.

Fig. 2. Effect of bdellovibrios and bacterial extract concentrations on rate of thymidine incorporation. Standard rate of incorporation assays were performed (see Methods). (A) Bacterial extract concentration constant at 1·0 mg. protein/ml. (B) Bdellovibrio concentration constant at $1{\cdot}5 \times 10^9$ organisms/ml.

Thymidine incorporation during host-free Bdellovibrio growth

Thymidine was incorporated into bdellovibrios during incubation in which long forms developed, but not under other host-free conditions. Upon incubation of Bdellovibrio, Pseudomonas extract and ^{3}H-thymidine (see Methods) there was a lag period of several hours with little incorporation, followed by a much more rapid incorporation. If ^{3}H-thymidine was added after the lag period, it was incorporated immediately (Fig. 1). In the latter case, the rate of incorporation was found to vary linearily with both the concentration of cell extract and the Bdellovibrio concentration over the ranges shown (Fig. 2). Extracts of *Micrococcus lysodeikticus* stimulated incorporation to approximately the same extent. Separation of organisms according to size, on a sucrose gradient, showed that the label was distributed broadly among large forms, with < 2 % incorporated into bdellovibrios of normal size (Fig. 3). Over 90 % of the radioactivity incorporated was in DNAse-sensitive material.

On no occasion in this work did we observe long forms without thymidine incorporation or thymidine incorporation without long forms. Neither was ever observed without prior addition of cell extract or an extract fraction. None of the following nutrients induced long forms or thymidine incorporation: nutrient broth, yeast extract, tryptone, brain heart infusion (each at 29 and 35 mg./ml. final concentrations);

bovine serum albumin, haemoglobin, yeast RNA, calf thymus DNA, each at 0·2, 1 and 5 mg./ml. final concentrations, in 4 mg. casein amino acids/ml. pH 7·5; yeast RNA and calf thymus DNA degraded by RNAse and DNAse at 0·2, 1 and 5 mg./ml., in 4 mg. casein amino acids/ml. pH 7·5.

In view of these results, we considered thymidine incorporation to be a useful supplement of microscopic examination as an indicator of the development of Bdellovibrio long forms. In the preliminary characterizations that follow, extract activity is quantitated in terms of the rate of ^{3}H-thymidine incorporated into bdellovibrios under standard conditions (Methods). For each assay, growth at three or more extract concentrations was examined microscopically after 12 to 14 hr. In every instance this semi-quantitative examination was consistent with the results of the incorporation assay.

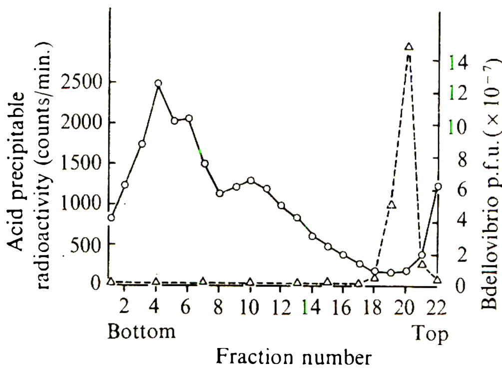

Fig. 3

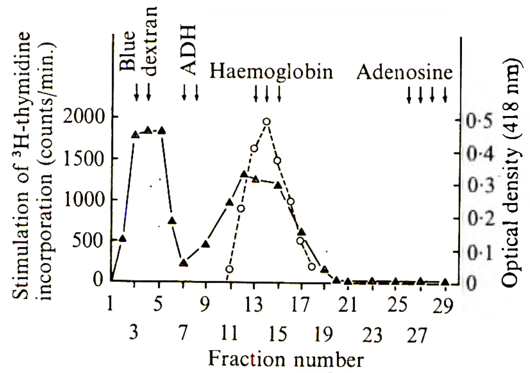

Fig. 4

Fig. 3. Sucrose gradient of Bdellovibrio organisms. ^{3}H-thymidine was added to a standard mixture of bdellovibrios and bacterial extract which had incubated for 12 hr. Following 3 hr additional incubation, 0·3 ml. of the mixture was layered on a 5 ml. gradient of 5 to 20 % sucrose in amino acid buffer. The gradient revolved at 2000 r.p.m. for 10 min. at 4° in a clinical centrifuge. The tube was then punctured at the bottom and drops were collected. Recovery in the drops was 70 % of the acid-precipitate counts applied to the gradient. ○——○, acid-precipitable counts; △-----△ Bdellovibrio plaque forming units.

Fig. 4. Sephadex G-150 chromatography of supernatant fraction of bacterial extract. A 3 ml. sample, containing 40 mg. of bacterial protein and added molecular weight markers was applied to a 100 ml. column of Sephadex G-150 (25 cm. × 4 cm.^{2}) equilibrated with 0·03 M tris, pH 7·5. Fractions of 2·2 ml. were collected and filter sterilized. Stimulation of ^{3}H-thymidine incorporation into bdellovibrios was measured by the standard rate of incorporation assay (see Methods), using 0·15 ml. fraction aliquots in 0·3 ml. reaction mixtures. Incorporation of 1000 counts/min. is 1 % of the ^{3}H-thymidine added. ADH is yeast alcohol dehydrogenase, MW 151,000. ▲——▲, thymidine incorporation; ○-----○, optical density (418 nm) due to haemoglobin marker.

An active factor in cell extracts is macromolecular and heat stable

A factor from cell extracts which induced long forms was non-dialysible. The activity in extracts of *Pseudomonas aeruginosa*, *Escherichia coli*, *Micrococcus lysodeikticus*, *Saccharomyces cerevisiae* and *Bdellovibrio bacteriovorus* increased 20 to 100 % upon dialysis (based on rate of thymidine incorporation). Upon centrifugation of a dialysed sonicated extract of *P. aeruginosa*, at 120,000 × g for 2 hr, 40 to 60 % of the activity was recovered from the pellet and an approximately equal amount from the supernatant. (The same distribution between supernatant and pellet was obtained from extracts of *P. aeruginosa* prepared by lysozyme-EDTA).

The supernatant material was fractionated by G-150 Sephadex chromatography. All recovered activity, representing 65 % of the total applied to the column, was in fractions of molecular weight ⩾ 50,000 (Fig. 4). No inhibitory material was detected in the fractions of lower molecular weight or the fractions between the two activity peaks. Long forms were induced by all fractions which stimulated thymidine incorporation (fractions 2 to 19), although the lengths were variable and, in the area between the peaks, considerably shorter than in the long forms induced by the unfractionated material. The other fractions did not induce long forms of any size. The same pattern of activity was obtained when the molecular weight markers were omitted.

The non-dialysible factor from extracts was heat stable. More then 90 % of the activity from diluted dialysed *Pseudomonas aeruginosa*, *Escherichia coli* and *Micrococcus lysodeikticus* extracts, or the supernatant fractions, remained after boiling for 20 min. When extracts were autoclaved or boiled at very high concentration, a heavy flocculent precipitate appeared, and 50 to 100 % of the activity was lost.

The factor was stable to DNAse, RNAse and pronase. Dialysed *Pseudomonas aeruginosa* extracts were first diluted to 5 mg. protein/ml. in 0·02 M-tris (pH 7·5) and heated for 5 min at 98°. To test DNAse and RNAse, $MgSO_4$ was added to 10 mM, the enzymes added each to 30 μg./ml., and the solution incubated for 30 min. at 25°. Full activity remained, both before and after dialysis. To test pronase, the enzyme (final concentration 100 μg./ml.) was added to the diluted heated extract, and the solution incubated at 45°. After 60 min. the mixture was heated for 5 min. at 98° to inactivate the pronase, and was assayed. Full activity remained. (If an extract was incubated at 45° without prior heating at 98°, 30 to 90 % of the activity was lost; the loss of activity varied between extract preparations, but was essentially the same whether or not RNAse, DNAse or pronase was present.)

An assay for division

^{3}H-thymidine was incorporated almost exclusively into long forms (Fig. 3). If division occurs, label should appear later in short forms, with a corresponding loss from long forms. Evidence of this was obtained by the direct filtration of long form cultures on cellulose ester (Millipore) filters. Long forms are retained on 1·2 μ (mean pore diameter) filters, while most short forms pass through. Fig. 5A shows that after 15 to 20 hr of incubation label appeared in material which passed through 1·2 μ filters. The results were basically the same when addition of ^{3}H-thymidine was delayed (Fig. 5B). (Levels of incorporation measured by 0·22 μ filter retained-material, and by acid-precipitable material, were indistinguishable.)

To examine more closely the size distribution of the divided forms, a Bdellovibrio mixture labelled as in Fig. 5B was displayed on a sucrose gradient after 14 and 24 hr incubation (Fig. 6). In the period of sedimentation of these gradients, the undivided long forms pelleted, so that only small forms were visible (compare Fig. 3). Figure 6 shows that after 14 hr little or none of the acid-precipitable radioactivity was in the region corresponding to small organisms, while at a later time 50 % of the label was in this region. The radioactivity peak was slightly ahead of the bulk of the bdellovibrio organisms probably because the newly divided bdellovibrios were more compact than the longer and thinner older organisms and therefore sedimented faster (see below).

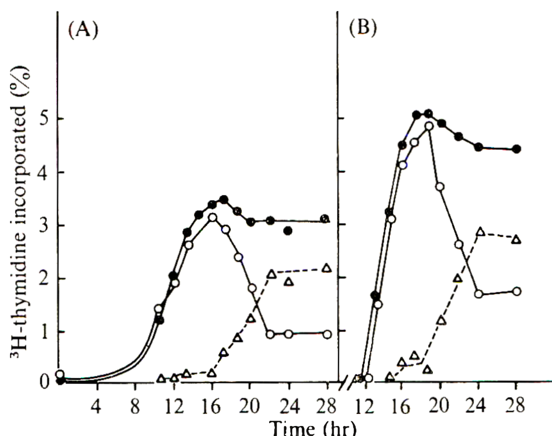

Fig. 5. Appearance of label in short forms. ^{3}H-thymidine was added to a standard incubation mixture at t = 0 (A) or t = 12 hr. (B). At various times 0·2 ml. samples were removed into 10 ml. of cold amino acid buffer and mixed for 1 min. Equal portions were filtered on 0·22 μ and 1·2 μ filters. Each filter was washed with 50 ml. nutrient broth. ●——●. Material retained on 0·22 μ filters, ○——○, material retained on 1·2 μ filters; △-----△, material passing through 1·2 μ but retained on 0·22 μ filters (by subtraction).

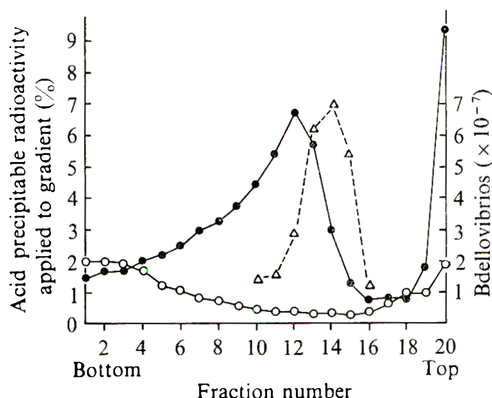

Fig. 6

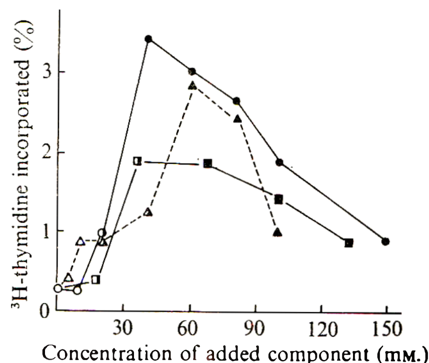

Fig. 7

Fig. 6. Sucrose gradients of Bdellovibrio organisms. ^{3}H-thymidine was added to a standard incubation mixture at t = 12 hr. At the specified times, 0·3 ml. of the mixture was layered on a sucrose gradient as in Fig. 3 and revolved at 2000 r.p.m. for 30 min. The tubes were then punctured and drops were collected. ○——○, Acid-precipitable counts of sample removed at t = 14 hr; ●——●, acid-precipitable counts of sample removed at t = 24 hours; △-----△, Bdellovibrio concentration determined by Petroff–Hausser counter.

Fig. 7. Effect of osmolarity on morphology and thymidine incorporation. Standard rate of incorporation assays were performed, except that the usual amino acid concentration of 5 mg./ml. was replaced by various amino acid, sucrose, or NaCl concentrations. After 13 hr incubation, cultures were examined by phase contrast microscopy. ○——●, Amino acid added; △ --- ▲, sucrose added; □——■, NaCl added. Open symbols, coiled long forms; closed symbols, segmented, uncoiled forms; half closed symbols, intermediate or both forms.

Two morphologically distinct long forms depending on osmotic conditions

In addition to the segmented long form already described, a tightly coiled non-segmented long form was induced when amino acids were omitted from the standard incubation mixture (Pl. 2, fig. 8–11). The effect was not due to nutrition, since the NaCl or sucrose concentrations also controlled which form appeared (Fig. 7). At intermediate concentrations, forms intermediate between tight-coil and segmented straight forms occurred. The addition of NaCl, sucrose or amino acids after 13 hr growth of long forms in their absence did not induce a conversion of coiled to segmented forms, nor did it stimulate increased thymidine incorporation.

Rich medium induces inclusion bodies in Bdellovibrio short forms

In testing various rich media for growth of long forms, we frequently observed that prominent inclusion bodies, previously unreported in Bdellovibrio, were induced in the short forms. A reproducible procedure which we adopted was to incubate bdellovibrios at 30° in cell-free dilute nutrient broth (DNB of Shilo & Bruff. 1965) in which *Pseudomonas aeruginosa* had been heated for 30 min at 65°. Under these conditions, over 70 % of the bdellovibrios developed one or more inclusion bodies within 4 hr (Pl. 2, fig. 12). Despite the small size of the bdellovibrios, these bodies were also visible by phase contrast microscopy.

DISCUSSION

Evidence had accumulated, from the enzymic content of Bdellovibrio (Simpson & Robinson, 1968) and its ability to grow equally well on live, heat-killed and u.v.-killed hosts (Varon & Shilo, 1969*b*), that the role of the host bacteria in Bdellovibrio infection was limited to satisfying nutritional and/or physical requirements. The growth of bdellovibrios in bacterial extracts reported here further confirms this view. Since extracts of both hosts and non-hosts support growth, it is apparent that factors other than nutrition prevent bdellovibrios from parasitizing certain organisms. A physical barrier against attachment and/or penetration is the likely limitation in many cases. Chemical defences may be relevant as well, however. We noted in this connexion that dialysed and undialysed extracts of *Bacillus cereus* and *B. subtilis* induced few or no long forms, and inhibited the development of long forms when added to other extracts.

Osmotic as well as nutritional conditions affected extracellular growth in our system. At low osmolarity only non-segmenting forms occurred. On the other hand, low osmotic strength was excellent for motility (Varon & Shilo, 1968) and respiration (S. C. Rittenburg, unpublished data) of normal extracellular bdellovibrios. This may reflect the natural dichotomy encountered by bdellovibrios in their normal life cycle. Intracellular growth causes only limited leakage of host material to the extracellular environment (Drucker, 1969), and so presumably occurs at high osmolarity. Motility and maintenance, on the other hand, occur in dilute extracellular environments.

Extracellular bdellovibrios demonstrate considerable metabolic activity (Simpson & Robinson, 1968; Varon & Shilo, 1969*b*). Differences were expected, however, between activities involving motility and maintenance, and those which involve growth. We looked for an activity specific enough to the growing forms to overshadow the corresponding activity in the majority population of the short forms. Experiments with radioactive uracil and leucine showed significant incorporation into both forms. Thymidine, however, was incorporated only into the long forms. Since growth of

long forms in bacterial extracts occurred whether or not thymidine was added for an incorporation measurement, other sources of thymidine were available in the incubation mixtures. Nevertheless, we found that ^{3}H-thymidine incorporation consistently was correlated with the amount of long forms.

We do not know to what extent the limited extracellular growth in our system is relevant to the intracellular parasitic growth of bdellovibrios. It could instead represent a facultative ability for extracellular growth, with characteristics and controls different from those of the intracellular cycle. There exist mutant Bdellovibrio strains which can multiply extracellularly in complex media with no requirement for a factor from bacterial extracts (Shilo & Bruff, 1965; Seidler & Starr, 1968; Dietrich, Denny, Hashimoto & Conti, 1969). The growing forms observed with such strains are significantly shorter than those in our system (M. Starr, personal communication). It is tempting to speculate that chains of up to 100 cells accumulate in our system because the bdellovibrios require a 'divide signal' which would normally occur intracellularly when nutrients were depleted, or when the pressure of the confining host cell wall was sensed. The osmotic requirement in our system also may suggest that growth is proceeding as if intracellularly. Additional studies are needed to clarify the relationship between the three demonstrable forms of Bdellovibrio growth (i.e., intracellular, extracellular in microbial extracts and extracellular by selected strains in complex media).

Intracellular parasites need access to the host materials. Many such parasites are permeable to molecules normally excluded from most organisms (Moulder, 1962; Hall & Claus, 1963). A particularly striking case is that of *Clamydaie psittaci*, which changes to a more permeable form during the intracellular stage of its life cycle (Tamura & Manire, 1968; Moulder, 1968). Bdellovibrios may well undergo a similar change to a more permeable intracellular form. Since the extracellular long forms reported here may closely resemble intracellular bdellovibrios, it would be of interest to measure the relative permeability toward various molecules of the long and short forms. Such a permeability change early in intracellular development could exert direct control over many metabolic processes. Thus, DNA synthesis might begin when precursors which are normally excluded from the organism are free to enter. It is possible that thymidine uptake and subsequent incorporation into DNA in our system is an immediate consequence of a permeability change.

Elongation of bdellovibrios after lysis of the host culture has been observed previously (Varon & Shilo, 1968). Typically, bdellovibrios, short and plump upon their release from the host, become thinner, rod-like and somewhat longer, sometimes doubling or tripling their original length. Burger, Drews & Ladwig (1968) briefly reported a different observation, elongation of bdellovibrios into spiral forms in host cell extracts. In none of these cases do the lengths approach those observed in our system, and in no case was segmentation reported.

One of us (A. M. R.) received a grant from the B. de Rothschild Fund for the Advancement of Science and Technology, and an Advanced Studies Fellowship from the Hebrew University. These are gratefully acknowledged.

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EXPLANATION OF PLATES

Figures 5-7 and 11-12 are electron micrographs taken by Dr Martin Kessel of this department. These specimens were prepared by negative staining by either 1 % potassium phosphotungstate (pH 7·0 to 7·2) or 1 % ammonium molybdate. Specimens were viewed in an AEI EM6B electron microscope operating at 60 kv.

PLATE 1

Bdellovibrio long forms grown on *Pseudomonas aeruginosa* extract. Photographs made after 16 hr incubation at standard conditions. Small bdellovibrios are visible in background.

Fig. 1-4. Phase contrast microscopy (× 1400).

Fig. 5. Electron microscopy (× 6000).

Fig. 6. Electron microscopy (× 7500).

Fig. 7. Electron microscopy (× 4500).

PLATE 2

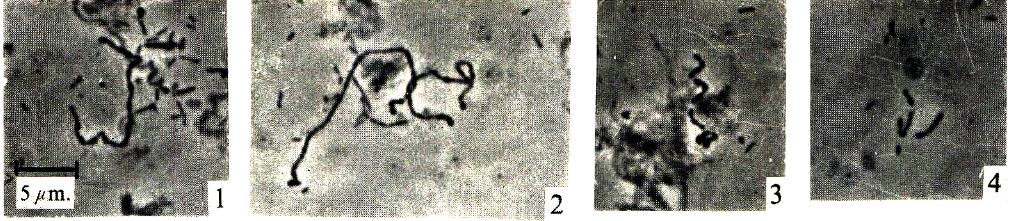
Fig. 8-11. Bdellovibrio long forms grown at low osmotic strength (see text). Photographs made after 16 hr incubation.

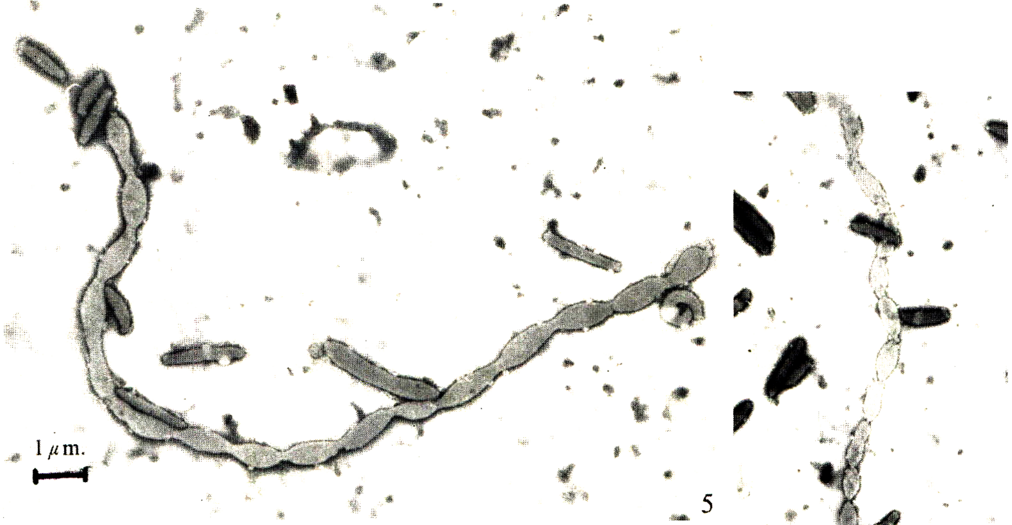
Fig. 8-9. Typical highly coiled forms. Phase contrast microscopy (× 1400).

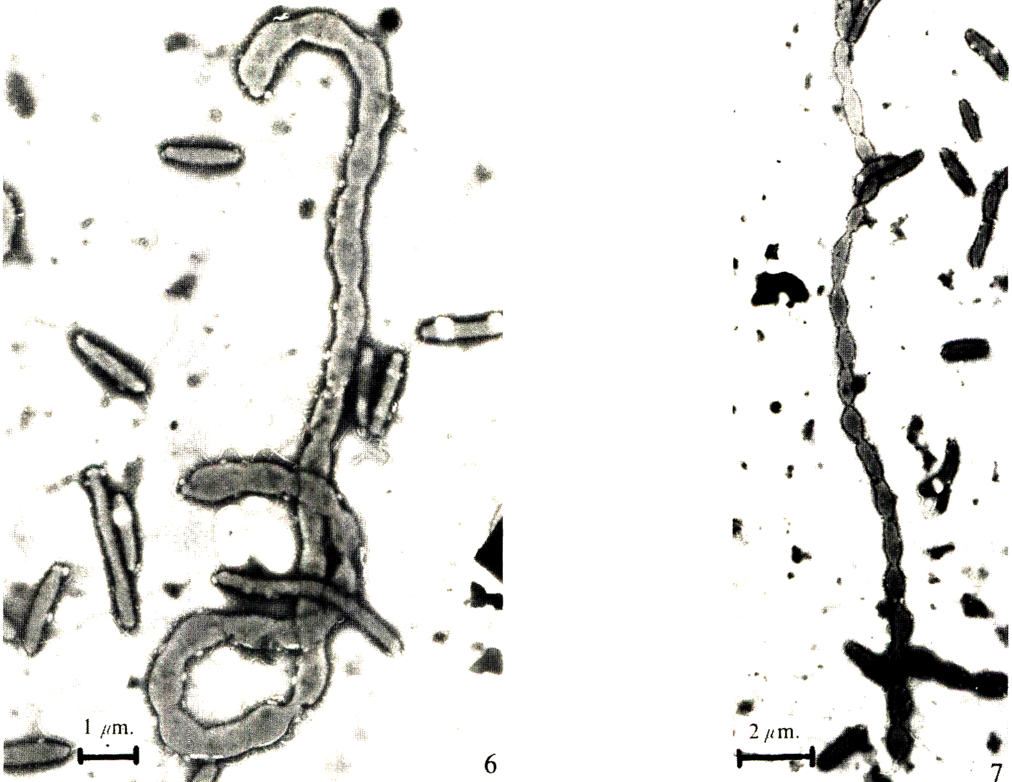
Fig. 10. Less typical, less coiled form, showing coils more clearly. Phase contrast microscopy (× 1400).

Fig. 11. Electron micrograph (× 17,500). Highly coiled forms appear partly unravelled in electron microscope preparations.

Fig. 12. Bdellovibrio with inclusion bodies (× 20,000).







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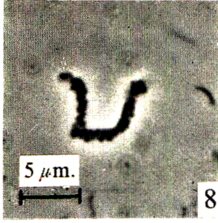
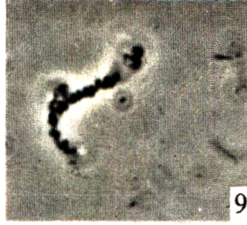
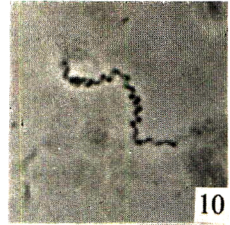
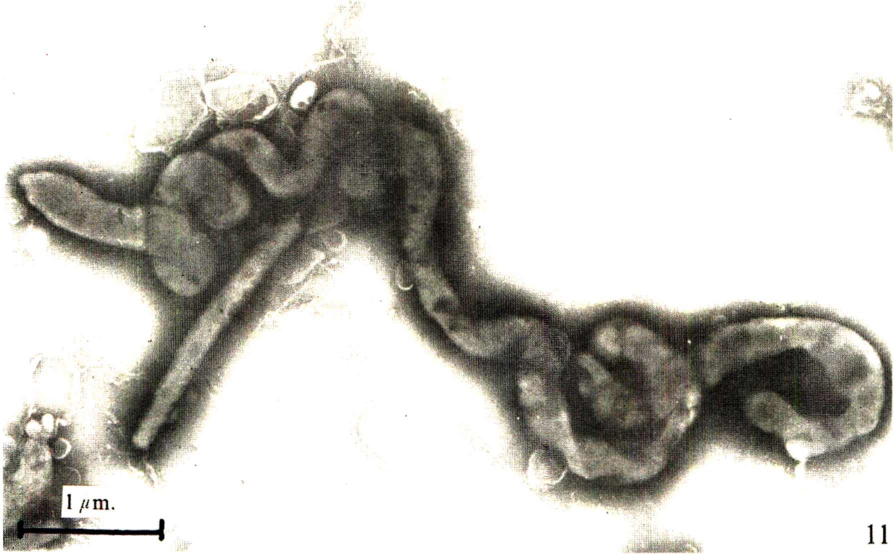
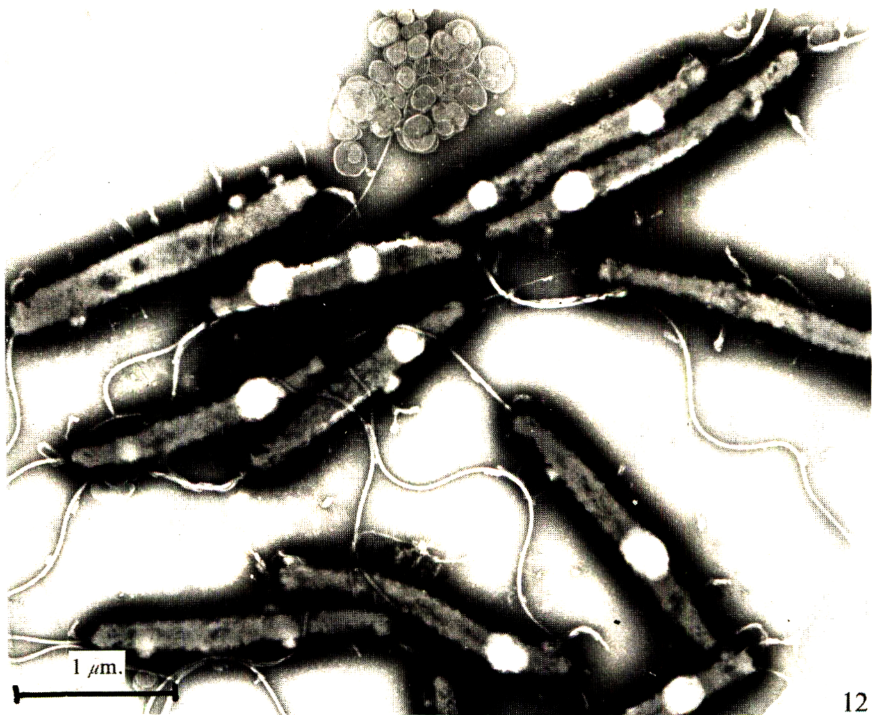
(*Facing p.* 410)











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J. gen. Microbiol. (1969), **59**, 411–425
Printed in Great Britain

The Somatic Antigens of Two Strains of *Rhizobium trifolii*

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(*Accepted for publication 26 September 1969*)

SUMMARY

Strain-specific lipopolysaccharide extracted by hot phenol from two strains of *Rhizobium trifolii* was fully antigenic in rabbits with an injection schedule using Freund's adjuvant, and highly active in gel diffusion. A minor second diffusion line was considered to be due to a smaller fragment of the main molecule, since the antigen was converted almost entirely to this form by sodium dodecylsulphate and such disaggregated material absorbed antibodies to both forms of the antigen. Chemically the lipopolysaccharide showed some features related to those of the Enterobacteriaceae; for example, the presence of firmly bound lipid, 2-keto-3-deoxyoctonate, glucose, mannose, fucose and, in one strain, a heptose. However, the lipopolysaccharide of *R. trifolii* was unusual in its very low phosphorus content and the presence of glucuronic acid. One strain diverged even further in its high content of carbohydrate, its lack of a heptose, its anionic behaviour and the gelatinous nature of some preparations; these properties might relate to the presence of a capsule-like structure which has been observed in old cultures of this strain.

INTRODUCTION

The composition and structure of the lipopolysaccharide (LPS) somatic antigens of Gram-negative bacteria has been most studied in the Enterobacteriaceae, in which the general pattern (Lüderitz, Staub & Westphal, 1966) is that of a glucosamine-containing lipid component, linked through a heptose phosphate core to a polysaccharide containing 2-keto-3-deoxyoctonate (KDO) and bearing highly specific sugar side chains. However, recent work on *Xanthomonas* spp. (Volk, 1966, 1968*a*, *b*) reveals that this is not invariable. The toxic phenol-extracted LPS from 20 Xanthomonas species contained KDO, uronic acid, glucose, mannose and various combinations of galactose, fucose, xylose and rhamnose; in no case was a heptose detected. The present work reports properties of the LPS extracted by hot aqueous phenol from two serotypes of *Rhizobium trifolii*, with the object of comparing them with the pattern established in the Enterobacteriaceae, and determining any differences which might contribute to the antigenic distinction between the two strains.

METHODS

Organisms. The strains of *Rhizobium trifolii* were SU297/31 and TA1, the latter being maintained as line SU329 in the Sydney University collection.

Preparation of lipopolysaccharide (*LPS*). The bacteria were grown for 72 hr at 26°, each in two 40 l. batches of a defined medium adequate in calcium (Vincent,

1962). The harvested bacteria, totalling about 200 g. wet weight for each strain, were disintegrated by ultrasonic treatment for 5 min. in an ice bath, and the bacterial debris centrifuged, washed and used for preparation of the somatic antigen. The modification by O'Neill & Todd (1961) of the hot phenol extraction method (Westphal, Lüderitz & Bister, 1952) was used to further decrease contamination by nucleic acids. This modification involves a first extraction in the cold with 0·25 % (w/v) trichloracetic acid (TCA) which dissolves nucleic acid besides liberating a portion of the somatic antigen in the form of a Boivin-type antigen (LPS attached to protein). The subsequent phenol extraction of the residue yields, in the aqueous phase, LPS low in protein and contaminating nucleic acid. This procedure allows two forms of the somatic antigen (Boivin-type antigen and phenol-extracted LPS) to be obtained from one batch of bacteria.

LPS extracted by the unmodified hot phenol method of Westphal *et al.* (1952) was used for some phosphorus analyses to permit comparison with figures published for other organisms.

Preparation of antisera. Rabbit antisera to whole or mechanically disintegrated organisms were prepared as described by Humphrey & Vincent (1965) by using one intramuscular injection of bacterial suspension in Freund complete adjuvant (Difco) followed 4 weeks later by an intravenous injection of bacterial suspension without adjuvant. A similar regimen was followed with phenol-extracted LPS or TCA-extracted Boivin-type antigen, using 100 μg. dissolved in saline for each injection. Blood was collected by exsanguination 1 week after the intravenous injection.

Gel diffusion and quantitative absorption. The technique for gel diffusion followed that of Dudman (1964) as detailed by Humphrey & Vincent (1965). The precipitinogen activity of preparations was assessed by determining the least concentration (μg./ml.) at which extracted material formed a precipitation line. Routinely, antisera to mechanically disintegrated organisms suspended in culture supernatant were used. In addition, gel immunoelectrophoresis was done in barbiturate buffer (pH 8·8), I = 0·05, for 2 hr at 350 V, which gave a current of 1 mA/cm. The method of quantitative absorption of agglutinins was as described in Vincent & Humphrey (1968).

Total phosphorus analysis. This was done by the method of Chen, Toribara & Warner (1956) with perchlorate digestion of the sample, and by the method of Ames (1966) with magnesium nitrate ashing.

Chromatography. The LPS, or the polysaccharide obtained after lipid extraction (see Results), was hydrolysed for 16 hr at 100° in N-HCl, and the HCl removed by repeated evaporation in vacuum over KOH pellets and concentrated H_2SO_4. Descending chromatography was used in solvents A (butanol + pyridine + benzene + water = 5+3+1+3 by vol.), B (butanol + acetic acid + water = 3+1+1 by vol.) and C (butanol + ethanol + water = 4+1+5 by vol.). Solvent A was favoured for routine analysis since it gave clear separation of glucose from galactose; the other solvents were used to confirm results from solvent A. Chromatograms were made visible with *p*-anisidine HCl for sugars, with naphthoresorcinol to confirm the presence of glucuronic acid, or with urea phosphate (Greene, 1958) to confirm the presence of heptose. For KDO, the antigens were hydrolysed for 8 min. at 100° in 0·25 N-HCl, and the chromatograms developed with solvent D (butanol + pyridine + 0·1 N-HCl = 5+3+2 by vol.), and the KDO located with sodium thiobarbiturate (Warren, 1960). For separation of glucosamine and galactosamine, solvent E

Pathogenesis of Respiratory Mycoplasma Studied in Human Embryo Trachea Cultures. By
M. Butler (*Department of Biological Sciences, University of Surrey*)

Recently trachea cultures have been shown to support the growth of mycoplasma (Butler, 1968, *Int. Symp. Mycoplasma Dis. Man., Erfurt, D.D.R.*), and evidence has since been collected which demonstrates that the cultures support growth of several mycoplasma species obtained both from clinical material from the respiratory tract and various laboratory adapted cultures. One of these laboratory cultures, *M. mycoides* var. *capri*, caused cytopathic effects in the trachea cultures. The first effect was cessation of the activity of the cilia when the colony count of mycoplasma was about 10^5 units/ml. In stained sections other changes were observed after a few days. The most striking effects were the rounding up and stripping of the cells of the ciliated epithelium and the epithelium of the mucous glands. Simultaneously the nuclei of the cartilage cells became densely stained, in contrast the cartilage matrix lost its capacity to retain stain. The cellular structure of the culture became progressively more disorganized and ultimately, after about ten days, the nuclei fragmented. Our observations raise the question discussed by Collier & Clyde ((1969), *Fed. Proc.* **28**, 616) whether cytopathogenesis in trachea culture is correlated with virulence of the mycoplasma in respiratory infections. In their studies, on *M. pneumoniae* in hamster embryo trachea, they found that avirulent strains were not cytopathogenic. It is here suggested that the trachea system may not only provide a suitable system for testing pathogenesis of respiratory mycoplasma but also of mixed mycoplasma and virus infections.

The Detection of Plasmids in a Multiple Resistant Staphylococcus. By
L. K. Dunican and M. M. Monaghan-Cannon (*Department of Microbiology, University College, Galway*)

Staphylococci which are resistant to many antibiotics have been reported in many countries. The genetic basis of multiple resistance is unknown, although the existence of the penicillinase plasmid is well documented (Richmond (1968), *Ad. Microbiol. Physiol.* **2**, 43). Cotransduction of other antibiotic resistances with the pencillinase plasmid occurs infrequently in the case of erythromycin (Hashimoto, H., Kono, K. & Mitsuhashi, S. (1964), *J. Bact.* **88**, 261) and tetracycline (Mitsuhashi, S., Oshima, H., Kawaharada, U. & Hashimoto, H. (1965), *J. Bact.* **89**, 967).

Resistance to penicillin, streptomycin and tetracycline, as well as other characters such as coagulase are removed at high frequency by growth of a *S. aureus* at 43° (Dunican, *Biochem. J.* (1967), **106**, no. 4, 51). This was considered to be a multiple plasmid. In this report it was found that the 50 % inhibition point with mitomycin C was 10 times higher in strain M (an induced strain showing plasmid removal) than in its multiple resistance parent strain *S. aureus* B. On treatment with mitomycin C prophages were induced from the B strain but not from the M strain. The induced prophages from strain B conferred on *S. aureus* ps 47 resistance to tetracycline. Mitomycin induction curves of ps 47 and *S. aureus* ps 47 Tc^r indicated that lysogenization accompanied the resistance to tetracycline. The % G + C ratios were found to be significantly different, strain B had a consistent 6 % increase in % G + C over strain M. Preliminary experiments of DNA fractionation on poly L-lysine kieselguhr showed multiple peaks in the B strain. Fewer peaks were seen in the strain which had lost the resistance. Evidence will be presented to show that these extra peaks in the B strain may be plasmids.

The financial assistance of An Foras Taluntais is acknowledged, as is the Department of Education for a grant.

Inhibition and Host Modification in a Staphylococcal Phage-Host System. By
P. R. Smith (*Department of Microbiology, University College, Galway*)

Typing phage 80, a group I phage, produces areas of inhibition when plated on a group III staphylococcus 757. Previous workers (Wentworth, B. & Romig, W. R. (1968), *Jap. J. Microbiol.* **13**, 309; Beard, M. A. & Rountree, P. M. (1965), *J. gen. Microbiol.* **40**, 207) have shown that phages produced in areas of inhibition are neither mutant nor modified by the non permissive host.

Although no phage active on PS 80 or 757 could be isolated from areas of inhibition such phage could be isolated from small plaques within the area. These were shown to have a higher EOP for group III strains and a reduced EOP for PS 80. The phage was shown not to be an induced prophage of 757 (Pether, J. V. S. (1968), *J. Hyg., Camb.* **66**, 605). Evidence from single step growth experiments on PS 80 suggest that the restricted phage is in fact phenotypically altered by passage on 757.

Strain 757 made doubly lysogenic by the introduction of prophage 134′ from a group I strain 134 was able to support the growth of PS 80 at low efficiency. This doubly lysogenic strain 757/134′ also conferred a modification on PS 80 which was different to that produced by 757 or 134.

The Chemical Estimation of Fungus in Plant Tissue. By J. P. RIDE and R. B. DRYSDALE (*Department of Microbiology, University of Birmingham*)

The methods currently available for measuring the quantity of a pathogen in plants infected by filamentous fungi are imprecise and lack reproducibility (Matta, A. & Dimond, A. E. (1963), *Phytopathology*, **53**, 574). An attempt is being made to develop a convenient and reproducible assay for this purpose by estimating colorimetrically the amount of glucosamine, derived from chitin, a constituent of many fungi but not of plants, in hydrolysates of infected plants. The method used (Ashwell, G., Brown, N. C. & Volk, W. A. (1965), *Arch. Biochem. Biophys.* **112**, 648) reliably estimates 2 μg glucosamine. *Fusarium oxysporum lycopersici* and tomato (*Lycopersicon esculentum*) are being used as a model system. Because of the possibility of variation in the composition of fungal cell wall with morphology and cultural conditions (Bartnicki-Garcia, S. & Nickerson, W. J. (1962), *Biochim. Biophys. Acta* **58**, 102), the glucosamine to dry-weight ratio has been determined for both filamentous and yeast-like forms of *F. oxysporum* grown on a variety of media for periods of up to 3 weeks. The maximum variation in the ratio for each of the morphological forms is twofold to threefold. In artificial mixtures of homogenized tomato and fungal suspension all of the added fungus can be measured as glucosamine. The results of experiments to measure the amount of fungus in infected resistant and susceptible tomato varieties will also be reported.

Extracellular Cationic Proteases of *Bacillus licheniformis*. By C. D. LITCHFIELD and J. M. PRESCOTT (*Department of Biochemistry and Biophysics, Texas A & M University, College Station, Texas, U.S.A.*)

Culture filtrates resulting from the growth of *Bacillus licheniformis* in glucose-peanut meal medium present a complex mixture of extracellular proteinases; at least two anionic and two cationic proteases have been separated. When the dialysed, ammonium sulphate precipitated culture filtrate was subjected to DEAE-Sephadex chromatography at pH 7·0, three major peaks of proteolytic activity appeared. The largest proteolytic fraction emerged from the column unretarded, whereas the other two fractions (the anionic proteases) required salt for elution from the anion-exchanger. Rechromatography on SE-cellulose of the two partially purified cationic proteases (C_I and C_{II}) resulted in subfractionation of both enzymes into two entities each. Fraction C_I'' was found to be homogeneous by polyacrylamide disc gel electrophoresis. This enzyme had maximum activity over the range of pH 6·5–9·5 and showed optimal activity at 55° during a 30 min exposure period; it was inactivated rapidly above 60°. C_I'' was found to hydrolyse haemoglobin, gelatin, and to a lesser extent casein. The most readily hydrolysed synthetic substrate of choice was *N*-acetyl-L-tyrosine ethyl ester (ATEE) for which a K_M of 50 mM was determined. Hydrolysis was observed with several other synthetic substrates in which leucine or phenylalanine contributed the carboxyl group to the bond. The presence of a serine in the active site of C_I'' was indicated by the inhibition of both esterolytic and proteolytic activities by phenylmethane sulphonylfluoride and diisopropylphosphorofluoridate. Peptide mapping of the hydrolytic products resulting from the cleavage of S-aminoethylated lysozyme for 24 hr at 37° showed 27 peptides of which 10 contained arginine and five contained histidine and/or tyrosine.

Kinetics of DNA Breakdown and of Inhibition of Cell Division Induced by Colizin E2 in *Escherichia coli*. By Eva M. Holland and I. B. Holland (*Department of Genetics, University of Leicester*)

Colicins, which are proteins, do not appear to penetrate sensitive bacteria but adsorb to specific cell surface receptors. The lethal intracellular damage induced by the colicin is presumably achieved through its interaction with the cytoplasmic membrane. The surface of *Escherichia coli* may contain up to 3000 colicin E2 receptor sites (Maeda & Nomura (1966), *J. Bact.* **91**, 685). Since the kinetics of colicin action always show single hit killing it appears that the attachment of one colicin molecule to a receptor may be sufficient to kill the cell. Previous reports have shown that E2 induces DNA breakdown in sensitive cells. We have studied the kinetics of this process in exponentially growing broth cultures at both saturating and non-saturating concentrations of E2. Under the latter conditions, where a maximum of 10 % of the receptors should be occupied, about 50 % of the cells receive a lethal hit but breakdown of DNA is not detected for up to 40 min. after addition of E2. With saturating concentrations of E2, however, the release of soluble ^{3}H-thymine may be detected within 4 min. This suggests that very few of the receptors allow, at any given moment, a lethal interaction between colicin and membrane. At high colicin concentrations this lethal connexion is made immediately but at subsaturation concentrations growth and division of most of the cells is required before a lethal interaction takes place. The inhibition of DNA synthesis by E2 appears to be a secondary effect and may only occur up to 30 min. after the onset of DNA breakdown. In contrast, inhibition of cell division occurs already 5–10 min. after the onset of DNA breakdown and so can be detected well before the inhibition of DNA synthesis. It is not yet clear whether inhibition of cell division results from DNA breakdown or whether E2 acts directly on the cell division machinery.

SYMPOSIUM: ASPECTS OF MICROBIAL PATHOGENICITY

Toxaemia in Experimental Cholera. By E. M. Vaughan Williams and A. N. Dohadwalla (*Department of Pharmacology, Oxford University*)

In the past decade, an enterotoxin of *Vibrio cholerae* responsible for the fatal loss of intestinal fluid in cholera has been recognized. Previous lack of progress was due to the absence of either an experimental animal or a biological test in which cholera could be simulated; mice were killed by living *V. cholerae* and its endotoxin, but the signs of cholera were not evident. Advances followed studies of living *V. cholerae* and its products in the following biological tests in which some effects of cholera were reproduced; intra-intestinal infection of suckling rabbits (Dutta, N. K. & Habbu, M. K. (1955), *Br. J. Pharmacol.* **10**, 153); a ligated rabbit gut technique (De, S. N., Ghose, M. L. & Sen, A. (1960), *J. Path. Bact.* **79**, 373); and oral infection of mongrel dogs (Sack *et al.* (1966), *Lancet*, **2**, 206). The thermolabile enterotoxin (cholerigenic toxin) is different from cell wall endotoxin. Questions now arise as to its nature, mode of action and relation to vaccination.

Protection against cholera by present vaccines is brief (3–6 months) and uncertain (40–80 %; (1969), *W.H.O. Rep. Ser.*, p. 414). The antibodies provoked are vibriocidal, but not antitoxic. Should improved vaccines evoke antitoxin, how effective would the latter be? If cholera is localized in the alimentary tract (Finkelstein, R. A. (1965), *Proc. Choler. Res. Sym.* U.S.G., Washington, p. 264) antitoxin may be ineffective unless it reaches the toxin intraluminally. An oral vaccine (live) might produce intestinal antitoxin, but such vaccines were actually found to produce less antibody in the intestine than in the blood and tissues (Bhattacharya, P. & Mukerjee, S. (1968), *J. Infect. Dis.* **118**, 271). If, however, in cholera the toxin is not confined to the alimentary tract but distributed in the blood, circulating antitoxin produced by a parenterally administered vaccine might be effective in neutralizing toxin before damage was done. We attempted to determine whether or not circulating cholerigenic toxin exists and is active.

'Thiry'-type isolated segments of intestine, with one end blind, the other opening on to the skin, were prepared in 10-day-old rabbits (Vaughan Williams, E. M. & Dohadwalla, A. N. (1967), *Nature, Lond.* **215**, 552). The isolated segment retained its blood, nerve and lymph supply, but its lumen did not communicate with that of the remainder of the intestine, the continuity of which was restored by anastomosis. *Vibrio cholerae* was instilled into the lumen of the segment 2 days after operation, and most animals died within 48 hr with diarrhoea and fluid accumulation in the main bowel, the cultured contents of which did not grow vibrios.

Although it seemed possible that a cholerigenic agent had spread from the isolated loop, there were two facts against this hypothesis. First, Finkelstein (see above) prepared an enterotoxin with no apparent effect when administered parenterally. Second, although the blood of cholera patients contains antitoxin (W.H.O. Rep., see above), direct evidence that absorbed toxin produces choleraic diarrhoea is lacking.

Recent experiments have eliminated both points in relation to cholera in the infant rabbit. First, a sterile lysate supernatant from *V. cholerae* [Inaba 569B, ultrasonically disintegrated (Oza, N. B. & Dutta, N. K. (1963), *J. Bact.* **85**, 497) centrifuged at 25,000 r.p.m. to remove cell walls] caused diarrhoea when given intravenously, in both infant and adult rabbits. Secondly, the appearance of a cholerigenic agent in the blood of infant rabbits infected intra-intestinally with *V. cholerae* has been proved by the following cross-perfusion experiments.

Intestinal 'rice-water' accumulation was estimated by removal of the whole intestine and calculation of its water content from wet and dry weights. In 32 controls, this was $83{\cdot}05 \pm 0{\cdot}33\,\%$; in animals infected intraintestinally with *V. cholerae*, it rose to 93 % at the time diarrhoea started, but did not increase further before death because fluid overflowed in the stools. Similar results were obtained when sterile lysate was injected intraintestinally. For the cross-perfusion experiments, cannulae were inserted into the heart-side of the left carotid artery, and into the head-side of the right, in a pair of litter-mates. Heparin was injected and blood was circulated through polythene tubes, drop-timers, and a roller pump (to equalize the outputs) from the heart of one rabbit to the head of the other, and vice versa. Instillation of vibrios or of lysate toxin into the intestine of one animal caused significant intestinal fluid accumulation in both. The water contents of the intestines in control cross-perfusions did not differ from that of normal animals.

Finally, serum from adult rabbits injected with increasing amounts of lysate for 1 month, gave significant passive protection when injected into infant rabbits infected intraintestinally with *V. cholerae*. If the human disease resembles cholera in infant rabbits, these experiments suggest a toxoid produced from lysate should be added to cholera vaccines.

The Enteropathogenicity of *Escherichia coli*. By H. WILLIAMS SMITH (*The Animal Health Trust, Stock, Ingatestone, Essex*)

Provided certain experimental conditions are observed, pigs, calves and lambs are suitable subjects for demonstrating enterotoxigenicity in strains of *Escherichia coli* by the ligated intestine technique. Rabbits are less reliable; rats and guinea-pigs are unsuitable. In pigs, calves and lambs the anterior part of the small intestine is the most susceptible to enterotoxin; the posterior part in some animals may be completely unreactive.

Strains of *E. coli* either isolated from diarrhoeic pigs under conditions suggesting that they play an aetiological role or belonging to serotypes commonly incriminated in diarrhoea in pigs consistently dilate ligated intestine in pigs, calves and lambs. Only some of them dilate ligated intestine in rabbits.

A minority of strains of *E. coli* isolated from the alimentary tract of calves and lambs suffering from diarrhoea dilate both calf and lamb intestine, but not pig and rabbit intestine. Severe diarrhoea can be produced in very young calves and lambs by the oral administration of these strains but not by the administration of dilatation-negative strains, irrespective of their source of origin. The oral inoculation of these animals with pig enteropathogenic strains does not produce diarrhoea despite the fact that these strains are strong dilators of calf and lamb ligated intestine. This is because they are unable to proliferate in the anterior small intestine of calves and lambs. Ability to proliferate in this region is an essential property of enteropathogenic strains; ability to produce enterotoxin of itself is not enough.

Strains of *E. coli* enteropathogenic for human babies dilate the ligated intestine in rabbits but not in pigs, calves and lambs. These strains, given orally, cause no harm in very young rabbits. One reason for this is their inability to proliferate in their small intestines.

By means of ligated intestine tests, enterotoxin can be demonstrated in cell-free culture fluids of pig enteropathogenic strains of *E. coli*. It can be removed from these fluids by precipitation with substances such as acetone. It is quite distinct from endotoxin. The oral inoculation of piglets with enterotoxin-containing culture fluids gives rise to severe diarrhoea.

In some porcine strains of *E. coli*, enterotoxin production appears to be governed by a plasmid transmissible, apparently during conjugation, to other strains of *E. coli* and to salmonellae. This fact has facilitated the study of enterotoxin.

The Role of Allergic Reactions in Microbial Pathogenicity. By R. R. A. COOMBS (*Department of Pathology, University of Cambridge*)

There is now a fair degree of understanding of the mechanisms whereby allergic reactions not only produce resistance to the spread of microbial infection (immunity) but also lead to host tissue damage and disease.

At the symposium emphasis will be laid on concepts that allergic reactions, mounted against microbial products, play a considerable role in the pathogenesis of certain infections. In some cases it is the allergic reactions which determine the manifestations of infection and the clinical picture.

Is There a Malaria Toxin? By B. G. MAEGRAITH (*Liverpool School of Tropical Medicine*)

The basic problem in malaria is how the asexual parasite going through schizogony inside the erythrocyte can influence the host animal and initiate and maintain the clinical state we call malaria. The link between the parasite and the host has not yet been fully determined, but there is evidence that physiologically active factors are released into the plasma of infected animals. Most of these agents are non-specific to malaria and are present in other acute infections and medical states (Maegraith, B. G. (1967), *Protozoology*, **2**, 65).

The existence of such pathogenic agents is indicated in several ways. For example, in simian malaria during parasitaemia there is gross abnormal membrane transport of sodium in both parasitized and non-parasitized erythrocytes, resulting in slowly progressive accumulation of sodium despite equimolar loss of potassium (Dunn, M. J. (1969), *J. clin. Invest.* **48**, April). This state of affairs, which predisposes to lysis, persists for some days after removal of parasites by chemotherapy. Erythrocyte ATP also increases as parasitaemia and sodium influx increase, indicating abnormally high energy phosphate metabolism in erythrocytes and the ATP remains elevated after therapy. The change in membrane cationic transport is not related to auto-immune processes; it is most easily explained by the existence of a diffusable toxic agent. Lysis of unparasitized erythrocytes has been demonstrated clinically and experimentally (by the use of radioisotopes) in *P. falciparum* infections in man (Devakul, K. (1969), *Ann. Trop. Med. Parasit.* **63**, in the Press). This loss of erythrocytes is augmented by very active phagocytosis in the spleen and elsewhere which is to some degree specific so far as infected cells are concerned, but evidence of haemolysis is demonstrable by the third day of infection with E parasites in man, i.e. before phagocytosis is fully established. The recently demonstrated increased permeability of the cerebral vessels to protein, leading to the escape of water and the production of local stasis, and the presence of factors inducing increased permeability of skin vessels are most easily explained by the presence of circulating active substances. These are probably non-specific and pharmacological rather than specific to the parasite.

We believe that these phenomena (and probably also the intense hyperactivity of the visceral sympathetic nervous system (reversible by adrenergic blocking drugs) which occur in *P. knowlesi* infection in *M. mulatta*) are caused by agents released during the process of development of the host reaction to the infection. We have evidence of the release of kinins, histamine, adenosine and other active substances to support this view (Tella, A. & Maegraith, B. G. (1966), *Ann. Trop. Med. Parasit.* **59**, 153; Maegraith, B. G. & Desowitz, R. S. (1967),

Ann. Trop. Med. Parasit. **61**, 515). What we would like to know is what initiates the expanding chain of reactions leading to the parasite-host: host-parasite responses producing clinical malaria and pathological changes in the host.

We have demonstrated a factor in the serum in *P. berghei*, *P. knowlesi* and *P. falciparum* infections which profoundly affects the basic metabolic processes of tissue cells by inhibiting respiration and oxidative phosphorylation in mitochondria. This agent has a molecular weight of less than 1000 and is free from non-esterified fatty acids, bilirubin, and large polysaccharides and contains very little N; it emerges from ion-exchange resins with a carbohydrate fraction not yet fully identified (Fletcher, K. A. & Maegraith, B. G. (1966), *Bull. Soc. Path. exot.* **59**, 526). It is undoubtedly a 'toxic' factor, but we do not know whether it is specifically created by the parasite-erythrocyte complex or is some endogenous product evoked from the host tissues elsewhere. Various extracts of parasite bodies in rodent and simian malaria have also been shown to be toxic, including a lethal agent and one which actively induces haemolysis *in vitro*. There is no evidence of the release of such substances *in vivo*.

If by 'toxin' is meant soluble poisonous substances released as a result of the infection, the answer to the question in the title is '*Yes*'. Whether any such agents are specific to the infection (and I would guess this is generally not the case), remains to be seen. The solution to the problem will come only after more vigorous attack at the physiopathological level.

The Biochemical Basis of Host and Tissue Specificity in Microbial Infection of Animals. By
J. H. PEARCE (*Department of Microbiology, University of Birmingham*)

The term 'host specificity' in infectious disease implies the capacity of an organism to infect a single host species; the concept may be broadened to include organisms which cause disease in a limited range of hosts when the pattern of infection is comparable. In infections where a tissue specificity is apparent the organism grows primarily, but not necessarily exclusively, in a particular tissue and typical disease is associated with infection of that tissue.

Leprosy, poliomyelitis and dysentery are primarily diseases of man; brucella and vibrio placentitis are diseases of cattle and sheep. Shigella infection and brucellosis in the mouse bear little relation to disease in the natural host. In man, growth of *Neisseria gonorrhoeae* is localized within the epithelium of the genital tract or in the conjunctiva in infants. In the pregnant cow or ewe growth of *Brucella abortus* or *Vibrio fetus* is largely confined to foetal tissues of the placenta.

The factors responsible for these specificities are largely unknown (Keppie, J. (1964), *Symp. Soc. gen. Microbiol.* **14**, 44). Ultimately they must be expressed in the capacity of the pathogen to resist defence mechanisms, multiply, and damage the host so that differential distribution, at the host or tissue level, of mechanisms restricting or enhancing microbial growth must be of prime importance. Factors such as temperature, route of infection or mechanical stress may influence tissue specificity; differential susceptibility to toxic microbial products is probably of greater importance for host specificity.

The many reports in the literature illustrate the variety of processes that can determine infection and examples relevant to specificity will be discussed. Very few studies have dealt directly with the problem of specificity but these indicate possible approaches in future investigation.

In placental infection of pregnant cows by *B. abortus* (Keppie, J. (1964), *Symp. Soc. gen. Microbiol.* **14**, 44; Smith, H. (1968), *Bact. Rev.* **32**, 164), the nutritional role of erythritol in localization was indicated by its growth stimulation in placental tissue extracts and high concentration in heavily infected tissues. Analogous studies with *V. fetus* are in progress (Lowrie, D. B. & Pearce, J. H. (1969), *J. gen. Microbiol.* in the Press). In kidney infections caused by the active urease-producers *Corynebacterium renale* and *Proteus mirabilis* a comparable role has been considered for urea (Lovell, R. & Harvey, D. G. (1950), *J. gen. Microbiol.* **4**, 493; Braude, A. I. & Siemienski, J. (1960), *J. Bact.* **80**, 171; Maclaren, D. M. (1968), *J. Path. Bact.* **96**, 45), but is less clear-cut.

In a quite different mechanism, local growth of *Fusiformis necrophorus*, in its interaction with *Corynebacterium pyogenes* in 'heel-abscess' of sheep (Roberts, D. S., Graham, N. P. H., Egerton, J. R. & Parsonson, I. M. (1968), *J. Comp. Path.* **78**, 1) appears to be stimulated by

a heat-labile, high molecular weight component elaborated by *C. pyogenes* (Roberts, D. S. (1967), *Brit. J. exp. Path.* **48**, 674).

In virus infection, modification of any of the processes leading to penetration, multiplication and release of infective particles could determine host or tissue specificity. Cell receptors appear important for poliovirus infection of primate tissues (Holland, J. J. (1964), *Symp. Soc. gen. Microbiol.* **14**, 257); correspondingly naked poliovirus RNA infects cells from a wide range of species (Holland, J. J., Mclaren, L. C. & Syverton, J. T. (1959), *J. exp. Med.* **110**, 65). Test of tissues in organ-culture, to minimize changes in susceptibility, with viral nucleic acid, might help in evaluating the importance of receptors *in vivo*. Localization of influenza in the respiratory tract occurs on intravenous as well as intra-nasal inoculation of ferrets and susceptibility of tissues *in vitro* was similar to that *in vivo* indicating the value of organ culture for this type of study (Basarab, O. & Smith, H. (1969), *J. gen. Microbiol.* in the Press). The variation in adenovirus and herpes infection in tissue culture associated with availability of arginine (Rouse, H. C. & Schlesinger, R. W. (1967), *Virol.* **33**, 513; Becker, Y., Olshevsky, U. & Levitt, J. (1967), *J. gen. Virol.* **1**, 471) offers a parallel for influence of such factors *in vivo*.

The Biochemical Basis of Host and Tissue Susceptibility in Plants to Fungal Infection. By R. J. W. BYRDE (*Long Ashton Research Station, University of Bristol*)

It is clear that many mechanisms are involved in host and tissue specificity among fungal and bacterial pathogens attacking plants. Necessary properties in a would-be pathogen include the ability to overcome the defence mechanisms of the host (for example by detoxification of resistance factors), and the necessary complement of extracellular enzymes and toxins to affect the tissue to be attacked. For example, the fruit-rotting fungus *Sclerotinia fructigena* secretes enzymes which degrade pectic substances in the cell walls of parenchymatous tissue, but lacks enzymes lysing cellulose, xylan and lignin. The cellular skeleton of infected fruits thus remains intact, and the fungus is unable to invade adjacent woody tissue unless this is unusually immature. By contrast, obligate parasites secrete little or no lytic enzymes or toxins, which would otherwise cause death of host cells and thus deprive the fungus of its nutrient source.

A pathogen must be supported nutritionally by its host, and inadequate nutrition has long been recognized as a possible limiting factor in host–parasite relations, as for example in the Balance Theory of Parasitism (Lewis, R. W. (1953), *Am. Naturalist*, **87**, 273). There is good evidence that, within a given host genotype, nutritional modification of the phenotype, for example in nitrogen and carbohydrate status, can greatly affect host susceptibility. In addition, the specific stimulatory role of plant exudates has been demonstrated.

It is nevertheless apparent that a combination of a pathogen able to secrete necessary lytic enzymes or toxins with a host able to support it nutritionally is not sufficient to ensure infection. Recent work has shown that specific resistance factors in plants are particularly important in determining host specificity, though perhaps not for individual fungal races. The presence in healthy plants of substances in concentrations sufficient to inhibit pathogens may give protection, but probably even more significant are 'dynamic' systems in which the resistance factors are formed in sufficient concentrations only as a result of infection or attempted infection. Such factors include the phytoalexins (see contribution by B. J. Deverall), which are products of host metabolism, and some products of fungal metabolism, e.g. the substituted benzoic acids formed from apple constituents by *Sclerotinia fructigena* (Fawcett, C. H. & Spencer, D. M. (1966), *Nature, Lond.* **211**, 548). Important in resistance to obligate parasites is the so-called 'hypersensitive reaction' in which infection is soon followed by death of the host cell, depriving the fungus of its nutrient supply, if not actually killing it. Use of the term 'hypersensitivity' in this context carries no immunological implication.

The relationship between host and obligate parasite is a delicate and subtle one, and the biochemical basis of recognition and interaction is not yet fully understood. The 'gene-for-gene' hypothesis of Flor ((1942), *Phytopathology*, **32**, 653), postulated for a 'rust' obligate parasite, has lead to the realization that, in other forms of parasitism as well, there is a critical relationship at the gene level. Clearly this must be expressed in the enzymic make-up of host and pathogen, and Stahmann *et al.* ((1968) in (ed.) Hirai, T., *Biochemical Regulation in*

Diseased Plants or Injury, p. 263, Phytopath. Soc. of Japan) have suggested a working hypothesis that, in a compatible host–parasite combination, 'the enzyme subunits of both host and parasite interact to give an enzyme hybrid having properties of activity, specificity and regulation consistent with the needs of the host–parasite metabolism and different from those of the host or parasite alone'. A more general unifying model system is due to Hadwiger, L. A. & Schwothau, M. E. ((1969). *Phytopathology* **59**, 223). Fortunately, current experimental methods should enable these interesting hypotheses to be evaluated.

Apple Scab: a Possible Aggressor Mechanism. By R. C. HIGNETT (*East Malling Research Station, Maidstone, Kent*)

The flow of solutes in normal apple plants has been shown (using radioactive tracers) to change shortly after inoculation with spore suspensions of *Venturia inaequalis* (Cke.) Wint., to a distinct pattern in which the interveinal areas of leaves were partially starved. Later in the course of development of the disease the solute flow changed again to favour specifically those parts of the leaf which contained developing lesions. The alteration of solute flow observed *in vivo* was attributed to the production of fungal metabolites within the host. Fungal products having similar effects on solute flow in disease-free plants were isolated from artificial cultures of *V. inaequalis*. The effects were produced by introducing the products in to the vascular system of the plant via petioles at the base of the shoot. The fungal products consist of a complex mixture of non-dialysable pigmented materials of a proteinaceous nature. It was also shown that the incidence of disease in susceptible host plants was increased when the spore inoculum contained small amounts of the pigments (Hignett, R. C. & Kirkham, D. S. (1967), *J. gen. Microbiol.* **48**, 269).

The specificity of the observed effects and the small amounts of pigment required suggested that the mechanism involved might be linked to hormone action. Effects on solute transport have been observed in various types of plant after treatment with phytohormones or their analogues (Mothes, K. & Engelbrecht, L. (1961), *Phytochem.* **1**, 58; Seth, A. K. & Wareing, P. F. (1967), *J. Exp. Bot.* **18**, 65).

In general the effects were such that solutes in parts of the leaf distant from the treated area were translocated to the treated region, thus demonstrating the alteration of solute flow by hormone action. The application of kinetin to inoculated test plants has now been shown to stimulate lesion development in a fashion similar to that observed after application of fungal pigment. Conversely, work with soybean callus cultures has indicated that pigment can maintain growth almost as efficiently as kinetin. Thus there appears to be a close analogy between the actions of hormones and those of fungal products. However, the chemical differences between the two classes of material are substantial. In addition, certain biological effects of fungal pigment applied to plants in increasing amounts, such as stunting of leaf growth, dehydration and eventual interveinal necrosis indicate that *V. inaequalis* produces material which under certain circumstances may mimic the action of hormones in the plant and possibly compete for the relevant site of action. In this respect, evidence has been presented which suggests that hormone action may be linked with the protein complement of ribosomes (Marsilii, G., Lorenzoni, C. & Crescioli, R. (1969), *6th Meeting Fed. Eur. Biochem. Soc.*, 1109). Silver labelled fungal pigment has been shown to stain ribosome-like particles in plant material *in vivo* which suggests that the protein synthesizing system of the host plant may be affected by the developing lesion (Hignett, R. C. (1969), *6th Meeting Fed. Eur. Biochem. Soc.*, 1113). The suggestion is made that *V. inaequalis* redirects the host metabolism partly by producing materials which influence the phytohormone system or its sites of action.

Phytoalexins. By B. J. DEVERALL and J. A. BAILEY (*Department of Botany, Imperial College of Science and Technology, London*)

Phytoalexins have been defined as 'antibodies' which are produced as a result of the interaction of two metabolic systems, host and parasite, and which inhibit the growth of microorganisms pathogenic to plants (Müller, K. O. (1956), *Phytopath. Z.* **27**, 237). Phytoalexins are produced by plants, not only in response to infection by fungi, but also following exposure

to a wide variety of chemicals (e.g. Perrin, D. & Cruickshank, I. A. M. (1965), *Australian J. biol. Sci.* **18**, 803). Characterized phytoalexins have been obtained from pea, french bean, orchids, sweet potato, carrot (Cruickshank, I. A. M. (1963), *Ann. Rev. Phytopath.* **1**, 351) and potato (Tomiyama, K. *et al.* (1968), *Phytopathology*, **58**, 115). Formation of a phytoalexin after infection of pepper by a bacterium is suggested in recent work (Stall, R. E. & Cook, A. A. (1969), *Phytopathology*, **58**, 1584).

The role of phytoalexins in resistance of plant tissues to invasion by fungi has still to be fully established. However, recent work indicates that phytoalexins may play a major role in limiting the growth of parasitic fungi to the localized lesions produced in some diseases (Pierre, R. E. & Bateman, D. F. (1967), *Phytopathology*, **57**, 1154). Chocolate spot of broad bean, *Vicia faba*, caused by *Botrytis* spp. is a typical example. *B. fabae* can grow through the leaf but *B. cinerea* usually remains limited to the initial brown infection sites. A phytoalexin is formed in the leaves following infection by both fungi. *B. cinerea* is inhibited by the concentrations of phytoalexin formed in the leaf, is only able to degrade the phytoalexin slowly, and as a result, growth is restricted. *B. fabae* is relatively insensitive to the phytoalexin, degrades it rapidly and is thus able to colonize the entire leaf (Deverall, B. J. & Vessey, J. C. (1969), *Ann. appl. Biol.* **63**, 449).

The ability of a fungus to grow in plants may not only depend on the nature of the infecting fungus but also on the physiological state of the tissue. A close association has been demonstrated between senescence of bean leaves and their susceptibility to aggressive infection by *B. fabae*. Work with pea leaves has suggested that this may be a consequence of a decrease in the ability of the leaves to produce phytoalexin (Bailey, J. A. (1969), *Ann. appl. Biol.* **64**). The concentration of the phytoalexin pisatin produced by pea leaves decreased to a very low level as they senesced. When senescence was delayed by treating the leaves with the plant growth hormone benzyladenine, pisatin production was maintained at a high level. Induction of phytoalexin formation by chemicals and maintenance of capacity to produce phytoalexins by application of plant hormones may be important in future crop protection.

In contrast, little is known concerning the role of phytoalexins in differential reactions of varieties of a host species to physiological races of a pathogen. Resistance and suceptibility in varieties of *Phaseolus vulgaris* infected with races of *Colletotrichum lindemuthianum* is being investigated. The production of the phytoalexin phaseollin, which occurs in resistant and susceptible tissues, is associated with the appearance of visible necrosis. In susceptible varieties intracellular growth takes place for several days before necrosis occurs. In resistant (hypersensitive) varieties cellular necrosis takes place on contact with the invading germ tubes, which grow no further. Phaseollin formation occurs much earlier and to a greater extent in resistant than in susceptible cells.

The Study of Virus Virulence Using Newcastle Disease Virus. By P. Reeve (*Department of Virology, Royal Postgraduate Medical School, DuCane Road, London, W.* 12)

Newcastle Disease Virus (NDV) has been suggested as a model for studying virus virulence (Waterson, Pennington & Allan (1967), *Br. med. Bull.* **23**, 138). Many strains are available, exhibiting a continuous spectrum of virulence from strains extremely virulent for chickens, chick embryos and cell cultures, to avirulent strains isolated from apparently healthy chickens. Accurate comparisons of virulence can be made by estimating the time taken to kill young chickens or chick embryos and the virus can be grown easily for morphological and chemical studies and assessed accurately by measuring infectivity, haemagglutination or neuraminidase activity.

Studies with isolated virions have shown a surprising uniformity. All strains look alike in the electron microscope, and no major serological differences have been found in neutralization, haemagglutination or gel diffusion studies (W. H. Allen, unpublished; T. H. Pennington, 1967, Ph.D. thesis, London). Different strains have equivalent amounts of haemagglutinin and neuraminidase, and after disruption with sodium dodecyl sulphate (SDS) show three major protein components in polyacrylamide gel electrophoresis.

Virulence appears directly related to cytopathogenicity; strains which kill chick embryos most rapidly produce the largest plaques on chick embryo fibroblasts. As to the reasons for

this, differences have been reported in the rate at which strains multiply. Mussgay ((1960) *Zbl. Bakt.* (I. Orig.), **177**, 437) compared the virulent strain Italien and the avirulent F strain; final extracellular titres were similar but Italien achieved maximum titres sooner. We found with chick embryo fibroblasts that all strains achieved substantially the same extracellular titres with only small differences in the length of the growth cycles. Differences were, however, found in intracellular multiplication in chick embryo chorioallantoic membranes. With seven strains cell-associated haemagglutinin increased exponentially from 4 to 12 hr after infection but then continued to increase only in those membranes infected with virulent strains. Virulence appeared to be associated with the continued production and accumulation of cell-bound haemagglutinin and for two virulent strains neuraminidase and viral ribonucleoprotein; with two avirulent strains no such accumulation of viral components was found. Avirulent strains may infect less cells than the virulent strains so the difference may be one of invasiveness rather than multiplication. In chick embryo fibroblasts strains produced major differences in cytopathology: with the virulent strain Herts haemadsorption was demonstrable 4 hr after infection, syncytia were visible after 6–8 hr and by 12 hr most cells were in large syncytial masses. With the avirulent strains F, Queensland and Ulster haemadsorption was always less than with virulent strains and syncytia were not seen. A major cause of cytopathogenicity appears to be the extent to which the cellular surface is modified and the subsequent transformation of cells in syncytia.

Strains of NDV differ in their effects on the synthesis of cellular macromolecules (Wilson (1968), *J. Virol.* **2**, 1; (1969), *J. gen. Virol.* **4**, 245) showed that 6 hr after infection with the virulent strain Texas G.B. protein synthesis in chick embryo fibroblasts was inhibited and cellular RNA degraded. The less virulent strain Beaudette C inhibited protein synthesis but by a different mechanism. Wheelock & Tamm ((1961), *J. exp. Med.* **114**, 617) claimed that in cells infected with NDV protein synthesis was inhibited before a cytopathic effect; our preliminary studies suggest that cytopathogenicity is a cause rather than an effect of cellular inhibition.

Baron ((1964), *Newcastle Disease Virus, an Evolving Pathogen*, University of Wisconsin Press, Madison) claimed that interferon plays little role in variation of virulence but Thiry ((1963), *Virology*, **9**, 225) showed that the virulence of NDV mutants to be inversely related to the amounts of interferon induced. It is unlikely that extremely virulent strains induce interferon: cytopathogenicity occurs rapidly and cellular protein synthesis, necessary for interferon synthesis, is inhibited. It remains to be seen if avirulent strains induce interferon production and to assess the role of interferon in virulence.

The Biochemistry of Virus Cytotoxicity. By J. STEPHEN and T. H. BIRKBECK (*Department of Microbiology, University of Birmingham*)

This contribution is restricted to consideration of intracellular biochemical events that take place after cells have been infected with virus and which lead to injury or death of those cells. It excludes consideration of such toxic effects as those that have been described in mice injected with massive doses of influenza (Westwood, J. C. N. (1963), In *Mechanisms of Virus Infection*, ed. Wilson Smith, p. 298, Acad. Press, Inc.).

There are three main problems regarding cytotoxicity. The first concerns the relation of the 'cut off' phenomenon, i.e. the cessation of host-cell macromolecular biosynthesis (Martin, E. M. & Kerr, I. M. (1968), *Symp. Soc. gen. Microbiol.* **18**, 15), to the cytopathic effect. Are these two phenomena synonymous or separate, and if the latter, sequentially or synergistically linked? The second relates to the probable existence in some cases (poliovirus, Bablanian, R. *et al.* (1965), *Virology*, **26**, 100; (1965), *Virology*, **26**, 114; mengo virus, Amako, K. & Dales, S. (1967), *Virology*, **32**, 184; influenza virus, Scholtissek, C. *et al.* (1967), *J. gen. Virol.* **1**, 219; Newcastle disease virus, Waterson, A. P. *et al.* (1967), *Br. med. Bull.* **23**, 138; and vaccinia virus, Bablanian, R. (1968), *J. gen. Virol.* **3**, 51) of cytopathic factors in addition to those viral components (be they structural or induced) responsible for cut off. The position on these two problems regarding poxviruses, our main interest, may be summarized as follows. It is claimed by some (Joklik, W. K. (1966), *Bact. Rev.* **30**, 33; Moss, B. (1968), *J. Virol.* **2**, 1028) that the early cytopathic and cut off effects are functions of the virion or virus-activated

host-enzyme, while others (Bablanian, R. (1968), Wilcox, W. C. & Cohen, G. H. (1969), *Current Topics in Microbiol. and Immunol.* **47**, 1) interpret the available evidence in favour of the cytopathic effect being caused by *de novo* protein synthesis. The relation between cut off, cytopathology and *de novo* protein/antigen production will be discussed.

The third problem concerns the need to devise a more direct experimental approach to the recognition of the factor(s) responsible for viral cytotoxicity thereby avoiding the ambiguity that characterizes much of the relevant literature. This involves the isolation of virus-specific components and testing these for the reproduction of toxic effects in uninfected cells. To achieve the latter requires techniques which will promote the aselective uptake of biologically active macromolecules by mammalian cells (i.e. cells other than those with specialized phagocytic or immunocompetent functions). Comparatively few quantitative studies have been made on uptake of proteins (Ryser, H. J. P. (1968), *Science, N.Y.* **159**, 390) and these indicate that cells take up preferentially basic molecules of high molecular weight. Pinocytotic mechanisms of uptake resulting in the efficient digestion of large proportions of the ingested molecules may have obscured positive results in all but a few cases (e.g. nucleic acids, Ledoux, L. (1965), *Prog. Nucleic Acid Res. Mol. Biol.* **4**, 231; interferon, Lockart, R. Z. (1967), *J. Virol.* **1**, 1158; adenovirus fibre antigen, Levine, A. J. & Ginsberg, H. S. (1967), *J. Virol.* **1**, 747; and diphtheria toxin, Bonventre, P. F. & Imhoff, J. G. (1967), *J. exp. Med.* **126**, 1079) where the expression of biological activity was itself the indicator of successful uptake. Work with biologically active substances eliminates the need for labelled derivatives and, the ensuing problems of interpretation regarding the meaning of intracellular label.

Experiments are in progress in which virus-free extracts, from HeLa/ERK (HERK) cells which had been infected with vaccinia virus, are being tested for their cytopathic effects on uninfected cells; preliminary results will be discussed. As a prelude to more definitive studies, HERK-cell antigens have been removed from vaccinia-specific antigens with disulphide-linked immunosorbents (Stephen *et al.* (1966), *Biochem. J.* **101**, 717) derived from anti-(HERK) sera and individual vaccinia-specific antigens have been isolated with appropriate monoprecipitin anti-vaccinia sera (Birkbeck, T. H. & Stephen, J., unpublished observations).

The Role of Immunological Responses in the Pathogenesis of Encephalitis. By Dr C. E. Gordon Smith (*M.R.E. Porton, Salisbury, Wilts.*)

Encephalitis is a relatively uncommon result of natural infection with even the so-called 'encephalitis viruses' and a rare complication of infection with a very large proportion of viruses. Perhaps the major puzzle is why it occurs so infrequently because many viruses enter the central nervous system during the course of infection, at least in the circulation, but seldom cause recognizable damage in terms of signs or symptoms of encephalitis. On the other hand, an infection of the central nervous system can cause damage (often quite extensive and sometimes progressive) without physical signs, and persistent latent infections of the central nervous system also occur—perhaps more frequently than is realized. The natural route of access of viruses to the central nervous system is through the circulation (with some exceptions such as rabies) and in such circumstances a systemic infection is well advanced or has even run its course before encephalitis becomes apparent: immunological responses have therefore begun and are often well advanced before the onset of encephalitis. In some circumstances the route of infection (respiratory or intracerebral) may short-circuit the systemic route and resulting lesions are different in character. It has become abundantly clear that the type of histological lesion found depends on the immunological state of the host—there is evidence that antibody (probably by forming antigen-antibody complexes) is an important factor in determining the type, severity and probably the outcome of a virus infection of the central nervous system. Whether cell-mediated responses are involved in pathogenesis is not clear, although they are almost certainly the main factors in recovery from and protection against virus infections. During a virus infection, antibody is formed not only to the mature virion but also (probably earlier and in much larger amounts) against virus components and virus-determined materials such as enzymes formed by infected cells. The relatively large amounts of antigen-antibody complexes formed from such material are probably the cause of inflammatory lesions. Sensitized (partially immune) individuals may have more severe disease than those

with no previous experience of the virus antigens—probably because antibody excess occurs earlier in the course of the infection. Maternal antibody may also be important in the pathogenesis of neonatal infections. The astrocytosis which follows infection and which can be made progressive and chronic by repeated infection suggests that other immunological factors not yet understood are also involved. Various immunological explanations have been advanced to account for subacute sclerosing panencephalitis—a late complication of measles infection.

In all cases the outcome of a virus infection probably depends on the temporal and quantitative relations between virus multiplication, interferon production, the immunological responses and perhaps other factors. The paper will attempt to focus attention on the mechanisms and factors which may combine to determine the disease picture.

Virulence of Respiratory Viruses. By D. A. J. Tyrrell (*Common Cold Research Unit, Salisbury, Wiltshire*)

A precise definition of virulence for these organisms is as difficult as it is for any others. As far as possible I shall deal only with the virulence of viruses affecting their natural hosts under natural conditions, and I shall define virulence as the capacity of the organism to cause disease or death.

Respiratory viruses affect primarily the mucous membranes of the nose, throat and lower respiratory tract. They can enter alveoli and cause damage directly and they may destroy epithelial cells, producing symptoms and decreasing resistance to bacterial infection. We know very little about the means by which virus multiplication gives rise to clinical effects like excess secretion, respiratory obstruction and so on. The means by which viruses damage cells are ill understood—they may include the production of proteins which inhibit cell synthesis. Certain factors probably increase the chance of serious lower respiratory tract involvement—these include the ability of the virus to survive in small droplets which can be inhaled deeply into the respiratory tract, its ability to multiply freely in the warmer conditions of the lower respiratory tract and possibly the ability to cooperate with or enhance the effect of bacteria. Certain viruses also seem to multiply more extensively in respiratory epithelium than do others and the former are more virulent. The means by which severe general symptoms are produced are not known—virus or cell toxins, and viraemia, could well be studied. In some conditions exposure early in life, while maternal antibodies circulate, may lead to immunity without disease, while in others, such as respiratory syncytial virus, it may lead to severe or even fatal illness. In others infection by a non-respiratory route may give rise to a silent infection. The full understanding of the situation requires a detailed knowledge both of the epidemiology and mechanism of infection and also of pathogenesis.

Virulence can only be defined in terms of the reaction between a specified virus and host species, but many factors have been shown to influence the susceptibility of individual hosts. The immune status is, by convention, ignored, but age, the possession of certain blood groups, the state of nutrition, the existence of physiological stress or chronic respiratory disease can all make the outcome for the host more serious.

Virulence may also be looked at from the point of view of evolution and ecology. Precise analysis is difficult, but it seems likely that viruses are rarely carried chronically in the respiratory tract. They must therefore spread efficiently and this requires that nasal or tracheal secretions be shed and inhaled, as swallowed virus would never be inhaled. There is thus probably a survival advantage for a virus which produces mild catarrhal disease, and rhinoviruses might have evolved from enteroviruses by exploiting this mode of transmission—it is theoretically important that they spread slowly so as to avoid exhausting the susceptibles and they are, in fact, relatively inefficient spreaders.

Attempts are being made to reduce the virulence of viruses in order to use them for live vaccines. Some success has been achieved with influenza and 'cold' mutants of some other viruses are being sought.

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(isopropanol + water = 4 + 1, v/v) was used, with the Elson-Morgan reagent (Smith, 1960, p. 252) to locate the sugars.

Spectrophotometric identification of sugars. The cysteine + H_2SO_4 reaction (Dische, 1955) was used to confirm the identities of hexoses, heptoses and methylpentoses eluted from chromatograms. The carbazole reaction (Bowness, 1957) was used to identify and estimate the glucuronic acid in eluted spots. With whole antigen, KDO was determined by the thiobarbiturate reaction as modified by Osborn (1963) and by the semicarbazide reaction (MacGee & Doudoroff, 1954). The method of Dische & Shettles (1948) modified by Gibbons (1955) was used for deoxysugars, and that of Rondle & Morgan (1956) for amino sugars. Total carbohydrate was estimated by the phenol + H_2SO_4 method (Dubois *et al.* 1956).

Materials. Authentic KDO, provided by Dr K. Knox (United Dental Hospital, Sydney, N.S.W.), had been obtained from the penta-acetyl derivative made available to him by Dr E. C. Heath (Johns Hopkins University, Baltimore, Maryland, U.S.A.). The standard heptose used was the 'α-D-glucoheptose' supplied by Light & Co. The agarose column was of Gelarose (Litex, Denmark).

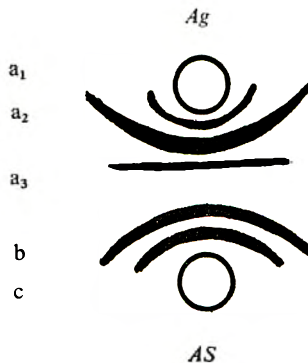

Fig. 1. Schematic representation of gel-diffusion pattern shown by a broken suspension of *Rhizobium trifolii* (*Ag*) diffusing against homologous antiserum (*AS*). The 'a' antigens are on the bacterial surface (somatic) and strain-specific. The 'b' and 'c' antigens are located internally and are common to all *R. trifolii* strains which have been tested.

RESULTS

Serological reactions of the whole bacteria

The two strains were distinctive in their somatic agglutination, although sporadically cross-reacting at 1/50 dilution of sera which gave a homologous reaction at 1/3200. This slight degree of cross-agglutination was not reflected in gel diffusion of surface antigens from intact bacteria. The 'a' line corresponding to these strain-specific somatic antigens (Humphrey & Vincent, 1965) was resolved at suitable concentrations into three lines (designated a_1, a_2, a_3 according to their relative proximity to the antigen well). The a_2 line was invariably visible even with a low concentration of bacteria, and developed early. The 'b' and 'c' lines, obtained only with broken or Ca-deficient bacteria, were common to both strains but unrelated to agglutination (Fig. 1).

Immunological characteristics of the isolated antigens

Precipitinogen activity of the extracted fractions. The Boivin-type antigen, precipitated from the TCA extract by 3 vol. ethanol, the concentrated dialysed ethanol supernatant fluid of the Boivin precipitate and the phenol extract of the supernatant fluid after the initial ultrasonically treated material had been centrifuged, all showed precipitation with specific antisera. Limiting concentrations varied between 4 and 1000 μg./ml. from batch to batch. About 0·8 % of the wet weight of the TCA-treated bacterial debris was extracted in the aqueous phase of the hot phenol, and this extract was consistently highly active, showing precipitation at 1 and 4 μg./ml. in the two batches of SU 297/31, and 8 and 16 μg./ml. with TA 1. For each strain, the two batches of these last phenol-extracted antigens were then pooled for subsequent analysis.

There was no cross-precipitation between the extracted antigens of SU 297/31 and TA 1. The TA 1 antigen, a more slowly diffusing, presumably larger, molecule than that of SU 297/31, formed its precipitation arc nearer the antigen well. The angle of precipitation line when antigen and antibody diffused at right angles to each other also indicated that the TA 1 antigen was a larger molecule (Crowle, 1961, p. 80).

After 24 to 48 hr, all extracts showed only a single line in gel diffusion corresponding to the major strain specific (a_2) line of whole bacteria; but in 5 to 7 days, at higher concentrations, a faint second line began to appear, nearer the serum well and apparently identical with the a_3 line (Fig. 1). Incubation of antigen solutions for 5 days at 26° before being placed in the antigen well did not result in a quicker appearance or greater quantity of the a_3 antigen precipitate. It was concluded, therefore, that the slow appearance of this line was due to the longer time necessary for a low concentration of a smaller fragment to accumulate a precipitate to visible limits, rather than to its slow liberation from the a_2 antigen either in the well or in the first-formed a_2 line. Diffusion of antigen and antiserum at right angles to each other showed that this was not a Liesegang effect (Crowle, 1961, p. 81). The following methods were used to attempt to purify the phenol-extracted antigen:

(i) *Ethanol precipitation in the presence of* Mg^{2+} (Osborn, Rosen, Rothfield & Horecker, 1962). One hundred mg. of LPS were brought as far as possible into true solution by shaking with 25 ml. distilled water, centrifuging the undissolved material at 12,000 *g*, resuspending in a further 25 ml. distilled water, shaking, etc., to a total of 100 ml. The supernatant solutions from the water treatments were poured into 3 vol. ethanol containing 0·025 M-$MgCl_2$, the flocculent precipitate collected, suspended in 20 ml. 0·02 M-ethylene diaminetetra-acetic acid (EDTA) and dialysed for 24 hr. The recovery of the dissolved LPS was 68 % for SU 297/31 and 40 % for TA 1. The activity of the recovered material in gel diffusion was unchanged with regard to the a_2 line, and the a_3 line still appeared at a concentration of 250 μg./ml. The insoluble residue remaining after the four water treatments was lyophilized, suspended in saline (1 mg./ml.) and tested for the limiting dilution of its precipitinogen activity. SU 297/31 yielded 1·5 mg. of inactive residue. The LPS of TA 1 was less soluble, since there remained after the four water treatments a gelatinous pellet weighing 11·6 mg. which was as active in gel diffusion as the starting material.

(ii) *Ultracentrifugation.* Twenty mg. phenol-extracted antigen were shaken vigorously in 10 ml. distilled water to obtain an evenly opalescent suspension which was then centrifuged at 100,000 *g* for 1 hr. Both supernatant solution and pellet were

lyophilized, yielding, in SU 297/31, 67 % in solution, 33 % in the pellet; in TA I, 27 % in solution, 73 % sedimented. The gel diffusion activity of the sedimented material was unchanged both for the a_2 and a_3 lines. The a_2 line appeared in the soluble fraction of both strains at a slightly lower concentration than with the original material (2, compared with 4 μg./ml. for SU 297/31, 8 compared with 16 μg./ml. for TA I). The limiting concentration of the a_3 line was also unchanged in this fraction, and chromatography of the two fractions showed no qualitative difference.

Effect of sodium dodecylsulphate (DDS) on diffusion patterns. Beer, Braude & Brinton (1966) found that DDS altered the proportion of the three diffusion lines produced by a Boivin-type preparation from *Escherichia coli.* In the present work with phenol-extracted antigens, it was found that DDS, at a concentration of 0·2 % in the antigen well, similarly altered the occurrence and density of the lines. In SU 297/31, the a_2 line virtually disappeared, the a_3 line was intensified, and an additional line, 'a_4', formed nearer to the serum well than the a_3 line. In TA I, increased density of a_3 was accompanied by a decrease in a_2, and both lines were displaced markedly towards the serum well; a_4 did not appear. In both preparations lines lost definition and reaction became less sensitive (visible) in the presence of the detergent. Prolonged treatment (14 days at 4°) with DDS disaggregated the antigens to such an extent that they no longer formed precipitin lines.

When antisera were absorbed 'in-well' (Humphrey & Vincent, 1965) with DDS-treated antigens and diffused against untreated LPS, the formation of the a_3 line was prevented completely, and only a fine tracing of the a_2 line remained. This indicated that only a small proportion of the antibodies responsible for this line were still free (i.e. unabsorbed by the DDS-treated antigen). The antigen responsible for the a_3 line and formed from the a_2 antigen in the presence of DDS carried in the main the same antigenic groupings.

The effect of cetyltrimethylammonium chloride (cetavlon). The cetyltrimethylammonium ion will precipitate highly acidic polysaccharides at neutral pH and less acidic polysaccharides at higher pH (Scott, 1960). Phenol-extracted antigens (100 mg.) were dissolved in water with shaking, and the fraction sedimented after centrifugation at 12,000 *g* removed. The solutions were made 2 % (w/v) with respect to cetavlon, and the pH value was adjusted progressively with alkali. The TA I antigen began to precipitate at pH 7 to 8; that of SU 297/31 at pH 10. The opalescent solution was left overnight, the precipitate separated by centrifugation, thrice dissolved in 5 M-NaCl (30 min., 37° with shaking) and precipitated in 10 vol. ethanol to displace the cetyltrimethylammonium ion (Jann *et al.* 1965). The precipitate was freeze dried and tested in gel diffusion. The supernatant fluid from the primary cetavlon precipitation was dialysed at 4° with stirring until free from foam (7 days), concentrated in vacuum at 35° and freeze dried. In the case of SU 297/31, 7 % of the starting material was recovered as precipitate (inactive), and 80 % as active material from the supernatant fluid. The latter gave a faster and more diffuse band which fused with a_3 of the parent material. It seemed that the effect of cetavlon on the SU 297/31 antigen was mainly as a detergent comparable with DDS, but that, under the conditions used, the LPS was not sufficiently acidic to be precipitated by cetavlon. In the case of TA I, 60 % of the starting material was recovered in the precipitate, fully active and identical with a_2. The 18 % recovered from the supernatant fluid formed a fast-moving diffuse precipitation line in the a_3 region.

Immunoelectrophoresis. The indication, from cetavlon precipitation, that the TA I antigen was more acidic than that from SU297/31, was confirmed by immunoelectrophoresis, both of the phenol-extracted LPS and of mechanically-disintegrated bacteria. The TA I antigen moved towards the anode, as did the a_2 component of the antigen mixture from disintegrated bacteria. The a_2 antigen of SU297/31 moved a short distance towards the cathode, probably by endosmosis. In neither case did the a_3 line become visible.

Antigenicity in rabbits. The phenol-extracted LPS and the Boivin-type antigen fractions yielded antisera showing a_2 and a_3 gel-diffusion lines, and having the following titres (determined by agglutination of homologous whole bacteria): SU297/31 phenol-extracted LPS, 3200; SU297/31 Boivin-type antigen, 3200; TA I phenol-extracted LPS, 800; TA I Boivin-type antigen, 3200. These compare well with titres of 3200 to 6400 usually obtained with antisera produced against whole bacterial cultures using the same regimen.

The antisera obtained from isolated antigen differed from those obtained from the bacteria themselves in that they lacked precipitin due to internal antigens (Humphrey & Vincent, 1965) and those agglutinins more efficiently absorbed by Ca-deficient bacteria as shown by the technique of quantitative absorption (Vincent & Humphrey, 1968). In the case of antisera produced from isolated antigen, the Ca-adequate and Ca-deficient bacteria were equally able to absorb all the antibodies, whereas with antisera to bacteria a portion of the total agglutinin resisted absorption by the Ca-adequate cells.

Chemical composition of the extracted antigens

Table 1 shows the chemical characteristics of the phenol-extracted LPS, and compares these with data published for *Xanthomonas* spp. and members of the Enterobacteriaceae. In many cases, the rhizobial antigens show similar values to other antigenic LPS which have been analysed. Notable exceptions are the low P value and the presence of glucuronic acid in both rhizobial strains; and in TA I, the high total carbohydrate content and failure to detect heptose.

Phosphorus content. It was thought that the extremely low phosphorus value (about one twentieth of the amount usually reported in the LPS of the Enterobacteriaceae), might be due to the preliminary extraction with TCA. To test this possibility, whole bacteria of SU297/31 were extracted directly by hot phenol according to the most commonly used technique (Westphal *et al.* 1952), and the resulting extract precipitated with ethanol. The precipitate was redissolved and reprecipitated twice. This material contained about 10 % nucleic acids (as estimated from the absorption peak at 260 mμ) and 1·2 % P. It was further purified successively by digestion with ribonuclease and deoxyribonuclease, dialysis and passage through a column of 8 % agarose. The column had a void volume of 50 ml. and the antigen as revealed by gel diffusion was located in fractions 12 to 23 (5 ml. fractions of 0·2 M-ammonium acetate). The nucleotides appeared in fractions 28 to 49. The antigen-containing fractions were pooled, dialysed, concentrated in vacuum and freeze dried. The recovered material (115 mg.) showed no absorption peak at 260 mμ and was highly active in gel diffusion (the a_2 line appeared at 0·5 μg./ml., a_3 at 64 μg/ml.). This antigen preparation contained only 0·045 % phosphorus. The fact that this highly active extract showed a lower content than the previous preparation

Table 1. *Chemical characteristics of phenol-extracted lipopolysaccharides**

	Rhizobium trifolii SU297/31	*Rhizobium trifolii* TA1	Xanthomonas species[a]	Enterobacteriaceae
Carbon	44	46	—	45[b]
Nitrogen	2·0	1·4	—	1·7[b]; 1·7[c]
Phosphorus	0·14; 0·045†	0·07	1·8 to 6·5	2·2[b]; 1 to 1·5[c]; 3 to 4[d]
Lipid (firmly bound)	13	10	22 to 47	46[b]; 10 to 16[c]; 32 to 50[e]
Total Carbohydrate‡	33	71	—	35 to 48[c]; 28 to 35[f]
Hexose	Glucose, mannose	Glucose, mannose	Glucose, mannose	Glucose, galactose, mannose
Heptose	10·8	n.d.§	n.d.‖	12 to 14[c]; 3 to 9[e]; 16 to 20[f]; 16 to 19[d]
Hexosamine	2·5 (one-third in lipid)	1·1 (half in lipid)	Glucosamine (in lipid only)	10 to 13[c]; 4 to 7[e]; 5 to 7[f]; 3·5 to 6[d]
KDO	1·5	1·2	1·5 to 3	9 to 10[c]; 2·3[g]; 3 to 5[e]; 3 to 6[f]; 1·7[d]
Methylpentose	Fucose 16	Fucose 20	Rhamnose 1–31	Rhamnose 0·7 to 1·5[g]
			Fucose 5–15	Fucose
Di-deoxysugar	Possibly present¶	Possibly present¶	n.d.	Often present
Uronic acid	Glucuronic acid 6	Glucuronic acid 6	Galacturonic acid 4 to 9	Rarely present[h]
Pentose	n.d.	n.d.	Xylose	Ribose[e]

* Numerical values are % dry wt.
† Prepared and purified by a different method (see text for details).
‡ Phenol-sulphuric acid method.
§ n.d. = not detected.
‖ Compare Adams *et al.* (1967).
¶ Unidentified sugar R_G 2·14 in Solvent A.

[a] Volk (1966, 1968*a*, *b*).
[b] Davies (1956).
[c] Kasai (1966).
[d] Work *et al.* (1966).
[e] Lüderitz, Galanos *et al.* (1966).
[f] Rapin and Mayer (1966).
[g] Knox (1966).
[h] Lüderitz *et al.* (1968).

made according to the method of O'Neill & Todd (1961) suggests that the small amount of P is a contaminant rather than an essential component of the antigen. The gelatinous nature of a similar direct phenol extract of TA 1 prevented its separation, due to its extremely slow penetration of the water phase of the agarose column. Contamination of the unfractionated TA 1 material with nucleic acid was estimated at 18 %.

Lipid content of the antigens. The phenol-soluble antigens (200 mg.) were extracted successively with ether, chloroform and chloroform + methanol (2 + 1). The antigens were first extracted without pre-treatment, then the residue was divided into two parts and each heated for 1 hr at 100°, either in buffered saline (pH 7·2) or in N-acetic acid. The extractions with ether, chloroform and chloroform + methanol were repeated on the heated material. The percentages of lipid material extracted at each step are shown in Table 2. The fractions heated in acid had lost both their ability to form a precipitate in gel diffusion and their ability to absorb the antibodies in 'in-well' absorption. The fractions heated at pH 7·2 and extracted with the three solvents were as active in gel diffusion as the starting material.

Table 2. *Lipid fractions extracted from rhizobial antigens*

	Rhizobium trifolii			
	Strain SU 297/31		Strain TA 1	
	% dry wt			
From unheated material	3·6		10·1	
From heated material	Neutral	Acid	Neutral	Acid
Ether extract	8	7	—	4·6
Chloroform extract	1·9	9·4	—	1·8
Chloroform + MeOH extract	7·7	14·5	4·8	9·4
Total lipid extractable after heating	17·6	30·9	4·8	15·1
Firmly bound lipid (liberated by acid hydrolysis)	13·3		10·3	
Total lipid in preparation	34·5		25·2	

Chromatography of sugars. Chromatography, in solvents A, B and C, was carried out on hydrolysates both of the original phenol-extracted LPS and the polysaccharide residue left after lipid extraction. The latter material gave cleaner separations, although both materials showed the same sugars when visualized with *p*-anisidine HCl.

The phenol-extracted antigens of both SU 297/31 and TA 1 appeared to contain glucose, mannose, fucose, glucuronic acid and its lactone and an unknown, fast-running spot ($R_{glucose}$ = 2·14 in solvent A) which reacted with *p*-anisidine HCl but not naphthoresorcinol. The presence of glucuronic acid and its lactone was confirmed by the use of naphthoresorcinol as dipping reagent. In addition, SU 297/31, but not TA 1, gave a spot in the galactose region shown to be a heptose by urea phosphate spray. A slow-moving spot in SU 297/31 was probably a hexose-heptose oligosaccharide (see below).

To confirm the identity of the sugars, and the absence of heptose in the TA 1 antigen, parallel strips of both polysaccharide hydrolysates and standard sugars were developed in solvent A, and then either made visible or eluted, the eluates

being tested by various identification procedures. Both the glucose spot in SU 297/31 and in TA 1, and the slow-moving spot in SU 297/31 showed, in the cysteine + H_2SO_4 reaction, a peak at 400 mμ identical with that from the glucose standard. The unknown slow-moving spot also showed a small peak at 510 mμ, indicating the presence of a heptose. The SU 297/31 spot in the galactose region showed only a clear heptose peak at 510 mμ, with no hexose peak. The TA 1 eluate from this region showed no sugars. The mannose and fucose spots from both antigens showed absorption typical of the standard sugars in the cysteine + H_2SO_4 reactions, i.e. mannose showed a flat peak at 400 mμ which did not change on standing to a peak at 600 mμ (as does glucose); fucose showed a very sharp peak at 400 mμ. The fast-running sugar also gave a sharp peak at 400 mμ, suggesting a deoxy- or dideoxy-sugar, but it was not rhamnose. Eluates of the glucuronic acid and glucuronolactone spots gave typical coloration with the carbazole reaction, and were estimated at about 4 % of the total polysaccharide residue, i.e. about 6 % of the original LPS.

Glucosamine was identified, and distinguished from galactosamine, in hydrolysates of LPS in 6 N-HCl (100°, 16 hr). Chromatograms were run in Solvent E and the amino sugars detected with the Elson–Morgan reagent.

KDO was detected on chromatograms after hydrolysis of the LPS for 8 min. at 100° in 0·25 N-HCl. The thiobarbiturate-positive spots in the hydrolysates developed in solvent D (48 hr) co-chromatographed with authentic KDO.

Estimation of carbohydrate constituents. These were made on phenol-extracted antigen prepared by the method of O'Neill & Todd (1961). The results are shown in Table 1. In heptose analyses, using Osborn's 10 min. modification (1963) of the cysteine + H_2SO_4 reaction, the SU 297/31 antigen developed a pink coloration with a maximum at 508 mμ, typical of heptose and equivalent to about 10 % heptose in the whole antigen. TA 1, on the other hand, gave a brownish solution with an atypical broad curve having a slight maximum at 530 mμ confirming the absence of heptose. Full colour developed in these reactions only after 16 hr.

In the thioglycollic acid reaction for estimating methylpentose (Gibbons, 1955), the SU 297/31 antigen developed a predominating pink colour, and showed not only a peak at 400 mμ for fucose but also a peak at 510 mμ for heptose (as did a heptose standard). The heptose content was probably high enough to interfere slightly with the fucose value (see Gibbons). TA 1 developed a yellow-brown colour with thioglycollic acid, and showed a large amount of fucose (as estimated by $A_{400} - A_{430}$), and in addition a low broad absorption curve with a small maximum at 530 mμ.

Glucosamine was estimated in the whole LPS and in the lipid and polysaccharide fractions after hydrolysis in N-acetic acid and extraction with chloroform and chloroform + methanol. For these analyses, all the solvent extractions were pooled to give a total lipid fraction. The hexosamine content of the rhizobial antigens, lower than that usually reported in LPS, was distributed between polysaccharide and lipid fractions.

KDO, estimated with thiobarbituric acid according to Osborn (1963) was detected in both antigens, even without prior hydrolysis (SU 297/31, 1·1 %; TA 1, 0·6 %). After hydrolysis with 0·1 N-acetic acid for 30 min. at 100° the amount of KDO detected in the SU 297/31 antigen increased to 1·5 %; more vigorous hydrolysis with 0·25 N-HCl for 8 min. at 100° did not augment this value. Prior hydrolysis of the TA 1 antigen with 0·1 N-acetic acid did not release more KDO but hydrolysis with 0·25 N-HCl

increased the amount detected to 1·2 %. The semicarbazide reaction of MacGee & Doudoroff (1954), although less specific, did not give higher values for the estimated KDO content of either antigen.

DISCUSSION

The lipopolysaccharides of two strains of *Rhizobium trifolii* studied in the present work were fully antigenic. Boivin-type antigens have always been considered to be capable of stimulating antibody production (Boivin, Mesrobeanu & Mesrobeanu, 1933). This property was ascribed to the presence of the protein moeity; but Webster, Sagin, Landy & Johnson (1955) have shown that purification of a Boivin-type antigen with successive decrease of nitrogen content, to 0·6 %, did not diminish the antigenicity. With phenol-extracted antigens, low in nitrogen content, some workers (e.g. Davies, 1956) were unable to produce antiserum unless the antigen was coupled with a foreign protein. Others who have used phenol-extracted LPS without adjuvant as antigen, have had to adopt a programme of as many as twenty injections to obtain satisfactory response (e.g. Taylor, Knox & Work, 1966). The antisera developed with the rhizobial antigens, by using one intramuscular injection with Freund complete adjuvant, had satisfactory titres when this was followed by a single intravenous injection. The use of adjuvant is known to reduce the lower limiting molecular size associated with antigenicity (White, 1967). In a previous study of the antisera developed by whole and broken rhizobia (Vincent & Humphrey, 1968) the disorganized surface of bacteria grown in a deficiency of calcium absorbed a portion of the antibodies from all antisera more readily than did the agglutinogens of calcium-adequate bacteria. In the present study, however, the antisera produced against the isolated LPS were absorbed as effectively by normal as by calcium-deficient bacteria. This indicates that any extra antigen revealed on the calcium-deficient surface is not part of the phenol-extractable somatic antigen, but part of some underlying layer of the wall which has been exposed as a result of calcium deficiency. The concentration of phenol-extracted rhizobial antigens produced precipitation lines in gel diffusion (between 0·5 and 16 μg./ml.), comparing well with values reported by other workers (e.g. 1 μg./ml. for Pasteurella antigen; Davies, 1956; 20 to 40 μg./ml. with an *Escherichia coli* LPS preparation; Kasai, 1966).

The two lines formed in gel diffusion by the rhizobial antigens probably represent particles of different molecular weight and configuration bearing the same set of antigenic sites, rather than two antigenically distinct particles. This concept is based on the observation that the antigen responsible for the major precipitation line (a_2) was readily converted to that producing the minor line (a_3) by DDS disaggregation, and the fact that this disaggregated material was able to combine with antibodies responsible for the a_2 precipitation line. The observation that a smaller fragment was also liberated, as revealed in the a_4 line, suggests that each particle size of antigen had an optimum precipitation zone at a different distance between the two wells. The methods of purification used, including column chromatography on agarose, were not able to separate the different particle sizes encountered in the present investigation. However, other workers (Beer *et al.* 1966), with a Boivin-type antigen from *Escherichia coli*, were able to separate on sucrose density-gradients a small particle responsible for two of the three diffusion lines shown by the parent preparation, and a large particle which formed the third diffusion line. The large particle could be

disaggregated with DDS to form small particles which then showed the two precipitation lines typical of the small particles in the parent mixture, while the line due to the large particle had disappeared. Beer *et al.* considered that the two precipitation lines obtained with the apparently homogeneous small particles (with some but not all antisera) were due to two different antibodies. In the present study of rhizobial antigens, with clear evidence of conversion of one form to another, and of the ability of one form to absorb the antibodies of the other, it seems reasonable to suppose that the extracted material, in all states of aggregation, carried the same antigenic determinants, but that different particle size or configuration may either have stimulated the production of several distinct antibodies or have been responsible for different zones of optimum precipitation with the same antibody.

Chemically the rhizobial antigens, particularly from strain TA 1, differed markedly from the established structure of the phenol-extracted O-somatic antigens of the Gram-negative bacteria (Lüderitz, Jann & Wheat, 1968). The most basic and significant departure from the structure proposed for any other somatic antigens which have been studied was the extremely low phosphorus content of both rhizobial preparations. In the Enterobacteriaceae (see, for example, Holme, Lindberg, Garegg & Onn, 1968), phosphate is taken as being part of the lipid fraction, and also as a link between di-heptose units in the polysaccharide core. The value usually found for LPS phosphorus is 1 to 4 % (see Table 1). In the rhizobial LPS the highest value found (0·14 % for SU 297/31) might be decreased to 0·05 % by a different method of preparation and purification, with no loss of activity in gel diffusion. It seems possible therefore that the phosphorus is a contaminant, and in any case it is unlikely that at these low concentrations it could play the major structural role assigned to it in the Enterobacteriaceae.

The other major departure from the usual pattern, and a distinction between the two rhizobial strains studied here, was the absence of heptose sugar from the antigen of TA 1. Various heptose-less mutants have been reported in Salmonella (e.g. Kasai & Nowotny, 1967), but the LPS from these strains, soluble in the phenol rather than the aqueous phase of extraction, is the lipid-KDO-phosphate-glucosamine core of a normal somatic antigen which has lost its O-specific polysaccharide by mutation. Volk (1966, 1968*a*, *b*) isolated from twenty Xanthomonas species an LPS which contained all the normal constituents of an O-somatic antigen, including lipid, KDO and phosphate, but in which no heptose was detected by the cysteine + H_2SO_4 reaction, or by chromatography. However, Adams, Quadling & Perry (1967), who used urea phosphate as visualizing reagent on paper chromatograms (and also by using gas-chromatography), found heptose in chromatograms of the phenol-extracted LPS of all bacteria tested, including two of the Xanthomonas species (but not necessarily the same strains) studied by Volk. In the rhizobial preparations studied here, heptose was clearly present in the SU 297/31 antigen but was not detected in the TA 1 antigen by any of the tests applied, including the urea phosphate procedure used by Adams *et al.* (1967).

In other respects, the antigens of the rhizobial strains resembled those extracted from Enterobacteriaceae, although firmly bound lipid, glucosamine and KDO were present in amounts less than those usually reported. The lability of the KDO bonding in the SU 297/31 antigen makes it possible that some of this compound may be involved in the type of polysaccharide-$(KDO)_n$-lipid linkage proposed by Osborn

(1963). The other sugar constituents common to both TA I and SU297/31, glucose, mannose and fucose, have been reported often as the constituents of cell wall LPS, but values for methylpentoses appear higher in these rhizobia and in the Xanthomonas species than in the strains of Enterobacteriaceae. In the rhizobial antigens it seems probable that the unknown fast-moving sugar component (possibly a di-deoxy-sugar) is contributing to the methylpentose value in analyses of the whole antigen. Uronic acids reported rarely in LPS of Enterobacteriaceae (see Lüderitz *et al.* 1968) are suspected of being of capsular polysaccharide origin with the suggestion that sometimes O-and K-antigens are bound together with unusual firmness. Volk (1968*a*) identified the uronic acid which he had reported earlier in Xanthomonas spp. as galacturonic acid. In our two rhizobial antigens it seems that the glucuronic acid of SU297/31, unlike that of TA I, is linked in such a way as to decrease any negative charge on the molecule.

The low phosphorus content of the rhizobial antigens places them in a different category from other O-somatic LPS antigens. Both strains studied here showed some features (low phosphorus content, presence of acidic sugars, and, in TA I, a high percentage of neutral sugar) which related them chemically to the K-antigens of capsulated *Escherichia coli* (Ørskov, Ørskov, Jann & Jann, 1963; Jann *et al.* 1965). They also differed chemically from the K-antigen capsular polysaccharides in that they contained KDO and a higher percentage of firmly bound lipid. When Jann *et al.* (1965) extracted a mixture of O-antigens and capsular polysaccharides from capsulated *E. coli* strains by the hot phenol method, they were able to centrifuge down the O-somatic antigen LPS from the aqueous phase of phenol extraction at 105,000 *g*, and then precipitate acidic K-polysaccharide from the supernatant fluid with cetavlon. In this study of the rhizobial antigens it was not possible to fractionate the material at 100,000 *g* or by cetavlon. The phenol extract of the *E. coli* capsulated strains showed a gel-diffusion line attributable to the O-somatic LPS and another (double) line from the acidic K-polysaccharide. The rhizobial preparation consisted of one antigen, albeit in two convertible molecular forms. The *E. coli* K-polysaccharides, unlike the rhizobial antigens, were not able to stimulate antibody production, even with Freund adjuvant. The high serological activity and homogeneity of the rhizobial antigens would seem to preclude the possibility that the preparations studied in this present work were mixtures of O-antigen and K-polysaccharide. Gel-immunoelectrophoresis in particular would be expected to separate a typically acidic K-polysaccharide from a typically neutral LPS.

However, the antigen of rhizobial strain TA I requires some further consideration. This LPS had a persistently gelatinous nature when prepared from whole bacteria without prior treatment with TCA. Although it was quite different in chemical constitution, solubility and antigenic activity, its physical appearance resembled extracellular gum (Humphrey & Vincent, 1959, 1965); TA I is one of the few rhizobial strains in which Dudman (1968) observed capsules. In confirming Dudman's observations on this strain, we have been able to distinguish on TA I, but not on SU297/31, capsules morphologically distinct from the extracellular gum. They were not seen in cultures less than 15 days old. We could not show any difference in agglutination or gel diffusion with such old capsulated cultures. The preparations of TA I antigens described in this paper were from 72 hr, twice washed, and in one case ultrasonically-treated, bacteria, at which age no capsules were detected. Any extracellular gum

would have been removed from the bacteria by the washings. The gelatinous nature of the antigen extracted by hot phenol from such bacteria suggests that even before the capsules are demonstrable the surface is covered with a gelatinous material which is the somatic antigen, and which accumulates as the culture ages until forming a visible layer. The surface as well as the isolated antigens of the strain TA 1 are probably atypical of the species. Marshall (1967), on the evidence of whole-cell electrophoretic mobility, found that its surface appeared to be charged exclusively with carboxyl ions (unlike most other *Rhizobium trifolii* strains which showed a carboxyl- and amino-charged surface) and its surface charge density was also unusually high. Our results with gel-immunoelectrophoresis of the isolated antigens of TA 1 and SU297/31 paralleled the relative mobility of the whole bacteria as determined by Marshall.

Thanks are due to Dr K. Knox (the Dental Research Institute, Sydney) for much helpful advice and for a sample of authentic KDO; and to Dr A. J. Wicken (School of Microbiology, University of New South Wales) for advice and assistance with column chromatography. We are grateful to Mr J. dal Pont, of the C.S.I.R.O. Division of Fisheries and Oceanography, for N, C and confirmatory P analyses. The work has been supported by the Australian Research Grants Committee, the Wheat Industry Research Council and the Rural Credits Division of the Reserve Bank of Australia.

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J. gen. Microbiol. (1969), 59, 427–428
Printed in Great Britain

SHORT COMMUNICATIONS

A Simple Device for Obtaining Axenic Cultures of Phototactic Unicells

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(*Accepted for publication 5 September 1969*)

A reliable technique was required for the cleaning of a contaminated stock culture of *Euglena gracilis* which would preclude the selection of subtle physiological mutants and provide sufficient euglenas for an inoculum in as short a time as possible.

The phototactic response exhibited by many unicells and coenobia has been exploited for years with varying success as a means of inducing cells to swim away from non-motile contaminants. The success of the simple device illustrated (Fig. 1) depends on the ease with which organisms can be injected into sterile media (with a minimum of disturbance) and then withdrawn under sterile conditions, following accumulation as a dense axenic suspension in a narrow beam of light. As the tube has a narrow bore, and is held perfectly still during separation, the organisms swim along it without inducing sufficient convection to contaminate the solution farther along the tube, permitting a high yield of 'cleaned' organisms. Thus the tendency to select physiological mutants does not arise to the same extent as with dilution separation techniques, particularly where these rely on establishing new clones from single-cell isolates. Originally devised for removing a persistent actinomycete from a laboratory stock clone of *Euglena gracilis*, this technique has been found equally suitable for 'cleaning up' the following: *Euglena gracilis* (two strains), *E. spirogyra*, *Ochromonas danica*, *Cryptomonas ovata*, *Amphidinium carteri*, *Chlamydomonas* sp., and the coenobia *Gonium sociale* and *Pandorina morum*.

Operation, The apparatus is made of Pyrex glass with the area shown solid in the diagram permanently masked with heat-resistant black enamel paint.

Before operation the ports A and B are plugged with new 'Suba-seal Turn-over Closures' (William Freeman & Co., Barnsley) the culture solution poured in, taking care to displace all the air, and the open end plugged with cotton-wool. The unit is then supported horizontally in an autoclave and sterilized intact.

To use the tube it is aligned horizontally on a burette stand, and left for a few minutes for convection to cease. A suitable quantity of the contaminated organism is then slowly injected with a syringe through seal A. The ports are the same diameter as the main tube so that any small bubbles or buoyant contaminants become trapped in the port. As the tube is masked except for the narrow zone around port B, the phototactic cells will accumulate as a dense suspension within a light beam shone onto this region. A sterile syringe is used to withdraw the cleaned suspension through seal B, after the top of the seal has been sterilized with ethanol.

The depression below port B will be found useful for accumulating sluggish species such as *Euglena spirogyra*, which tend to settle out after arriving in the light beam. That below port A will trap heavy contaminants.

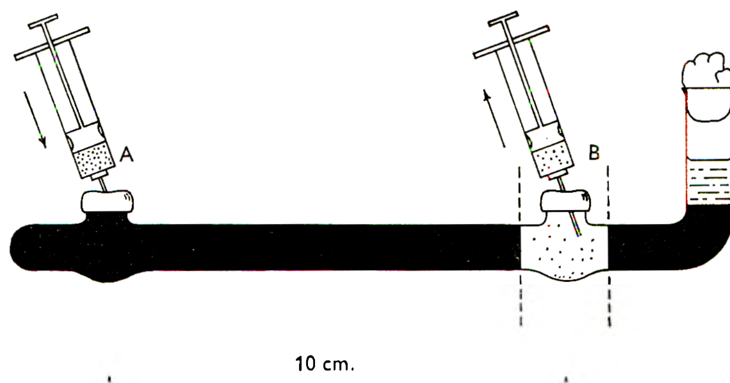

Fig. 1. Tactic Unicell Separator. For explanation see text.

The dimensions are probably not very critical but a ratio of 10:1 for the tube length/diameter has given satisfactory results. With a 10 cm. swimming path, *Euglena gracilis* should arrive in the collecting zone after 30 min. A single run is generally adequate to provide bacteria- and fungus-free cultures, but if several runs are necessary new culture solution is required. This is most readily achieved by preparing a number of the units to be used in sequence. In *E. gracilis* and possibly other organisms the phototactic response is lost in continuous light; a 6 to 10 hr dark pretreatment may be required to induce a maximum response.

Other uses. This device could be used for a variety of phototactic and chemotactic separations. Phototactic species with differing swimming rates could be separately concentrated by repeated passes through the tube. Negatively chemotropic responses could be utilized by injecting the contaminated suspension at B, the inducing agent at the open end, with final axenic recovery at A. For a positive chemotactic response inject the suspension at A, the attractant at the open end and recover at B.

Where a culture is heavily contaminated a sample should be withdrawn as soon as the first organisms arrive at the recovery port. For cleaning organisms with contaminated mucilage sheaths a dilute agar solution might prove suitable as a swimming medium.

J. gen. Microbiol. (1969), **59**, 429–430
With 1 *plate*
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Selection of Mutant Bacterial Sex Factors Determining Altered Sex Pili

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(*Accepted for publication 18 September 1969*)

Bacterial sex factors determine the synthesis of sex pili that act as receptors for donor-specific phages, so that many sex factors can be conveniently identified by the phage-sensitivity of their hosts (see Meynell, Meynell & Datta, 1968). Gross differences distinguish the two major groups of F-like and I-like pili and their factors, while minor differences are evident within the F-like group, as with the drug-resistance factor, R100-1, and the plasmid, F_0-*lac* (Nishimura, Ishibashi, Meynell & Hirota, 1967; Lawn, Meynell, Meynell & Datta, 1967; Meynell *et al.* 1968). However, for phage sensitivity to be generally useful as a genetic marker, one must be able to select mutant sex factors that confer sensitivity to only one class of phage, e.g. to isometric RNA phage but not to filamentous DNA phage. Valentine and his colleagues have shown that such mutants exist in mutagenized Hfr cultures of *Escherichia coli*, by infecting with RNA phage, plating for isolated colonies, spraying with the same phage and picking unlysed colonies (see Valentine, Silverman, Ippen & Mobach, 1969). Only a minute proportion (2/900) of such colonies contain mutant factors (Silverman, Mobach & Valentine, 1967), because phage resistance less often arises in this way than from poor expression of the pilus-forming ability of parental factors or from a complete mutational block in pilus synthesis. We have therefore tested an alternative method of selection which appears more efficient. It depends on the fact that, of the three classes of phage-resistant cell in the culture—unexpressed parental type, blocked mutant and the mutants to be selected—only the last will be phage-resistant yet able to synthesize sex pili and to transfer their sex factors by conjugation. A mutagenized donor culture is accordingly treated with phage, mated and the factors acquired by the recipient examined.

An overnight culture of *Escherichia coli* strain 24 carrying the de-repressed *fi*+ (F-like) R factor, R1*drd*-16 (Meynell & Datta, 1967) was treated with 0·4 M-ethylmethane sulphonate for 20 min. at 37°, diluted 1/100 into 10 ml. broth and grown overnight without shaking. Next morning 10^{10} particles of the RNA F phage, MS2, were added. After allowing 30 min. at 37° for adsorption, the infected culture was diluted 1/100 in broth, incubated overnight and then again diluted 1/100 in broth and incubated overnight to encourage pilus formation and consequent killing of cells with parental factors. The second stationary phase culture was diluted 1/20 in broth, incubated on a turntable for 2 hr at 37° and mixed with equal numbers of the recipient strain, *E. coli* 712, and left overnight at 37°. The recipient was finally isolated by streaking on glucose-salts agar containing the appropriate antibiotic and growth factors. The colonies obtained in two independent experiments were purified and tested for sensitivity to phage MS2, the unrelated RNA phage, Qβ, and the DNA phage, fd.

Some colonies were sensitive to all three phages like the parental strain; others were resistant; and about 50 % were mutants resistant to MS2 but sensitive to Qβ and fd.

Electron microscopy showed that, although the mutants appeared resistant to MS2 in spot tests, their pili could adsorb the phage, albeit far less efficiently than the pili of their parent. These mutants may therefore resemble the type A mutants of Valentine *et al.* (1969). Plate 1 shows a mixture of mutant and parental pili after addition of a high multiplicity of phage MS2. To prepare the specimens a mutant and a parental culture were mixed and phage added. After 20 min. at room temperature, the suspension was fixed with formalin and the cells collected on a membrane filter, from which they were transferred to an electron microscope grid and stained with uranyl acetate. As Pl. 1 shows, the parental pili were heavily coated with phage whereas relatively few particles attached to the mutant pili. Moreover, the mutant pili had a conspicuously abnormal appearance in that they formed bundles and showed numerous vesicular structures resembling the 'knobs' previously seen on the tips of both F and I sex pili (Lawn, 1966; Meynell & Lawn, 1967).

Despite their abnormal appearance, these mutant pili evidently allowed conjugation and gene transfer, since this provided the means by which the mutants were isolated. Mating experiments showed that 2 to 25 % and 0·7 to 3 % of cells in the two mutant strains could transfer their drug resistance in 20 min., compared to 50 % for their parent. The abnormal morphology of the mutant pili therefore suggests that the alteration in the pilus protein that leads to poor adsorption of phage MS2 also results in structural instability of the pilus.

The electron microscopy was kindly undertaken by Dr A. M. Lawn.

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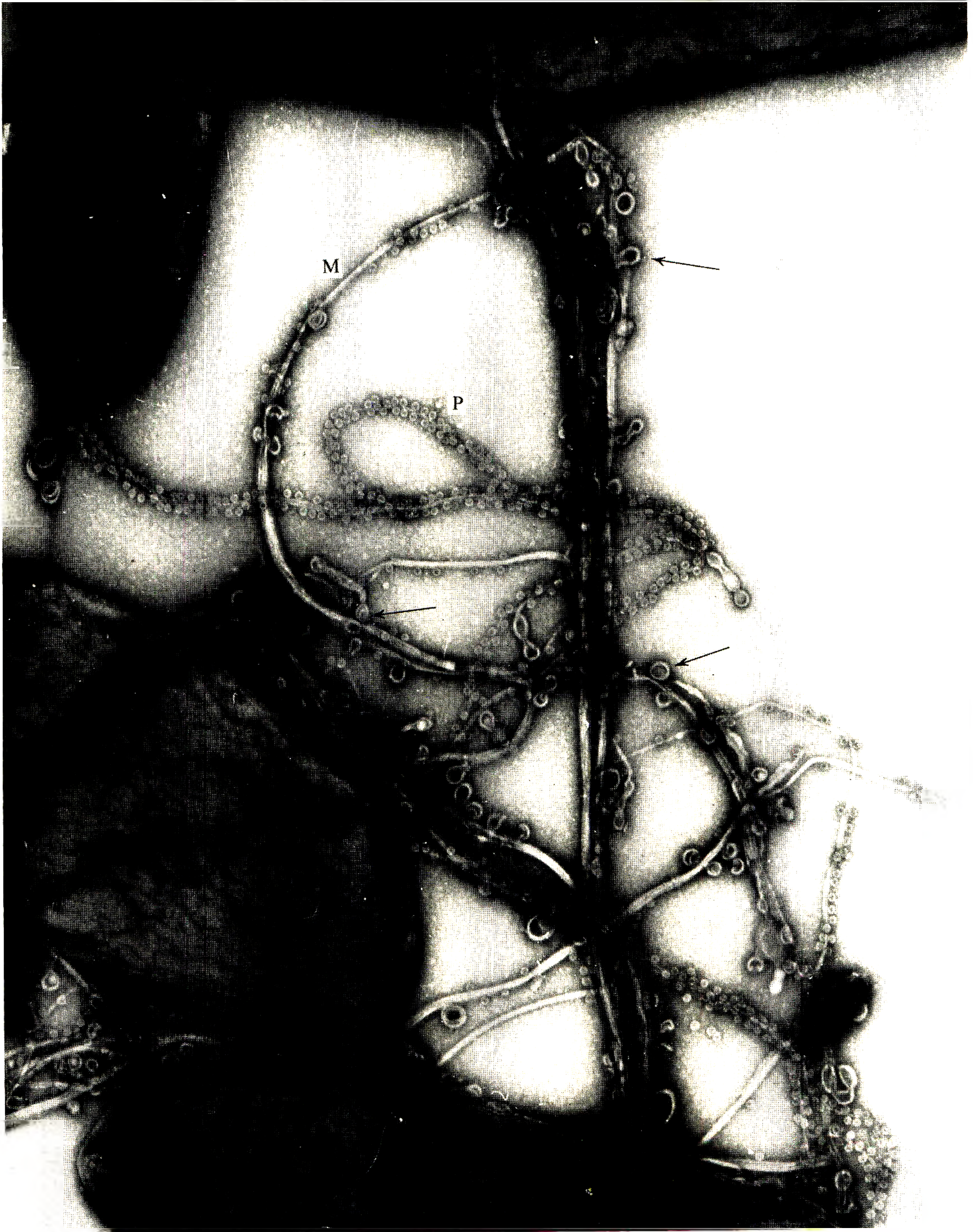
EXPLANATION OF PLATE

PLATE 1

A mixture of mutant (M) and parental (P) pili mixed with phage MS2. The vesicular structures associated with the mutant pili are marked by arrows.


M
P

J. gen. Microbiol. (1969), **59**, 431
Printed in Great Britain

Books Received

Antimicrobial Agents and Chemotherapy—1968. Proceedings of the Eighth Interscience Conference on Antimicrobial Agents and Chemotherapy, New York, N.Y., 21–23 October 1968. Edited by GLADYS L. HOBBY. Published by The Williams and Wilkins Company, 428 E. Preston Street, Baltimore, Maryland 21202, U.S.A. 556 pp. Price $15.00.

The Comparative Endocrinology of the Invertebrates. By KENNETH C. HIGHNAM and LEONARD HILL. Published by Edward Arnold (Publishers) Limited, 41 Maddox Street, London W.1. 270 pp. Price £4 (paperback £2).

The Cytology and Life-history of Bacteria. By K. A. BISSET. Published by E. & S. Livingstone Limited, Nos 15–17 Teviot Place, Edinburgh. 147 pp. £2 10*s*.

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THE SOCIETY FOR GENERAL MICROBIOLOGY

The Society for General Microbiology held its fifty-sixth General Meeting at the University of Birmingham on Thursday, Friday and Saturday morning, 18, 19 *and* 20 *September* 1969. *The following communications were made:*

ORIGINAL PAPERS

Immunogenic Fragment Antigens from Tissue Cultures Infected with the Virus of Louping-ill (Arbovirus group B). By J. G. BROTHERSTON and J. B. BOYCE (*Moredun Research Institute,* 408 *Gilmerton Road, Edinburgh, EH*17 7*JH*)

Subunit vaccines against arboviruses have been suggested as the ultimate answer for vaccines (C. E. Gordon Smith (1969), *Br. med. Bull.* **25**, 142). Virus fragments have been reported as haemagglutinins in group A arboviruses (Mussgay, M. & Rott, R. (1964), *Virology* **23**, 573) and as subunits of vesicular stomatitis virus (Brown, F. & Cartwright, B. (1966), *J. Immun.* **97**, 612), but their immunogenicity is uncertain.

In our experience, sheep-kidney tissue culture fluids with high titres of louping-ill virus inactivated with 0·001 % formalin and injected into animals, do not produce a good antigenic response unless two or three doses are given. However, the treatment of such tissue cultures before or after inactivation with formalin, with lower aliphatic alcohols (e.g. methanol) to a final concentration of 31 %, in one step releases or unmasks antigens which induce haemagglutinating inhibiting (H.I.) antibodies and neutralizing protective antibodies when injected into sheep. The active fractions are easily obtained by centrifugation after alcoholic precipitation and re-suspended in buffered saline as a tenfold concentrate. The effects of a single dose of 0·25 ml. are long lasting with or without the incorporation of adjuvant mineral oils. The serological response is equal to that which results from natural infection and the immunity induced protects against challenge with live virus. A single dose of vaccine 28 days before the end of pregnancy is capable of inducing in ewes sufficient maternal antibody to protect lambs against challenge two months after birth. Maternal antibodies are detectable in the blood of lambs within 7 days of birth. (Brotherston, J. G. & Boyce, J. B. (1969). *Vet. Rec.* **84**, 514).

The results of alcohol treatment are not wholly dependant upon concentration or purification but may be due to the release of non-infective fragments of virus which can be separated in the ultra-centrifuge.

Dietary Lactobacilli and Amine Production in the Alimentary Tract of the Pig in relation to Weaning and Postweaning Diarrhoea. By I. R. HILL, R. KENWORTHY and P. PORTER (*Unilever Research Laboratory, Colworth/Welwyn, Colworth House, Sharnbrook, Bedford*)

The inclusion into diets of milk products soured by lactobacilli has long been considered beneficial in microbially associated enteritis of man. It is thought that their curative action involves the maintenance of acidic conditions in the intestine, which suppresses growth of enteropathogens. For this reason some workers have advocated chemical rather than microbiological control of intestinal pH. However, experiments which have reported successful therapy by reducing the numbers of enteropathogens have not been convincing. It was therefore considered worthwhile to look for changes in potentially deleterious metabolites following the use of lactobacilli preparations.

Pigs were abruptly weaned at 3 weeks on to cows milk reconstituted from spray dried powder, fed unmodified or after 48 hr incubation with *Lactobacillus acidophilus*. Intestinal

amines were investigated in animals autopsied in the immediate pre- and postweaning period. Urinary heterocyclics were used to monitor *in vivo* formation of diamines, due to microbial action in the intestine.

Excretion of urinary heterocyclic amines increased in the immediate postweaning period, and again if an animal was diarrhoeic, the increases being smaller in pigs fed lactobacillus milk. Overall production of diamine, within the alimentary tract, was no indication of a predisposition to diarrhoea since clinically normal and scouring animals could excrete similar levels. However, the site of amine production might be important. The small intestine was the main site of production in diarrhoeic animals, whereas in clinically unaffected animals only low levels of amines were present. In animals fed dietary lactobacilli a transfer of activity from the small intestine to the colon was apparent during the diarrhoeic phase. Concomitantly diarrhoea in these animals was reduced in severity and duration.

Lactobacillus administration failed to induce any consistent changes in the small intestinal microflora.

A Growth Stimulant for *Fusarium graminearum* in Wheat Anthers which Promotes Head Infection. By R. N. STRANGE and H. SMITH (*Department of Microbiology, University of Birmingham*)

Epidemics of cereal headblight caused by *Fusarium graminearum* (*Gibberella zeae*) occur in warm humid regions of the world. Heads prior to anthesis and emasculated heads appeared resistant to infection but non-emasculated heads seemed susceptible (Anderson, A. L. (1948), *Phytopathology*, **38**, 595; McKay, R. & Loughnane, J. B. (1945), *Sci. Proc. R. Dublin Soc. N.S.* **24**, 9). Confirmation of the importance of anthers in the infection process on wheat was obtained by two different techniques: (1) emasculated and non-emasculated plants were exposed to an aerosol of fungal conidia; and (2) droplets of conidia suspensions were deposited on various parts of non-emasculated heads. After incubation at high humidity for 48 hr and for 5 days in a greenhouse the degree of infection was obtained by plating out the partially dissected head and scoring those parts yielding colonies of the fungus. In the second series of experiments additional data were obtained by homogenizing the inoculated spikelet and plating on a selective medium for fungal colony counts. In both experiments high rates of infection were obtained only when conidia had been deposited on anthers.

A growth stimulant in wheat anthers was demonstrated when growth of the fungus on an agar medium (Vogel's salts with 2 % sucrose for 18 hr at 25°) and in liquid culture (Vogel's salts with 2 % sucrose for 36 hr at 25°) was enhanced by aqueous extracts of anthers more than by extracts of any other tissue. The anther extract contained a number of amino acids (chromatography) and preliminary work on the extraction of the growth factor(s) has shown that it passes a millipore membrane and is dialysable, heat stable and not destroyed by freeze drying.

The Role of Pectic Enzyme Production in the Resistance of Tomato to *Fusarium oxysporum lycopersici*. By P. LANGCAKE, R. B. DRYSDALE and H. SMITH (*Department of Microbiology, University of Birmingham*)

While pectic enzymes are widely held to be involved in vascular wilt diseases (Wood, R. K. S. (1967), *Physiological Plant Pathology*, Blackwell, Oxford) their precise role is uncertain and has been investigated from a number of different points of view (Patil, S. S. & Dimond, A. E. (1968), *Phytopathology*, **58**, 676). Deese, D. C. & Stahmann, M. A. ((1962), *Phytopathology*, **52**, 255) found that pectic enzyme activity increased in susceptible tomato plants infected with *Fusarium oxysporum lycopersici*, while in infected resistant plants the level of pectic enzymes was less than in uninfected controls. We have studied three possible mechanisms for this difference between susceptible and resistant plants: (1) susceptible and resistant plants differ in their ability to induce fungal pectic enzymes following infection, (2) the fungal pectic enzymes formed differ in ability to use the pectic substances in resistant and susceptible plants as substrates, (3) resistant plants produce an inhibitor of pectic enzymes. No evidence has been obtained for a difference in the induction of pectic enzymes by

susceptible or resistant plants or for a difference in the specificity of these enzymes once induced. The possibility that the resistant variety produces an inhibitor of pectic enzyme activity is still being investigated.

An Experimental System for Studying Tissue Specificity of Influenza Virus. By O. BASARAB and H. SMITH (*Department of Microbiology, University of Birmingham*)

A quantitative survey for infective virus in relevant organs of ferrets inoculated with influenza virus revealed that the agent localized and grew significantly only in nasal mucosae after direct inoculation into the blood stream and predominantly in that site after intranasal inoculation. In the latter case, lung and trachea also became infected, but they always contained less virus than nasal mucosae. Virus was sometimes recovered from the oesophagus of infected animals. However, direct inoculation of this organ without concurrently establishing respiratory infection produced no oesophageal infection after 1–3 days. Thus, the virus recovered from the oesophagus of animals with respiratory infection represented spill over.

In organ culture, infection followed the pattern *in vivo* for nasal mucosae, lung, trachea, oesophagus and aorta, namely virus grew in the first three, particularly well in nasal mucosae, but did not grow in oesophagus and aorta. The resistance of oesophagus to infection both *in vivo* and *in vitro* is striking, considering that this organ is exposed to large amounts of infective virus in the course of disease.

Unexpectedly, bladder and oviduct which were not infected in the original experiments *in vivo* supported virus growth in organ culture. Direct injection of virus into the bladder or oviducts of ferrets resulted in local infection. This urogenital infection recalls reports of virus recovery from human urine in influenza and indicates that the virus is more ubiquitous than usually believed. These results also show that route of infection plays a role in localization of influenza virus.

The parallel between infection *in vivo* and the growth patterns of the virus in organ cultures establishes the feasibility of the latter system for studying factors influencing tissue susceptibility or resistance to infection with this virus.

Studies of Tumour Malignancy by Analogy with Microbial Virulence. By R. A. KILLINGTON, A. E. WILLIAMS, N. A. RATCLIFFE, T. P. WHITEHEAD and H. SMITH (*The Department of Microbiology, University of Birmingham*)

Investigation of differences between high and low malignancy sublines of the same tumour may allow identification of determinants of malignancy just as comparison of high and low virulence strains of pathogenic bacteria has led to the characterization of virulence attributes such as toxins and aggressins. With this aim in view differences in malignancy, analogous to increased bacterial virulence on passage, have been produced between separate passage generations of a rat ascites tumour (WBP 1); WBP 1 (A) (7th passage) kills rats in *ca.* 36 days and WBP 1 (V) (21st passage) in *ca.* 16 days (1×10^7 cells i/p). Amounts of these tumours and distribution of metastases did not differ, when compared at the same time after injection but more A than V tumour was present at death. Thus WBP 1 (V) may be more toxic than WBP 1 (A). In attempts to identify specific changes due to the tumours, the concentrations of 14 serum components were measured in normal rats and, during tumour growth, in rats injected with A or V tumour cells. Changes occurred in several components but the most striking were those affecting glucose (normal rat, 200 mg./100 ml. serum). In rats injected with WBP 1 (V) (1×10^7 cells i/p), serum glucose concentrations decreased after 8 days and at death were < 10 mg./100 ml. Similar levels occurred terminally in rats with WBP 1 (A) but the rate of decrease was less. Reduction of serum glucose to < 10 mg./100 ml. in normal rats, by insulin injections, proved fatal. Conversely, injections of glucose into rats dying of WBP 1 (V) tumour produced significant prolongation of survival. Thus the toxic effects and the differences in malignancy between WBP 1 (V) and WBP 1 (A) appeared related to their abilities to induce a fatal hypoglycaemia. The mechanism of this effect is being investigated.

Virulence Mechanisms of Staphylococci. By C. ADLAM, J. H. PEARCE and H. SMITH *(Department of Microbiology, University of Birmingham)*

The mechanisms by which staphylococci resist cellular bactericidins of the host remain uncertain. The factors responsible should be most readily recognized by study of organisms isolated directly from the infected host (Smith, H. (1964), *Symp. Soc. gen. Microbiol.* **14**, 1) and by comparison of related strains of differing virulence. To investigate these possibilities a strain of *Staphylococcus aureus* has been passaged in rabbits to increase virulence and organisms grown *in vivo* have been isolated from rabbits infected with the passaged strain.

The virulence of the original (O) and passaged (P) strains grown *in vitro* and the organisms grown *in vivo* (V) was compared by LD50 for rabbits; V was 25 times more virulent than P and 6000 times more virulent than O. In parallel with the virulence differences the three strains differed in their susceptibility to killing by both serum and lysates of polymorphonuclear leucocytes. Whereas strains O and P were killed off (strain O more rapidly), strain V multiplied. With intact polymorphonuclear leucocytes the three strains were equally susceptible to phagocytosis; strains O and P were similar in their susceptibility to intracellular killing but survival of strain V was substantially greater. No strain showed evidence of capsular material when examined in indian ink films.

The significance of these findings for virulence of staphylococci will be discussed.

The Placental Localization of *Vibrio fetus*. By D. B. LOWRIE and J. H. PEARCE *(Department of Microbiology, University of Birmingham)*

Vibrio fetus causes abortion in cattle and sheep following extensive multiplication of the organism within the placental tissues (McFadyean, J. & Stockman, S. (1913), *Rep. Dep. Comm. Bd. Agric. & Fish. Lond.*: Part III—Abortion in sheep; Jensen, R., Miller, V. A. & Molello, J. A. (1961), *Am. J. Vet. Res.* **22**, 162). Such local multiplication also causes abortions of cattle and sheep resulting from infections with *Brucella* spp. The growth of *Brucella* spp. is localized within the placental tissues and correlates with the presence of the specific growth-enhancing nutrient erythritol (Smith, H., Williams, A. E., Pearce, J. H., Keppie, J., Harris-Smith, P. W., Fitz-George, R. B. & Witt, K. (1962), *Nature, Lond.* **193**, 47).

In our work the extent of the placental localization in vibrionic placentitis has been examined following experimental infection, then evidence has been sought for a possible nutritional basis for the localization. Pregnant sheep were infected intravenously with *V. fetus*. After 1–4 weeks the animals were slaughtered and the distribution of organisms in aliquots of the maternal and foetal tissues was determined. Organisms were confined to the uterine contents: the placenta and the allantoic fluid contained 84 % of the total organisms present ($2 \cdot 4 \times 10^{12}$); the remainder were associated with the chorion, uterine wall and uterine exudate, with traces in the internal organs of the foetus.

Foetal and maternal tissues of several pregnant sheep were extracted by maceration in phosphate buffered saline. The supernatants from centrifuged extracts were adjusted to pH 6·0, sterilized by membrane filtration and used as growth media. Growth rates in extracts from foetal cotyledons, maternal liver and occasionally maternal kidney were significantly greater than those in extracts from other tissues. Preliminary experiments indicate that material responsible for high growth rates in these tissues is diffusible. Erythritol did not, however, enhance the growth rate of *V. fetus*.

Virulence and Host Specificity of TRIC Agents. By J. E. MOORE, M. S. GRIFFITHS and J. H. PEARCE *(Department of Microbiology, University of Birmingham)*

TRIC agents show a marked host specificity, causing conjunctivitis in primates only, the natural disease being restricted to man (Jawetz, E. (1964), *Ann. Rev. Microbiol.* **18**, 301). Primary cultures of baboon conjunctival cells have been set up to examine various stages in the developmental cycle which might influence host specificity. However, attempts to infect cells *in vitro* were unsuccessful, although cells infected *in vivo* subsequently developed inclusion bodies when cultured *in vitro*. Failure to obtain infection *in vitro* was not due to

inactivation of the agent nor to non-adsorption on to the cell surface since centrifugation (Reeve, P. & Taverne, J. (1967), *Ann. J. Ophthal.* **63**, 1167) of TRIC agent on to cells did not result in the formation of inclusions.

Baboons infected with a laboratory strain of TRIC agent developed a moderate disease and relatively few inclusions were seen in conjunctival smears. Thus, failure to obtain infection *in vitro* could have been due to reduced virulence following passage in eggs. Passage of agent in baboons was therefore carried out and considerable increase in virulence resulted after three passages as indicated by the number of infected cells and the severity of clinical signs (Collier, L. H. & Blyth, W. A. (1966), *J. Hyg., Camb.* **64**, 513). In the first passage no more than 12 inclusions per smear were seen compared with up to 750 inclusions per smear in the third passage. The severity of inflammatory signs paralleled the number of inclusion bodies observed. Agent isolated from the conjunctiva of heavily infected animals was much reduced in infectivity for the baboon conjunctiva after re-passage in eggs.

The significance of these results for studies of host specificity will be discussed.

Isoelectric Focusing of *Clostridium perfringens* α- and θ-toxins. By C. J. SMYTH and J. P. ARBUTHNOTT (*Department of Microbiology, University of Glasgow*)

The method of isoelectric focusing (Vesterberg, O. & Svensson, H. (1966), *Acta Chem. Scand.* **20**, 820), allows the separation of proteins on a preparative scale in a natural pH gradient of carrier ampholytes (low molecular weight aliphatic polyamino-polycarboxylic acids), each protein being focused at its isoelectric point (pI). The possibility that *Clostridium perfringens* α-toxin consists of three or four components, as detected by starch block electrophoresis has been suggested (Ispolatovskaya, M. V. (1964), *Biokhimiya*, **29**, 869). Recently two forms of α-toxin, having pI's of 5·2 and 5·5 respectively were detected by isoelectric focusing (Bernheimer, A. W. (1968), *J. Bact.* **95**, 2439); there are no known reports of isoelectric focusing of θ-toxin. In the present study partially purified α-toxin, prepared from *Cl. perfringens* Type A (strain S107), was focused in a gradient pH 4–6. The carrier ampholytes were inhibitory, but after dialysis of the fractions three main components were found. These had pI's of 5·20, 5·27 and 5·39; in each case hot cold haemolytic activity paralleled phospholipase C activity as detected by release of phosphocholine or egg-yolk turbidity.

By contrast partially purified θ-toxin prepared from *Cl. perfringens* strain BP6K focused in a gradient pH 3–10 showed a single peak of haemolytic activity with a pI of 6·5. Approximately 65 % of input activity was recovered without dialysis. The haemolytic peak could be refocused in a broad pH gradient without much loss of activity. However when refocused in a narrow gradient, pH 5–8, most of the activity disappeared. Similar findings have been reported for cereolysin and streptolysin O each of which had a pI of 6·5 (Bernheimer, A. W. (1968), *J. Bact.* **95**, 2439).

These findings emphasize the basic similarity of these oxygen labile haemolysins.

The Proteolytic System of *Microsporum canis*. By J. O'SULLIVAN and G. E. MATHISON (*Department of Microbiology, Queen Elizabeth College, University of London*)

The association of *Microsporum canis* with keratinized tissues in animals and man has led to an interest in the proteolytic system of this fungus. It has been shown by Chattaway, F. W., Ellis, D. A. & Barlow, A. J. E. ((1963), *J. invest. Derm.* **41**, 31) that aqueous mycelial extracts of *M. canis* exhibit four peaks of peptidase activity at pH 6·3, pH 7·3, pH 8·7 and pH 8·8. Further work on the proteolytic system of this fungus has not been reported. This communication deals with the growth characteristics of the fungus, partial characterization, purification and localization of the proteolytic enzymes.

The fungus is grown on a defined mineral medium with 2 % (w/v) casein as carbon and nitrogen source and buffered with phosphate (0·05 M, pH 6·5). The growth rate (μ) is 0·05 hrs^{-1} and the doubling time (t_d) is 14 hr. Proteinases are assayed by the method of Kunitz ((1947), *J. gen. Physiol.* **30**, 291) and their secretion into the growth medium is followed; proteolytic activity reaches a maximum after 75 hr. Using the same mineral medium with 2 % (w/v) casein hydrolysate in place of casein, μ = 0·06 hrs^{-1} and t_d = 11 hr and no proteolytic activity

is detected. The purification of the extracellular proteolytic enzyme is complicated by the presence of casein in the medium and column chromatography using Sephadex G-100 and G-200 failed to separate the enzymes from the casein fraction. By lowering the pH of the medium to pH 4·6, the casein is precipitated with only slight loss of proteolytic activity from the supernatant. A 40 % ammonium sulphate fractionation of the supernatant results in a twofold increase in the specific activity of the proteolytic system. This preparation is applied to a DEAE cellulose column and eluted with phosphate buffer (0·01 M, pH 7·0) and a sodium chloride molarity gradient is applied, resulting in two peaks. Each of these peaks is applied to a Sephadex G-100 column and are eluted as single peaks.

The proteolytic activity was localized using sucrose density gradient centrifugation showing activity at three sites in the cell in fraction corresponding to 43 %, 21 % and 10 % (w/w) sucrose. Alkaline phosphatase has also been localized.

Structure and Composition of Resistant Layers in Bacterial Spore Coats. By G. W. Gould and J. M. Stubbs (*Unilever Research Laboratory, Colworth/Welwyn, Sharnbrook, Bedford*)

Spores of various bacteria treated with β-mercaptoethanol (10 %, w/v) in urea (8 M) for 1 hr at 37° became lysozyme-sensitive, and further treatment with alkali (0·1 N-NaOH; 15 min at 4°) increased their lysozyme-sensitivity still more. It was found that the alkali treatment removed an unusual acid-precipitable protein from the spores (7 % of residues tyrosine, 12 % glutamic acid, 8 % aspartic acid, 9 % lysine in *B. coagulans*) similar to a coat protein isolated by Kondo & Foster ((1967), *J. gen. Microbiol.* **47**, 257). A variety of mercapto-ethanol-treated *Bacillus* and *Clostridium* spores tested all released similar proteins in alkali.

Electron microscopy of freeze etched and thin sectioned preparations suggested that the alkali soluble protein was an outer spore coat component which contributed to a strikingly regular 'bandage' pattern on the surface of some spores, made up of parallel spaced (57 Å) striations. Although increasing lysozyme-sensitivity of spores, the mercaptoethanol-alkali treatments did not alter spore resistance to heat or to cobalt 60 γ-radiation.

Physiological Changes in Mice Infected with *Streptococcus pneumoniae* Type III. By T. B. Dick and C. G. Gemmell (*Department of Microbiology, University of Glasgow*)

Studies of the physiological changes following intraperitoneal injection of *Streptococcus pneumoniae* Type III (N.C.T.C. 7978) into Porton white mice have been undertaken. Attempts have been made to correlate these changes with the number of bacteria present in the blood during the infection.

The results of the experiments can be summarized as follows: (1) There was a fall in blood glucose to around 40 % of the level found in control mice. There was no evidence of glycosuria nor of changes in inorganic phosphate which often accompanies derangement of glucose metabolism. (2) There was a rise in plasma enzyme levels (glutamate oxalo-acetate trans-aminase to four times normal, glutamate pyruvate transaminase to four times normal and lactic dehydrogenase to eight times normal), suggesting a degree of hepatic damage. (3) There was a marked rise in plasma potassium as the infection progressed with levels up to ten times normal being recorded at death. It was thought that this was due to leakage of ions perhaps through the erythrocyte membrane as there has been a report (Shumway & Pollock, *J. Lab. Clin. Med.* **65**, 432) of increased erythrocyte osmotic fragility brought about by the pneumo-coccus in rabbits. (4) There was a fall in the white blood cell count during the infection: the value at death was often as low as 30 % of normal.

It is unlikely that the changes are due simply to the presence of a large number of bacteria in the blood and it is thought that the pneumococcus may produce a toxin *in vivo*. This toxin may not be lethal *per se* but may cause some of the changes described, e.g. release of intracellular enzymes and loss of white blood cells.

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CAMBRIDGE UNIVERSITY PRESS

Bentley House, 200 Euston Road, London, N.W. 1

American Branch: 32 East 57th Street, New York, N.Y. 10022

Price 45*s*. net ($7.50 in U.S.A.). Annual subscription £25 net ($85.00 in U.S.A.)

Printed in Great Britain at the University Printing House, Cambridge

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THE JOURNAL OF GENERAL MICROBIOLOGY

AND

THE JOURNAL OF GENERAL VIROLOGY

ARE CONDUCTED BY THE

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