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THE
JOURNAL OF GENERAL MICROBIOLOGY

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

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

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

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

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

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

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

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A New Property of Phage Group II *Staphylococcus aureus* Strains: Host Restriction of Phage K14

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(*Received* 10 *December* 1962)

SUMMARY

Various strains of *Staphylococcus aureus* which type exclusively with phages of lytic group II were found to modify phage K14 so that its ability to form plaques on host K1N was lessened. The restricted phage formed plaques with high efficiency on all strains of lytic group II. In general, it plated at lower titres on strains of lytic groups I, III, IV, and on some strains of miscellaneous typing characteristics; however, there were some variations among separate cultures of the same strains. For example, the restricted phage plated at high titre on strains 52A/79^a, 73, and 44A, but formed significantly fewer numbers of plaques on strain 52A/79^b and on a second culture of 44A. Strain K1N was found to dissociate into apt (K1H1) and non-apt (K1N2) forms. The probability of plaque development by restricted phage on strain K1N was dependent upon the nutritional state of the cocci and also upon the proportion of apt and non-apt cells. The restriction of phage K14 was eliminated during its propagation on all strains other than lytic group II. The unrestricted progeny particles tended to assay at equal titre on all the indicator strains. In all cases the genotype of the phage—susceptibility to host-control—remained unchanged. The observations add to existing data which indicate that strains of phage group II form a genetically distinct group. The suggestion is made that this phenomenon might help in taxonomic classification of strains of *S. aureus*.

INTRODUCTION

Passage of staphylococcal phage K14 through host *Staphylococcus aureus* strain 51, formerly designated 145 (Ralston & Krueger, 1954), results in a host-controlled restriction of plaque forming ability for host *S. aureus* strain K1 (Ralston & Krueger, 1952, 1954). Only 1 or 2% of the phage particles released from each infected host 51 bacterium initiates a successful infection and forms a plaque on strain K1. Passage of this successful phage through host K1 produces phage particles of equal ability to initiate plaques on both hosts. The parent phage K1 from which the phage K14 was isolated is not host alterable. Phage K14 is indistinguishable from phage K1 on the bases of adsorption capacity, latent period, burst size, thermolability, susceptibility to phage K1 rabbit antiserum and ability to induce the formation of a wall-dissolving lysin, virolysin (Ralston, Baer, Liberman & Krueger, 1961). The restricted form of phage K14 adsorbed to and killed host K1. It caused no lysis and formed no phage in the majority of K1 cocci to which it adsorbed. Otherwise this phage is indistinguishable from its unrestricted counterpart. Phage K14, like phage

K1, had a wide host range and was highly virulent. Both phages belong to the serological D group of Rippon (1956).

In the present studies propagating strains for many of the *Staphylococcus aureus* typing phages and several miscellaneous strains of staphylococci were employed in an attempt to determine which strains could support infection with the restricted phage K14 and which strains could impose a restriction on the unaltered phage K14. The data supply evidence to show that strains which have the ability to restrict phage K14 are sensitive to group II typing phages.

METHODS

Media. The various media employed for these studies and referred to in the text, usually by abbreviation, were as follows: tryptose-phosphate (TP) broth and agar (Difco Detroit, Mich., U.S.A.); trypticase soy (TS) broth and agar (Baltimore Biological Laboratories, Baltimore, Md., U.S.A.); Medium III, composed of TS broth, TP broth, 15 g., yeast extract (Difco), 5 g., distilled water, 1000 ml.; Brucella agar (Albimi Laboratories, Brooklyn, New York, U.S.A.).

Phages. The production and plaque assay of the phages K1 and K14 were described previously (Jones & Krueger, 1951; Ralston & Baer, 1960). The designations P1 and P14 used in former publications are the same as the K1 phage of Krueger and its variant K14. Duplicate sets of typing phages were obtained from the late Dr A. Browne (California Dept. of Public Health, Berkeley, Calif., U.S.A.) and from Dr J. E. Blair (New York Hospital for Joint Diseases, New York, N.Y., U.S.A.). They were stored at 4° for 1 year prior to use.

The slide assay plaque procedure of Jones & Krueger (1951) which has been rapid and accurate for estimate of the K1 and K14 phages was not completely adaptable to these phages; therefore the assays were made by the usual Gratia technique on TS agar containing calcium in concentrations according to the known requirement of each phage.

Bacterial strains. Staphylococcus aureus K1 was isolated from a case of multiple furunculosis in 1926 by Dr A. P. Krueger. It has been maintained on laboratory media for 37 years, the last 10 years on TP agar. A separate culture of this organism, *S. aureus* no. 11987, was obtained from the American Type Culture Collection (Washington, D.C., U.S.A.). Strain K1 is typically quite resistant to restricted phage K14 and was the source for single colony isolates of strains K1S (cf. Results and Table 4). Strains K1H1 and K1S2 were derived from strain K1S. Unless otherwise stated, the strain K1S used in the titrations was equivalent to K1S2 in its resistance to restricted phage.

The specific propagating strains of *Staphylococcus aureus* obtained from Dr Blair included PS 51 (formerly designated 145), 52A/79^{b} (formerly 925) and 31/44 (formerly 18), received in 1950; 3A, 3B, 3C, 55, 71, 29, 52A/79^{a}, 47, 80, 6, 7, 42E, 53, 54, 75, 77, 42D, 81, 42B, 44A and 73, received in 1959; and 187, received in 1961. Strains PS 39, 44A, 52, 70, 82 and 523 were obtained from Dr Browne in 1959 to replace those which were no longer viable in the Blair set. Dr Blair also supplied some antibiotic-resistant strains, recorded by the patient name's (see Table 1). A penicillin sensitivity test strain (probably FDA 209P), designated WR, was obtained from the Western Regional Research Laboratory (Albany, Calif., U.S.A.). Strains 8511, s 68

$Sr^{R}E^{R}N^{R}Lys^{+}$ (streptomycin resistant, erythromycin resistant, novobiocin resistant, lysogenic for phage 53) and s83 $L^{-}M^{-}$ (lactose non-fermenting, mannitol non-fermenting) were obtained from Dr M. L. Morse (University of Colorado, Denver, Col., U.S.A.).

Phage typing. The method recommended by Blair & Carr (1960) was used. All of the standard propagating strains of staphylococcus were tested with phages at 1 RTD (routine test dilution). The following phages were used: Group I includes phages 29, 52, 52A, 79, 80; group II includes phages 3A, 3B, 3C, 39, 55, 71, 523; group III includes phages 6, 7, 42B, 42E, 47, 53, 54, 70, 73, 75, 77, 83; group IV includes phage 42D; miscellaneous group includes phages 44A, 81. A few strains were tested by a procedure modified from that of Hood (1953) in order to determine broad phage group patterns. Microscope slides were layered with 0·5 ml. TP agar (0·3%) containing 3×10^{7} cocci/ml. Mixtures of typing phages at 10^{-2} dilution were then spotted on the agar surface with a loop or cotton swab. The phage mixtures were as follows: group I (81) contained phages 29, 44A, 52, 52A, 79, 80, 81; group II contained phages 3A, 3B, 3C, 39, 55, 523; group III (IV) contained phages 6, 47, 53, 54, 73, 77, 83, 42D, 42B. The slides were incubated at 28° in moist chambers for 24 hr. The most prominent areas of clearing were recorded. In some instances the strain was retested with individual members of the phage pools.

Plaque isolations. Phage from isolated plaques was suspended in 10 ml. sterile 0·85% NaCl. Known volumes were then mixed with a suspension of the desired strain of staphylococcus and assayed by plaque count.

Detection of restricted phage. Detection was accomplished by simultaneous assay of phage K14 on each of two hosts, one being a test indicator strain and the other being host K1N.

Further description of the detection of restricted phage is presented in the text.

Terms used. The terms describing the observed changes in phage K14 preparations are not intended to express underlying mechanisms. 'Restricted' phages are those which are highly infective for cocci of group II, but are significantly less infective for host K1N. 'Unrestricted' phages are those having a potential for infecting strain K1N equal to that for infecting strains of group II. The symbols K14/51 and K14/K1N represent phage K14 produced on host strains 51 and K1N respectively. 'Restrictor' hosts are those which reduce the infectivity of phage K14 for host K1N. 'Releasor' (or derestrictor) hosts are those which yield unrestricted phage K14 after being initially infected with restricted phage. Indicator strains exhibited high or low susceptibility, i.e. were apt or inapt for infection by phage K14. The infection potential refers to the ability of phage K14 to produce plaques on a given assay strain.

RESULTS

Correlation of phage pattern of Staphylococcus aureus strains with their ability to serve as efficient indicator strains for restricted phage K14/51

Previously we reported that when phage K14 was restricted by host 51 it formed about 40-fold fewer plaques on host K1 than on host 51 and that it also had a low efficiency of plating for a large number of strains of *Staphylococcus aureus*. In fact, when restricted phage K14/51 was assayed by plaque count on various *S. aureus* it appeared to divide the strains into two groups: on one group (highly susceptible)

Table 1. *Correlations between specific phage patterns of Staphylococcus aureus and their susceptibilities to restricted and unrestricted forms of phage K14 and to the non-host-controllable parent phage K1*

Phage group	Assay strain	Broad phage pattern	Phage stock used: K1/K1* non-controllable *a*	K14/K1 unrestricted *b*	K14/51 restricted *c*
		Propagating strains			
I	29	I	$< 10^6$	$< 10^2$	$< 10^4$
	52	I	$5{\cdot}4 \times 10^9$	$2{\cdot}0 \times 10^9$	$7{\cdot}5 \times 10^7$
	52A/79a	I	$5{\cdot}3 \times 10^9$	$2{\cdot}3 \times 10^9$	$2{\cdot}2 \times 10^9$
	52A/79b	I	$6{\cdot}3 \times 10^9$	$1{\cdot}3 \times 10^9$	$4{\cdot}3 \times 10^7$
	80	I, III, 81	$4{\cdot}4 \times 10^9$	$5{\cdot}8 \times 10^9$	$9{\cdot}0 \times 10^7$
II	3A	II	$6{\cdot}7 \times 10^9$	$4{\cdot}6 \times 10^9$	$6{\cdot}3 \times 10^9$
	3B	II	$7{\cdot}0 \times 10^9$	$5{\cdot}0 \times 10^9$	$6{\cdot}4 \times 10^9$
	3C	II	$9{\cdot}8 \times 10^9$	$4{\cdot}2 \times 10^9$	$6{\cdot}9 \times 10^9$
	39	II	$8{\cdot}8 \times 10^8$	$5{\cdot}2 \times 10^9$	$9{\cdot}4 \times 10^8$
	51	II	$1{\cdot}1 \times 10^{10}$	$7{\cdot}6 \times 10^9$	$9{\cdot}0 \times 10^9$
	55	II	$3{\cdot}1 \times 10^9$	$8{\cdot}9 \times 10^9$	$2{\cdot}6 \times 10^9$
	71	II	$5{\cdot}7 \times 10^9$	$6{\cdot}9 \times 10^9$	$5{\cdot}7 \times 10^9$
	523	II	$7{\cdot}0 \times 10^9$	$1{\cdot}0 \times 10^9$	$6{\cdot}7 \times 10^9$
III	6	III, 81	$3{\cdot}6 \times 10^9$	$1{\cdot}2 \times 10^9$	$8{\cdot}2 \times 10^7$
	7	III, IV, 81	$2{\cdot}0 \times 10^9$	$< 10^6$	$< 10^4$
	42B	III, 81	$8{\cdot}5 \times 10^9$	$2{\cdot}0 \times 10^{10}$	$1{\cdot}3 \times 10^8$
	42E	III, 81	$1{\cdot}8 \times 10^9$	$7{\cdot}8 \times 10^8$	$2{\cdot}8 \times 10^7$
	47	III	$< 10^6$	$< 10^2$	$< 10^4$
	53	III	$4{\cdot}0 \times 10^9$	n.t.	$2{\cdot}2 \times 10^7$
	54	III, 73	$4{\cdot}7 \times 10^9$	$1{\cdot}0 \times 10^9$	$4{\cdot}5 \times 10^7$
	70	III, 81	$< 10^6$	$< 10^2$	$< 10^4$
	73	I, II, III	$7{\cdot}8 \times 10^9$	$2{\cdot}0 \times 10^9$	$2{\cdot}2 \times 10^9$
	75	III	$1{\cdot}7 \times 10^9$	$1{\cdot}3 \times 10^9$	$1{\cdot}5 \times 10^8$
	77	III	$< 10^6$	$< 10^2$	$< 10^4$
	83	III, 81,	$< 10^6$	$< 10^2$	$< 10^4$
IV	42D	IV	$2{\cdot}0 \times 10^9$	$3{\cdot}6 \times 10^8$	$3{\cdot}7 \times 10^7$
Misc.	44A	I, III, IV	$3{\cdot}4 \times 10^9$	$2{\cdot}1 \times 10^9$	$2{\cdot}0 \times 10^9$
	187	187	n.t.	n.t.	$8{\cdot}7 \times 10^7$
	81	I, 81	$2{\cdot}7 \times 10^9$	$5{\cdot}2 \times 10^9$	$7{\cdot}3 \times 10^7$
		Non-propagating strains			
Other	KIN	I, II, III, IV, 81	$6{\cdot}3 \times 10^9$	$2{\cdot}9 \times 10^9$	$8{\cdot}6 \times 10^7$
	WR	I, IV	$4{\cdot}7 \times 10^9$	$4{\cdot}8 \times 10^9$	$7{\cdot}2 \times 10^7$
	Gold	I	$5{\cdot}0 \times 10^9$	n.t.	$5{\cdot}1 \times 10^7$
	Sullivan	I	$5{\cdot}7 \times 10^9$	n.t.	$1{\cdot}0 \times 10^8$
	Gillespie	III	$5{\cdot}3 \times 10^9$	n.t.	$1{\cdot}8 \times 10^7$
	Bradley	III	$5{\cdot}5 \times 10^8$	n.t.	$< 10^4$
	Morse s68	III	$< 10^6$	n.t.	$< 10^4$
	Morse s83	III†	$2{\cdot}1 \times 10^9$	n.t.	$1{\cdot}3 \times 10^7$
	Morse 8511	III	$1{\cdot}4 \times 10^9$	n.t.	$6{\cdot}5 \times 10^7$
	Dodge	I	$5{\cdot}7 \times 10^9$	n.t.	$3{\cdot}7 \times 10^8$

* K1'K1 designates phage K1 produced on host, K1, etc.
n.t. = not tested.
† Broad phage pattern determined by testing with pools.

phage K14/51 formed plaques roughly equivalent in number to those produced on host 51, and on the other group, the phage formed many fewer plaques, their number being equivalent to those produced on strain K1 (Ralston & Krueger, 1954). In the present study this apparent division of response of staphylococcal strains to restricted phage K14 has been further investigated to determine whether it might be related to a particular pattern of susceptibility to individual typing phages. For this analysis over 40 coccal strains were classified as to phage-typing pattern and then were tested for their relative susceptibility to infection and plaque formation by restricted phage K14/51, by unrestricted phage K14/K1, and by the non-host controllable parent phage K1/K1N. In the data shown in Table 1, the strains are grouped according to the broad phage-typing patterns as determined in our laboratory by the method of Blair & Williams (1961). These patterns were in general agreement with the results reported by Williams & Rippon (1952), Blair & Carr (1960), Wallmark & Finland (1961), and Blair & Williams (1961).

As indicated in Table 1, the non-controllable phage K1/K1N tended to form equivalent numbers of plaques on all susceptible strains, the titres being mainly in the range of 10^9/ml. (column *a*). The unrestricted phage K14/K1 was much like the parent phage K1/K1N (column *b*). In these tests certain strains were resistant to all preparations of the K1 or K14 phage: PS 29, 47, 70, 77 and 83. The restricted phage K14/51 (column *c*) divided the strains of *Staphylococcus aureus* into two groups, and these groups were correlated with their typing patterns as follows: (*a*) all strains susceptible to group II phages (i.e. forming lytic patterns with group II phages at 1 RTD) were highly susceptible to the restricted K14/51 phage-titres in the range of 10^9/ml.; whereas (*b*) most strains not typing with group II phages were significantly less susceptible—titres in the range of 10^7/ml. We suggest that the group II strains possess a property which confers on them a high degree of susceptibility to plaque formation by group II typing phages and that this property also controls their susceptibility to restricted phage K14/51. However, there are some unexplainable exceptions, since strains PS 73, 52A/79* and 44A did not type solely with group II phages but underwent good plaque formation with phage K14/51, the phage titre being equivalent to that on host 51.

Restriction of phage K14 by other strains of Staphylococcus aureus, a property of phage group II cocci

The restrictor host, *Staphylococcus aureus* 51, used for the studies of Table 1 is known to belong to phage group II. Tests were next performed to determine which of the other staphylococcal strains would restrict or fail to restrict phage K14, and which strains would serve as efficient indicator hosts for the restricted phage. A phage lysate of unrestricted phage K14/K1N prepared in medium III broth was diluted in saline and plated with each *S. aureus* strain in TP agar. The contents of one or two typical phage plaques from each passage were suspended in saline, diluted, and assayed on hosts representative of the broad lytic phage groups. Upon each plaque passage the titre of the phage for a given indicator host was compared to the titre for host K1N. The plaques/ml. formed on the indicator host (H) were then divided by the plaques/ml. formed on host K1N. The resultant quotient represented the efficiency of plating (EOP) H/K1N of phage from a given passage for any given assay host, relative to host K1N. This procedure also served as a means of detecting

Table 2. *Restriction of phage K14 by Staphylococcus aureus strains of phage group II; non-restriction by other strains*

Restricted phage K14/51 was propagated by plaque passage on TP agar with hosts of different phage groups. After each passage material from isolated plaques was suspended in 10 ml. 0·85% saline and the phage was assayed by plaque count technique on a series of indicator strains (H). EOP H/K1N values were calculated from the titres/ml. and then were grouped into categories: Code 7 = EOP H/K1N from 120 to 45; Code 6 = 44 to 15; Code 5 = 14 to 5; Code 3 = 4·9 to 2·0; Code 1 = 1·9 to 0·5; Code 0 = 0·4 to 0·1; Code 0* = < 0·1. Samples grouped under codes 5, 6 and 7 were considered to contain significant amounts of restricted phage K14.

Indicator strain (H)		Relative EOP phage H/K1N* of K14 after plaque passage on propagating strain (coded values)																						
		Phage group... I				II								III					IV	Miscellaneous			Other	
Phage group	H	52	52A/79a	52A/79b	80	3A	3B	3C	39	51	55	71	523	6	7	42B	42E	73	42D	44A	81	187	K1N	WR
I	29	R†	R	—‡	—	R	R	R	R	R	R	R	—	R	—	R	—	R	—	R	—	—	R	—
	52	1	1	1	3	1	1	0	1	0*	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	52A/79a	1	1	1	1	5	5	7	6	6	—	0	1	1	1	1	1	1	1	1	1	1	1	1
	52A/79b	1	1	3	3	1	1	1	—	1	0	1	0	1	—	1	—	1	1	1	5	1	1	0
	80	1	1	1	1	1	1	1	—	1	1	1	3	1	1	1	1	1	1	—	1	1	1	1
II	3A	1	1	1	5	5	5	6	—	6	5	6	5	1	—	1	0	1	1	1	3	1	1	1
	3B	1	1	1	3	5	5	6	—	5	5	6	5	1	1	1	1	1	1	1	1	1	1	0
	3C	1	1	1	3	5	5	6	—	6	5	5	5	1	—	1	1	1	1	1	1	1	1	0
	39	1	1	—	—	5	—	—	7	—	6	—	—	1	1	1	1	1	—	1	—	—	1	—
	51	1	1	1	3	5	5	7	6	6	5	6	5	1	1	1	1	1	1	1	3	1	1	0
	55	1	1	1	1	5	5	6	—	5	5	6	5	1	—	1	1	1	1	1	1	1	1	1
	71	1	1	1	3	5	5	6	—	6	5	6	5	1	1	1	1	1	1	1	1	1	1	0
	523	0	1	1	1	5	5	7	—	6	5	—	5	1	—	1	—	1	1	1	1	1	1	0

Table 2 (*cont.*)

Relative EOP phage H/K1N* of K14 after plaque passage on propagating strain (coded value)

Indicator strain (H)		Phage group... I				II								III					IV	Miscellaneous			Other	
Phage group	H	52	52A/79a	52A/79b	80	3A	3B	3C	39	51	55	71	523	6	7	42B	42E	73	42D	44A	81	187	K1N	WR
III	6	1	1	1	1	0*	0	0*	1	1	1	0	0*	1	1	1	0	0	1	1	1	1	1	0
	7	0*	0*	0*	0*	0*	0*	0*	0*	0*	0*	0*	0*	0*	0*	0*	0*	0*	—	0*	0*	0*	0*	1
	42E	1	1	1	1	1	1	0*	0*	0	1	1	—	1	0	0	1	1	1	1	1	1	1	0
	47	R	—	—	—	R	R	R	R	R	R	R	—	R	—	R	—	R	—	R	—	R	R	—
	53	1	1	—	—	—	—	—	0*	0*	—	—	—	3	—	1	1	1	1	1	—	1	1	—
	54	1	0	1	1	1	0	1	R	1	0	0	1	1	1	1	—	0	1	1	1	1	1	0
	70	R	—	—	—	R	R	R	—	—	R	—	—	R	—	R	—	R	—	R	—	R	R	—
	75	0	1	1	1	0	0	1	—	0*	0	1	0	1	—	0	—	0	1	1	0	1	0	1
	77	R	—	—	—	R	R	R	R	—	R	—	—	—	—	R	—	R	—	R	—	R	R	—
	83	R	—	—	—	R	R	R	R	—	R	—	—	—	R	R	—	R	—	R	—	R	R	—
IV	42D	1	0	1	1	0*	0*	0*	1	0*	0	0	0	1	0*	1	0	0	1	1	1	1	1	1
Misc.	42B	1	3	1	3	1	1	3	1	3	—	1	1	0*	1	1	1	1	1	1	3	1	1	1
	44A	1	1	1	1	5	5	6	6	6	5	6	3	1	1	1	1	3	1	1	3	1	1	0
	73	1	1	1	3	6	5	6	6	6	—	6	5	1	1	1	1	1	1	1	1	3	1	1
	81	1	1	1	1	1	1	0	—	3	1	1	—	1	—	—	—	—	1	—	1	1	1	0
	187	—	—	—	—	—	—	—	—	0	—	—	—	—	—	—	—	—	1	—	1	1	0	—
Other	K1N	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	WR	1	1	1	1	1	1	1	1	—	1	1	1	—	—	1	—	—	1	1	1	1	1	1

* EOP phage H/K1N = Efficiency of plating, calculated from $\frac{\text{plaques per ml. on assay strain}}{\text{plaques per ml. on host K1}}$.

† R designates a strain resistant to phage K14 when plaque contents were plated at 1:10 final dilution with a given test strain.

‡ Sample not tested.

the presence of restricted phage particles. For example, when the phage K14 formed a number of plaques on any given indicator strain equal in number to the plaques produced by the same volume of phage on host K1N, the EOP H/K1N of the phage for the test indicator host was equal to 1. This indicated there were no restricted particles. When more plaques were produced on the test strain than on host K1N, the EOP H/K1N, was greater than 1, reflecting the presence of restricted phage particles.

To simplify the comparison of the numerous plaque passages and the subsequent ability of the phage K14 to form plaques on different assay strains, the various EOP H/K1N values were grouped into categories, coded 7, 6, 5, 3, 1, 0, 0*, as recorded in Table 2. An EOP H/K1N assigned to group 5 is considered to represent a significant restriction of the phage; it indicates that a range of from 5 to 14 plaques had appeared on a given indicator strain for each plaque on host K1N. By comparing the titres of phage K14 from single plaque passages for a series of indicator strains with the titres for host K1N, we could tell at a glance which strains behaved like strain 51 and which strains behaved like host K1N. In Table 2 the strains of *Staphylococcus aureus* that were used to propagate and to assay phage K14 have been classified according to their broad phage-typing patterns. The results may be summarized as follows: (1) In addition to strain 51, all other strains of phage group II were capable of restricting phage K14 for host K1N (code numbers 7, 6 and 5). The ability to host-restrict phage K14 appears to be confined to group II strains. (2) When phage K14 had been produced on and restricted by a given strain of phage group II, all other strains within phage group II served as effective indicator hosts although there were differences in their relative susceptibilities. (3) Propagating strains that were not members of phage group II yielded unrestricted phage K14 (code 3, 1, 0, 0*). When these 'non-group' II strains were tested for their ability to serve as indicator host for the assay of restricted phage K14, they proved to be relatively inapt, i.e. the restricted phage produced many fewer plaques on these strains than on strains belonging to phage group II. Strains 44A, 52A/79[a] and 73 were exceptions, being highly susceptible to the restricted phage regardless of the group II host employed as the source of the phage. In contrast to the group II coccal strains, however, none of these three hosts restricted phage K14. (4) In general, hosts classified as restrictors of phage K14 reduced but did not abolish the ability of the phage to propagate on host K1N, as evidenced by the fact that samples containing several hundred particles of restricted phage generally formed a few plaques on host K1N. During these studies certain bacterial hosts have tended to be slightly more resistant than others to plaque formation by unrestricted phage K14/K1N: PS 6, 7, 42E, 75 and 42D. Of these, strain 7 showed quite a variation, sometimes being highly resistant, the phage forming no plaques even in concentrated (10^8/ml.) amounts. We have noticed that four out of five of these strains are susceptible mainly to group III phages and that several other strains which are completely resistant also show lytic group III typing characteristics, i.e. PS 47, 70, 77 and 83. Perhaps group III cocci possess metabolic patterns which tend to be unfavourable for successful infection by phage K14. If so, then the change brought about by group II restrictor hosts might reflect a capacity to lower the ability of phage K14 to replicate in a generally unfavourable metabolic environment.

The interaction of restricted phage K14 with cocci from strains other than phage group II

Most of our information concerning the interaction between restricted phage and *Staphylococcus aureus* strains on which it does not form plaques efficiently has been obtained from studies with the K1N strain. Suggestive evidence that an early step in phage infection is blocked as a result of the restriction was obtained from the following preliminary observations: (1) Phage-adsorbed cocci showed no detectable increase in ultraviolet adsorption at 260 or 280 mμ, indicating that no net synthesis of protein or nucleic acid had occurred, in contrast to the increases normally following injection of phage material into the cell. (2) The lytic enzyme, virolysin, was not detected; normally this enzyme is produced early in the latent period (Ralston *et al.* 1961). (3) The phage-adsorbed cocci remained susceptible to infection with the unrestricted phage K14, indicating that during the early steps after adsorption the restricted phage did not cause the cell to produce materials which might inhibit the synthesis of unrestricted phage. There is as yet no evidence as to the exact step past adsorption which has been blocked.

We have found that several variables determine whether restricted phage K14 can initiate a plaque. For example, with the K1N strain, aptness for infection with the restricted phage K14/51 increases during the logarithmic phase of growth (Ralston & Krueger, 1954). The number of cells that can support infection are also influenced by changes in the medium of the cells prior to infection. In these studies *Staphylococcus aureus* K1 ('N' form) was grown for 24 hr periods at 37° either on TP agar or on Brucella agar. After 10 and 20 daily transfers the culture that had been carried on Brucella agar was transferred to TP agar. Cells from each transfer were stored at 4°, and finally a transfer was made from each passage, thus resulting in a series of cultures which had been incubated at 37° for 24 hr. These cultures were then tested for their relative susceptibilities to restricted phage K14/51. Simultaneously the phage was assayed on strain 51. The data in Table 3 record the number of plaques/ml. developing on each culture and the EOP 51/K1 values calculated with respect to each K1 preparation. During the first transfer on Brucella agar the susceptibility of K1N cocci decreased significantly, the EOP 51/K1 changing from 62 to 280. After continued transfer on Brucella agar, K1 cocci showed a slight increase in susceptibility to restricted K14, but not to the level of K1 cocci maintained on TP agar. When the culture was returned to TP agar after the 10th and 20th transfer on Brucella medium, it immediately increased in susceptibility to the restricted phage K14, to equal that of cocci maintained continuously on TP agar.

Detection of apt and non-apt forms of Staphylococcus aureus K1. We have recognized that with any restricted phage-host system, either the phage or the host cells—or both—might contain mixtures of infective (plaque-forming) and non-infective particles or apt and non-apt cells. An early observation that identical amounts of phage K14/51 plates with low but approximately equal efficiencies on a large number of strains of *Staphylococcus aureus* prompted us to suggest that lysates of restricted phage contained a low percentage of phage particles possessing a high potential for infecting strain 51 mixed with a large percentage of particles possessing a low potential for forming plaques on many other strains (Ralston & Krueger, 1954). However, since it is now known that most of these relatively insusceptible

strains type mainly with group III phages, this observation might be explained equally as well by differences in the general metabolic patterns of susceptible and less susceptible hosts. If there exist mixtures of particles in stock preparations of restricted phage, their physical separation would require the use of a highly sensitive technique; for we have been unable to detect their presence by methods based on adsorption rates, temperature stability, on rates of inactivation by specific antiserum, or by ultraviolet irradiation.

Table 3. *Effect of growth medium on susceptibility of Staphylococcus aureus* K1 *to restricted phage K14/51*

K1 (ATC culture no. 11987), previously incubated on TP agar, was grown on each medium for 24 hr at 37°, for 3 series of 10 transfers; and each transfer was stored at 4°. After each 9th transfer a 24-hr culture was prepared from each stored sample; the cocci were resuspended in TP broth to 6×10^7/ml., mixed with 0·5 ml. of phage/K14/51 diluted in saline, and 2·5 ml. TP 0·75% agar, and plated for relative plaque counts. Simultaneously assays were made with strain 51 on TP agar. The EOP 51/K1 values were calculated from the plaque titres.

	Growth medium prior to infection with 5×10^9/ml. particles of phage/K14/51			
	Tryptose-phosphate agar		Brucella agar	
Transfer no.	Plaques/ml. $\times 10^7$	EOP 51/K1	Plaques/ml. $\times 10^7$	EOP 51/K1
1	8·0	62	1·8	280
2	n.t.	n.t.	1·8	280
3	n.t.	n.t.	3·3	150
4	n.t.	n.t.	2·6	190
5	n.t.	n.t.	3·1	160
6	n.t.	n.t.	2·7	185
7	n.t.	n.t.	4·6	109
8	n.t.	n.t.	2·2	228
9	n.t.	n.t.	2·3	218
10	10·0	50	2·8	178
20	5·7	87	3·0	167
21	8·0	62	2·5	200
22	10·0	50	2·5	200
23	9·0	55	1·8	280
24	9·6	53	1·8	280
25	9·2	54	1·6	312
26	8·2	61	3·7	135
27	8·0	62	1·4	358
28	6·2	80	1·6	312
29	5·8	86	2·0	250
30	5·6	89	1·3	383

(Arrows indicate transfers discussed in test.)

In contrast to the lack of evidence for a mixture of particles in restricted phage preparations, we have recently succeeded in separating apt and non-apt cell types from an atypical K15 strain. Both types were obtained from a stock K15 culture which had changed from low to high susceptibility to phage K14/51. The change was first noticed after the culture had undergone numerous daily transfers on TP agar (Table 4). Since their isolation each of these two types has maintained its charac-

teristics with respect to phage K14/51. One culture, designated K1H1, is a susceptible indicator strain. On this strain phage K14/51 forms plaques at titres almost equal to strain 51. The second culture, K1N2, is less susceptible; on this strain the phage 14/51 plates at significantly lower titres.

There may be other strains of *Staphylococcus aureus* which undergo changes similar to the K1 culture. For example, strains 73, 44A, and 52/79[a] were efficient indicators for restricted phage K14, the titres on these strains being equivalent to titres on strain K1H1 or on hosts belonging to phage group II. In contrast, strain 52A/79[b] and one other culture of strain 44A tested 9 years previously were resistant to the restricted phage K14 and, therefore, were similar to the typical K1N2 culture. Possibly strains 52A/79[b] and 44A represent forms arising in the populations by mutation. We have noted that 52A/79, 44A and 73, like both types of strain K1, are susceptible to phages from group I (although the K1 culture and strain 73 are susceptible to additional phages). Perhaps strains typable with group I phages possess a metabolism which allows a greater percentage of successful infections with restricted phage.

Table 4. *Apt and non-apt colonies of Staphylococcus aureus* K1N *as shown by differences in EOP* 51/K1N *values with restricted phage K*14/51

Strain K1N represents a single colony isolated from the parent K1 stock culture. During transfer it became highly sensitive to the restricted phage K14, the ratio 51/K1N changing from 65 to 7.

Isolate no.*	EOP 51/K1N isolate
1	7
4	43
12	7
13	14
14	65
A	1
B	15
C	42
Stock culture	7

* Isolates on TS agar were all off-white, round, slightly raised centres; they were typical staphylococci in morphology when viewed at 900×. Strain K1N2 came from no. 4; strain K1H1 from A.

Release of restriction by strains other than group II

Hosts other than group II which had been infected with unrestricted phage K14/K1N continued to produce the unrestricted form, as evidenced by the fact that the phage progeny from these hosts generally formed equivalent numbers of plaques on all the strains (Codes 3, 1, 0, Table 2). Whenever these hosts were infected with the restricted form of K14 (Table 5), they yielded phage which subsequently formed equal numbers of plaques on assay strains of all the various phage groups. Experimental data showed that similar effects occurred when restricted phage infected strains K1N2, K1H1 as well as PS 52A/79[a], 73 and 44A. In effect, then, all these cultures, once infected, can raise the plaque-forming potential of restricted phage K14 despite any differences in their relative susceptibility to restricted particles. This can be regarded as a second kind of host-control—release of the restriction which had been imposed by strains of phage group II. From this it may be con-

Table 5. *Release of restriction of phage K14/51 by non-group II strains of Staphylococcus aureus*

Phage K14/51 was propagated by plaque passage on TP agar. Material from isolated plaques was suspended in 0·5% (w/v) saline and assayed by plaque count technique on a series of *S. aureus* assay strains (H) of known phage group. From the relative titres/ml. the EOP H/KIN values were calculated. Prior to passage on each propagating strain, the restricted phage had an EOP 51/KIN = 40.

		EOP H/KIN of phage K14/51 after one plaque passage				
		I		III		
Phage group of strain	Assay strain (H)	52A/79[a]	44A	42E	73	Other KIN
I	52	1·0	0·7	0·2	1·0	0·1
	52A/79[a]	1·6	1·8	0·8	1·0	1·6
II	39	1·6	0·8	0·5	0·5	1·0
	51	0·6	1·3	0·4	0·5	1·5
III	6	1·0	1·5	0·1	1·0	0·2
	7	0·1	0·1	0·1	0·1	0·6
	42E	0·4	0·6	0·5	0·5	0·3
	53	1·6	1·0	0·6	1·0	1·7
	42B	3·0	2·4	1·0	1·0	1·9
	73	1·6	1·4	0·7	1·0	1·0
Other	KIN	1·0	1·0	1·0	1·0	1·0

Table 6. *Phenotypic changes of phage K14 during serial passage on strains of Staphylococcus aureus in TP agar*

Phage K14 was propagated by plaque technique. Material from isolated plaques was suspended in saline and assayed on strain 51 (group II), on host KIN, and on representative strains of known phage group. Phage K14 was considered to be restricted when the titre/ml. for strain 51 was greater than five-fold (usually 20-fold), the titre/ml. for strain KIN; it was considered to be unrestricted when the titre/ml. for strain 51 was equivalent to the titre/ml. for strain KIN.

Stock phage		Passage no. 1		Passage no. 2		Passage no. 3	
Initial host	Effect on phage K14	Host	Effect on phage K14	Host	Effect on phage K14	Host	Effect on phage K14
KIN	Unrestricted	→ 523	Restricted	—	—	—	—
		→ 51	Restricted	—	—	—	—
		→ 3C	Restricted	→ 31/44	Unrestricted	—	—
				→ KIN	Unrestricted	—	—
				→ 3A	Restricted	—	—
51	Restricted	→ 39	Restricted	→ 39	Restricted	—	—
		→ 7	Unrestricted	→ 42B	Unrestricted	—	—
		→ 73	Unrestricted	→ KIN	Unrestricted	—	—
				→ 51	Restricted	—	—
		→ KIN	Unrestricted	→ 42B	Unrestricted	—	—
				→ 42E	Unrestricted	—	—
				→ 52	Unrestricted	—	—
				→ 52A/79	Unrestricted	—	—
				→ 73	Unrestricted	—	—
				→ 7	Unrestricted	→ 42B	Unrestricted
						→ KIN	Unrestricted
						→ 51	Restricted
						→ 71	Restricted

cluded that the ability of a strain to release the restriction is distinct from that property which confers on it a susceptibility to infection and plaque formation by restricted phage K14. (Of course, were any group II hosts able to reduce the potential of phage K14 for other hosts to zero, these non-apt hosts could not be tested for their ability to release the restriction.)

In additional tests phage K14 was propagated serially by plaque passage on strains selected at random from all groups and tested for its relative ability to infect K15, the host of propagation, and strains of phage group II. The phage was restricted and released from restriction according to the phage group of the host. The phage remained phenotypically alterable, regardless of the propagating host (Table 6).

With hosts K15 and 51, control over the plaque-forming potential of the phage K14 has been demonstrated to occur within each single burst cycle of an infected cell (Ralston & Krueger, 1954). While single burst studies have not been performed during the present studies, the results of single plaque passages indicate that the same changes are involved.

DISCUSSION

Strains with group II phage patterns, a genetically distinct group of Staphylococcus aureus

Strains of *Staphylococcus aureus* can be divided into broad but often overlapping groups based upon their ability to be lysed by specific phages (Williams & Rippon, 1952), lysogenic interrelationships (Williams Smith, 1948*a*; Rountree, 1949*a*), the nature of surface antigens (Cowan, 1939; Oeding & Williams, 1958), the ease with which antibiotic resistance develops (Barber & Whitehead, 1949; Barber & Burston, 1955; Wallmark & Finland, 1961), and the antigenic specificity of their free coagulases (Barber & Wildy, 1958). Phage-typing studies have established the fact that group II strains of human origin behave as a distinct group, rarely forming patterns with phages from other groups (Rippon, 1956) or carrying phages for other groups (Rountree, 1949*a*; Della Vida, 1957). According to our present data, strains of *S. aureus* in phage group II can be distinguished from those in other groups by their unique ability to host-restrict the polyvalent phage K14 for most hosts other than those of group II. It appears, however, that sensitivity of the hosts to group II phages is not the sole prerequisite for the host-controlled restriction to occur because strains K1 and 73 are susceptible to the group II phages but are incapable of restricting phage K14. Perhaps the ability to exclude phages other than those of group II is a second requirement in order for the host to possess the ability to restrict phage K14. The host-controlled restriction may reflect an interaction with a specific basic structure or region of the group II genome (or some product under its control), and therefore knowledge of this phenomenon may be useful in the taxonomic description of staphylococci, particularly of untypable strains.

Patterns of host-control of staphylococcal phages

Host-directed changes in staphylococcal phages occur both with phages of high virulence and wide host range (such as phage K14) and with the more temperate and specific typing phages. Data from host-control experiments with staphylococcal

phage Col of B serology, phage 47C of A serology (Rountree, 1956), and phage K14 of D serology show that phages of widely different serology may be subject to host control.

The controls described by Rountree (1956) involved specific restrictions of host range for strains typing with phages of groups I and III and with 42B and 47C (now classified as miscellaneous). These changes appear to have been confined to specific organisms within the strains of groups I and III and miscellaneous typing characteristics which had been selected for investigation. The K14 controls, on the other hand, have involved changes *en bloc* of its infectivity for group II and groups other than group II. In other investigations we have observed still another group II–non-group II control: Phages of the group II series were phenotypically altered by passage through strains KIN and 73, so that the group II phages could no longer easily infect group II hosts. At the same time their potential for infecting group III strains was increased (Ralston & Baer, 1964*a*, *b*). Using the phage 3C (serology A), we found that on the first burst from host KIN, the progeny phage 3C/KIN particles no longer could form plaques on strain 3C but plated at high titre on strains KIN, 6, 42E, 53, 54 and 47. A similar result was obtained by passaging the phage 3C directly on those group III strains for which it already had an infection potential (extremely low—in the range of 10^{-6} to 10^{-8}). Other members of the group II phage series, including phage 55 (serology B), underwent similar controls, the chief differences lying in the potentials for infecting specific strains of groups II and III (Ralston & Baer, 1964*b*).

Mechanism of host-control

The exact nature of the host-controlled restriction of staphylococcal phages is not yet known. Neither the alteration of phage K14 nor of phage 3C (Ralston & Baer, 1964*b*) appears to involve interference with their abilities to adsorb to the host bacterium. This is not unexpected because both the polyvalent phage K14 and the specific typing phages (Rountree, 1947) adsorb to a wide range of staphylococci regardless of their ability to form plaques. Phage specificity in these systems seems likely to involve mechanisms for penetration of DNA and establishment of phage replicating machinery. Probably the changes which bring about the restriction concern phage structures (protein, DNA, or both) which function in these early steps.

On the part of the restricting host, it would seem logical to investigate the possibility that the restriction results from an interaction between a carried phage—or cellular product thereof—and the phage K14 particle. In general, the hosts of group II are susceptible to and are lysogenic for A, B and F phages (Rountree, 1949*b*; Rippon, 1956), and their specific phage-typing patterns are in part determined by their carried phages (Williams Smith, 1948*b*; Rountree, 1949*a*). However, the K14 phage infects all the group II strains tested by us and is restricted by all, irrespective of their known lysogeny. Therefore, if a prophage-phage K14 genome interaction occurs, it probably involves one carried in common by all group II hosts rather than one which confers strain specific patterns within the group. It would be equally logical to study the possibility that prophages are involved in protecting a given host from infection by a restricted phage particle, or that they might contribute to the formation of a particle with increased plaque-forming

abilities, should infection be established, thus releasing the restriction. Evidence to support both possibilities was presented by Christensen (1961) for a *Shigella dysenteriae*-phage P2 and T1 system. However, the staphylococcal host KIN does not appear to be lysogenic, yet it is capable of releasing the restriction.

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Propagation of Staphylococcal Typing Phages on a Common Host, *Staphylococcus aureus* K1, and Host-Controlled Changes in Their Lytic Range

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SUMMARY

A broad range of typing phages belonging to lytic groups I, II, III, IV and those classified as miscellaneous produced plaques on *Staphylococcus aureus* strain K1. Two variant forms of this strain were compared, K1N and K1HI. Phages belonging to lytic group II, of both A and B serology, had low efficiencies of plating (EOP) for the K1N variant but had high EOP values for the K1HI variant. In general, of phages from other lytic groups, those of B serology tended to plate with low EOP values on both variants, whereas those of A serology plated with high EOP values. Propagation of several of the phages on both K1 hosts resulted in host-modifications of the phage progeny. Similar host-induced modifications were produced by propagation of the phages on PS 73. The host-modified phages showed striking losses in plating efficiency for their usual propagating hosts. The significance of these findings with respect to genetic classification of the phages and hosts is discussed.

INTRODUCTION

This investigation has given information on the relative efficiencies of plating (EOP) of a series of staphylococcus phages for a common host, *Staphylococcus aureus* K1N. This host exhibits a broad susceptibility to members of all groups of typing phages. These phages are classified as members of groups I, II, III and IV, or are placed in a category designated miscellaneous (Blair & Williams, 1961). By comparative titration of these phages on their common propagating strain and on the K1N strain, we have detected interesting patterns of behaviour which seem to be related in some cases to their lytic group and in others to their antigenic structure.

This study is part of a series designed to outline the nature and extent of host modifications of phages brought about by various staphylococci. In previous work we have shown that two forms of the K1 strain released the host-induced restriction of the polyvalent K14 phage (also designated P14) which occurs during replication on staphylococci typing with group II phages (Ralston & Krueger, 1952, 1954; Ralston & Baer, 1964*a*). Evidence is presented in this paper to indicate that propagation of the typing phages on these cultures of strain K1 results in marked host-modified changes in phage host range so as to mask the original high efficiency of plating for their usual propagating host strain, thus altering the lytic group of the phages.

In future publications it will be shown that the K1 strain is only one of a series of staphylococci which can profoundly host-modify the lytic specificity of phages.

MATERIALS

Bacterial strains and phages. Staphylococcus aureus K1 cultures were maintained on Difco-tryptose phosphate agar. This strain was isolated from a case of multiple furunculosis in 1929 by Dr A. P. Krueger. When originally tested it was a golden pigmented, slightly haemolytic, coagulase-positive strain; it fermented mannitol, reduced nitrate, and liquefied gelatin. It has been subcultured frequently on laboratory media, and at present is non-pigmented, mannitol positive, coagulase negative, produces no detectable amounts of hyaluronidase, haemolysin, or extracellular deoxyribonuclease, and has lost its pathogenic potential for rabbits. This strain has been the propagating host for the well-known polyvalent phage K, now designated phage K1 in this laboratory, and for the host-alterable variant phage K14—also referred to as the P1 and P14 phages in former publications (Ralston & Krueger, 1952). Two forms of the K1 strain were used in these studies: K1N and K1HI. The host-restricted phage K14 plates with a low efficiency of plating (EOP) on strain K1N but with a high EOP on strain K1HI.

Phages and bacterial hosts of the typing series were obtained through the courtesy of Dr J. E. Blair (N.Y. Hospital for Joint Diseases, New York City, U.S.A.) and from the late Dr A. Browne (Calif. State Dept. of Public Health, Berkeley, Calif., U.S.A.), who originally obtained his set from Dr Blair. Stock bacterial cultures were maintained on trypticase soy (hereafter TS) agar (Baltimore Biological Laboratories, Baltimore, Md., U.S.A.), and were stored at 4°. Twenty-four hr cultures were used in all experiments. The stock suspensions of phage were stored at 4°.

Phage assay. Phage titres were determined by plaque count by a typical Gratia technique. All dilutions were made in TS broth. Suitable dilutions of phage were mixed with 5×10^7 cocci/ml. in 0·3% (w/v) TS agar supplemented with 400 μg. calcium ion/ml. One ml. amounts of the phage + cocci assay mixtures were spread over a basal layer of 2·5% (w/v) TS agar. The plates were incubated at 28°. Freshly poured basal agar was essential for obtaining plaque counts.

RESULTS

Efficiencies of plating of individual typing phages for Staphylococcus aureus K1 *cultures as compared with their respective propagating strains*

The individual phages tested in this series represented members of the major lytic groups as defined by Blair & Williams (1961). These include phages of A, B, F and L serology. The phage K14 (not a typing phage) is of D serology. It was used in its host-restricted form after modification by propagation on host PS 51. The phages were assayed by plaque count on host K1N and on their usual propagating hosts (H). A comparison was made of the relative plaque-forming ability of each phage stock for the two hosts by calculating an efficiency of plating: EOP K1N/H = titre/ml. on strain K1N divided by the titre/ml. on the usual propagating host for a given phage (H). Table 1 shows that 23 of 28 phages could produce plaques on the K1N strain; and these showed patterns of EOP K1N/H values as follows: (1) In general, serological type B phages from all lytic groups had low EOP K1N/H values, i.e. they formed many fewer plaques on strain K1N than on their respective

Table 1. *Differences in plaque-forming abilities of typing phages of known serological type as determined by comparative assays on their propagating hosts, on Staphylococcus aureus* K1N *and Staphylococcus aureus* K1Hi

Stock phages, prepared on their respective hosts, were diluted in TS broth and assayed on their propagating host, on strain K1N and K1Hi. The EOP values were calculated from the resultant titres. Titres for the assays on K1Hi, which provided data for the calculations of EOP K1N/K1Hi, are not included in the table.

Typing phage phage group	Phage	Serotype of phage	Titre/ml. *S. aureus* K1N	Titre/ml. Propagating host (H)	EOP* K1N/H	EOP K1N/K1Hi
I	29	B	$7{\cdot}3 \times 10^2$	$7{\cdot}3 \times 10^7$	0·00001	n.t.†
	11A	B	$3{\cdot}1 \times 10^2$	$6{\cdot}2 \times 10^6$	0·00005	n.t.
	52	B	$1{\cdot}4 \times 10^4$	$1{\cdot}0 \times 10^7$	0·001	1.0
	52A	B	$< 1{\cdot}0 \times 10^2$(R)‡	$1{\cdot}7 \times 10^4$	—	0(R)
	79	B	$3{\cdot}1 \times 10^6$	$3{\cdot}6 \times 10^6$	0·9	1·0
	80	B	$7{\cdot}4 \times 10^4$	$5{\cdot}0 \times 10^6$	0·01	2·5
II	3A	A	$4{\cdot}0 \times 10^5$	$1{\cdot}0 \times 10^7$	0·04	0·001
	3B	A	$1{\cdot}5 \times 10^8$	$3{\cdot}5 \times 10^9$	0·04	0·001
	3c	A	$3{\cdot}6 \times 10^5$	$2{\cdot}0 \times 10^7$	0·02	0·002
	71	B	$2{\cdot}9 \times 10^2$	$4{\cdot}1 \times 10^4$	0·007	0·005
	55	B	$< 1{\cdot}0 \times 10^2$(R)	$2{\cdot}0 \times 10^6$	—	0(R)
	39	B	$< 1{\cdot}0 \times 10^2$(R)	$5{\cdot}0 \times 10^7$	—	0(R)
	523	B	$< 1{\cdot}0 \times 10^2$(R)	$1{\cdot}0 \times 10^7$	—	0(R)
III	6	A	$2{\cdot}4 \times 10^9$	$3{\cdot}9 \times 10^8$	6·3	1·0
	7	A	$1{\cdot}8 \times 10^7$	$8{\cdot}8 \times 10^6$	2·4	1·0
	42B	A	$4{\cdot}4 \times 10^9$	$7{\cdot}4 \times 10^9$	0·60	1·0
	42E	A	$2{\cdot}4 \times 10^9$	$1{\cdot}2 \times 10^9$	2·0	0·67
	47	A	$2{\cdot}6 \times 10^4$	$3{\cdot}0 \times 10^6$	0·008	5·0
	54	A	$1{\cdot}0 \times 10^8$	$4{\cdot}7 \times 10^6$	5·0–20·0§	1·0
	70	A	$5{\cdot}0 \times 10^9$	$1{\cdot}0 \times 10^9$	5·0	1·0
	73	A	$6{\cdot}0 \times 10^6$	$6{\cdot}0 \times 10^6$	1·0	1·0
	75	A	$1{\cdot}7 \times 10^9$	$2{\cdot}0 \times 10^9$	0·8–1·6	1·0
	53	B	$4{\cdot}0 \times 10^5$	$5{\cdot}0 \times 10^7$	0·008	1·0
	83	B	$5{\cdot}0 \times 10^5$	$5{\cdot}0 \times 10^7$	0·01	1·0
	77	F	$1{\cdot}5 \times 10^4$	$1{\cdot}5 \times 10^8$	0·0001	1·0
IV	42D	B	$1{\cdot}6 \times 10^6$	$4{\cdot}0 \times 10^6$	0·3–3·0	1·0
Misc.	81	A	$6{\cdot}0 \times 10^6$	$1{\cdot}2 \times 10^7$	0·5–8·0	1·0
	187	L	$< 1{\cdot}0 \times 10^2$(R)	$2{\cdot}5 \times 10^7$	—	0(R)
Non-typing phages	K14 (Produced on group II hosts)	D	$2{\cdot}0 \times 10^7$	$1{\cdot}0 \times 10^9$	0·02	0·02
	K14 (Produced on non-group II hosts)	D	$1{\cdot}0 \times 10^9$	$1{\cdot}0 \times 10^9$	1·0	1·0

* EOP designates efficiency of plating, obtained from the ratio: titre/ml. on host K4N ÷ titre/ml. on host (H), etc.

† Designates no test carried out.

‡ R designates resistant to all concentrations of phage tested. In this test 10^{-2} dilution was the highest concentration plated. In other tests higher concentrations of phage failed to form any plaques. No EOP was calculated because the phages were considered to be incapable of forming plaques on K1N (designated R). In the case of phage 187, failure to form plaques was found to be due to failure to adsorb; in the case of phage 55, adsorption was so slow as to be almost insignificant. Failures of the other phages have not been analysed further.

§ A range of variation in values is included. All calculations are to the first significant decimal place.

propagating hosts. In addition, of five phages which failed to form any plaques on strain KIN, four were of B serotype: 52A, 55, 39, 523 (the fifth was phage 187, L serotype). Phage 79 (lytic group I) and phage 42D (lytic group IV) were exceptions and tended to plate with high EOP KIN/H values. (2) Regardless of serotype, and like the B phages, all phages of lytic group II also had low EOP KIN/H values, indicating a low potential for forming plaques on host KIN. This low plaque-forming ability resembles that of the restricted form of the host-modifiable phage K14 (Ralston & Baer, 1964*a*). (3) Most of the lytic group III phages and those classified as miscellaneous of A serology tended to plate with EOP KIN/H values close to or greater than 1. Phage 47 (lytic group III, of A serotype) was different, however, and formed 100-fold more plaques on strain PS 47 than on KIN. (This phage produced a mixture of plaques on both hosts—90% large and 10% small—the significance of which has not been ascertained.) (4) Only one serotype F phage was tested, phage 77 (lytic group III). This phage had a low EOP KIN/H value, i.e. formed relatively few plaques on host KIN as compared with host PS 77.

Because of the proven lysogenicities of many of the parent cultures from which the phage stocks were obtained, a possibility existed that high-titre phage stocks which formed low numbers of plaques on strain KIN were really contaminated with phages carried lysogenically by the propagating hosts. However, a number of observations make this quite unlikely: (1) separate stocks of the same phage + host system showed fairly constant EOP KIN/H values. The EOP KIN/H values remained constant when assays were performed on lysates whose titres had dropped several-fold on storage or on phage suspensions from single plaques. It would seem unlikely that contaminants would be present in constant proportions under so many conditions. (2) Different hosts had produced each phage; and therefore, to produce low titres on host KIN, all the different hosts would have to be lysogenic and carry a phage capable of forming plaques on host KIN. Tests of several propagating strains failed to indicate that they all harboured phages capable of forming plaques on host KIN. (3) According to Rippon (1956) spontaneously induced contaminants are rarely present in titres as high as 10^4 in lysates containing 10^8 particles/ml. Several of our phage stocks had titres for host KIN which were 10^{-2} to 10^{-3} of the titres for their propagating host. This would indicate an unusually high percentage of lysogenic phage in the phage stocks. Because of these considerations we are more inclined to consider that phage stocks which plated at comparatively low titres on strain KIN contained a single phage which possessed a low potential for plating with strain KIN rather than a mixture of phages one of which was capable of forming plaques on this strain.

Comparative assays on strain KIN *and* KIHI. Upon performing titrations of the restricted form of phage K14 on the two types of *Staphylococcus aureus* KI, we found previously that this phage plated with low efficiency on strain KIN but with high efficiency on strain KIHI (Ralston & Baer, 1964*a*). We have now determined the EOP KIN/KIHI values of several of the typing phages. All group II typing phages of both A and B serology behaved similarly to the host-restricted phage K14, forming many plaques on strain KIHI and few plaques on strain KIN, whereas the other phages tended to form plaques equally well on both hosts, their EOP values approaching 1. At present we do not know what metabolic characteristic distinguishes the two KI forms or what property causes both phage K14 (a broad host-range

phage) and the lytic group II phages (narrow host-range phages) to exhibit similar relationships in their plaque-forming potentials for the two hosts. We wish to point out that it may be significant that these phages have all undergone at least one cycle of infection in a host of phage lytic group II and that phage K14 has been shown to be host-restricted by all strains belonging to this phage group.

Restrictions of host range of typing phages by Staphylococcus aureus K1 *cultures*

Notwithstanding its wide susceptibility to many typing phages, the K1 strain is capable of producing restrictions in the host range of many phages. Strain 73, which is also susceptible to plaque formation by a wide number of phages, produces similar host-modifications as demonstrated by the following tests. Several typing phages were plated to obtain isolated plaques on their propagating host (H), on strain K1N and on strain 73. The progeny from single plaques were each suspended in TS broth and assayed again on the three hosts (Table 2). From the titration values, two calculations were made: (1) EOP K1N/H, to determine what happened to the ability of the phage to form plaques on its common propagating host after it had been produced on (H), on host K1N, and on host 73; and (2) EOP K1N/73, to compare the plating efficiency of phage from each passage for two hosts each known to be susceptible to many phages. The calculations of EOP K1N/H indicated that: (1) passage of a phage on its usual propagating host resulted in progeny with an EOP K1N/H much like the parent stock; (2) passage of each phage on host K1N resulted in host-modified particles. These had a low efficiency of plating for their respective hosts (H). Phage 70 appeared to be an exception; (3) passage of each phage on PS 73 also resulted in host-modified particles. These particles, like those produced on host K1N, also had a low efficiency of plating for their usual propagating host. (Phage 70 was host-restricted by strain 73.) The EOP K1N/73 values indicated that in most instances the phages formed equal numbers of plaques on both hosts, but there were enough exceptions to make it appear that strains K1N and 73, while very similar, often differed with respect to their responses as phage modifiers and as indicator hosts.

In other studies, strain K1Hi was tested for ability to host-modify the typing phages. Lysates were prepared on this strain in TS broth and samples were titrated on the propagating host (H) and on strain K1Hi. From the titres, an EOP K1Hi/H was calculated. The following values were obtained: 3C = 16,000; 79 = 10,000; 70 = 500; 77 = > 5000; 53 = 33; 42D = 1,000,000. It can be concluded that both forms of strain K1 host-restrict many different typing phages. In these tests phage 70, which was not altered during passage on strain K1N, was host-restricted by strain K1Hi. In each instance of host-restriction the lytic specificity of the phage was either greatly reduced or abolished, i.e. the phage no longer possessed a high efficiency of plating for its usual propagating host.

In the present studies, one-step burst tests were not performed on the phages during their replication in the K1 and 73 hosts. These tests would have provided more reliable evidence for a true host-controlled modification of the phages. Our conclusions are based upon changes occurring within a single plaque passage. However, a detailed study of phage 3C will be presented separately (Ralston & Baer, 1964*b*), showing that a phenotypic modification of the phage occurs within a single

burst on cocci of strain KIN and KIHI, and that the host range of the phage is changed from one predominantly lytic for hosts of phage group II to one almost exclusively lytic for host KIN, KIHI, strain PS 73 and for certain *Staphylococcus aureus* strains of

Table 2. *Host-induced modifications of the plaque-forming abilities of specific staphylococcal typing phages, as shown by single plaque passage on strains* KIN *and* 73

Stock phages were propagated on TS agar on each of three strains. Contents of isolated plaques were diluted in TS broth and assayed on each of the three strains. The relative efficiencies of plating (EOP) were calculated from the resultant titres.

Phage group	Phage	Serotype of phage	Passage on host	Plaque contents assayed (plaques/ml. on strain) (H)†	KIN	73	EOP* (approximate values) KIN/H	KIN/73
I	79	B	79	125	244	55	2	5
			KIN	0	25	0	> 25	> 25
			73	0	5,000	466	> 5000	10
II	3A	A	3A	5,000	16	10,000	0·003	0·0001
			KIN	0	9	5	> 9	1
			73	0	5,000	5,000	> 5000	1
	3C	A	3C	191	13	200	0·07	0·06
			KIN	0	170	199	> 170	1
			73	n.t.	5,000	5,000	n.t.	1
III	7	A	7	124	149	91	1	1
			KIN	2	37	41	18	1
			73	13	2,000	2,000	154	1
	42E	A	42E	3,000	5,000	5,000	2	1
			KI	45	10,000	10,000	220	1
			73	312	> 10,000	> 10,000	> 30	1
	70	A	70	50,000	70,000	70,000	1	1
			KIN	30,000	30,000	192	1	15
			73	100	15,000	15,000	150	1
	53	B	53	1,500	10	596	0·006	0·02
			KIN	n.t.	988	922	n.t.	1
			73	44	22	> 50,000	> 0·5	< 0·0004
	83	B	83	100,000	1,000	3,000	0·01	0·3
			KIN	0	190	303	> 190	0·6
			73	0	5,000	5,000	> 5000	1
	73	A	73	10,000	10,000	—	1	1
			KIN	—	588	517	1	1

* EOP designates efficiency of plating, determined from the ratio: titre/ml. on host KIN ÷ titre/ml. on host H, etc. Values are preceded by signs > or < where plaque contents provided insufficient phage to make more accurate calculations.

† H designates propagating host, commonly used for propagating the specific phage.

phage lytic group III. Yet its group II lytic host range is restored within a single burst cycle by those few cocci of strain 3C which are successfully infected by the host-modified particles of phage 3C. Most of the changes of Table 2 are probably similar in basic mechanism.

DISCUSSION

According to the present typing scheme (Blair & Williams, 1961) certain phages are considered to be related and are placed in broad lytic groups because they tend to form patterns of lysis in common. The extent to which the classification relates to phage or host genetic structure is not yet apparent. Most of the phages originated from lysogenic strains or were obtained by 'adaptation' (Fisk, 1942; Williams Smith, 1948) and are temperate (Rippon, 1956). In general they lyse strains only within their broad lytic group, but each lytic group of phages embraces a variety of kinds. Their serology does not correlate with their lytic specificities; neither does the serology of the host surface structure relate to their lytic range (Oeding & Williams, 1958), though vague relationships have been reported (Hobbs, 1948; Wahl & Fouace, 1952). Phages of a given serotype, however, share characteristics in common, and our studies make evident certain patterns of plaque-forming abilities with respect to a common host, *Staphylococcus aureus* K1N. These patterns appear to be dependent upon phage serology but are also related to the lytic classification and previous passage history. It has also become clear from our tests that, for these characteristics to be demonstrable, the phages need to be propagated on their recommended hosts; for, immediately after they are produced on strain K1N, they lose their lytic specificity for their usual propagating host.

Hosts which type primarily with phages of lytic group II have been shown to modify (restrict) the phage K14 for host K1N (Ralston & Baer, 1964*a*), and these same hosts have recently been found capable of restricting the plaque-forming efficiency of phage 3C for host K1N. The present studies indicate that all phages of group II show low potentials for infecting host K1N. Based on these considerations, it may be speculated that group II cocci possess an unusual ability to modify certain phages. Certain differences between the group II and group III phages, particularly those of A serotype, may depend upon changes imposed by the different propagating hosts.

It has long been recognized that staphylococcal phages are subject to host-controlled modifications (Rountree, 1956), but, in general, the changes reported heretofore have been confined to hosts within a given broad lytic phage group. Results of our tests with these phages indicate that by propagation on cultures of *Staphylococcus aureus* K1 many phages can be modified so that their original group lytic specificity is abolished. This is not the only kind of change which can be imposed by the K1 host, for this strain is known to release the polyvalent K14 phage from the restriction contributed to it by hosts of phage group II (Ralston & Baer, 1964*a*). It may be significant that strain K1, which performs both restrictive and release modifications, is, itself, susceptible to a broad range of phages. More detailed analyses of the kind and extent of restrictions that can be performed by different hosts might aid in further defining the genetic structure of both phage and host and bring more meaning to the present staphylococcal classification schemes.

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Host-controlled Changes of Staphylococcal Phage 3C Affecting its Broad Group Typing Pattern

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SUMMARY

Propagated on a group II host, phage 3C behaves as a typical group II phage; but after passage on hosts of wide group patterns, *Staphylococcus aureus* K1 and 73, 3C phage is host-restricted, losing its potential for infecting group II hosts and gaining an increased potential for infecting group III hosts. Such a phage would be considered a group III phage. The change occurs in the first burst of every infected cell. The phage released retains its A serology. Phage 3C can be propagated directly on several group III cocci and in these hosts a similar host-restriction occurs. These observations constitute evidence that host-induced modifications can alter the group lytic spectrum of a specific typing phage.

INTRODUCTION

This paper is part of a series in which the host-controlled changes of staphylococcal phages have been examined with reference to their broad group lytic patterns. Host-directed changes in the infectivity patterns of phages have been established for several systems. They have been described for the polyvalent K14 phage and staphylococcal hosts (Ralston & Krueger, 1952). In this system phage K14 is host-modified by all group II cocci so that it exhibits a decreased potential for infecting most non-group II strains. Successful infection of these non-group II hosts results in a release of the restriction and the phage then exhibits its typical broad lytic pattern with hosts of groups I, II, III, IV and with propagating hosts for phages of miscellaneous typing patterns (Ralston & Baer, 1964*a*).

Host-induced modifications of phage Col and 47C were described by Rountree (1956). These also occur on the first round of infection. In general the changes occurring in these specific, temperate phages do not involve any major change in their infection potentials for strains outside of their original group patterns (I and III). We have recently reported that most of the specific typing phages can infect a common host, *Staphylococcus aureus* K1, and that the progeny particles from the first plaque passage exhibit different host ranges from the original hosts. In general the infectivity for the homologous host tends to disappear after replication in strain K1 (Ralston & Baer, 1964*b*) and the phages show high infectivity for host K1.

In the present paper the changes attendant on passage of one of these phages, the serotype A, group II 3C phage, have been investigated in greater detail. It is shown that phage 3C can be converted from a phage specific for group II hosts to one with a greatly increased potential for infecting group III hosts and a decreased ability

to infect group II hosts. This change can be demonstrated when phage 3C is produced on strain K1 and also when produced on strain 73, both cultures of wide group lytic pattern. The alteration can be demonstrated to occur in the first burst from singly-infected *Staphylococcus aureus* K1 cells. The progeny particles exhibit typical A serology and show an extremely low potential for reinfecting host 3C. Those which succeed behave like typical 3C phage in that they have a lowered ability to plate on strain K1N and are still host-alterable.

The 3C phage has a low potential for infecting group III strains. When lysates are plated directly on these hosts, the phage undergoes changes which are similar to those produced by the K1 and 73 cultures. If the group II origin of the 3C phage were not known, it would appear that the progeny were typical specific group III phages.

METHODS

Phage. The typing phage 3C used throughout these studies was obtained from the late Alcor Browne, California State Dept of Public Health. The Browne stock was originally obtained from Dr J. E. Blair, New York Hospital for Joint Diseases, Madison Ave, N.Y. On two occasions a stock of phage 3C sent directly by Dr Blair was compared with the Browne phage, once to ascertain its host range, and once to determine the potency of phage serotype A antiserum.

For production of phage, trypticase soy (hereafter, TS) broth (Baltimore Biological Laboratories, Baltimore, Md, U.S.A.) was used. To this was added sterile $CaCl_2$ to a final concentration of 400 μg./ml. (hereafter TS–Ca broth). In some experiments a combined medium was used, designated medium III–Ca, of the following composition: TS, 5 g.; Difco-tryptose phosphate (hereafter TP), 5 g.; Difco-yeast extract 3 g.; distilled water, 1000 ml. To this was added $CaCl_2$ to 0·002-M. There were no differences in the behaviour of the phage on the two media, with the exception that 3C phage tended to be more stable on storage at 4° when prepared in the TS–Ca broth

Plaque isolations. Contents of isolated plaques on agar media were obtained by removing an agar core from the middle of the plaque with a sterile capillary pipette. The contents were suspended in 5–10 ml. broth, depending on the experiment. On several occasions the material was filtered through pads of Super-Cel (Johns Manville, standard grade) to remove bacteria.

Bacterial strains. A series of propagating strains for the specific typing phages was maintained on TS agar slants. The strains were obtained in 1959 from either Dr Browne or Dr Blair and were combined to form one set. They included strains PS 3A, 3B, 3C, 39, 55, 71, 523, 6, 7, 42B, 42E, 47, 53, 54, 70, 75, 77, 83, 73, 42D, 81, 187, 29, 52, 52A/79, 44A, 80. Strain 51 (originally designated 145) was obtained in 1950 from Dr Blair. For purposes of maintenance and storage of cultures, the strains were transferred on TS agar for 24 hr at 37° and then stored in the refrigerator. For experimental work, they were transferred to fresh TS or TP agar and grown for 16–24 hr at 37°. *Staphylococcus aureus* strain K1 was isolated in 1929 from a case of multiple furunculosis by Dr A. P. Krueger. When first tested it was a golden pigmented, coagulase positive culture. It has been maintained in these laboratories on agar media, for the past 10 years having been grown on TP agar. At present it is coagulase negative and white. It is susceptible to a large number of typing phages,

and with them it shows characteristic patterns of lysis dependent upon both the serology of the phage and the lytic group of phage specificity (Ralston & Baer, 1964*b*). Strains K1N2 and K1Hi represent variants of the parent culture (Ralston & Baer, 1963*a*) isolated from single colonies, and exhibiting different degrees of susceptibility to host restricted K14 phage. The K1N2 is of low susceptibility, the K1Hi is of high susceptibility. It has been found that these responses also occur with all group II phages derived from group II strains (Ralston & Baer, 1964*a*, *b*).

Phage assays. Phage was assayed by two methods. For plaque assay, phage was diluted in TS–Ca broth. Then 0·5 ml. phage was mixed with 2·5 ml. host cells at 5×10^7 cocci/ml. and 2·0 ml. hot TS–Ca agar (7·5%, w/v). One ml. of the soft agar mixture was layered on the surface of a small Petri plate containing TS–Ca in 2·5% agar. The plates were incubated at 37°, but incubation at 30° gave equally good counts. The slide method of Jones & Krueger (1951) did not produce uniform results with the 3C phage. The phage formed good plaques with host 3C, less readable plaques with the K1 strain, and no plaques with strain 73 by the slide method, but good plaques with all three by the plate procedure.

For spot test assays, the procedure was similar to that recommended for phage typing (Blair & Williams, 1961). Host cells suspended to $2{\cdot}0 \times 10^8$ cocci/ml. were spread with a moistened cotton swab in an ordinary size Petri plate on a hard agar layer of TS–Ca or Medium III–Ca. Phage was dropped on the surface from the tips of 24-gauge needles on 1 ml. syringes. The clearing due to lysis or number of plaques was recorded after 20 hr at 37°. Complete clearing was designated 4; > 100 plaques, 3; 50–100 plaques, 2; 1–50 plaques, 1; no plaques, 0. This procedure proved to be at least 10- to 1000-fold less sensitive than the plaque count technique. Reactions showing up by the plaque method were missed by the spot procedure, especially when the test phage contained less than 10^6 particles/ml.

Production of phage lysates. Phage was inoculated into cells suspended in TS–Ca broth, generally at initial phage:bacterium (P:B) ratios of 1:100. A total of 50 ml. medium was placed into 125 ml. flasks; 100 ml. medium into 250 ml. flasks; and 200 ml. medium into 500 ml. flasks. Occasionally phage was prepared in 50 ml. volumes in 500 ml. flasks. The phage-host mixtures were incubated in a reciprocal shaker at 37° until lysis. Some lysates were prepared at 30° in a rotary shaker. Higher titres were obtained at 37°. The initial cell concentration varied from 1×10^7 cocci/ml. to 1×10^8 cocci/ml.

Symbols and abbreviations. Phage 3C prepared on host 6 is designated 3C(6). Phage 3C passaged on a series of hosts, as K1Hi, then 6, is designated 3C(K1Hi/6), etc. P designates phage. B designates bacterium. IS designates indicator strain. H designates homologous host, i.e. the bacterial strain commonly used to propagate a specific phage. EOI designates efficiency of infection.

Antiserum neutralization tests. Serotype A, B and F antisera were kindly provided by Dr Phyllis Rountree (Fairfax Inst. of Pathology, Sydney, Australia). Preliminary experiments showed that a 1:100 dilution of serotype A serum in TS–Ca broth inactivated phage 6(6) and 3C(3c) and a 1/100 dilution of B antiserum inactivated phages 71(71) and 53(53) and 83(83). The sera were tested by mixing phage and antiserum in broth at room temperature for 2 hr. Then microdrops of the mixtures were spotted on lawns of the test hosts. It was ascertained that the antiserum alone caused no cellular inhibition and that the phage control caused

complete clearing of the host, whereas serum-inactivated phage produced no clearing, or a greatly reduced number of plaques.

RESULTS

Strains *Staphylococcus aureus* K1 and 73 are of interest because they show a wide susceptibility to the typing phages (Ralston & Baer, 1964*b*). Strain K1 forms plaques with 23 of 28 specific phages, including members of lytic groups I, II, III, IV. The 'N' form of strain K1 tends to assay at low titres with all phages from lytic group II and with most phages of B serology, whereas the 'Hi' form tends to assay at as high titres with these phages as do their respective homologous hosts. Most of the present studies have been concerned with the changes in phage 3C after propagation on either form of host K1, but it is to be emphasized that this strain is not

Table 1. *Changes in plating efficiency of 3C phage after plaque passage on hosts* K1*N*, 3*C and* 73

3C phage passage history	Titre/ml. on indicator strain (IS)		
	K1N*	3C	73
3C/73†	$5{\cdot}2 \times 10^4$	< 10	$3{\cdot}1 \times 10^4$
3C/3C	$1{\cdot}0 \times 10^4$	$3{\cdot}4 \times 10^4$	$3{\cdot}7 \times 10^4$
3C/K1N	$3{\cdot}8 \times 10^4$	< 10	$3{\cdot}1 \times 10^4$
73/73	$9{\cdot}0 \times 10^4$	10	$7{\cdot}0 \times 10^4$
73/K1N	$1{\cdot}2 \times 10^5$	< 10	$6{\cdot}8 \times 10^4$
K1N/73	$2{\cdot}5 \times 10^5$	10	$1{\cdot}5 \times 10^5$

* Shortly after this assay, the K1N strain dissociated into K1N and K1Hi forms. Isolated colonies were selected for stock cultures, K1N2 and K1Hi. They plated with a typical 3c(3c) phage as follows: 3C = 1×10^9/ml., K1Hi = 4×10^8/ml., K1N2 = $1{\cdot}3 \times 10^6$/ml.

† 3C/73 designates 3C phage first plated with 3C host; a plaque then being isolated, the contents being replated on strain 73; and from this strain, contents of a single plaque being plated with the three indicator strains.

unique in its behaviour and that similar effects also occur with host 73. As shown in Table 1, 3C phage was propagated on agar on hosts K1 and 73 and the progeny particles were assayed on indicator strains 3C, K1N and 73. Propagated on strain 3C, phage 3C formed plaques equally well on 3C and 73, but typically showed a lower titre on strain K1N. Whenever phage 3C was propagated on K1N or 73, by single plaque passage or serially from strain K1N to 73, the progeny 3C phage had little or no ability to reinfect strain 3C.

Changes in the broad lytic spectrum of 3C phage after propagation on hosts Staphylococcus aureus 3C and K1Hi. Broth lysates were prepared as described under Methods. The 3C lysate was prepared directly from the phage stock received from Dr Browne; the lysate on strain K1Hi was made from a phage inoculum derived from the second plaque passage on strain K1N. Table 2 shows the titre of each lysate when assayed by plaque count. By comparing the titre of 3C on each indicator strain to that on the host of immediate propagation, we could estimate the relative EOI. Phage 3C from host 3C plated at a high EOI with group II hosts, with K1Hi and 73. It showed a definite but low ability to infect a number of group III hosts: PS 6, 42B, 53, 54, 70 and 85, with EOI values ranging from 10^{-5} to 10^{-7}. (Low titres for several group III hosts have been reported previously (Blair & Carr, 1960).) Phage 3C assayed about

100-fold lower on strain K1N than on 3c, therefore the EOI = 10^{-2}. (This value has varied in some of our tests because the N culture developed a proportion of Hi cells. From this culture we reisolated fresh K1N2 and K1Hi cultures. These were maintained at 4° and used for preparation of 24-hr growth. The K1N2 culture tended to plate at an EOI = 10^{-4} to 10^{-5} with 3C(3c), whereas the K1Hi plated at values ranging from 10^{-1} to 10^{0}.)

Table 2. *Plating potential of 3C phage in broth lysates of strains Staphylococcus aureus 3c and K1Hi*

		Produced on strain 3c		Produced on strain K1Hi	
Group	IS	Plaques/ml.	Approx. EOI (IS/3c)	Plaques/ml.	Approx. EOI (IS/K1Hi)
I	29	—*	—	—	—
	52A/79	—	—	—	—
	52	—	—	—	—
	44A	—	—	—	—
	80	—	—	—	—
II	3A	$1{\cdot}0 \times 10^{7}$	10^{-2}	$1{\cdot}0 \times 10^{1}$	10^{-7}
	3B	$2{\cdot}0 \times 10^{9}$	1·0	$1{\cdot}0 \times 10^{1}$	10^{-7}
	3C	$2{\cdot}0 \times 10^{9}$	1·0	$2{\cdot}0 \times 10^{3}$	10^{-5}
	39	†	—	$3{\cdot}0 \times 10^{3}$	10^{-5}
	51	$2{\cdot}0 \times 10^{9}$	1·0	$2{\cdot}0 \times 10^{3}$	10^{-5}
	55	$5{\cdot}0 \times 10^{9}$	2·5	$2{\cdot}0 \times 10^{2}$	10^{-6}
	71	$8{\cdot}0 \times 10^{8}$	0·3	—	—
	523	—	—	—	—
III	6	$5{\cdot}0 \times 10^{3}$	10^{-6}	$1{\cdot}0 \times 10^{5}$	10^{-3}
	7	n.t.‡	—	—	—
	42B	$1{\cdot}0 \times 10^{3}$	10^{-5}	$7{\cdot}0 \times 10^{3}$	10^{-5}
	42E	—	—	—	—
	47	—	—	—	—
	53	$2{\cdot}5 \times 10^{1}$	10^{-8}	$1{\cdot}0 \times 10^{2}$	10^{-6}
	54	$2{\cdot}5 \times 10^{2}$	10^{-7}	$4{\cdot}0 \times 10^{3}$	10^{-5}
	70	$2{\cdot}5 \times 10^{2}$	10^{-7}	—	—
	75	—	—	$7{\cdot}0 \times 10^{3}$	10^{-5}
	77	—	—	—	—
	83	$2{\cdot}5 \times 10^{1}$	10^{-8}	$1{\cdot}0 \times 10^{2}$	10^{-6}
IV	42D	—	—	—	—
Misc.	81	n.t.	n.t.	—	—
	187	—	—	—	—
Wide	K1Hi	$2{\cdot}0 \times 10^{9}$	1·0	$2{\cdot}0 \times 10^{8}$	1
	K1N	$1{\cdot}5 \times 10^{7}$	10^{-2}	$4{\cdot}0 \times 10^{8}$	1
	73	$3{\cdot}0 \times 10^{9}$	1·0	$3{\cdot}0 \times 10^{8}$	1

* Dash (—) means no plaques with undiluted phage. † Mottled plaques in background.
‡ Indicates not tested.

When phage 3C was produced on K1Hi, the progeny could no longer easily infect 3c or other group II hosts, the EOI (IS/K1Hi) for these strains ranging from 10^{-5} to 10^{-7}. It showed a definite ability to infect group III hosts, with EOI values ranging from 10^{-3} to 10^{-6}, and it plated well on K1N and 73. In addition an infectivity for strain 75 could be detected. This might be related to an increase in potential for

75 rather than to an acquired new property since phage 3C has been shown to plate with strain 75 at low titres (Blair & Carr, 1960).

Host-dependent nature of the 3C modification. Figure 1 shows the changes which could be detected when 3C phage was propagated serially by plaque procedure on host 3c, then on K1Hi or K1N2 and back on 3c. Both forms of strain K1 could restrict

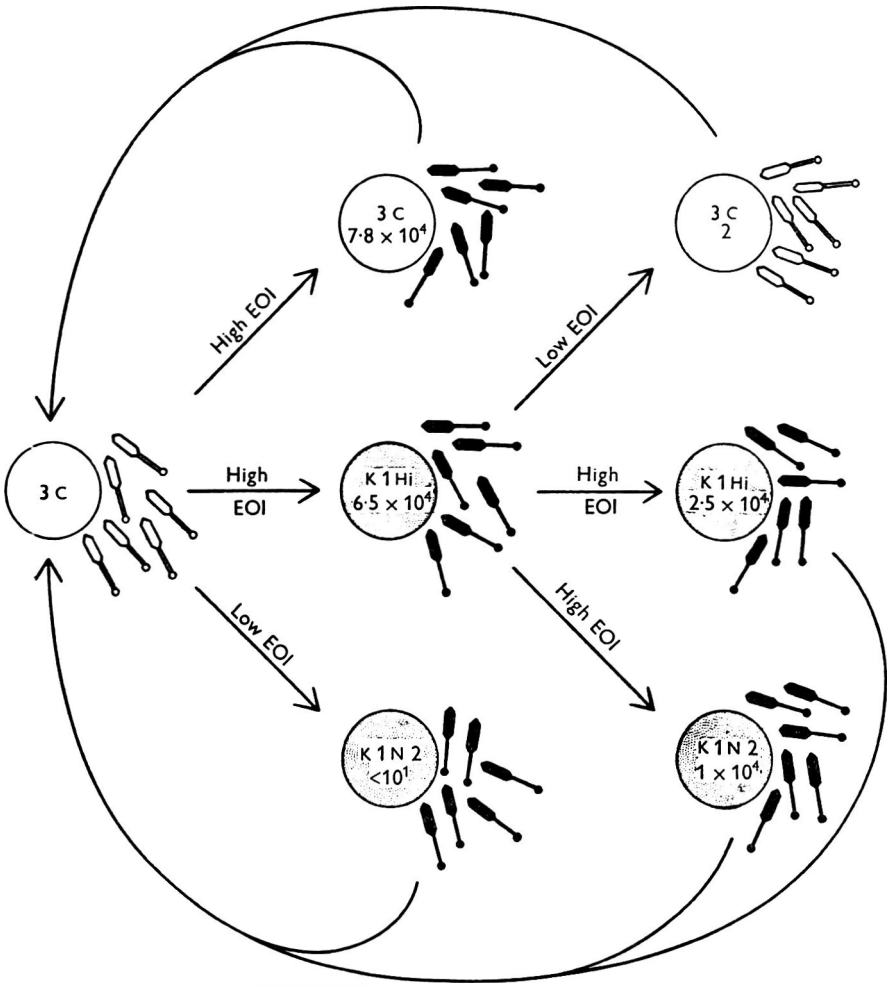

Fig. 1. Changes in EOI of phage 3C when passaged serially through *Staphylococcus aureus* K1N, K1Hi and 3c.

phage 3C for strain 3c. The few successful infections on host 3c again yielded typica 3C phage. Although these changes seemed to be a host-directed change, we considered several alternative possibilities: (1) that 3C lysates contained an induced phage contaminant in high titre which did not infect 3c but could adsorb to and multiply in strain K1Hi; (2) that the 3c culture could release spontaneously a phage which was lysogenic for host K1Hi; and (3) that phage 3C induced a phage carried lysogenically by host K1. To test for the presence of unusual particles in the 3c lysates, samples of 3C(3c) were adsorbed to host 3c or to host K1Hi and the EOI of

the residual free phage was determined. A similar test was performed with 3C(K1Hi) phage. In each case the phage 3C behaved similarly before and after adsorption with respect to its infectivity for the indicator strains. Therefore the tests failed to select out any fraction of particles which exhibited significantly different EOI values (Table 3).

Table 3. *Plaque-forming efficiency of* 3C(3C) *and* 3C(K1Hi) *phage in supernatant material after adsorption to Staphylococcus aureus* 3C *and* K1Hi

Adsorption mixture	Phage assay	Plaques/ml. on IS			EOI	
		3C	K1Hi	K1N	K1N/3C	K1N/K1Hi
3C(3c) + host 3c	Total phage input	$7{\cdot}5 \times 10^7$	$2{\cdot}5 \times 10^7$	$1{\cdot}0 \times 10^5$	0·0013	0·004
	Unadsorbed phage 30 min., 37°	$4{\cdot}1 \times 10^7$	$9{\cdot}2 \times 10^6$	$4{\cdot}9 \times 10^4$	0·0012	0·005
3C(3c) + host K1Hi	Total phage input	$1{\cdot}0 \times 10^9$	$4{\cdot}0 \times 10^8$	$1{\cdot}3 \times 10^6$	0·0013	0·003
	Unadsorbed phage 20 min., 37°	$2{\cdot}0 \times 10^6$	$1{\cdot}5 \times 10^6$	$1{\cdot}0 \times 10^4$	0·005	0·006
3C(K1Hi) + host K1Hi	Total phage input	$1{\cdot}8 \times 10^3$	$1{\cdot}3 \times 10^8$	$1{\cdot}2 \times 10^8$	$6{\cdot}0 \times 10^4$	1·0
	Unadsorbed phage 30 min., 37°	$2{\cdot}4 \times 10^2$	$1{\cdot}8 \times 10^6$	$2{\cdot}4 \times 10^6$	$1{\cdot}0 \times 10^4$	0·5
	Unadsorbed phage after readsorption with fresh cells of K1Hi 30 min., 37°	$< 1{\cdot}0 \times 10^1$	$2{\cdot}2 \times 10^3$	$2{\cdot}2 \times 10^3$	—	1·0

Adsorption mixtures contained $2{\cdot}5 \times 10^8$ cocci/ml. in TS–Ca broth and phage, as designated. Samples were centrifuged to remove phage-adsorbed cells at times indicated. The total free phage remaining unadsorbed were then assayed on the three hosts. In all tests performed phage 3C adsorbed to K1Hi cells at a much faster rate than to 3c cells.

Table 4. *Change in phage* 3C *after first burst on Staphylococcus aureus* K1Hi

Assay performed	Plaques/ml. on IS			EOI
	3C	K1Hi	K1N	K1N/3C
1. Input phage	$1{\cdot}0 \times 10^8$	$4{\cdot}0 \times 10^7$	$1{\cdot}3 \times 10^5$	0·0013
2. Unadsorbed phage	$2{\cdot}0 \times 10^6$	$1{\cdot}5 \times 10^6$	$1{\cdot}0 \times 10^4$	0·005
3. Infected cells at 10 min.	$< 1{\cdot}0 \times 10$	$1{\cdot}2 \times 10^7$	$1{\cdot}2 \times 10^7$	$> 10^7$
4. Yield after 50 min.	$< 1{\cdot}0 \times 10$	$8{\cdot}2 \times 10^7$	$1{\cdot}0 \times 10^8$	$> 10^7$

Burst study was performed with 24 hr cocci suspended in TS–Ca broth to 2×10^8/ml. from TS agar. All titrations were reported in terms of the undiluted cell-phage mixtures. After adsorption for 20 min. at 37°, free phage was determined by centrifugation of the mixture at 6500 *g* for 10 min. and assay of the supernatant material. At 15 min. assays represent infected cocci since no free phage could be detected in Super-Cel filtrates. At 50 min. lysis was complete, i.e. phage was all free in Super-Cel filtrates.

To test for lysogenicity, cocci from strain 3c were grown in Ts–Ca broth for 3 hr at 37°, under conditions similar to those used to produce phage lysates. They were stored in the refrigerator at 4° overnight and then centrifuged at low speed. The supernatant material was spotted on strain K1Hi and on the group III hosts for which 3C showed a plating potential. No plaques developed, indicating that this strain did not carry any phage active for these hosts. Similar tests were made with strain

Table 5. *Plaque-forming potential of 3C phage after four passages on Staphylococcus aureus K1Hi in TS–Ca broth*

Lysate no.	Theoretical residual activity for host 3c in inoculum (plaques/ml.)	Titre of phage 3C lysates (plaques/ml.) on IS			EOI*
		K1Hi	K1N2	3C	3C/K1Hi
1	1300†	$1{\cdot}9 \times 10^{8}$	$2{\cdot}8 \times 10^{8}$	$1{\cdot}3 \times 10^{3}$	$6{\cdot}8 \times 10^{-6}$
2	$4{\cdot}0 \times 10^{-1}$	$1{\cdot}6 \times 10^{7}$	$1{\cdot}8 \times 10^{7}$	$6{\cdot}0 \times 10^{1}$	$3{\cdot}1 \times 10^{-6}$
3	$2{\cdot}0 \times 10^{-3}$	$2{\cdot}6 \times 10^{8}$	n.t.‡	$3{\cdot}2 \times 10^{2}$	$1{\cdot}2 \times 10^{-6}$
4	$9{\cdot}0 \times 10^{-7}$	$4{\cdot}0 \times 10^{8}$	n.t.	$3{\cdot}2 \times 10^{2}$	$8{\cdot}0 \times 10^{-7}$

* Several other lysates also showed similar EOI values.

† This value assumes 1300 particles/ml. were derived from the original 3C(3c) phage used to prepare lysate no. 1.

‡ Indicates not tested.

Table 6. *Changes in host range at 3C phage after propagation on group II hosts*

Group	IS	Phage 3C propagated on group II hosts*					
		3A	3B	3C	51	55	71
I	29	0	0	0	0	0	0
	44A	0	0	0	0	0	0
	52	0	0	0	0	0	0
	52A/79	0	0	0	2	0	0
	80	0	0	0	0	0	0
II	3A	4	4	4	0	4	2
	3B	0	4	2	0	4	4
	3C	1	4	4	2	4	4
	39	0	4	4	0	0	4
	51	1	4	2	4	4	4
	55	0	4	2	0	4	4
	71	0	2	2	0	0	2
	523	n.t.†	n.t.	n.t.	n.t.	n.t.	n.t.
III	6	0	0	0	0	0	0
	7	0	0	0	0	0	0
	42B	0	0	0	0	0	0
	42E	0	1	0	0	0	0
	47	0	0	0	0	0	0
	53	0	0	0	0	0	0
	54	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
	70	0	0	1	0	0	0
	75	0	0	0	0	0	0
	77	0	0	0	0	0	0
	83	0	0	0	0	0	0
IV	42D	0	0	0	0	0	0
Misc.	81	0	0	0	0	0	0
	187	0	0	0	0	0	0
Wide	K1Hi	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
	K1N1	0	4	1	2	0	2
	73	0	4	4	4	4	4

* Contents of individual plaques assayed by spot test technique. Samples spotted undiluted on indicator lawns after filtration of plaque contents through Super-Cel.

† Indicates not tested.

K1Hi, also without success. These negative results reduce the likelihood that a carried phage could be responsible for the gross changes in the 3C lysates. In further support of these findings, the serotype of the phage 3C remained typically A after propagation on K1Hi and there was no relatedness to B or F sera.

Table 7. *Changes in host range of 3C phage after propagation on hosts of phage group III*

		3C phage propagated on group III hosts (contents of isolated plaques, per ml.)						3C/3c parent source
Group	IS	6/6	42B/42B	53/53	54/54	70/70	83/83	(plaques/ml.)
I	29	—*	—	—	—	—	—	n.t.†
	44A	—	—	—	—	—	—	n.t.
	52	—	—	—	—	—	—	n.t.
	52A/79	—	—	—	—	—	—	n.t.
	80	—	—	—	—	—	—	n.t.
II	3A	—	—	—	—	—	—	n.t.
	3B	—	—	—	—	—	—	n.t.
	3C	—	—	—	—	—	—	$3{\cdot}6 \times 10^9$
	39	—	—	—	—	—	—	n.t.
	51	—	—	—	—	—	—	n.t.
	55	—	—	—	—	—	—	n.t.
	71	—	—	—	—	—	—	n.t.
	523	—	—	—	—	—	—	n.t.
III	6	$1{\cdot}1 \times 10^6$	$5{\cdot}6 \times 10^4$	$4{\cdot}0 \times 10^6$	$1{\cdot}0 \times 10^6$	$4{\cdot}8 \times 10^2$	$5{\cdot}0 \times 10^6$	$5{\cdot}0 \times 10^3$
	7	—	—	$2{\cdot}8 \times 10^3$	—	—	$1{\cdot}4 \times 10^5$	n.t.
	42B	$5{\cdot}2 \times 10^2$	$5{\cdot}0 \times 10^6$	$5{\cdot}2 \times 10^2$	—	—	$1{\cdot}8 \times 10^3$	$1{\cdot}0 \times 10^3$
	42E	—	—	—	—	—	—	—
	47	$8{\cdot}0 \times 10^3$	—	$1{\cdot}8 \times 10^6$	$3{\cdot}0 \times 10^5$	—	$2{\cdot}0 \times 10^7$	n.t.
	53	$1{\cdot}0 \times 10^5$	$2{\cdot}9 \times 10^5$	$2{\cdot}0 \times 10^5$	$1{\cdot}4 \times 10^5$	—	$4{\cdot}8 \times 10^4$	$2{\cdot}5 \times 10^1$
	54	$9{\cdot}0 \times 10^6$	$6{\cdot}4 \times 10^4$	$3{\cdot}0 \times 10^6$	$8{\cdot}4 \times 10^3$	$8{\cdot}0 \times 10^2$	$2{\cdot}0 \times 10^6$	$2{\cdot}5 \times 10^1$
	70	$3{\cdot}4 \times 10^4$	$2{\cdot}0 \times 10^5$	$4{\cdot}1 \times 10^3$	$1{\cdot}5 \times 10^5$	$8{\cdot}8 \times 10^4$	$9{\cdot}6 \times 10^4$	$2{\cdot}5 \times 10^2$
	75	—	—	—	—	—	—	—
	77	—	—	—	—	—	—	n.t.
	83	—	—	$7{\cdot}5 \times 10^3$	—	—	$4{\cdot}0 \times 10^6$	$2{\cdot}5 \times 10^1$
IV	42D	—	—	—	—	—	—	—
Misc.	81	—	—	—	—	—	—	n.t.
	187	—	—	—	—	—	—	—
Wide	K1Hi	$1{\cdot}1 \times 10^6$	$5{\cdot}0 \times 10^6$	$4{\cdot}9 \times 10^6$	$8{\cdot}2 \times 10^5$	$4{\cdot}8 \times 10^4$	$1{\cdot}8 \times 10^6$	$2{\cdot}0 \times 10^9$‡
	K1N2	$1{\cdot}1 \times 10^6$	$5{\cdot}0 \times 10^6$	$2{\cdot}6 \times 10^6$	$8{\cdot}2 \times 10^5$	$5{\cdot}2 \times 10^4$	$2{\cdot}0 \times 10^6$	$1{\cdot}5 \times 10^7$‡
	73	$9{\cdot}2 \times 10^6$	$5{\cdot}0 \times 10^6$	$3{\cdot}4 \times 10^6$	$1{\cdot}2 \times 10^6$	$3{\cdot}6 \times 10^4$	$2{\cdot}0 \times 10^6$	$3{\cdot}0 \times 10^9$‡

* Dashes = < 10 plaques/ml. † Indicates not tested. ‡ Assay was made with a separate lysate of equivalent 3C titre.

A test was next made to determine whether the change in infectivity of 3C was produced on the first round of infection on K1Hi cocci. A total of 2×10^8/ml. cocci was mixed with 1×10^8 phage 3C(3c)/ml. (P/B = $\frac{1}{2}$) for 20 min. at 37°. The unadsorbed free phage was determined after filtration through Super-Cel. At 20 min. the infected cells were also diluted 10^{-4} in TS–Ca broth and incubated at 37° to allow a single burst to occur. The newly formed phage assayed at high titre on strains K1N and K1Hi and low titre on strain 3c (Table 4), indicating the change had occurred in a single-burst cycle. Platings of the phage-adsorbed K1Hi cells prior to burst indicated that a very low proportion of cells yielded phage active for host 3c,

although the same cells allowed to burst in the presence of assay hosts K1Hi or K1N2 produced many plaques.

When large concentrations of 3C(K1Hi) were plated with host 3c, it was observed that the lysates contained a few particles which could successfully infect host 3c. We considered the possibility that these represented some residual 3C phage which had escaped adsorption on to host K1Hi and were therefore available for infection of 3C. We prepared a series of broth lysates of K1Hi in TS–Ca medium, representing a serial dilution of the hypothetical 3C(3c) which might have been present in the original inoculum (Table 5). By the fourth lysate (passage) this theoretical residual titre for host 3c was less than 9×10^{-7}/ml., yet there was significant activity for host 3c. We concluded that each lysate produced a 3C(K1Hi) phage possessing a relatively constant probability of infecting host 3c, i.e. 10^{-5} to 10^{-6}, representing an assay of approximately 10^2 to 10^5 plaques/ml. on host 3c as compared with 10^8 plaques/ml. on hosts K1Hi or K1N. From this it is not possible to tell whether the low assays represented a few unusual host cells or a few unusual particles.

Table 8. *Comparison of 3C phage pattern after passage on hosts K1N and 73 with pattern after passage on group III hosts*

	Lytic pattern of phage 3C after propagation on host									
Group	73	K1N	K1N/70	73/70	K1N/6	73/6	K1N/53	73/53	K1N/83	K1N/42B
I	—	—	—	—	—	—	—	—	—	—
II	—	3C+	—	—	3C+	—	—	—	—	—
III	6	6	70	6	6	6	6	6	6	42B
	53	53	—	53+	53	53	53	53	83	83
	70	70	—	70	—	70	—	70	47	—
	83	83	—	—	—	—	—	—	—	—
IV	—	—	—	—	—	—	—	—	—	—
Misc.	81−	—	—	—	—	—	—	—	—	—
Wide pattern	K1N1	K1N1	K1N1	K1N1	K1N1	K1N1	K1N1	K1N1	K1N1	K1N1
	73	73	73	73	73	73	73	73	73	73

Spot test procedure was used. For phage 3C passaged on 73 and K1N, broth lysates containing 10^9 particles/ml. were used. For other assays plaque contents were used after filtration through Super-Cel. Hosts tested included all strains listed in Table 6 except for 54 and 75. In other studies 3C phage from host K1N has produced low titres for 54 and 75. Numbers indicate clear lysis; symbol − = 0–50 plaques per spot; dash (—) designates no lysis in any host of group.

Changes in 3C phage on passage through hosts of group II. When the phage 3C was propagated on members of group II the progeny particles behaved as a typical II phage. However, there were differences in their relative infectivity for each of the susceptible group II strains—an apparent intra-group host control (Table 6). These changes were similar to those previously described by Hood (1953).

Changes in 3C phage on passage through group III strains. High titre 3C(3c) phage lysates have shown a low but definite infectivity for certain group III strains. Contents of isolated plaques from each of the susceptible hosts were suspended in 5–10 ml. TS–Ca broth and assayed by plaque technique on a series of indicator hosts (Table 7). They showed typical group III lytic patterns. Phage 3C propagated

first on strains K1N or 73, then on members of group III, also behaved like a group III phage (Table 8). It was not possible to compare accurately the absolute host range for the phage derived from each series because one set of passages (Table 7) was tested by plaque count and the other (Table 8) by spot test, several-fold less sensitive. Nevertheless, the results demonstrated that the host range of phage 3C was changed from that of a group II to one essentially group III. In addition, minor differences of group III host range could be produced by individual members of group III. For example, phage 3C propagated on strains 83, 53 and 54 (Table 7) tended to infect strain 47 with high efficiency; but produced on strains 6, 42B and 70, it exhibited little or no infectivity for strain 47. Phage 3C produced first on K1N and then on 83 again showed high infectivity for strain 47 (Table 8), but after passage on other group III strains, it failed to produce lysis of strain 47. None of the passages produced phage with strong ability to lyse non-group III strains.

All the changes from group II to group III patterns occurred within one plaque passage on group III hosts. Although the possibility has not been eliminated that these effects are the result of lysogenic contaminations, nevertheless, the results so strongly resemble those established already for the 3C-K1 system that it seems safe to assume that a true host modification is involved. These studies constitute evidence that host-induced alterations can change the broad lytic pattern of a phage.

DISCUSSION

While use of specific typing phages has aided in epidemiological identification of *Staphylococcus aureus* strains, it has not been clear how phage patterns could reflect genetic units in bacterial hosts. There is no distinct correlation between phage type and surface antigens (Oeding & Williams, 1958). Hosts susceptible to phages of group II share in common mainly their ability to carry phages which produce plaques on other group II cocci (Rippon, 1956) and their ability to exclude phages which are not of group II typing specificities. Our recent studies have shown that besides the 3C control, group II cocci also possess the ability to host-restrict the polyvalent K14 phage and a number of specific phages (Ralston & Baer, 1964*a*, *b*). Since the changes imposed on a phage are directly the result of the genetic structure of the host, strains of group II phage pattern must share a common genetic unit, possibly of use in taxonomic classification.

Our observations of the host-controlled change of phage 3C from group II to group III typing characteristics provide a theoretical explanation for the origin of some of the group III specific phages, since a phage of A serotype from group II hosts could, by infecting a host of wide lytic pattern, or by chance infection of an exceptional group III host, be converted into a phage with a group III lytic range.

Since with the staphylococcal phages host specificity does not reside in differences between receptor materials (Rountree, 1947), the phenomenon of host-controlled alteration in specificity must be related to such factors as penetration, survival of phage genes, to their integration with host structures, to synthesis and maturation of virus and to virus release. In studies of the phage λK–*Escherichia coli* K12 (P1) and *E. coli* B systems, Dussoix & Arber (1962) have shown that successful replication of host-restricted phage can be dependent upon a DNA recognition system in the indicator cells, for DNA was rapidly degraded by non-infectible hosts. It has also

been shown that restricted phage can be 'rescued' by DNA from a superinfecting phage (Christensen, 1962). If a similar relationship holds for the staphylococcal phage-host systems, this would imply that the phage DNA from group II hosts is constructed in such a manner as to be non-degradable in group II hosts but susceptible to destruction by other cocci. Thus the modification might consist in the inheritance of a group II gene-like structure. It further would imply that the DNA-degrading powers of strains of wide phage susceptibility would be relatively potent for group II DNA units but poor with others. It is also possible that the extent to which a DNA component is degraded depends upon a host contribution to phage protein which can exert effects on the DNA depolymerases in a given host. Activation of deoxyribonuclease has been shown to be dependent upon the particular infecting phage (Wormser & Pardee, 1957)—an observation that is of significance with respect to the DNA recognition theory of Dussoix & Arber.

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Observations on the Labelling of a Strain of *Staphylococcus aureus* with Phosphorus-32

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SUMMARY

The best labelling of *Staphylococcus aureus* (var. *pyogenes*) with ^{32}P was achieved when the organisms were grown in broth containing 33 μg. orthophosphate/ml. and 150–200 μc./ml. ^{32}P. Cocci labelled in this way were as virulent to mice as control cocci. The phosphorus content of cocci was proportional to the orthophosphate content of the medium over the range 5 μg.–300 μg. PO_4/ml. ^{32}P was released from cocci when they were incubated with phosphate buffer and other media which did not support cell division. ^{32}P was released from labelled dividing cocci grown in non-radioactive media. About 80% of the ^{32}P was released during division; a maximum of about 55% was released from non-dividing cocci over a similar time period. Most of the ^{32}P released from dividing cocci was present in the form of molecules non-diffusible in dialysis (80%); most of the ^{32}P released into phosphate buffer was diffusible. Cocci deficient or rich in phosphorus were fractionated with trichloroacetic acid (TCA), ether, ethanol and perchloric acid. Cocci containing low amounts of P lacked ^{32}P in the fraction soluble in cold TCA, about 80% of which was orthophosphate. Cocci deficient in phosphorus incorporated ^{32}P from $^{32}PO_4$ solutions more rapidly than enriched cocci. The distributions of ^{32}P in cocci previously rich or deficient in P were similar, but labelling in deficient cocci was increased in the RNA-containing fraction; and that part of the fraction soluble in hot TCA which had a barium salt insoluble at pH 4. The phosphorus level in the cells had no effect on the amount of phosphorus released into phosphate buffer solution.

INTRODUCTION

Ely (1942) labelled *Staphylococcus aureus* with unstated amounts of ^{32}P and Thorbecke & Benacerraf (1959) labelled *S. pyogenes* with ^{32}P by growing the organisms in the medium containing 20 μc. ^{32}P/ml. The labelled organisms were used for tracer studies in animals and for that purpose the specific activity of the bacterium should be as high as is compatible with normal functioning of the cell. Experiments were made to discover the factors which affected ^{32}P incorporation and those conditions which gave good labelling and viable organisms. Stonier (1956) had previously labelled *Agrobacterium tumefaciens* with ^{32}P and studied the behaviour of the label; the following experiments were made along similar lines with *S. aureus*.

METHODS

Strain. *Staphylococcus aureus*, strain 1531, isolated in Melbourne in 1957 from meninges, was used in these experiments. It has been maintained on nutrient agar under paraffin oil.

Inoculum. Cocci from a 24 hr culture on nutrient agar were suspended in sterile distilled water (about 10^9 cocci/ml.) and 1 drop of this solution from a Pasteur pipette was used as an inoculum.

Media. The cocci were grown in nutrient broth from which orthophosphate had been removed by precipitation with ammonium molybdate. The nutrient broth contained (NaCl, 5·0 g., peptone, 10·0 g., Lab. Lemco, 10·0 g., distilled water, 1000 ml.). The orthophosphate was removed by boiling the broth with 2 ml. concentrated HNO_3 for 15 min. and adding a hot, saturated solution of ammonium molybdate drop by drop until no further precipitate was obtained in the filtered broth. The medium was filtered and adjusted to pH 7·8 with N-NaOH. The phosphate content of the broth, determined by Briggs's (1922) method, was less than 5 μg. PO_4/ml. Untreated nutrient broth contained 700 μg. PO_4/ml. The excess ammonium molybdate was not removed from the broth and the molybdenum present, determined by the method of Sandell (1953), was 320 μg./ml. ^{32}P-labelled orthophosphate (from the Commonwealth X-Ray and Radium Laboratories, Melbourne, who obtained it from Radiochemical Centre, Amersham) and PO_4 as Na_2HPO_4 were added to this broth as required. The solution was dispensed in 2–3 ml volumes into 50 ml. conical flasks and sterilized at 121° for 15 min.

Treatment of cocci. Cocci were separated from the supernatant fluid by centrifuging at 11,000 rev./min. for 30 min. at 4°. Cocci were washed three times either with a physiological saline solution (0·85 g. NaCl/100 ml.) containing 0·025% human serum albumin or with distilled water. Washing with distilled water was used when the specific activity of cocci was determined. Solutions in which cocci were suspended for ^{32}P release studies were placed in 40 ml. centrifuge tubes, previously sterilized with alcohol, and left in a 37° water bath for appropriate times.

Fractionation of cocci. Cocci were frozen and thawed twice in about 1 ml. water. Phosphorus-containing compounds were fractionated according to the method of Mudd, Yoshida & Koike (1958). Each fraction was not analysed chemically but the probable content of each was as follows:

Phosphorus compounds that were soluble in 5% trichloroacetic acid (TCA) after 1 hr at 4°. Hancock (1958) showed that inorganic phosphorus and amino acids were present in a similar extract prepared from *Staphylococcus aureus*. Phosphorus in this solution was precipitated with barium acetate at pH 4 and the remaining soluble phosphorus was precipitated at pH 7·4 with barium acetate. Metaphosphates are normally precipitated from TCA solutions at pH 4. Metaphosphate has not been identified from *S. aureus* and the precipitate obtained in these experiments has not been definitely characterized. The phosphorus precipitated at acid pH with barium acetate (2–20% total) has been tentatively called 'metaphosphate' since others have found metaphosphate in similar extracts of Mycobacteria (Mudd *et al.* 1958); 80–85% of the phosphorus remaining in acid solution was precipitated with barium acetate at pH 7·4. The remaining phosphorus compounds were not characterized.

P compounds that were soluble in alcohol and 3 + 1 *alcohol+ether mixtures.* Park & Hancock (1960) showed that lipid and polypeptides were present in this extract and the phosphorus extracted was probably combined in these compounds.

P compounds that were soluble in 5% *TCA after* 5 *min. at* 95°. Park & Hancock (1960) showed that nucleic acids were present in this extract with virtually all the teichoic acid from the cell wall. This fraction was treated at pH 4 with barium acetate and the precipitate obtained tentatively called 'polyphosphate', although polyphosphate was not characterized. The supernatant fluid from acid barium acetate precipitation was treated with barium acetate at pH 7·4. 50–80 % of the phosphorus present was precipitated with this reagent. Some PO_4 was probably present with organically bound phosphorus since Mudd *et al.* (1958) found that hydrolysis of polyphosphate occurred during extraction.

P compounds that were soluble in cold N*-perchloric acid after* 24 *hr.* Ribonucleic acids were extracted by Mudd *et al.* using this technique and the phosphorus present in this extract probably represented mostly RNA phosphorus.

P compounds that were soluble in 5% *TCA after* 15 *min. at* 95°. Deoxyribonucleic acid was extracted from mycobacteria by Mudd *et al.* (1958) using this technique. It seems likely from the studies of Park & Hancock (1960) that some nucleic acid-P would also have been extracted with TCA at 95° for 5 min. The P extracted would then be less than the total P present in DNA.

Phosphorus which remained in the cocci after the extraction procedure described was extracted after 2 hr hydrolysis with N-H_2SO_4 at 100°.

Radioactivity measurements. ^{32}P-containing solutions were counted in a Geiger–Müller counter for liquids attached to a high-tension power supply and scaler. Appropriate corrections were applied to measurements for paralysis time, radioactive decay and counter efficiency. The specific activity of cocci was determined by drying the cocci to constant weight on a watch glass and dissolving them in 10 ml. concentrated HNO_3.

Counts of viable cocci and measurements of turbidity. Viable bacterial counts were measured after the method of Miles & Misra (1938). Optical extinctions of the bacterial suspensions were read on a Unicam SP 600 spectrophotometer at 470 mμ and standardized against direct haemocytometer counts.

RESULTS

Phosphate requirements and uptake

The number of cocci harvested from broth was a maximum when 6 μg. PO_4/ml. was present. When less than this amount was added per flask the yield decreased slightly but the harvest of about 10^8 cocci ml. was not increased when the phosphate was increased in 6 μg./ml. increments to 36 μg./ml. Viability of the cocci grown in broth containing less than 6 μg./ml. was poorer, 7·6% total cocci compared with 9·3% when cocci were grown in broth containing PO_4 in excess of that amount. An aqueous suspension of cocci from a nutrient agar slope required only small amounts of PO_4 to increase to about 10^8 cocci ml.

Experiments were made to discover whether the cocci would accumulate more phosphorus than the minimum amount required for growth and viability. The incorporation of PO_4 into the cocci was studied using tracer amounts of $^{32}PO_4$ added

with orthophosphate. Cocci were grown in media which contained 70–450 μg. $^{32}PO_4$/ml. (Fig. 1). The cocci appeared to be saturated with phosphorus after growing in a medium containing 300 μg./ml. The percentage of ^{32}P in the cocci remained at 4–5% of that present in the broth when 70–300 μg. PO_4/ml. were present, but decreased to about 3% at higher concentrations. The cocci were able to accumulate more phosphorus than was necessary for normal growth and viability. The nutrient agar used to grow cocci for inoculation contained 700 μg. PO_4/ml. and the inoculum cocci probably contained reserve phosphorus. This may have enabled them to grow in broth containing less than 6 μg. PO_4/ml.

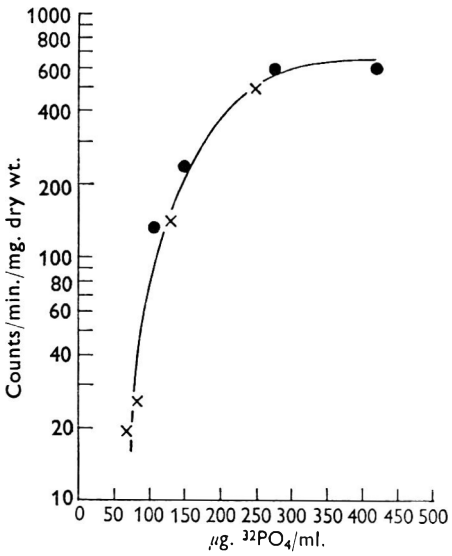

Fig. 1. The relationship between cell ^{32}P and concentration of $^{32}PO_4$ in the medium.

The effect of the specific activity of $^{32}PO_4$ on growth and viability

The following experiments were made to discover the maximum amount of ^{32}P a cocci s could contain without detriment to viability and growth and also the amount of $^{32}PO_4$ necessary in the broth to achieve this labelling. The total number of cocci/ml. and the total number viable were compared in cultures which contained PO_4 only and $^{32}PO_4$ with various specific activities. Replicate cultures contained $^{32}PO_4$ with specific activities of 3·7 μc./μg.; 5·5 μc./μg., 13·8 μc./μg., 1 mc./μg. and 67 μg., 33 μg., 18 μg., 0·25 μg. PO_4/ml. respectively. Control cultures contained the same amount of PO_4. No growth occurred in the culture containing 250 μc. ^{32}P and 0·25 μg. PO_4/ml.; the control culture contained $2{\cdot}1 \times 10^8$ cocci about 8% of which were viable. Growth of cocci was 60% that of the control culture when the specific activity of the $^{32}PO_4$ was 13·8 μc./μg. and the viability was decreased from 0·3% (control) of total cocci to 0·0003%. Growth was about 25% of the control when the specific activity was 5·5 μc./μg. but viability was unaffected; 0·23% of total cocci were viable compared with 0·1% (controls). Growth and viability were not affected when the specific activity of the $^{32}PO_4$ was 3·7 μc./μg. The total number of cocci was 8×10^9 in control and experimental flasks and the number viable was

0·5% total (control), 0·6% total (^{32}P-containing broth). The greatest $^{32}PO_4$ uptake, 44% of that present in the broth, occurred when the specific activity of the $^{32}PO_4$ was 13·8 μc./μg. and 18 μg. PO_4/ml. were present. However, these cells had a low viability (0·0003% of total cells) and were thus unsatisfactory for tracer experiments in animals. When the specific activity of the $^{32}PO_4$ was 5·5 μc./μg. with 33 μg. PO_4/ml. present in the medium, 14% of the $^{32}PO_4$ present in the broth was incorporated into the cocci. Cocci grown under these conditions were as viable as control cocci and were as virulent in mice as non-radioactive cocci. They had a specific activity of $3{\cdot}3 \times 10^{-8}$ μc. ^{32}P/coccus, which was similar to the specific activity of the cocci with reduced viability from cultures containing 13·8 μc./μg. $^{32}PO_4$. The specific activity of cocci from cultures containing 3·7 μc./μg. PO_4 and 67 μg. PO_4/ml. was $2{\cdot}4 \times 10^{-9}$ μc./coccus and only 4% of the $^{32}PO_4$ in the broth was incorporated in the cocci. Cocci labelled under these conditions were not used for tracer experiments in animals.

Distribution of ^{32}P in coccal fractions after uptake during growth

Cocci were labelled with ^{32}P by growing them in broth containing 18–500 μg. PO_4/ml. and 150–200 μc. ^{32}P/ml. They were washed three times with a solution of 0·025% albumin in 0·85% NaCl. Cocci washed in this way lost about 0·2–0·3% of their ^{32}P if they were suspended in the albumin-saline solution for 1 hr. They lost about 50% of their ^{32}P content when they were suspended in PO_4 buffer for 1 hr. Therefore, albumin saline was assumed to be a safe washing medium for cocci later

Table 1. *Fractionation of cocci deficient and rich in phosphorus to show the distribution of ^{32}P under these conditions*

The relative amounts of phosphorus in the cocci were controlled by the phosphate content of the broth.

		Fractionation procedure					
		TCA 1 hr 4°	Ethanol, ethanol + ether (3+1)	TCA 5 min. 95°	Perchloric acid 24 hr 4°	TCA 15 min. 95°	N-Sulphuric acid for 2 hr 100°
		Form of phosphorus in fraction					
		Polyphosphate and inorganic phosphate	Lipids and polypeptides	Teichoic acid, nucleic acid and polyphosphate	RNA	DNA	Insoluble
Phosphate content of broth (μg./ml.)	Treatment of cocci after washing	Percentage of total coccal ^{32}P in each fraction. The values for percentage of polyphosphate in each fraction are in parentheses					
33	Albumin saline 1–5 hr	38 (2·3)	2·6	54	1·5	4·7	0·6
33	M/15 phosphate buffer 1·5 hr	30 (26)	2·0	51 (92)	1·2	4·1	0·8
33		29 (4·0)	19	41 (50)	0·4	6·2	3·3
1000	None	79	0·7	14 (8·5)	1·4	1·4	0·1
2500	None	73 (4·5)	4·5	17 (4·8)	0·6	4·0	1·0

fractionated. Cocci grown in media with different PO_4 contents were analysed to discover the distribution of ^{32}P in the various cell compounds. Previous experiments showed that a coccus was saturated with phosphorus when it was grown in a medium containing more than 400 μg. PO_4/ml. The proportion of ^{32}P in the various fractions was influenced by the available PO_4 (Table 1). When small amounts were available the hot TCA fraction contained the most; when 1000 μg. PO_4 or more were present in the medium the cold TCA fraction contained most ^{32}P. This would indicate that the hot TCA fraction was preferentially labelled and that when adequate phosphate was available then the cold TCA fraction was replenished with ^{32}P. This agrees with Albaum's *et al.* (1952) studies in plant seeds, when phosphorus was incorporated preferentially in certain compounds in states of phosphorus deficiency and appeared in greater amounts in other compounds in normal embryos. In phosphorus-rich cocci most of the ^{52}P was present in the inorganic fraction (cold TCA). Phosphorus-rich cocci contained proportionally less ^{32}P in the 'polyphosphate' fractions, especially in the acid-insoluble 'polyphosphate' fractions. In one experiment placing cocci in phosphate buffer caused an increase in ^{32}P in 'polyphosphate' but not in another (Table 1). Polyphosphate has been shown to increase and decrease according to the division cycle of the cocci (Sall *et al.* 1958) and may be influenced only at certain stages by immersion in phosphate buffer. Since the percentage of 'polyphosphate'-^{32}P did not increase in phosphorus-rich cocci it does not appear to serve as a PO_4 reservoir.

The uptake of $^{32}PO_4$ by non-dividing cocci and the distribution of ^{32}P in coccal fractions under these conditions

Mitchell & Moyle (1953) found that ^{32}P was taken up by non-dividing *Staphylococcus aureus* and that 91% of the ^{32}P appeared in the inorganic fraction. Since Mitchell & Moyle's growth medium was not treated to remove orthophosphate their cocci were probably not phosphorus deficient. Experiments were made to discover the effect of the concentration of coccal P on $^{32}PO_4$ uptake. Cocci after these experiments were washed with water at 4° since Hancock (1958) showed that washing with water did not cause a large release of intracellular compounds. The specific activity of a portion of the cocci was determined after washing with water.

Staphylococcus aureus when suspended in a phosphate solution containing carrier-free $^{32}PO_4$ incorporated ^{32}P into all coccal fractions. The rate at which $^{32}PO_4$ entered the cocci increased with decreasing cocci P (Table 2). When maximal $^{32}PO_4$ concentration was reached the ^{32}P in the cocci then decreased; how long absorption proceeded before release occurred was related to the concentration of $^{32}PO_4$/ml. in the surrounding medium and the level of P in the cocci.

Cocci were fractionated after 1 hr in the suspending medium; excess medium was removed by washing twice with water. ^{32}P entered all the cell fractions, but most of it was present in the inorganic fraction soluble in cold TCA (Table 3). These observations are similar to those of Mitchell & Moyle (1953) but the rate of phosphate exchange depended on the concentration of cell P. The RNA phosphorus fraction was enriched more in the cocci which contained the smallest amount of P originally.

Table 2. *The relationship between the coccal phosphorus content and the uptake of $^{32}PO_4$ from non-nutrient solutions for phosphorus-deficient cocci*

The concentration of coccal phosphorus was related to the phosphate content of the culture fluid. See Fig. 1.

	Uptake of ^{32}P by cocci from a non-nutrient solution of $^{32}PO_4$		
Phosphate concentration of broth (μg./ml.)	Concentration of ^{32}P (μc./ml.)	Period of suspension in ^{32}P solution (min.)	^{32}P uptake by cocci (counts/min./mg. cocci dry wt.)
< 1·0	0·01	15	$9{\cdot}65 \times 10^2$
< 1·0	0·01	60	$1{\cdot}73 \times 10^3$
< 1·0	5·5	15	$8{\cdot}30 \times 10^5$
< 1·0	5·5	60	$3{\cdot}83 \times 10^5$
16·5	0·01	15	$1{\cdot}85 \times 10^2$
16·5	0·01	60	$1{\cdot}83 \times 10^2$
16·5	5·5	15	$8{\cdot}66 \times 10^4$
16·5	5·5	60	$4{\cdot}54 \times 10^4$
33	0·01	15	58
33	0·01	60	$1{\cdot}27 \times 10^2$
33	5·5	15	$3{\cdot}07 \times 10^4$
33	5·5	60	$1{\cdot}26 \times 10^4$

The addition of 10 μg./ml. of phosphate to the 0·01 μc./ml. $^{32}PO_4$ solution resulted in the coccal ^{32}P content reaching a maximum in 15 min.

Table 3. *The fractionation of cocci to obtain the distribution of ^{32}P taken up by non-dividing cocci containing different amounts of phosphorus*

The ^{32}P incorporated by the cocci was proportional to the coccal phosphorus content. The fractionation procedure is as given in Table 1.

		Fraction					
Phosphate content of broth (μg./ml.)	Concentration of ^{32}P in cocci (counts/min./mg. dry wt.)	Polyphosphate and inorganic phosphate	Lipids and polypeptides	Teichoic acid, nucleic acid and polyphosphate	RNA	DNA	Insoluble
		Percentage of total coccal ^{32}P in each fraction. The values in parentheses are the percentages of ^{32}P in the fraction as polyphosphate					
1	$2{\cdot}3 \times 10^5$	84	1·5	11·4 (16)	0·41	1·7	0·93
16·5	$1{\cdot}5 \times 10^5$	85	1·2	10·2 (2·5)	0·22	0·75	0·85
33	$1{\cdot}14 \times 10^5$	86	1·5	8·2 (6·0)	0·18	0·92	0·86
400	$4{\cdot}0 \times 10^4$	92 (2·0)	2·5	2·5 (5·1)	0·09	1·1	0·90
400	$4{\cdot}3 \times 10^4$	83 (2·4)	2·3	8·8 (9·3)	0·12	3·9	0·80

Release of ^{32}P from the cocci

(*a*) *Release in solutions not supporting growth*

Cocci placed in M/15 phosphate buffer for 1½ hr at 37° released between 13 and 30 % of the ^{32}P they contained. When the cocci were washed and resuspended in M/15 phosphate buffer they lost about 5–20% more of the ^{32}P they contained. This subsequent release of ^{32}P after 1½ hr in M/15 phosphate buffer was not prevented by suspending in 2% glucose solution, borate buffer or 0·85% NaCl solution containing 0·025% serum albumin. Phosphate buffer (M/15) caused more ^{32}P to be released from the cocci in the first 2 hr than albumin saline (13 % compared with 1%). Only 1·6–1·8 % of the ^{32}P in cocci was released into human serum during this period. Phosphate exchange with the external medium accounted for some of the release of ^{32}P as indicated by increased ^{32}P in the presence of PO_4.

The phosphate buffer in which cocci had been suspended was dialysed and treated with barium acetate solution at pH 7·3 to discover how much ^{32}P was present in the form of orthophosphate. Dialysis showed that only a proportion of the ^{32}P was diffusible; the amount diffusible varied with the experiment from about 90 to 50 %. Precipitation of the phosphate buffer with barium acetate showed that the $^{32}PO_4$ released also varied in a similar way. Precipitation figures for $^{32}PO_4$ were usually 5–10 % higher than the dialysis figure, which would indicate the presence of other

Table 4. *The effect of the specific activity of growth medium on the release of ^{32}P from labelled cocci into* M/15 *PO_4 buffer, pH 7·2*

Cocci were grown in broth containing 33 μg. PO_4/ml. with $^{32}PO_4$, washed three times and resuspended in non-radioactive buffer.

Specific activity of the culture medium (μc./mg.)	^{32}P released (% total) after 1 hr	24 hr
5·8	16·0	—
0·58	24·0	57·0
0·13	23·0	49·0
0·0003	21·0	57·0

materials in the PO_4 buffer which reacted similarly to PO_4 with barium acetate but had a high molecular weight. These results indicated that orthophosphate and other P-containing compounds were released from cocci into phosphate buffer, but that the amount of non-orthophosphate-P was variable. When cocci were suspended in an albumin-saline solution the proportion of dialysable ^{32}P released was lower than in phosphate buffer, 10–11% compared with 50–90 %. Solutions which contained glucose and released ^{32}P were Nesslerized after digestion with sulphuric acid and potassium persulphate to test for the release of intracellular N as well as ^{32}P. All solutions contained about 30 μg. N/ml., whether glucose was present with phosphate buffer or not.

Mitchell & Moyle (1953) found that the movement of $^{32}PO_4$ into the medium ceased when the cocci were respiring on glucose added to a buffer solution. In these

experiments the addition of glucose to the phosphate buffer solution did not prevent the outward movement of PO_4.

The simultaneous release of $^{32}PO_4$ and substances absorbing light at 260 mμ from *Staphylococcus aureus* treated with CTAB has been described by Salton (1951). Hancock (1958) showed that these substances were purines and pyrimidines and their corresponding nucleosides and nucleotides. Amino acids and phosphorus were also released from cells. A similar release of nitrogenous material with $^{32}PO_4$ occurred when cocci were suspended in phosphate buffer.

The effect of the specific activity of cocci on the release of ^{32}P compounds into PO_4 buffer M/15 was investigated. The amount of radioactivity in the coccus did not appear to influence the amount of ^{32}P released (Table 4).

The effect of total amount of P a coccus contained on the release of phosphorus was determined (Table 5). Analysis of the variance shows that the P content of the coccus had no significant effect on the amounts of ^{32}P released.

Table 5. *The effect of coccal phosphorus content on the release of ^{32}P into phosphate buffer*

Cocci were grown in broth with 1–10 μc. $^{32}PO_4$/ml., washed and resuspended in M/15 PO_4 buffer pH 7·2. The ^{32}P level in the cocci increased with increasing $^{32}PO_4$/ml. in the broth (Fig. 1). Each result was calculated from five experiments.

$^{32}PO_4$ in broth (μg./ml.)	Total ^{32}P released in 1 hr (%: mean and S.E.M.)
33	26·5 ± 12·8
100	24·5 ± 10·9
400	35 ± 15·7

Variance estimate, $F = 0{\cdot}631$

(*b*) *Release of ^{32}P from dividing cocci*

Cocci labelled with ^{32}P by growing them in broth containing 150–200 μc. ^{32}P/ml. and 33 μg. PO_4/ml. were inoculated into nutrient broth which had not been dephosphated. After washing with albumin-saline solution the distribution of ^{32}P between the cocci and the medium was measured. 80–81% of the ^{32}P originally present in the cells appeared in the medium after overnight growth at 37°. Only 20–21% of the ^{32}P released was diffusible on dialysis. Analysis of the ^{32}P distribution after growth of the labelled inoculum in the unlabelled medium showed small changes had occurred in the relative amounts of ^{32}P in each fraction. The hot TCA fraction contained less ^{32}P, 41% compared with 54% in the original inoculum; the cold TCA fraction contained 35% after growth; the original inoculum contained 38%. The content of the DNA fraction increased after growth from 4·7 to 9%; the phosphoprotein and lipid fraction was 9·0% after growth and 2·6% in the original inoculum. The organic fraction was most severely depleted.

Autoradiography of labelled cocci. Good autoradiographs of *Staphylococcus aureus* labelled with ^{32}P in the skin of the mouse were obtained when AR 10 (Kodak) stripping film was exposed to cocci containing $3{\cdot}3 \times 10^{-8}$ μc./coccus for 3 days.

DISCUSSION

Staphylococcus aureus may be satisfactorily labelled with ^{32}P for autoradiographic purposes and tracer experiments in animals by growing it in broth containing 150–200 μc. ^{32}P/ml. and 33 μg. orthophosphate/ml. The specific activity of the bacteria was about $2{\cdot}0\text{–}3{\cdot}5 \times 10^{-8}$ μc./coccus. It is not possible to label the cocci more heavily without severely decreasing their growth and viability. Cocci labelled in this way are as virulent in mice as non-labelled ones. These findings are in agreement with Stonier's (1956) *Agrobacterium tumefaciens* labelled with ^{32}P. He found a similar specific activity compatible with normal viability in bacteria grown on a defined medium. It would appear that in both *S. aureus* and *A. tumefaciens* the specific activity of the organism decides whether they will survive. The level of radioactivity per ml. (150–200 μc.) did not affect growth when sufficient non-radioactive phosphate was present (about 67 μg./ml.); metabolic disturbance was probably caused by the decay of ^{32}P to ^{32}S.

The interrelation of phosphate in the growth medium and phosphorus in fungi was previously recorded by Rennerfelt (1934), who worked with *Aspergillus niger*. As the phosphate concentration of the medium was increased from 25 μg./ml. to 750 μg./ml. the phosphorus in the mycelium increased almost linearly. Maximum uptake of phosphate from the medium was 20 %, decreasing to 17 % when maximal amounts of phosphate were present. The relationship between phosphorus in the cells and phosphate in the medium is very similar for *Staphylococcus aureus*. The maximal amount of phosphate in the medium is about half the maximal amount for *A. niger* and the percentage uptake is also less.

Park & Johnson (1949) analysed *Staphylococcus aureus* and found that 60 % of the P was present as inorganic phosphate, 5 % as labile phosphate and 30–35 % as stable phosphate. This analysis is similar to the one for P-rich cocci in these experiments. The cold TCA fraction contained 73–78 % of coccal ^{32}P, 80 % of which gave an orthophosphate reaction by forming an insoluble Ba salt at pH 7·4. Polyphosphate has not been described in *S. aureus* but has been found in other organisms, in *Corynebacterium diphtheriae* by Sall *et al.* (1958), in Mycobacteria by Winder & Denneny (1957) and in *Azotobacter vinelandii* by Zaitseva, Belozerskii & Novozhilova (1960). In these organisms it serves as a reservoir of P for RNA synthesis. In *S. aureus* part of the cold TCA and hot TCA fractions formed an insoluble Ba salt at pH 4, a reaction which is typical of metaphosphate. However, *S. aureus* contains large amounts of teichoic acid (Archibald, Armstrong & Baddiley, 1961), and if this compound forms an acid-insoluble Ba salt it might have been confused with metaphosphate.

The release of non-diffusible ^{32}P compounds on dialysis into phosphate buffer from *Staphylococcus aureus* may have been the result of osmotically fragile cells autolysing (Mitchell & Moyle, 1957). This release of large molecular weight materials may also be the result of degradation of RNA and other phosphorus-containing compounds present in non-viable cocci. The release from dividing cocci might be the result of an exchange reaction, autolysis and the degradation of P-containing compounds. The relatively low amounts of $^{32}PO_4$ in the broth, about 20% compared with the original content of the cocci, about 35%, suggests that some of the released material might have been re-utilized by the growing population. If this has occurred the cocci

utilizing the ^{32}P would have a higher proportion of ^{32}P in the cold TCA fraction and less in the hot TCA fraction, since the broth contained about 700 μg. PO_4/ml. The population would then contain less ^{32}P in the hot TCA fraction than the original inoculum. This change did take place but may also be explained on the basis of greater dilution of the hot TCA fraction with ^{32}P taken from the medium and the movement of ^{32}P from the organic fraction to compounds with a low P turnover. Possibly several factors caused a depletion of ^{32}P in the hot TCA fraction after growth in an unlabelled medium. Stonier (1960) found that the release of ^{32}P from *Agrobacterium tumefaciens* was caused by a lytic phage. No such phage was demonstrated in this strain of *S. aureus*.

Elek (1959) described the formation of extracellular compounds by *Staphylococcus aureus*: some of the ^{32}P appearing in the growth medium could be present in the extracellular enzymes and proteins excreted as the result of non-autolytic processes.

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Electron-microscope Studies of Nodule Development in Some Clover Species

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SUMMARY

In this account of the fine structure of a nitrogen-fixing clover nodule the interactions between host and bacteria have been particularly considered. It is suggested that the bacterial infection is located between the paired membranes of the endoplasmic reticulum, i.e. within the intrareticular space. It is shown that the plant membrane around the infection thread is continuous with the plasmalemma, but that it is fragmentary around the thread tip. Bacteria emerging from the infection thread remain surrounded by a plant membrane, and their subsequent fate (division or rapid swelling) is controlled by the condition of the host cytoplasm. Structural changes occurring in the cytoplasm, nuclear region and wall of emerged bacteria during their transition to the bacteroid form are described, and the development of peripheral carbohydrate platelets in the host cell is illustrated. Supporting observations on two types of ineffective nodules are discussed.

INTRODUCTION

At the level of the light microscope the general histology of leguminous nodules is by now well known, but at the level of the electron microscope only some aspects, notably the structure of the bacteroids, have been studied. A more general account of bacteroid development in barrel medic and subterranean clover (Dart & Mercer, 1963*b*, *c*) has been published since this paper was written. The fine structure of rhizobia in culture was investigated by Baylor, Appleman, Sears & Clark (1954) in the early days of electron-microscopy, and has been studied with present-day techniques by Vincent, Humphrey & North (1962). Bergersen & Briggs (1958) and Jordan (1962) examined the structure of bacteroids in crushed nodule fractions. In soya bean nodules Bergersen & Briggs (1958) have described membranes of plant origin enclosing groups of bacteria, and Bergersen & Wilson (1959) suggested that these membranes are the primary site of nitrogen fixation. Similar membranes enclosing single bacteroids have recently been described in other leguminous nodules (Dart & Mercer, 1963*a*; Mosse, 1963; Jordan, Grinyer & Coulter, 1963); their origin is described in this paper, and the exact location of the infection within the host cell is discussed. Other observations concern the structure of the infection thread, the way in which the bacteria emerge from it, the changes in bacterial structure that accompany the transition from the rod-shaped to the bacteroid form, and the influence of the host on this transition.

The histology of nodules of the type examined in the present work is well illus-

trated by Thornton (1930*a*, *b*), McCoy (1932) and Schaede (1940, 1941) on whose descriptions the following summary is based. Fåhraeus (1957) and Nutman (1959) have described the early stages of root hair infection. In the root hair the bacteria remain enclosed in a hypha-like structure—the infection thread—within which they multiply. The infection thread which depends for its growth on the close proximity of the nucleus, extends into the inner cortex where its approach stimulates certain tetraploid cells to divide (Wipf & Cooper, 1940), thus initiating the meristem of the nodule. The mature infection thread is surrounded by a sheath, which reacts histochemically like the host cell wall and contains cellulose, probably laid down by the host cytoplasm. At its tip the infection thread appears to be without a sheath, and is thought to consist of a zooglocal strand containing bacteria suspended in a mucilaginous matrix secreted by them. The formation of the thread wall is regarded by most observers as a typical defence reaction against an invading organism. In a somewhat different interpretation Nutman (1956, 1963) suggested that the infection thread may never penetrate into the plant cell cytoplasm, but that its matrix may represent an extension of the primary cell wall modified by bacterial action.

Bacteria emerge from the thread in one of three ways: as coccoid rods from the young unsheathed infection thread, by the bursting of vesicle-like swellings which arise on older sheathed parts of the thread, or, in some species, by a delayed release into cells already filled with bacteroids. Observations with the light microscope suggest that the bacteria released into the cytoplasm grow into elongated rod-shaped organisms with a banded structure. In this form they may continue to divide, but eventually enlarge and develop into the much swollen, sometimes branched forms described as bacteroids. The criteria distinguishing rods from bacteroids are ill-defined. The distinction is based mainly on morphological characters (see reviews by Jordan, 1962; Raggio & Raggio, 1962), and the present observations suggest that in clover nodules the transition point would be difficult to define. The generally accepted concept is that rods can divide and have a single nuclear region, whereas bacteroids cannot divide, have several discrete nuclear regions or do not react with Feulgen reagents, and are associated with nitrogen fixation. Eventually the bacteroids collapse. Unchanged bacteria remaining in infection thread fragments become parasitic, and their multiplication in the middle lamella probably causes the collapse of the nodule tissue.

METHODS

Nodules from seedlings of *Trifolium parviflorum* L., grown under sterile conditions in a nitrogen-deficient medium containing mineral salts and inoculated with an effective nitrogen-fixing strain (SU297) of *Rhizobium trifolii*, were used in the main investigation. A less detailed examination was also made of the pink bacteroid-containing zone in nitrogen-fixing nodules of *T. repens* L., *Vicia faba* L. and *Vicia hirsuta* L., and of non-fixing nodules in *T. parviflorum* (inoculated *R. trifolii* Cl.F.) and *T. repens* (inoculated *R. trifolii* H.K.C.) The seedlings were grown in a glasshouse during autumn and winter, with supplementary artificial light. The nodules of *T. parviflorum* have a single apical meristem and are elongated when mature. Four main regions (Fig. 1) representing different developmental stages in the symbiotic process are macroscopically distinguishable.

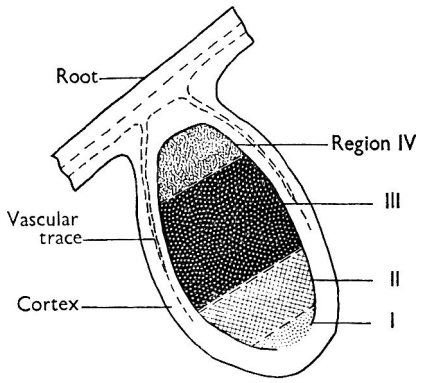

Fig. 1. Zones representing different stages of development in a nitrogen-fixing nodule of *T. parviflorum*. I, Meristematic zone containing dividing cells with large nuclei and very young infection threads; II, zone of expanding cells containing many rod-shaped bacteria and proliferating infection threads; III, pink-coloured zone with vacuolated cells containing bacteroids; IV, greenish-coloured zone containing collapsing cells and disintegrating bacteroids.

Electron microscopy. In the main investigation nodules of two different ages were examined: 5-day-old nodules from 2-week-old plants, and 4-week-old nodules from 8- and 10-week-old plants. Tissue slices (0·5 mm. thick) of the four zones were prepared for electron microscopy. Most of the material was fixed in 2% (w/v) potassium permanganate at room temperature for 2 hr. Some was also fixed in Palade's fixative (Mercer & Birbeck, 1961) for 1½ hr, followed by 1% (w/v) uranyl acetate for ½ hr. The material was then dehydrated slowly and embedded in araldite, with xylene as a solvent. Sections were cut with a Huxley ultramicrotome and examined with a Siemens electron microscope.

Light microscopy. Two techniques were used to assist in the interpretation of structures seen by electron microscopy. Comparable nodules were sectioned on a freezing microtome and the sections stained and mounted in lactophenol containing a little cotton blue. Additional observations were made on small slices of the araldite-embedded tissue cut off with a razor blade and mounted on a slide in unpolymerized araldite. Infection threads, bacteria and cell walls were stained brown by the permanganate and were clearly visible.

RESULTS

The infection thread

Plate 1, fig. 1, shows a typical infected cell from the meristematic tip (region I) of a nodule. The cell contains an infection thread (cut transversely) and many bacteria distributed throughout the cytoplasm, as well as the usual cell organelles (proplastids, mitochondria, Golgi bodies, endoplasmic recticulum) and a central nucleus. Plate 1, fig. 2 (region I), shows an infection thread (L.S.) passing through a cell and shedding bacteria near the nucleus. In the material examined such direct passage through cell walls was uncommon; more often threads appeared to originate from a distended part of the wall and to terminate within the cell. Plate 2, fig. 3

(region I), shows a thread that apparently originated in the wall and extended into two different cells.

The characteristic structure of a fully developed infection thread in region I is shown in Pl. 3, fig. 4. The electron-dense central area consists of bacteria surrounded by a substance (here called the thread matrix), which looks unevenly dense after permanganate fixation but is homogeneous and opaque when fixed with osmium tetroxide. This matrix, which resembles the bacteria in its histochemical reactions (Schaede, 1940), is surrounded (Pl. 3, fig. 4) by the middle lamella, from which it differs, by the cell wall and by a cytoplasmic membrane continuous with the plasmalemma of the host cell. The cytoplasmic membrane and the area that will develop into thread wall are clearly shown in the small thread protrusion (Pl. 3, fig. 4, bottom right), but the middle lamella is not visible. Plate 3, fig. 5, shows the mature wall of two infection threads (T.S.) from an older part of a nodule (region II), where the host cells were already highly vacuolated. Cell cytoplasm, confined between the plasmalemma and tonoplast membranes, is limited to a thin peripheral layer lining the cell walls and surrounding the infection threads. A pocket of cytoplasm containing a mitochondrion is attached to the right-hand thread. One bacterium is embedded in an extension of wall substance, another lies in the pocket of cytoplasm and the others lie within the thread matrix. These pictures show that mature parts of infection threads are surrounded by a wall continuous with that of the host cell. They confirm the view, based on histochemical tests, that this wall is of host origin.

There is no wall around the growing tip of the infection thread. The close association between the host nucleus and the extending thread has been mentioned, and often the thread tip was seen lying in a deep indentation of the nuclear surface. This is shown in Pl. 4, fig. 6 (region I), where a thread tip is surrounded by the nucleus on three sides. Some bacteria are present quite close to the tip. In this region the thread matrix appears to lie freely within the host cytoplasm (see also Pl. 4, fig. 7) and only some rudimentary fragments of an enclosing membrane are visible. The only structural evidence of the special relationship between thread growth and the proximity of the nucleus are the very fine fibrils radiating from the thread surface towards the nucleus (see also Pl. 15, fig. 27). Near the growing point of a thread the normal cytoplasmic pattern of the cell is often much disturbed, with endoplasmic reticulum in the form of short pieces with relatively wide lumen and an accumulation of mitochondria and Golgi bodies (Pl. 2, fig. 3).

Release of bacteria from thread and vesicles

Bacteria can be released from infection threads at different times (in region I, II or III) and in different ways, but they always emerge without adhering thread matrix and immediately become surrounded by a cytoplasmic membrane of host origin. Plate 4, fig. 7, shows emergence in region I from very young threads not yet enclosed by a wall. It seems that the movement of bacteria into the cytoplasm cannot, as was thought, be attributed merely to internal pressures caused by bacterial multiplication and slime formation in the thread. The cap of thread matrix being pushed aside by the emerging bacterium (B) in the main picture apparently does not penetrate into the cytoplasm but remains enclosed within the plant

membrane, and the thread wall develops behind it. Such caps were seen attached to the surface of young threads, and also of larger structures (Pl. 5, fig. 9).

As the thread ages, direct emergence is prevented by the thickening wall, but continues from two auxiliary structures: localized knob-like protruberances or swellings of the thread (Pl. 5, fig. 8) and delicate blister-like vesicles that arise laterally (Pl. 6, fig. 10). The structure illustrated in Pl. 5, fig. 9 (region II) is essentially a distended thread. Its wall is thinner than that of a normal thread, and disappears almost completely where bacteria are touching it. The thread matrix is less dense and granular, and the electron-transparent area around the bacteria is larger, than in very young threads (Pl. 4, figs. 6, 7). A bacterium on the point of emergence can be seen on the left. A true vesicle, shown in Pl. 6, fig. 11 (region II), has no enclosing wall and is interpreted as an extrusion of thread contents—matrix (now even less dense), and suspended bacteria (now surrounded by an even larger electron-transparent area)—into the host cytoplasm, from which they are separated by only a plant membrane. The bacterium shown in longitudinal section in Pl. 6, fig. 11, is near to emergence. During this process, further illustrated in Pl. 7, fig. 12 (region II), the thread matrix is characteristically left behind.

The interpretation of the electron-transparent zone, which increases around the bacteria with prolonged retention in the thread, is problematical. It may represent a polysaccharide secretion of the bacteria, an area from which some substance has been leached during processing, or less probably a lysogenous cavity. In light-microscope studies of *Neptunia oleracea*, Schaede (1940) noted unstained areas around the bacteria in infection threads, but was uncertain whether they were real or artifacts of fixation. Bacteroid development does not begin till the bacteria have emerged also from the transparent zone. An example of delayed or arrested emergence, into a host cell situated in the pink zone (region III) of a nodule and already containing mature bacteroids is shown in Pl. 7, fig. 13. Several groups of bacteria are contained in electron-transparent areas enclosed by a cytoplasmic membrane. Where the bacteria touch the membrane they are swollen and, as in earlier emergence the membrane is distended locally (Pl. 5, fig. 9). Eventually, protruding bacteria become completely surrounded by the plant membrane and pass into the cytoplasm by a process of apparent ingestion (also shown in Pl. 13, fig. 23).

The swelling of bacteria that approach the vesicle or thread boundary so close that there is no intervening matrix, and the subsequent distortion of the plant membrane, appear to be fundamental steps in the process of emergence. Schaede's (1941) view that the thread matrix protects the bacteria from the lytic effects of the host cytoplasm is supported by these observations.

The development of the enclosing membrane system

Figure 2 shows the interrelation between the membrane systems of the nucleus, the cytoplasm and its organelles, and the position the infection thread and bacteria are thought to occupy within this system. The bacterial infection is shown as developing between the paired membranes of the endoplasmic reticulum which will be referred to as the intra-reticular space. The now widely accepted concept of a continuous intra-reticular space, stretching from the cell surface to the nucleus, is based on the observed continuity between the nuclear membrane and the endo-

plasmic reticulum and its continuity through the plasmodema (Robertson, 1959; Whaley, Mollenhauer & Leech, 1960; Setterfield, 1961; Buvat, 1963). Irregularly shaped areas, interpreted as expanded intra-reticular space, were common in the cytoplasm of meristematic nodule cells (Pl. 1, fig. 2; Pl. 2, fig. 3). Characteristically such areas were enclosed by a single unit membrane which at the sharply pointed corners appeared to merge into the normal endoplasmic reticulum (Pl. 1, fig. 2, middle right), and contained small round vesicles 50–130 mμ in diameter. These

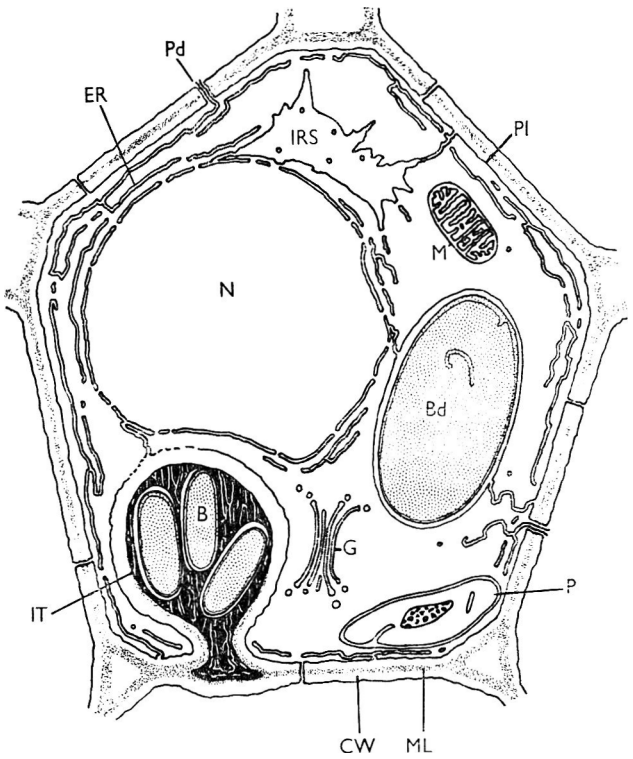

Fig. 2. Diagrammatic representation of the position of the bacterial infection in a nodule cell. B, Bacteria; Bd, bacteroid; CW, cell wall; ER, endoplasmic reticulum; G, Golgi body; IRS, intra-reticular space; IT, infection thread; M, mitochondrion; ML, middle lamella; N, nucleus; P, proplastid; Pd, plasmodesma; Pl, plasalemma.

areas resemble the pre-vacuolar bodies described by Whaley, Mollenhauer & Kephart (1962) in maize cells, but neither the very early stages in which prevacuolar bodies are more electron dense than the surrounding cytoplasm, nor later stages (seen by kind permission of Dr B. E. Juniper) in which their contents more resembled those of true vacuoles, were seen in clover. Whaley *et al.* (1962) consider that 'the pre-vacuolar body change to the vacuole is a specific organelle transformation'; other views about the origin of plant vacuoles are discussed by Buvat (1963) and Marinos (1963). In clover, these areas contained a substance similar to the cytoplasm and quite unlike vacuolar sap, and occurred in young meristematic cells, while true vacuoles developed much later. A separate investigation would be

needed to determine whether they are forerunners of true vacuoles; for the purpose of this paper, they will be considered as expanded intra-reticular space.

In Pl. 1, fig. 2 (top left), an enlarged intra-reticular space is seen apparently continuous with a very large gap in the nuclear membrane. A large opening in the nuclear membrane also occurs in the central nucleus in Pl. 1, fig. 2 and similar pores were common in nuclei in region I. In older parts of the nodule the nuclear pores were of more normal size, 50–100 mμ according to Setterfield (1961). Very large pores, associated as in clover nodules with abnormal configurations of the nuclear membrane and adjacent endoplasmic reticulum, have also been illustrated by Whaley *et al.* (1960) and by Leech, Mollenhauer & Whaley (1963) in root tip cells of maize. If such large gaps in the nuclear membrane are a characteristic of the polyploid nucleus in a meristematic tissue they may have some connexion with the role of the tetraploid nucleus in nodule development.

The cytoplasmic membranes surrounding the infection thread are unit membranes in the sense of Robertson (1959), averaging 7 mμ in width. Continuity between the thread membrane and the plasmalemma is illustrated in Pl. 1. fig. 2, Pl. 2, fig. 3; and Pl. 3, figs. 4, 5; but near a thread tip (Pl. 4, figs. 6, 7) or in other parts of a young thread close to the nucleus (Pl. 1, fig. 2) the membranes are fragmentary, possibly because of active synthesis, and their origin and connexion with the membrane system of the host cell are difficult to follow. Whether the thread membrane is regarded as a spontaneous new formation, possibly originating from Golgi bodies which are often nearby (Pl. 4, fig. 6), or as a continuous extension of the plasmalemma, which around the thread tip loses the appearance of cohesion, depends on one's views about the origin of cytoplasmic membranes. Plate 8, fig. 14, provides evidence that the plant membrane around recently emerged bacteria is continuous with the normal endoplasmic reticulum. Whether such continuity persists when the enclosed bacteria begin to swell is difficult to determine. Although there is often a strong suggestion of continuity, undoubted connexions are rare in the later stages, and the envelopes may separate completely from the endoplasmic reticulum. Open connexions between the enclosing membrane and the endoplasmic reticulum were common in one type of ineffective nodule (*Trifolium repens* inoculated with *Rhizobium trifolii* H.K.C.; Pl. 8, fig. 15).

The origin of membranes around bacteria emerging later from vesicles or distended threads can more easily be traced. The existing membrane around these subsidiary structures simply extends and closes up behind the emerging bacterium. During subsequent development this membrane, which originated in the host cytoplasm, behaves more like a bacterial than a plant membrane and is further considered below.

Changes in rhizobial structure associated with their transition from the rod-shaped to the bacteroid form

Rhizobia undergo two important developments in the nodule; they multiply and they increase in volume more than tenfold. The swelling is associated with changes in the nuclear region, the cytoplasm and the bacterial wall. These changes are illustrated diagrammatically in Fig. 3.

Rhizobia in the rhizosphere. Plate 8, fig. 16, shows some rhizobia near a root hair. They have a large irregularly shaped nuclear region in the centre, surrounded by a

narrow region of denser cytoplasm. Some bacteria contain a small electron-transparent vacuole near one end. The protoplast is surrounded by two unit membranes averaging 7–9 mμ in diameter, separated by a less dense inter-membrane area. By analogy with the structure of other Gram-negative bacteria (Martin, 1963; Salton, 1960) and in agreement with the interpretation of Dart & Mercer (1963*a*, *b*, *c*) and Vincent *et al.* (1962) the inner is regarded as the cytoplasmic membrane and the outer as the outside layer of the rigid bacterial wall. The inter-membrane area is wide (of the order of 50 mμ) and very irregular. This may be attributable in part to shrinkage or natural invagination of the cytoplasmic membrane but, as pointed out by Vincent *et al.* (1962), this space is by no means devoid

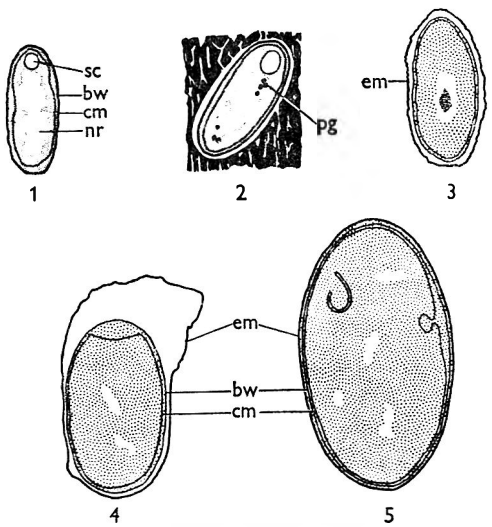

Fig. 3. Changes in bacterial structure during nodule development. 1, Bacteria in rhizosphere; 2, bacteria in infection thread; 3, bacteria recently emerged into the host cytoplasm and still dividing; 4, final stage in bacteroid formation; 5, bacteroids. bw, Bacterial wall; cm, cytoplasmic membrane of bacterium; em, enclosing membrane of plant origin; nr, nuclear region; sc, storage carbohydrate; pg, polyhedral granule.

of electron-dense material. Since it persists and becomes much more regular and wall-like during subsequent developments, it probably has some structural reality and must presumably be regarded as an interior non-rigid layer of the bacterial wall. The considerable enlargement of the inter-membrane area at the bacterial poles, which was observed by Vincent *et al.* (1962) in cultured rhizobia, was not seen in those from the rhizosphere.

Rhizobia in threads and vesicles. The rhizobia undergo only minor changes while they are enclosed in the infection thread. Those found in immature parts of the thread and likely to emerge early (Pl. 4, figs. 6, 7) have a clearly defined cytoplasmic membrane nearly parallel to that of the bacterial wall. Rhizobia left in the thread divide, mainly by constriction (Pl. 3, fig. 4), and those in vesicles by a process more like budding (Pl. 6, fig. 11). In such bacteria the nuclear area condenses while the

cytoplasm enlarges correspondingly, and the round or oval vacuoles within it increase in size (Pl. 3, fig. 4; Pl. 6, fig. 11). Their ontogeny (to be described elsewhere) and their similarity to deposits of the storage carbohydrate poly-β-hydroxybutyric acid (PHBA) in *Hydrogenomonas* sp. (Schlegel, Gottschalk & von Bartha, 1961) make it probable that they are of a similar nature, but they might be normal cytoplasmic vacuoles. PHBA has been isolated from leguminous nodules (Forsyth, Hayward & Roberts, 1958; Schlegel, 1962) and from rhizobia in culture (Vincent *et al.* 1962), although the latter authors observed only few vacuoles in cultured rhizobia containing up to 50 % (w/v) PHBA.

Electron-dense approximately polyhedral particles of about 50 mμ diameter appear in the bacteria retained in threads and vesicles, mainly in regions II and III (Pl. 6, fig. 11; Pl. 3, fig. 4; Pl. 4, fig. 7; Pl. 7, figs. 12, 13). They bear much resemblance to precursor-phage particles in *Escherichia coli* (Kellenberger, 1961), but are not necessarily confined to the nuclear region. In nodules the particles rapidly disappear when the bacteria emerge into the host cell, except occasionally where an emerged bacterium filled with such particles has developed no further. Investigations in progress may show whether these particles are connected with phage infection, and if so whether this is a peculiarity of the strain SU 297 or has any general significance for nodule development.

Rhizobia in the cytoplasm. Plate 1, figs. 1, 2, and Pl. 8, fig. 14, show bacteria in region I recently emerged into the host cytoplasm. After emergence the bacterial cytoplasm immediately begins to swell, accompanied by loss of the carbohydrate vacuoles and further shrinkage of the nuclear region. This is now clearly separated into a dense central portion, possibly indicating a more condensed form of DNA (Kellenberger, 1962), and a less dense perinuclear area. These trends are further illustrated in Pl. 9, fig. 17, which shows some bacteria about to divide. The host cell still contains a prominent nucleus and dense cytoplasm with Golgi bodies and structure characteristic of a meristematic cell.

The next stage (Pl. 9, fig. 18) occurs when the cytoplasm in the expanding host cell (region II) becomes dispersed and much less electron dense, and the bacteria no longer divide. Contrary to the widely accepted view that rod-shaped bacteria divide until they reach the bacteroid stage in region III, there was no convincing evidence that division occurred in vacuolating cells. The implications of this observation relating bacterial multiplication to the state of the host cytoplasm are further discussed below.

Plate 9, fig. 18, also illustrates changes in the spatial relationship of the bacterial wall and its associated membranes. The enclosing host membrane becomes more prominent and begins to fold. The two bacterial membranes approach one another closely so that the original inter-membrane space narrows, and the inner cytoplasmic membrane develops characteristic invaginations. The various stages of invagination illustrated in Pl. 9, fig. 18, are consistent with the explanation that the bacteria in region II are imbibing materials from the host cytoplasm by a process of pinocytosis (Suganuma, 1961). Plate 10, fig. 19 (osmium tetroxide fixation), shows the further expansion of the plant membrane enclosing the bacteria. This membrane now differs from the other plant membranes around vesicles of the host cytoplasm by the absence of the small electron-dense particles (about 5 mμ diameter) thought to be ribosomes, that give to the vesicular membranes the characteristic appearance

of rough reticulum. The two mitochondria identify the ground tissue as host cytoplasm.

The final stage in bacteroid formation (Pl. 11, fig. 20) is associated with the formation of a central vacuole in the host cell; the transition to the haemoglobin-containing tissue (region III) occurs very quickly. The host membrane around the bacteria is now fully expanded leaving a large space between it and the bacterial wall which will be occupied by the developing bacteroid. This space is occupied by a substance resembling vacuolar fluid. As the central vacuole develops in the host cell, the cytoplasm and enclosed bacteria are pushed towards the periphery, and as the bacteria expand the cell organelles are compressed and begin to disintegrate. At this stage the bacteria no longer have a single recognizable nuclear area; small, irregularly shaped, less dense areas in the cytoplasm probably represent the fragmented nucleus.

Swollen bacteria similar to those in the host cell were sometimes seen in intercellular spaces (Pl. 13, fig. 23), which also contained unidentifiable tissue fragments. Characteristically such bacteria had only two membranes and lacked the one produced by the host cytoplasm.

Plate 12, fig. 21, shows fully developed bacteroids from region III. These are surrounded by three unit membranes (Pl. 12, fig. 22) of which the plant membrane (about 10 mμ across) is now the most clearly defined. The ground tissue of the bacteroids looks exactly like that of the surrounding cytoplasm, and apart from the electron-transparent fragmented nuclear material, occasional invagination of the inner cytoplasmic membrane and some internal vesicles surrounded by compound membranes, they have no organized structure. No Golgi bodies, only small fragments of endoplasmic reticulum and very few mitochondria remain in the host cytoplasm.

The structural changes undergone by the bacteria in the cytoplasm, viz. swelling fragmentation of the nuclear region, formation of a uniformly granular cytoplasm and the folding and expansion of the outer enclosing membrane, have some similarity to those associated with the early stages of phage infection in *Escherichia coli* (Kellenberger, 1961).

Degenerative changes

Plate 14, fig. 25, shows the final disintegration of bacteroids and host cell in region IV. The cell wall breaks down, often by splitting at the middle lamella as shown; in later stages no continuous wall structure is left. The bacteroid membranes disintegrate and the original bacteroid shapes are only just discernible in the clumped tissue mass (Pl. 14, fig. 25). Young, actively dividing bacteria similar in size and structure to those of the rhizosphere are found in the split wall cavities and also among the collapsing bacteroids and in the cell vacuole. Only the faintly visible electron-dense particles within them suggest the influence of the host environment.

Cell carbohydrates

Raggio & Raggio (1962) reviewed information about the storage carbohydrates of nodules and their possible link with nodule metabolism. In effective nodules of *Trifolium parviflorum* and some other species, uninfected cells contain compound starch grains while adjacent infected cells do not. In several clover species a more

soluble polysaccharide, tentatively identified as a dextran by Bergersen (1957), occurs as a temporary deposit in a narrow transition zone between regions II and III. This carbohydrate occurs in the shape of rectangular platelets characteristically arranged opposite the intercellular spaces and around the cell periphery.

Plate 1, fig. 1, shows proplastids in region I with starch grain precursors (oval, densely granular bodies) in an uninfected cell, and with what appear to be eluted remains of such precursors in adjacent cells containing bacteria (see also Pl. 1, fig. 2; Pl. 2, fig. 3). In older infected cells in region II the empty proplastids tend to occur near the cell periphery; often they appear to be compressed by the expanding bacteria. In these peripheral plastids the rectangular carbohydrate platelets develop, appearing first as densely granular bodies (Pl. 17, fig. 20) and later becoming electron-transparent (Pl. 13, fig. 23). Well-developed mitochondria frequently occur near them. In their development the platelets closely resemble normal starch grains, but they differ in their characteristic rectangular shape.

In some nodules from an 8-week-old seedling of *Trifolium repens* a system of parallel lamellae, each a unit membrane, occurred in the otherwise empty plastids (Pl. 13, fig. 24). These structures somewhat resemble the grana of chloroplasts. The ribbon-like structure below the plastid is a swollen cell wall split at the middle lamella and filled with some secretory or breakdown products from a nearby group of intercellular bacteria like those in Pl. 13, fig. 23.

Additional observations on other material

It is as yet unknown to what extent symbiotic nitrogen fixation is linked to the structural organization of the bacteroid zone. More information about the nodules of other species will help to determine which developmental stages are important in establishing the symbiotic relationship. Some further observations on the structure of the bacteroid zone in other nitrogen-fixing combinations, and of general nodule structure in two non-fixing combinations, are given in this section.

Effective nodules. The structure of bacteroids in the pink zone (region III) of *Vicia faba*, *V. hirsutum* and *Trifolium repens* was essentially similar to that of *T. parviflorum*; each bacteroid was surrounded by a unit membrane of plant origin. A similar arrangement was found by Dart & Mercer (1963*a*) in *Medicago tribuloides*, *T. subterraneum* and species of Acacia, Vicia, Vigna, and Viminaria. Jordan *et al.* (1963) pictured bacteroids of *Medicago sativa* enclosed in a double membrane not apparently present when the bacteria first emerged from the thread, but preservation of bacterial structure in these pictures was not good, and the theories advanced for the origin of the enclosing membrane are curious.

Ineffective nodules. Genetic and anatomical investigations (Nutman, 1959) have shown that ineffectiveness—the inability to fix nitrogen symbiotically—can be based on different factors and that normal nodule development can be interrupted at different stages. Two types of ineffective nodules were examined. Neither contained bacteroids, but the structural patterns of ineffectiveness differed.

In nodules of *Trifolium parviflorum* inoculated with *Rhizobium trifolii* Cl.F. infection thread contents and cell organelles looked normal (Pl. 15, fig. 27), except for a notable lack of cytoplasm even in quite young cells near the nodule meristem. Possibly because of this, the thread wall was frequently rudimentary or lacking, as in Pl. 15, fig. 27, although the size of the electron-transparent area around the bac-

teria suggests that this was a mature infection thread. Emergence was much delayed, and when it occurred (often from vesicles) the thin cytoplasmic lining around the vesicle was broken, so that the bacteria emerged into the vacuole where they did not develop further. A few rod-shaped bacteria were seen in freshly cut sections; it is assumed that these emerged into small pockets of cytoplasm like those shown in Pl. 15, fig. 27, and developed normally, but none was encountered in the electron-microscope preparations. In older parts of the nodule the bacteria often multiplied in the distended cell wall which sometimes ruptured, as in Pl. 15, fig. 28; in the lower cell both nucleus and cytoplasm are disintegrating. These observations show that without the stimulation of cytoplasmic activity which the approaching infection thread normally induces in prospective host cells (Thornton, 1930*b*), few bacteria emerge from the thread, and there is no habitat in which they can multiply and swell. Evidently the central vacuole, even in an apparently normal cell, does not supply the necessary environment.

A much more drastic reaction occurred between *Trifolium repens* and *Rhizobium trifolii* (H.K.C.), both bacteria and host cells showing advanced collapse. Although the cell nucleus appeared normal and enlarged as in effective nodules, the cytoplasm even near the meristematic nodule tip was again scarce, and all the cell membranes (tonoplast, plasmalemma, endoplasmic reticulum) were fragmentary (Pl. 14, fig. 26). The matrix of young infection threads was much more electron transparent than in any of the other nodules examined, and in older parts of the nodule no trace of it remained. In some cells of the meristem there was enough cytoplasm for apparently normal release of bacteria, but degenerative changes appeared very quickly in both cytoplasm and bacteria (Pl. 16, fig. 29). Enclosing membranes were initiated but rarely completed, and after an initial swelling the bacteria soon looked lysed. Tonoplast, plasmalemma and even the cell wall disintegrated completely. In some cells in regions I and II a quite extensive system of membranes was found around and between collapsing bacteria, but never enclosing them completely (Pl. 16, fig. 30). In older parts of the nodule there was a vigorous development of bacteria in the quite abnormally distended cell walls, together with a complete collapse of all cellular contents including any bacteria accidentally released into the cell. The structural pattern of ineffectivity in this combination strongly suggested a pathogenic condition.

DISCUSSION

The present investigation has provided some information about the structure and development of the infection thread by means of which the bacteria enter new cells, and it has demonstrated that bacterial development in the host cell, and perhaps also emergence from the thread, are closely linked with the state of the host cytoplasm.

Although some bacterial multiplication occurs in infection threads and vesicles, the bulk probably occurs after emergence into the host cytoplasm. The accumulation of carbohydrate reserves in bacteria prior to emergence suggests, by analogy with Hydrogenomonas (Schlegel *et al.* 1961) and other bacterial growth studies, that such bacteria have reached a stationary growth phase. Schaede (1940) also noted that bacteria immediately after entry into the cytoplasm divided much faster than before. According to electron-microscope evidence emerged bacteria divided

only in the 'active' cytoplasm of physiologically young cells, whereas they swelled quickly and stopped dividing in the more 'dilute' cytoplasm of expanding cells. This disagrees with the widely accepted view, based on isolation experiments, that rod-shaped bacteria continue dividing until they reach the bacteroid form. Since, however, emergence is evidently a continuous process, and some recently emerged bacteria with relatively unchanged structure were found even in the bacteroid zone (region III), samples used for isolation experiments almost certainly contained bacteria at different stages of development. It is also possible that rod-shaped bacteria from region II can reproduce in culture and yet fail to do so in the nodule environment. Since the rate of vacuolation of the host cell must to some extent be influenced by the parent plant some observations relating growing conditions and nodule structure could also be interpreted on this basis. Schaede (1941) observed that nodules from rapidly growing plants contained relatively few large bacteroids in cells with large central vacuoles, whereas those from poorly growing plants had cells with small vacuoles containing many smaller bacteroids. Also Thornton's (1930*b*) observation that darkened lucerne seedlings contained many infection threads with a tendency to swell into zoogloeal masses, could be related to the known effects of etiolation on cell expansion and vacuolation. If then the important phase of bacterial multiplication occurs in cells with an 'active' cytoplasm, the cytoplasmic activity induced in prospective host cells by the approaching infection thread, and the early emergence from immature threads are of particular importance in the formation of an effective nodule. The host cytoplasm may also play a role in emergence. The retention of bacteria within the infection thread in some ineffective nodules that conspicuously lacked 'active' cytoplasm suggests this, and the electron micrographs of emerging bacteria do not suggest that internal pressures within the thread, to which emergence has been attributed, are likely to be a primary cause. If the host cytoplasm performed some active function in emergence, this would explain how the infection thread passes from the root hair to the nodule site without shedding bacteria in the passage cells of the cortex which are highly vacuolated and have only a narrow peripheral layer of cytoplasm.

How rhizobia enter a root hair and how the infection thread passes across cell walls has long presented a problem, because in culture rhizobia have neither pectolytic nor cellulolytic ability. Sahlman & Fåhraeus (1964) have obtained electron micrographs of entry into root hairs which, in their opinion, support an invagination hypothesis. From the present work no definite conclusion can be reached until more is known about the nature and origin of the thread matrix. If this is a bacterial secretion entry is probably brought about by bacterial action, direct or indirect, on the cell wall. The unexpected frequency of pockets of bacteria and thread matrix in distended splits of the middle lamella, also noted by Jordan *et al.* (1963) in alfalfa, suggests that, within the nodule, bacteria can exert some modifying influence on cell wall structure, especially when in a confined space. If, on the other hand, the thread matrix is some modified cell wall constituent, this would support invagination as a means of entry. Plasmodesma pores are clearly too small to offer any assistance as mechanical openings, although they may serve as channels of conduction for secretions from an infection thread, especially if the intra-reticular space is the site of the infection.

The possible significance of membranes isolating the symbiont or parasite from its host has been discussed by Nutman (1963) and Garrett (1963). It is argued that intra-cellular symbionts and avirulent parasites may invaginate the cytoplasmic membrane without actually penetrating it, and so be less damaging to the host cell. In nodules the membrane at the tip of an infection thread is fragmentary so this concept may over-simplify the issue. That membranes by themselves are not always sufficient barriers against mutually destructive effects was demonstrated in one type of ineffective nodule where the uncontrolled formation of such membranes was a feature. There is, however, in the normal nodule enough affinity between the host cytoplasm and the bacterial surface to allow the enclosing plant membrane to function as if it were part of the bacterial wall, and to allow new membranes to become reorganized rapidly around the maturing infection thread and emerging bacteria without more disturbance than occurs in the formation of a new membrane at cell division.

Whether the membrane enclosing the bacteroid is indeed the primary site of nitrogen fixation remains to be proved. Since nitrogen fixation is generally regarded as confined to the bacteroid zone whilst enclosing membranes are formed immediately the bacteria emerge from the infection thread, these membranes can hardly be more than a necessary prerequisite for fixation, with other factors initiating the process.

This investigation was started at the suggestion of Dr P. S. Nutman and owes much to his continued interest and enthusiasm. I also acknowledge with grateful thanks the advice of Mr H. L. Nixon, and the work of Mr R. D. Woods and Mr A. A Welch who did all the photography. Their technical knowledge and patient help were of the greatest assistance.

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EXPLANATION OF SYMBOLS ON PLATES

B	Bacterium	Pd	Plasmodesma
Bd	Bacteroid	Pl	Plasmalemma
CP	Carbohydrate platelet	RR	Rough reticulum
CW	Cell wall of host cell	TM	Thread matrix
Cy	Cytoplasm of host cell	Tmb	Thread membrane
ER	Endoplasmic reticulum	TW	Thread wall
G	Golgi body	V	Vacuole of host cell
IRS	Intra-reticular space	Ve	Vesicle
IS	Intercellular space	bw	Bacterial wall
IT	Infection thread	nr	nuclear region of bacterium
M	Mitochondrion	cm	Cytoplasmic membrane of bacterium
ML	Middle lamella	cm	Enclosing membrane of plant origin
N	Nucleus of host cell	sc	Storage carbohydrate (PHBA?) of bacterium
Nml	Nuclear membrane		
P	Proplastid		

EXPLANATION OF PLATES

Unless otherwise stated all micrographs are from tissue of nitrogen-fixing nodules of *Trifolium parviflorum* (inoculated with *Rhizobium trifolii* strain SU 297, fixed in 2% (w/v) potassium permanganate.

PLATE 1

Fig. 1. Meristematic cell (region I) containing an infection thread (cut transversely) and many bacteria distributed through the cytoplasm. The uninfected cell (above) contains proplastids with normal starch grain precursors; in the infected cell the precursors are shrunk or absent.

Fig. 2. Meristematic cell (region I) with an infection thread (cut longitudinally) passing across two cell walls and shedding bacteria in the vicinity of the nucleus. An intra-reticular space (top left), is apparently connected with a large pore in the nuclear membrane. Other large pores occur in the central nucleus. The membrane of the intra-reticular space (middle right) is continuous with the endoplasmic reticulum.

PLATE 2

Fig. 3. An infection thread spreading from the cell wall into two adjacent cells. Around the thread tips and the small thread protrusion (middle right) the plant membrane enclosing the thread is fragmentary. In the lower cell the normal cytoplasmic pattern is much disturbed and there are two large intra-reticular spaces. At Pd the endoplasmic reticulum appears to pass through a plasmodesma pore. Bacteria marked B are about to emerge from the infection thread.

PLATE 3

Fig. 4. An infection thread lying in a distended cell wall. The thread matrix (TM) is distinct from the substance of the middle lamella (ML). Bacteria about to divide are indicated by arrows.

Fig. 5. Mature infection threads in highly vacuolated cells (region II).

PLATE 4

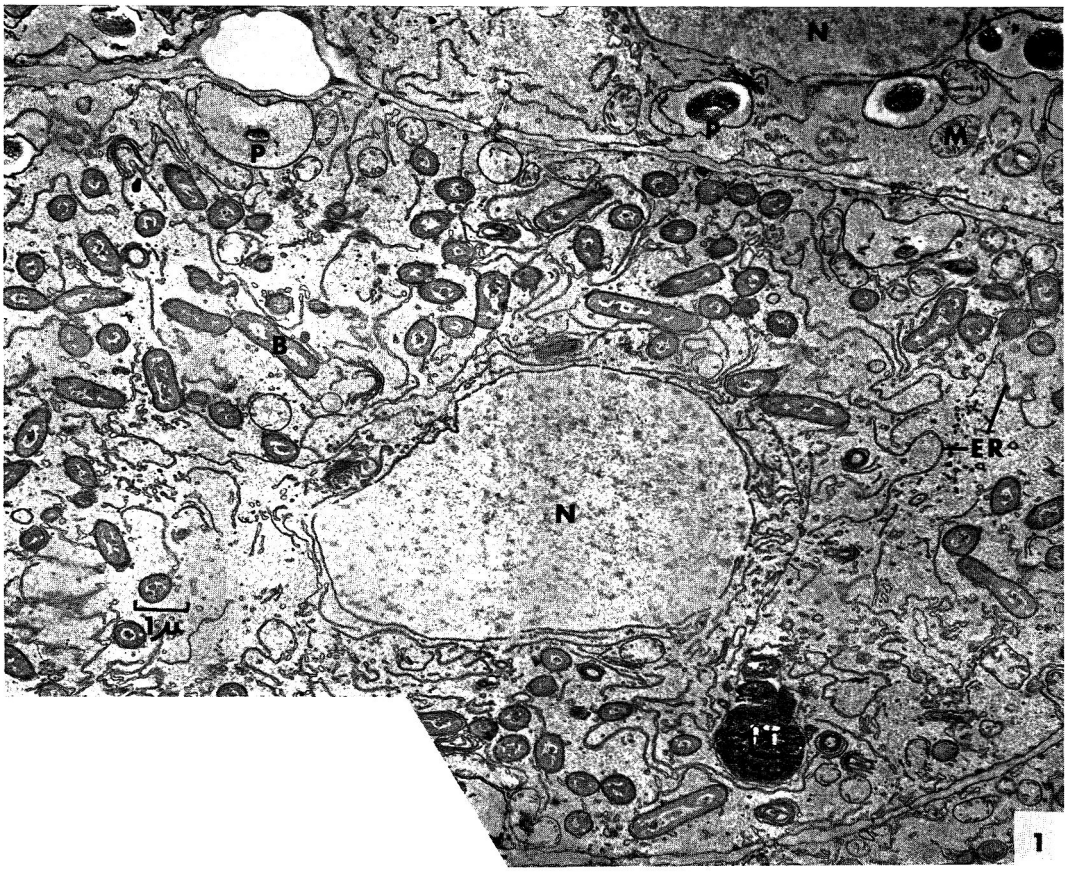
Fig. 6. Tip of an infection thread lying in an invagination of the host nucleus. The enclosing membrane of host origin is fragmentary or absent.

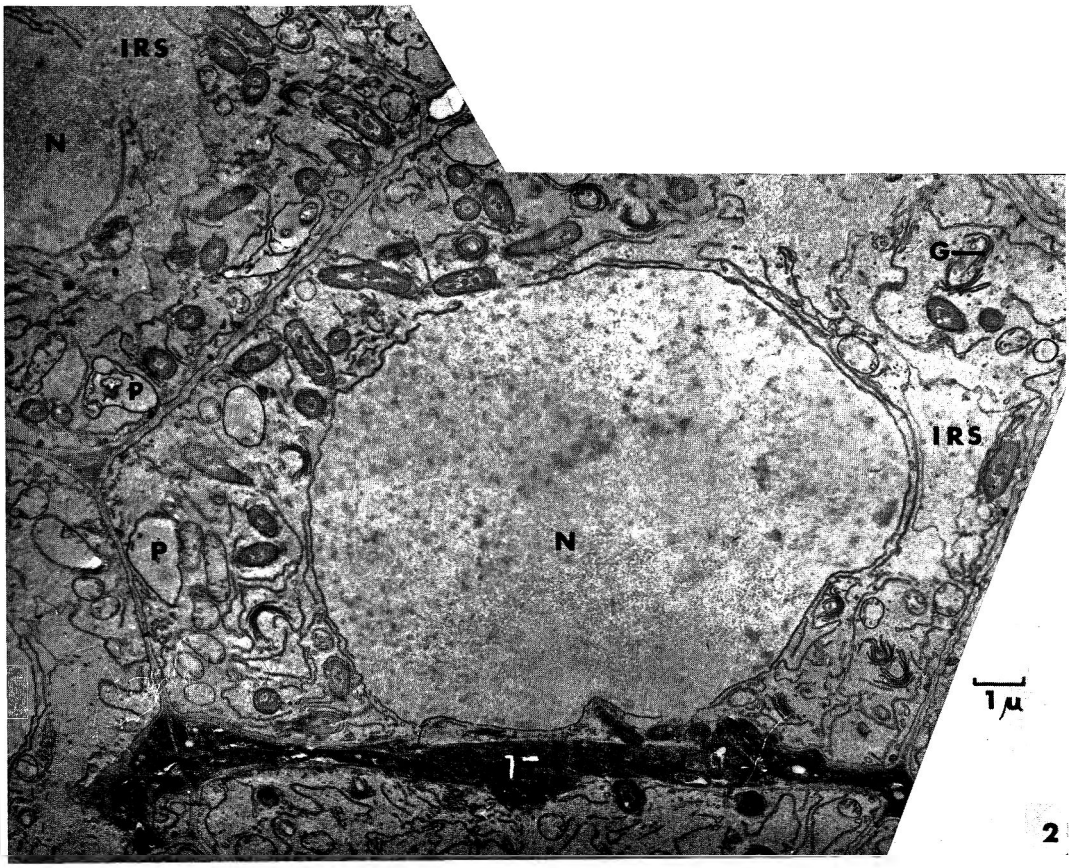
Fig. 7. Infection thread tip, situated near a nucleus not shown on the micrograph. The bacterium (B) is emerging, leaving behind an electron-transparent area that is not immediately filled up with thread matrix. (See also inset.)

PLATE 5

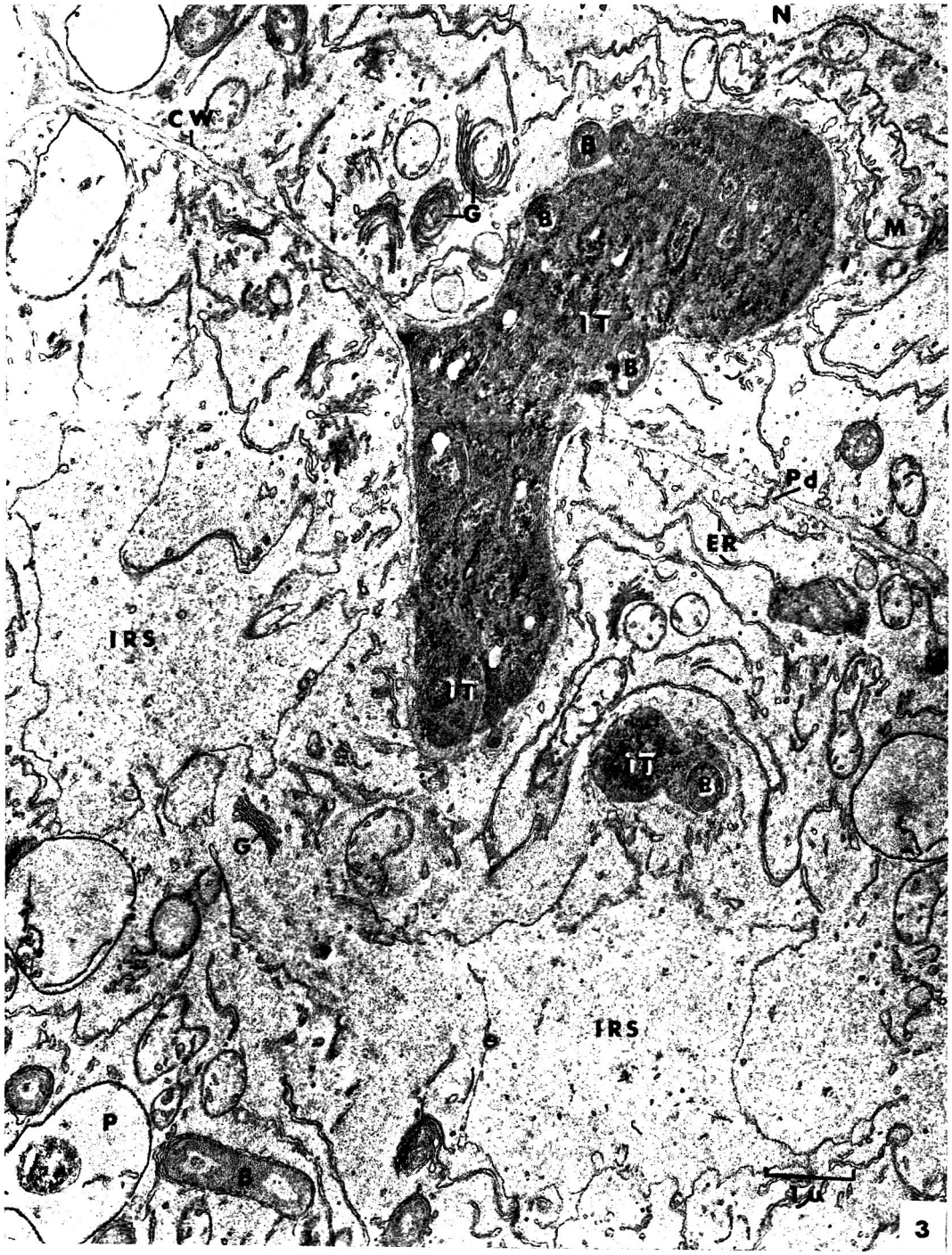
Fig. 8. Localized swellings of an infection thread containing bacteria, region II. (Freezing-microtome section.)

Fig. 9. Distended part of an infection thread in region II. The bacterium indicated by the arrow is near to emergence. Caps of thread matrix attached to the outside of the infection thread remain within the enclosing plant membrane.





(*Facing p.* 64)



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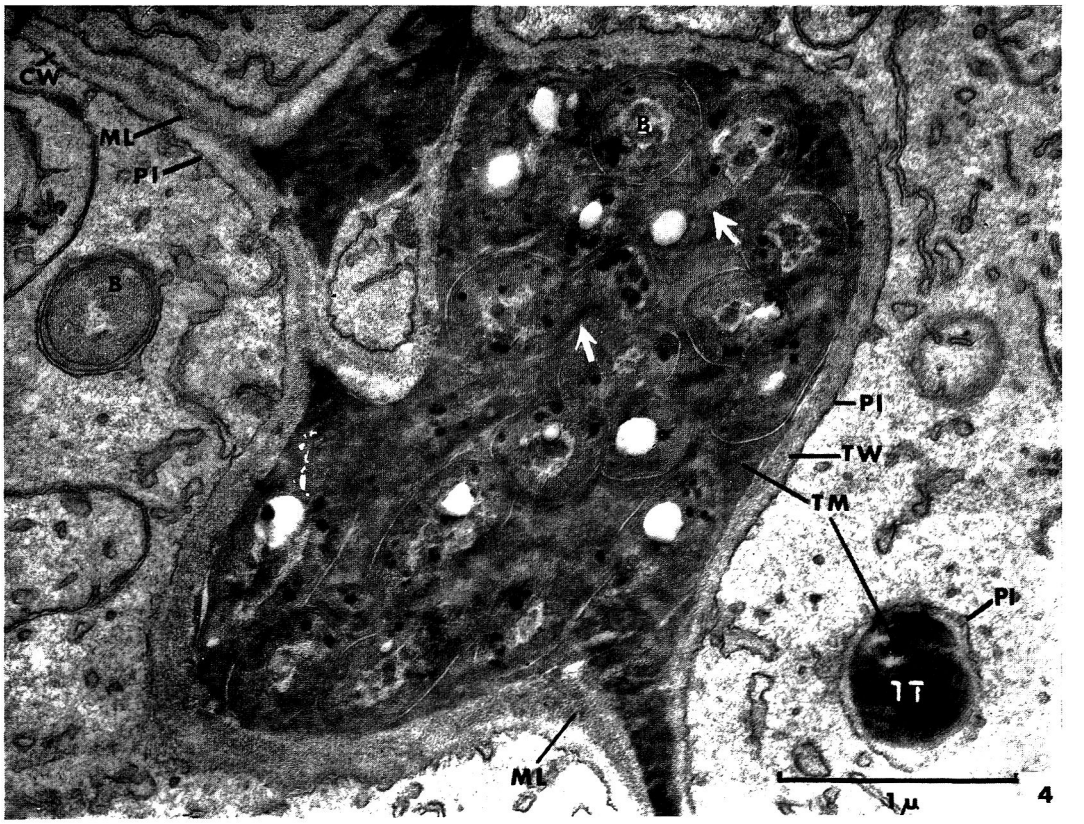
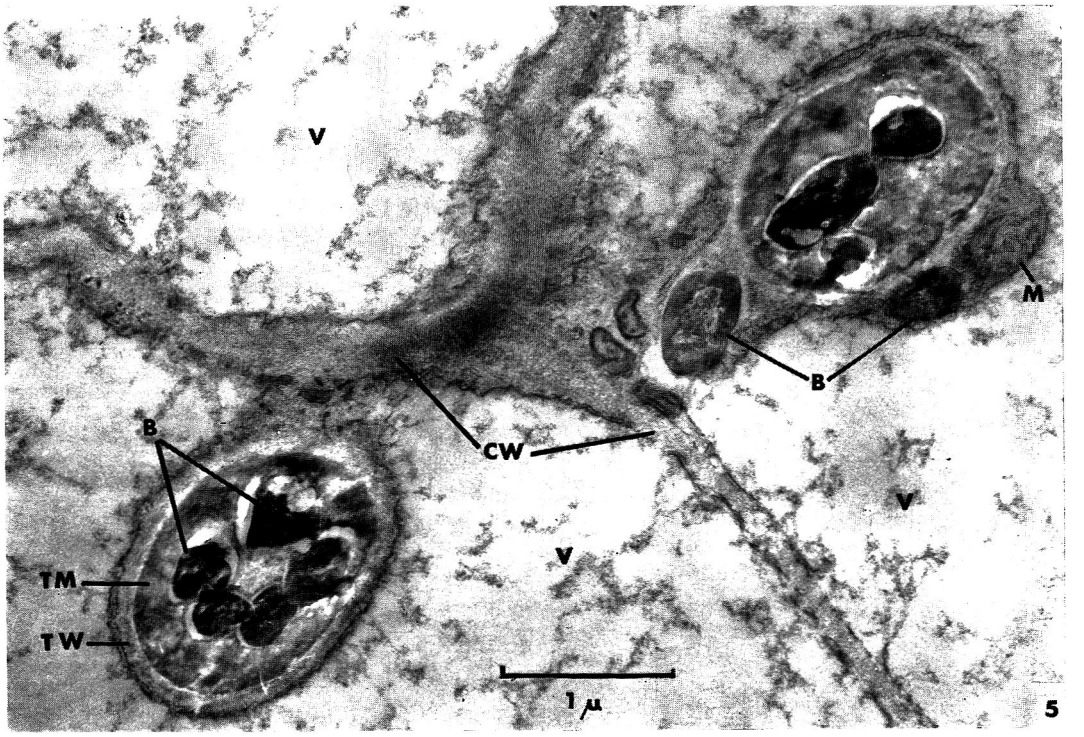
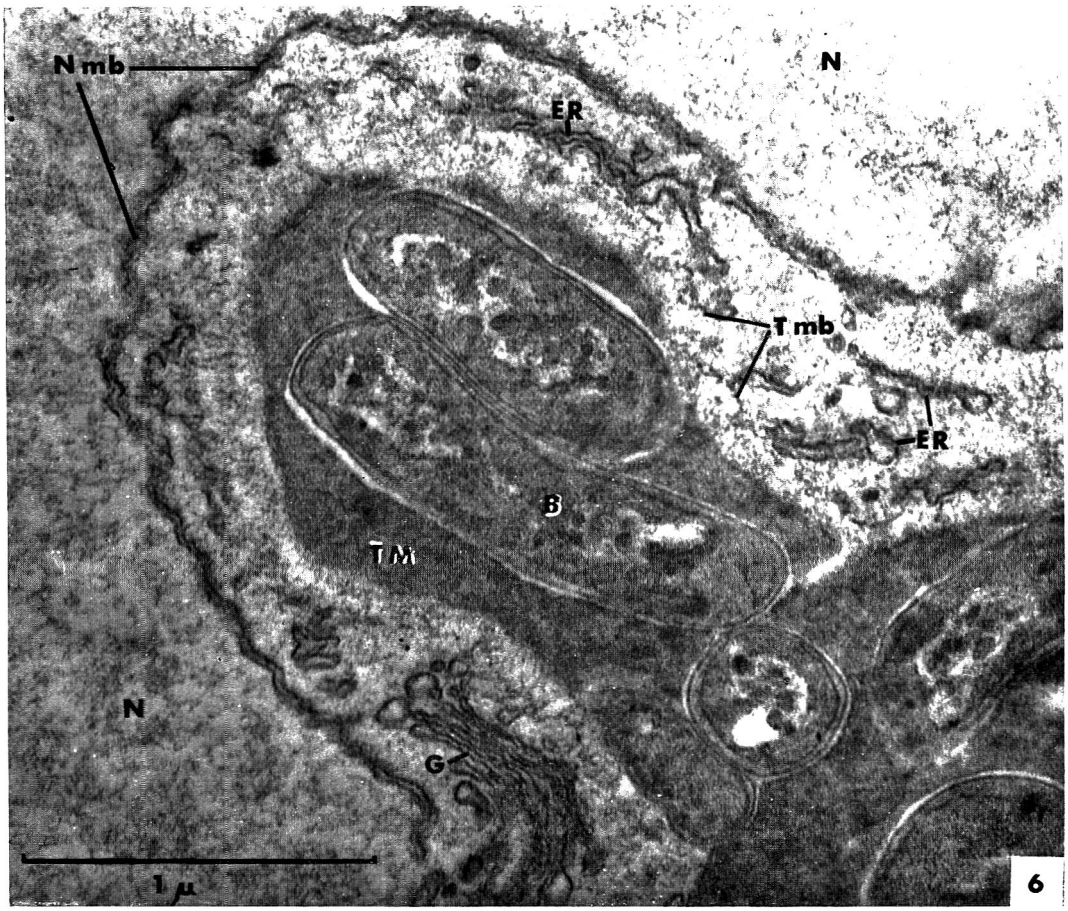
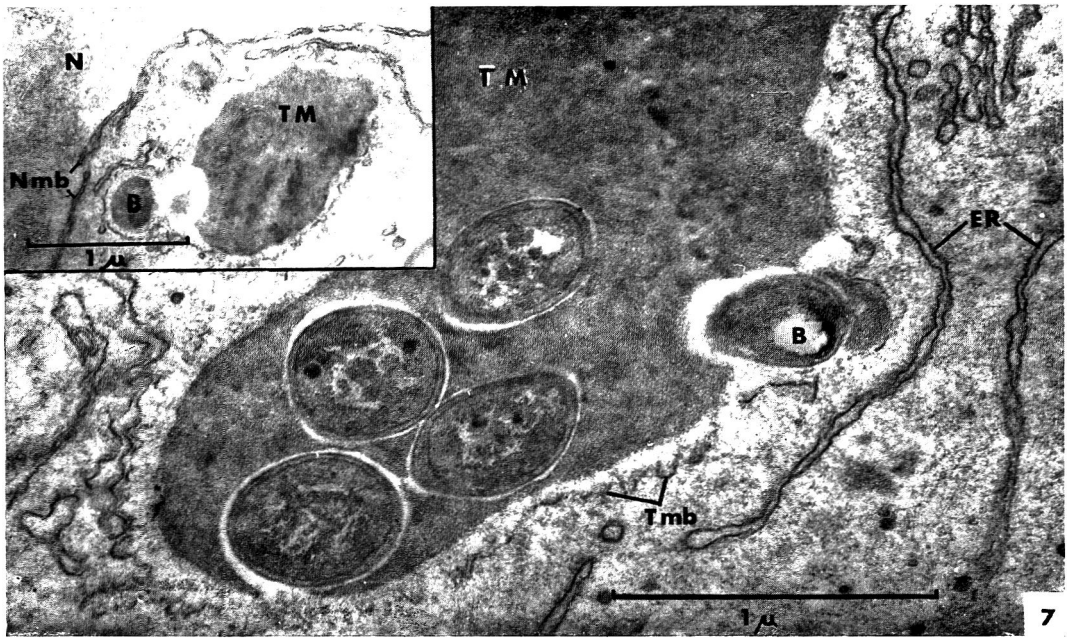

CW
ML
PI
B
B
PI
TW
TM
PI
TT
ML
1 μ
4


V
M
B
B
CW
V
V
TM
TW
1 μ
5


N mb
N
ER
T mb
ER
B
TM
N
G
1 μ
6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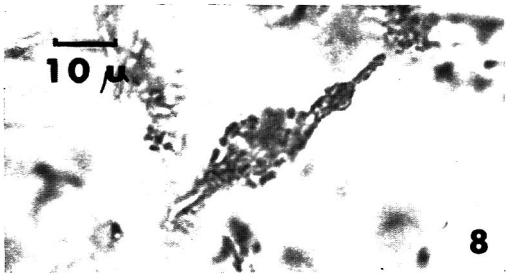
TM
Nmb
B
1 μ
TM
ER
B
T mb
1 μ
7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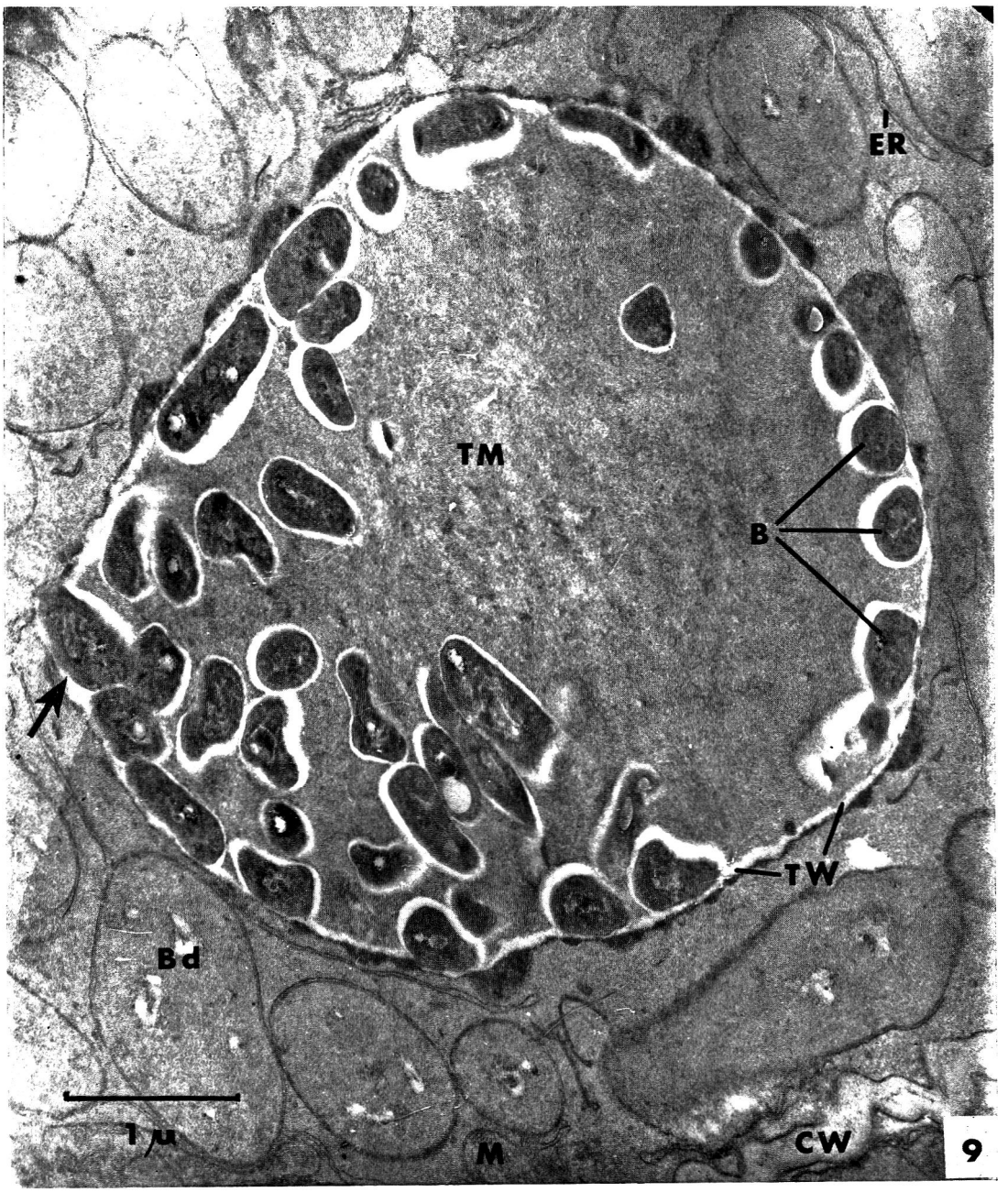
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TW
Bd
1 μ
M
CW
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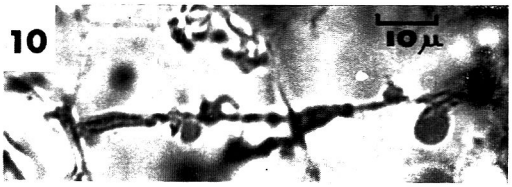

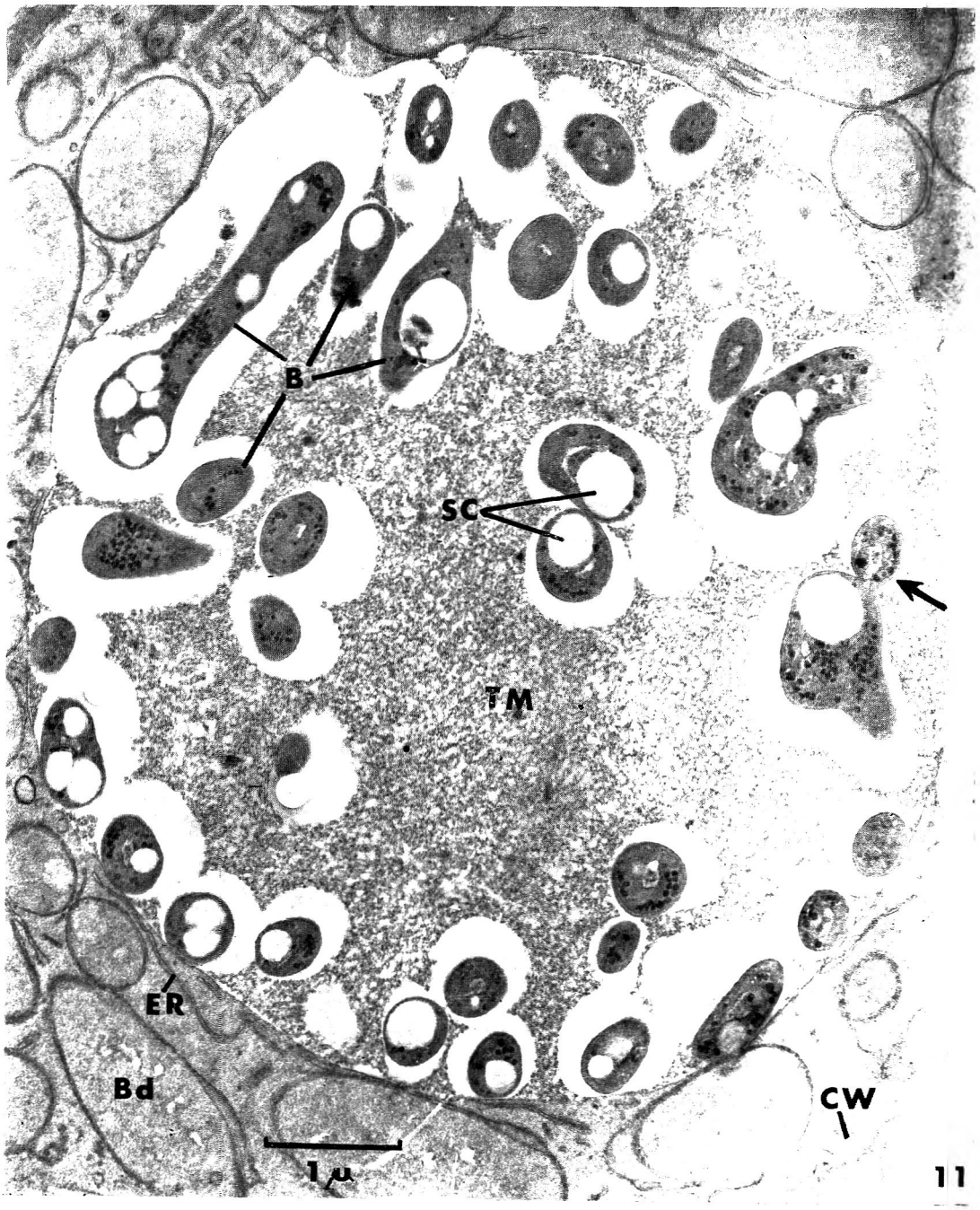

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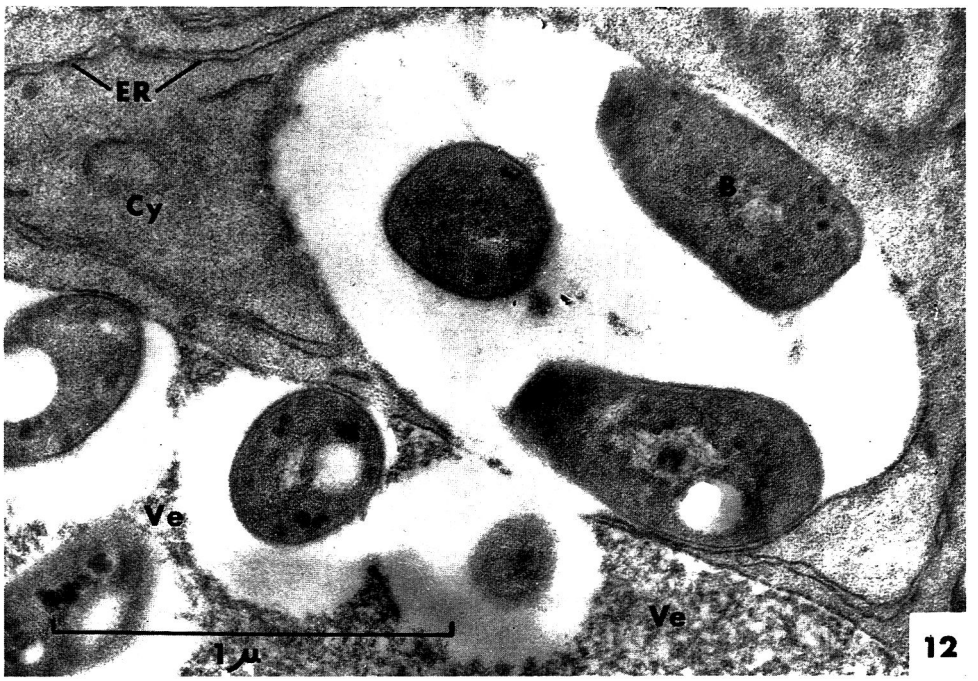
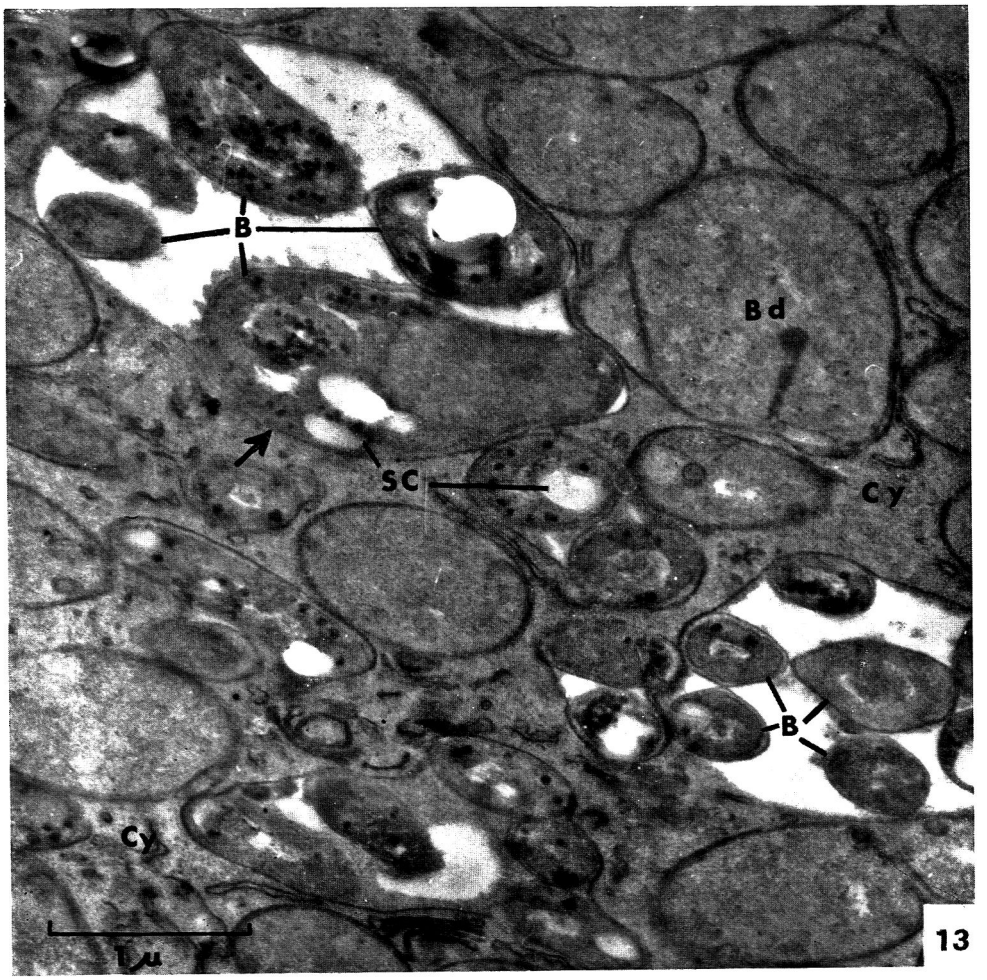

ER
Cy
B
Ve
Ve
1 μ
12


B
Bd
SC
Cy
B
Cy
1 μ
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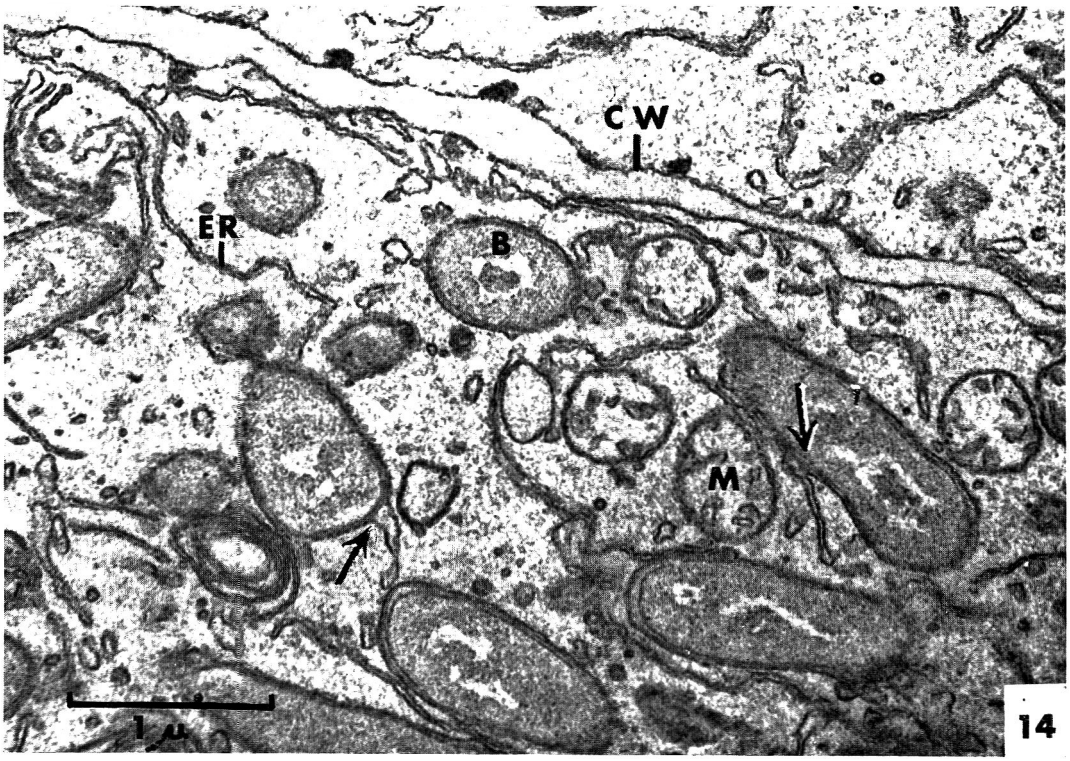
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ER
B
M
1 μ
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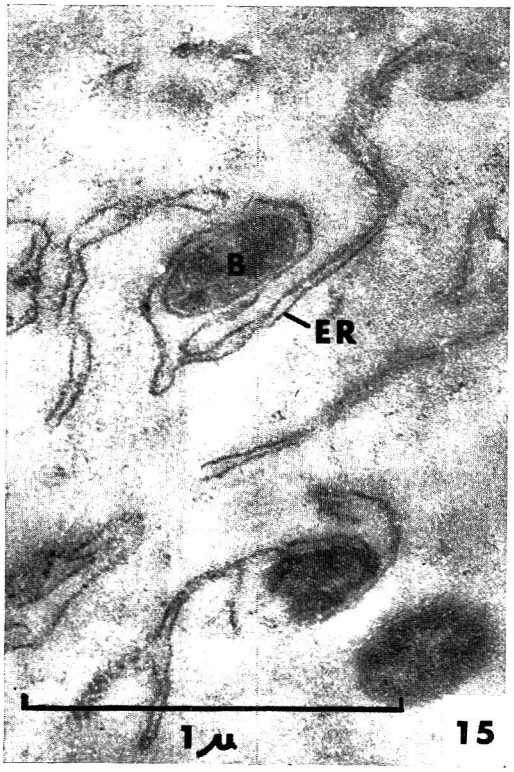
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ER
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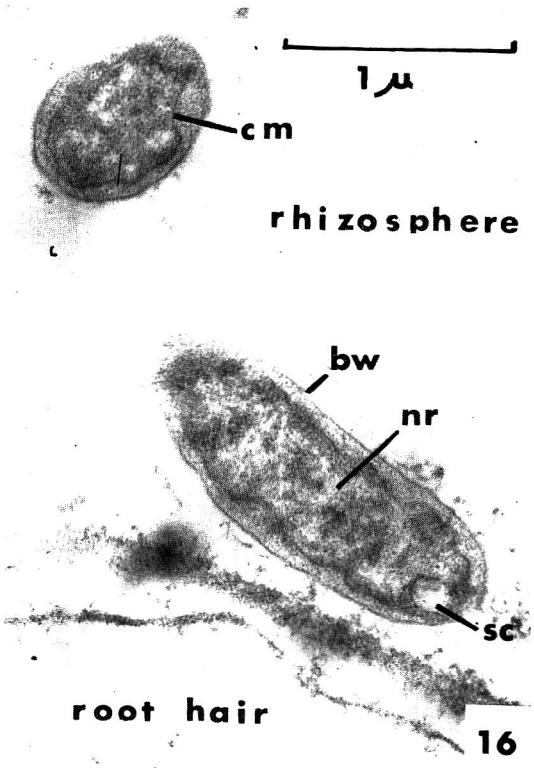
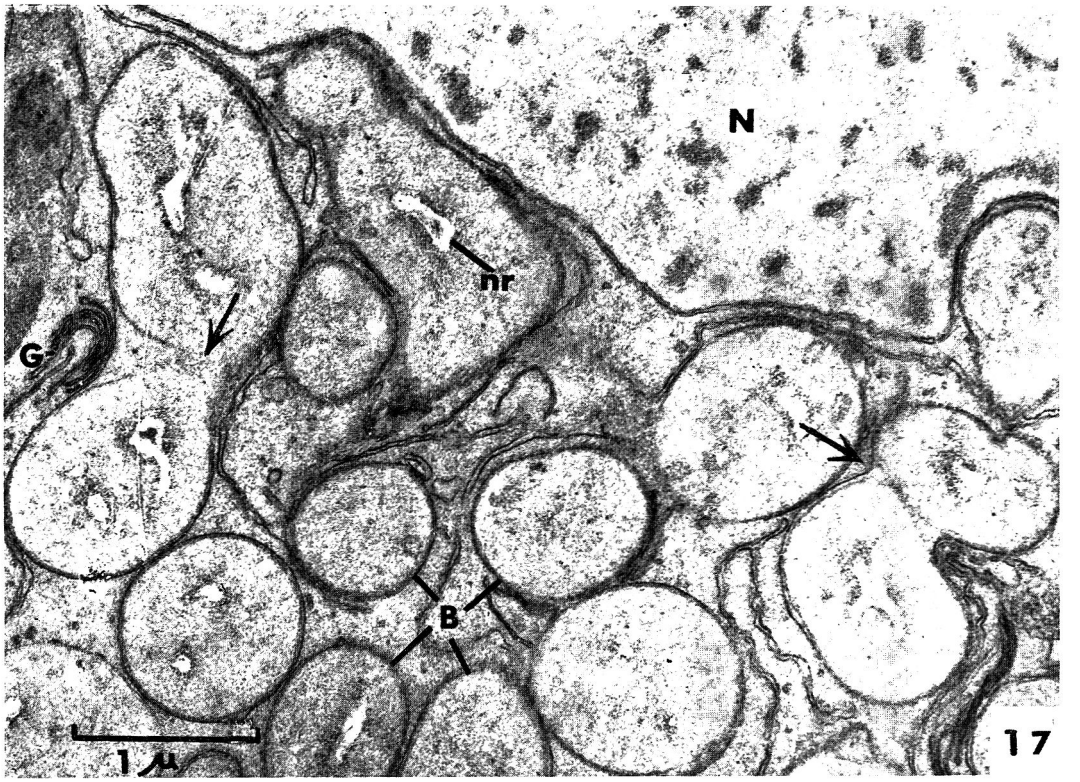

1 μ
cm
rhizosphere
bw
nr
sc
root hair
16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nr
G
B
1 μ
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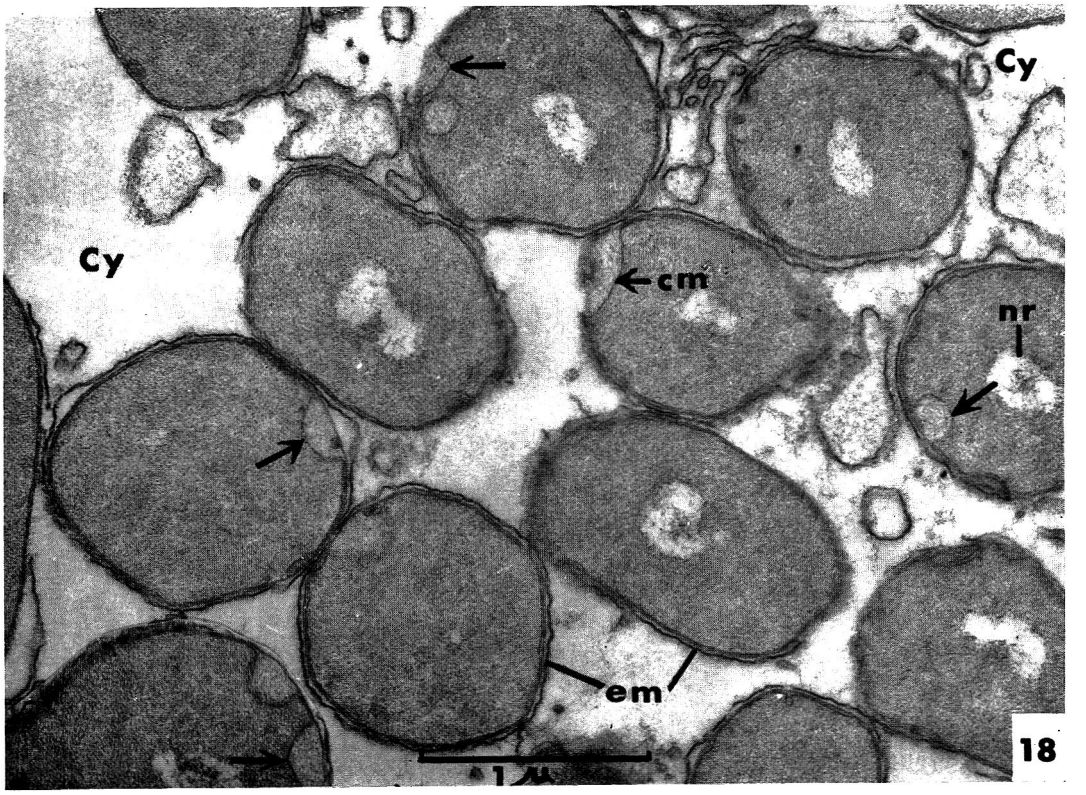
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cm
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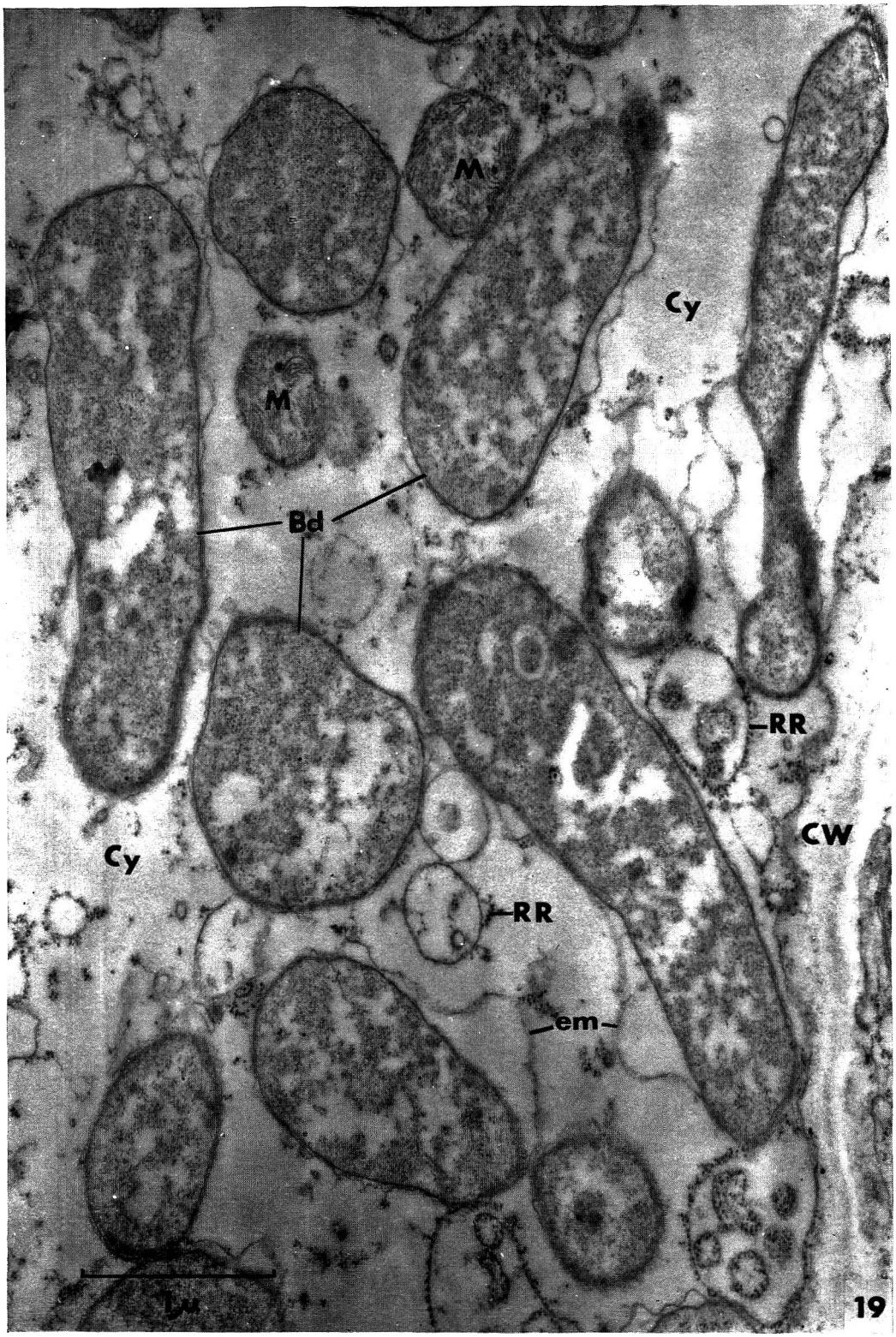

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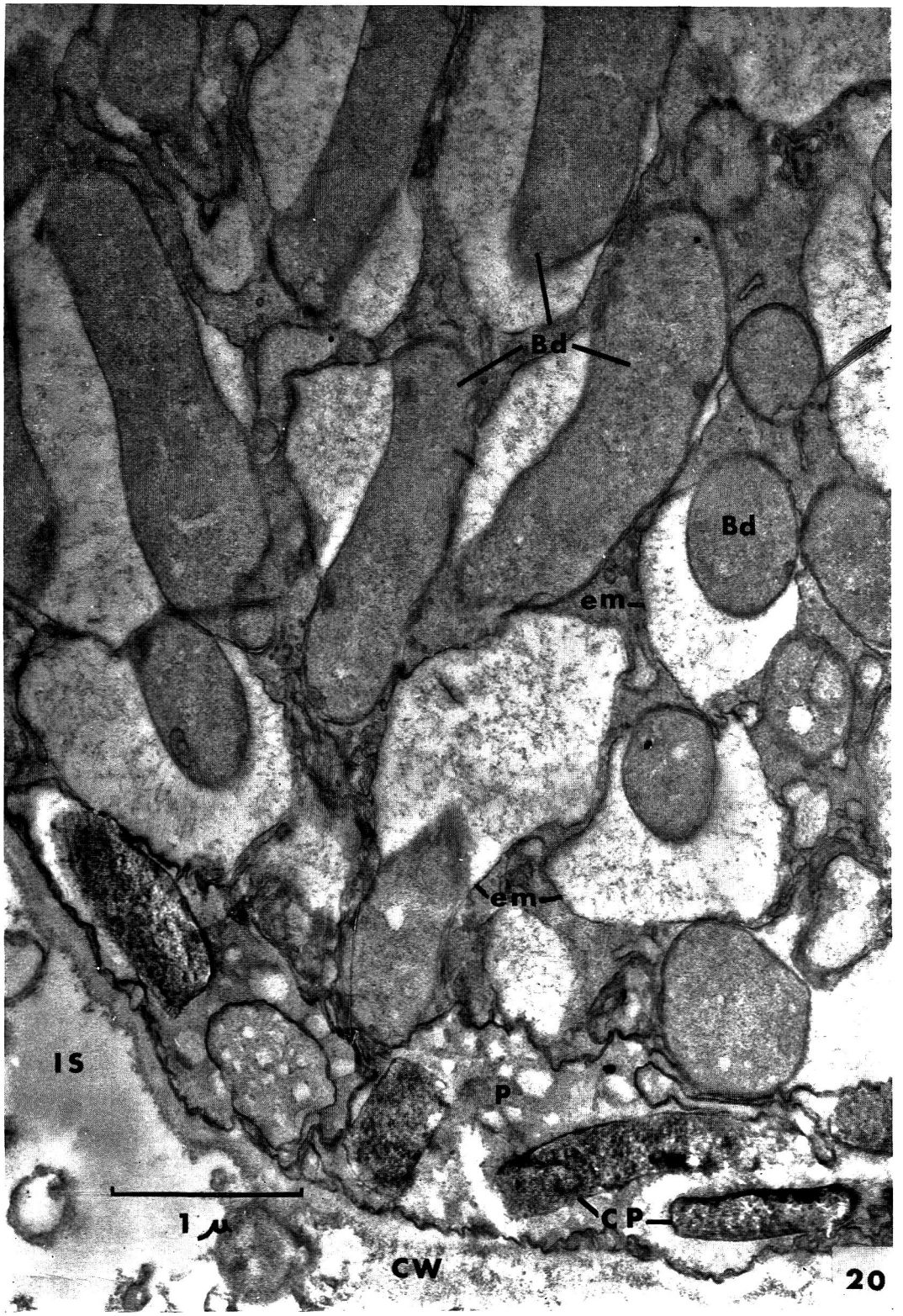

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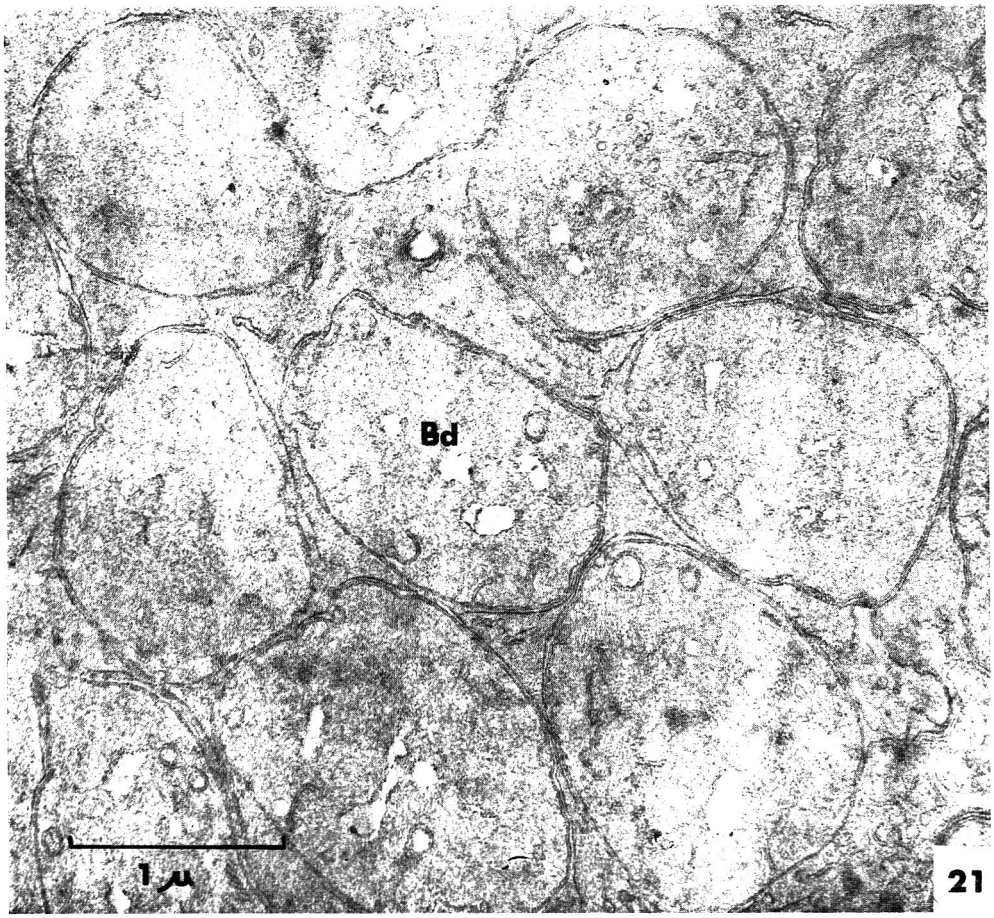
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1 μ
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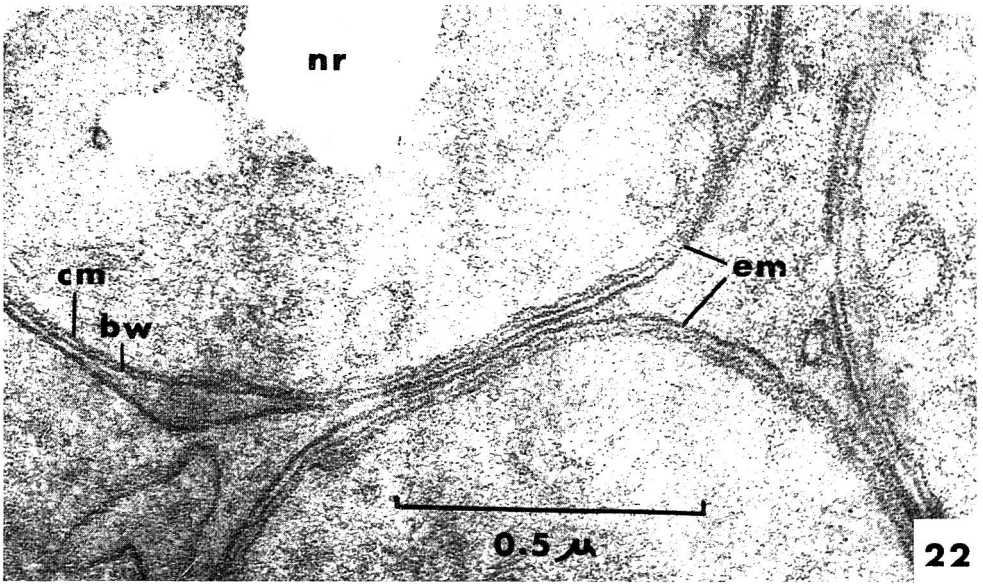
nr
cm
bw
em
0.5 μ
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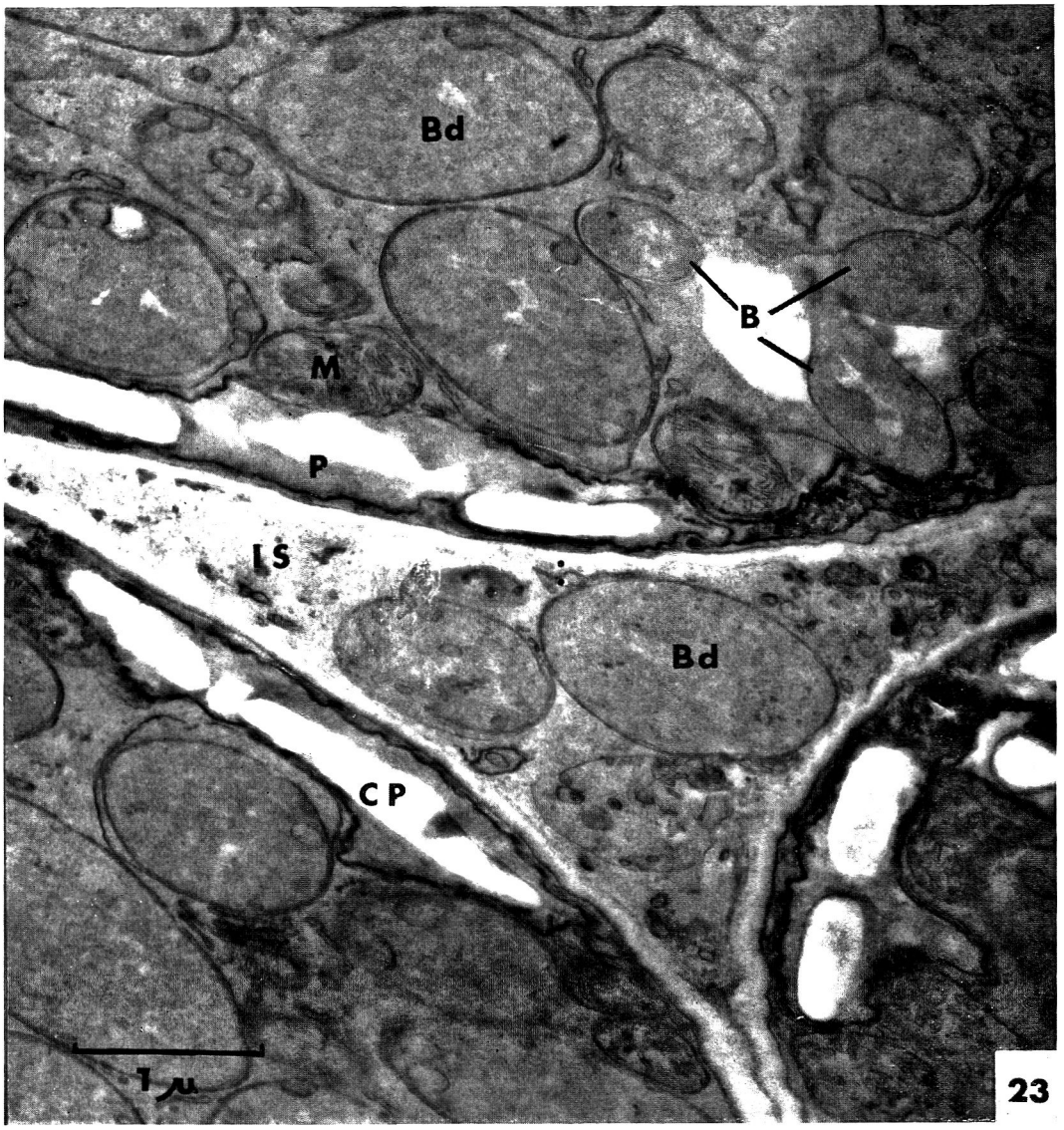
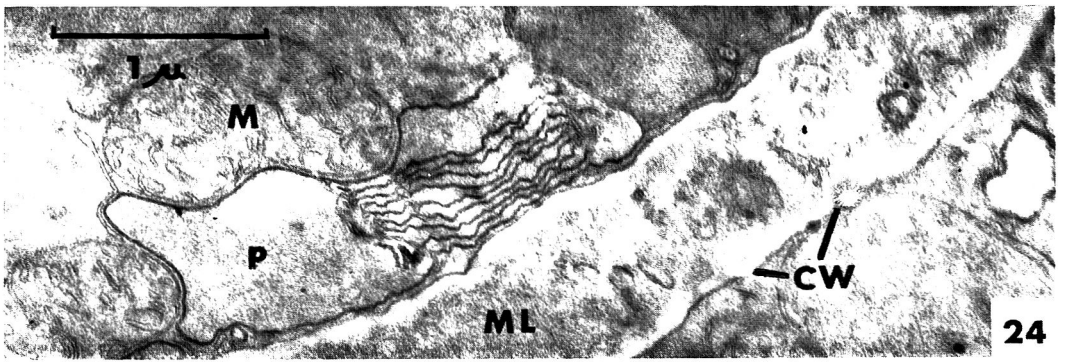

Bd
B
M
P
IS
Bd
CP
1 μ
23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M
P
CW
ML
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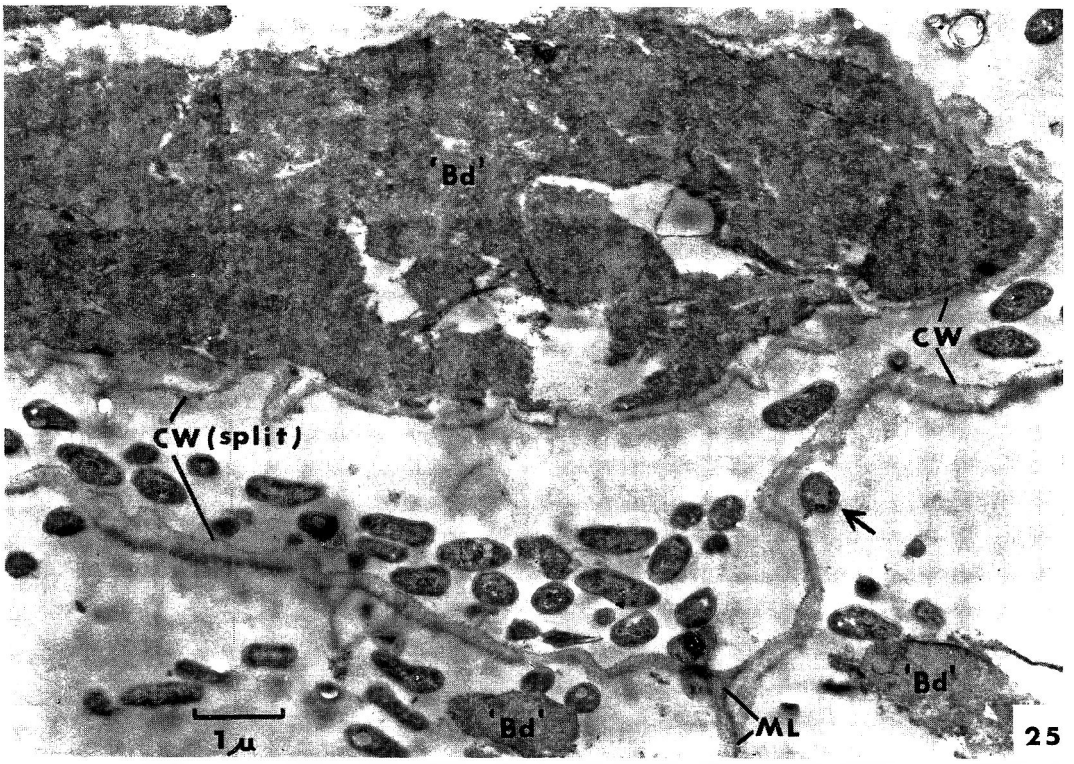
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CW
CW (split)
'Bd'
'Bd'
ML
1 μ
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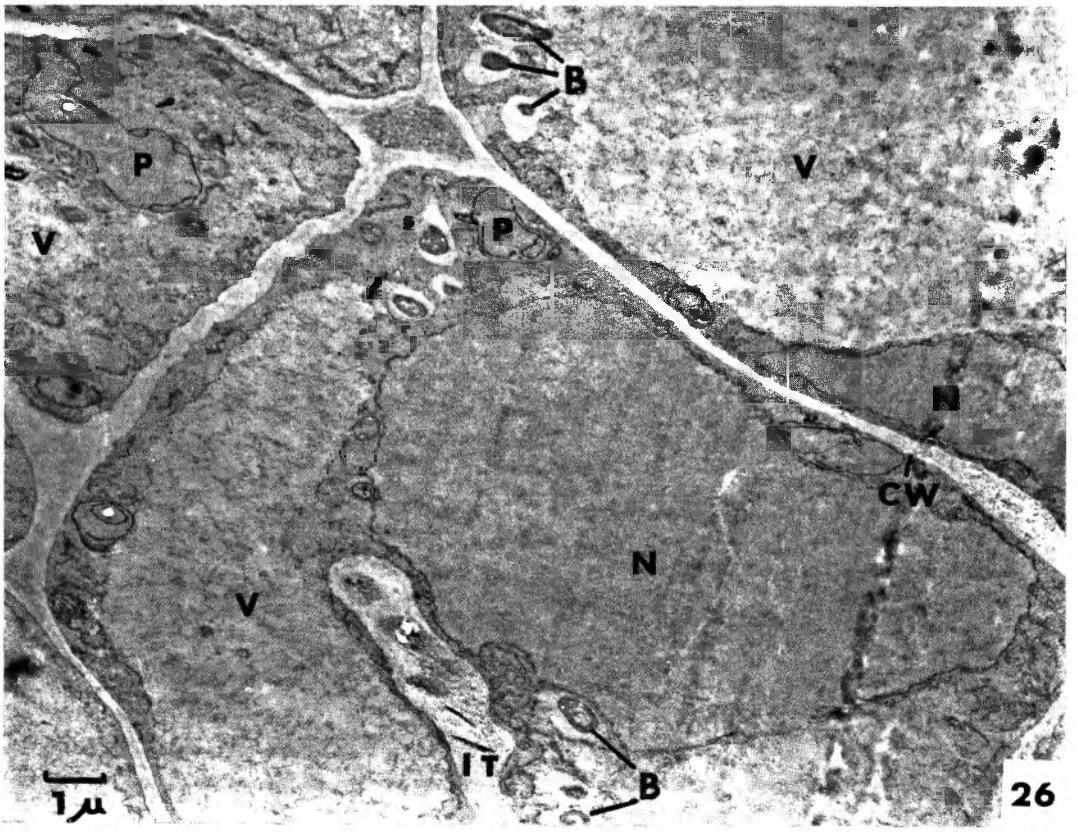
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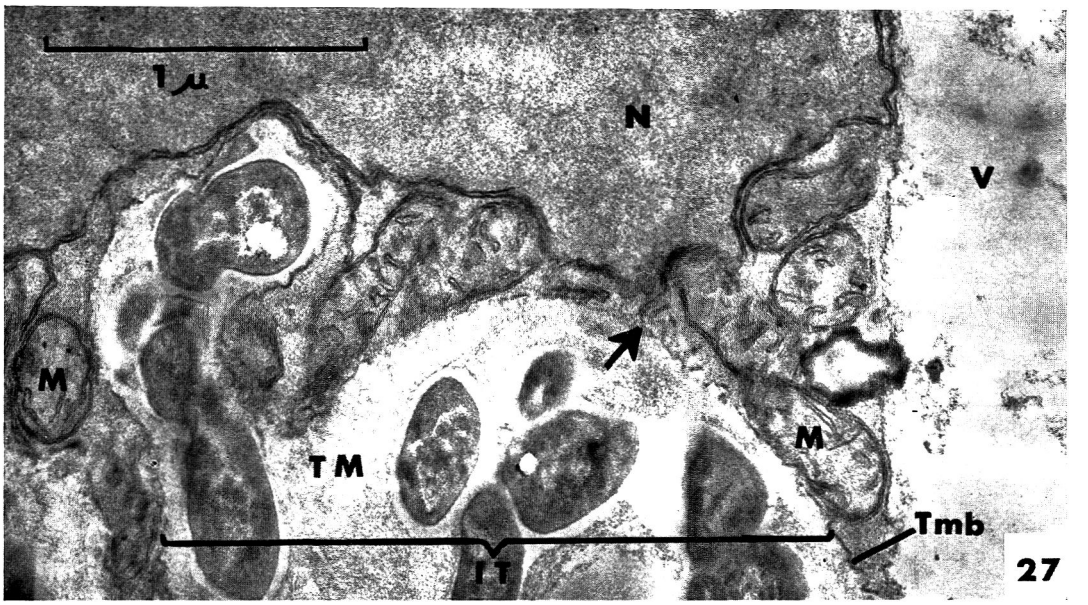
P
V
P
V
N
CW
N
V
IT
B
1 μ
26


1 μ
N
V
M
TM
M
Tmb
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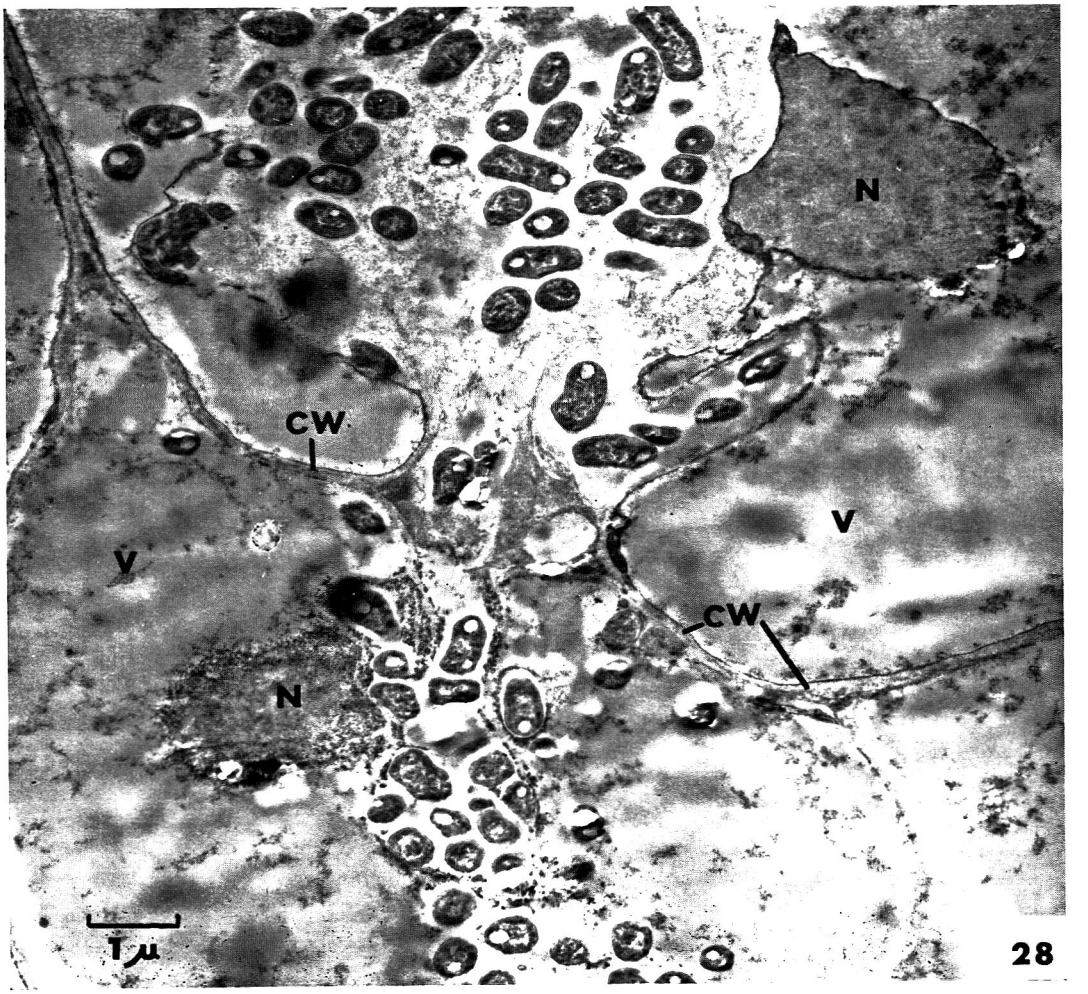
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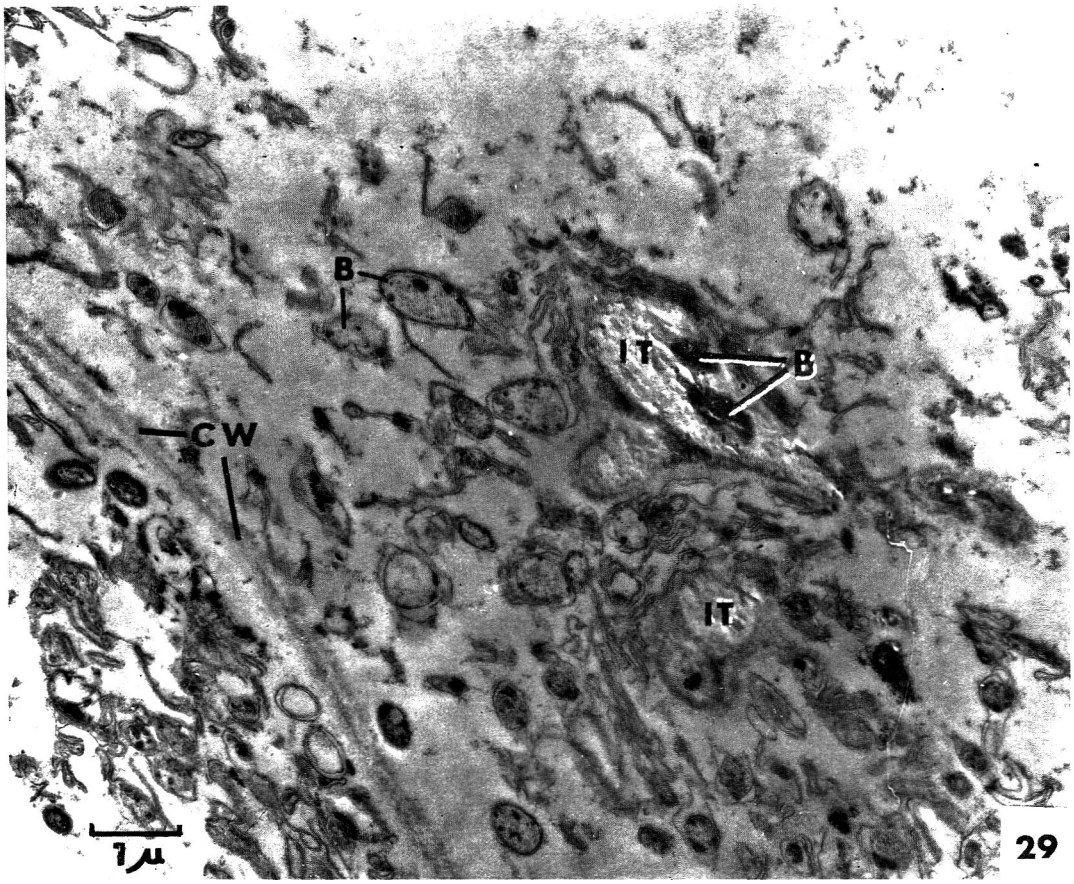
CW
V
V
CW
N
1 μ
28


B
IT
B
CW
IT
1 μ
29

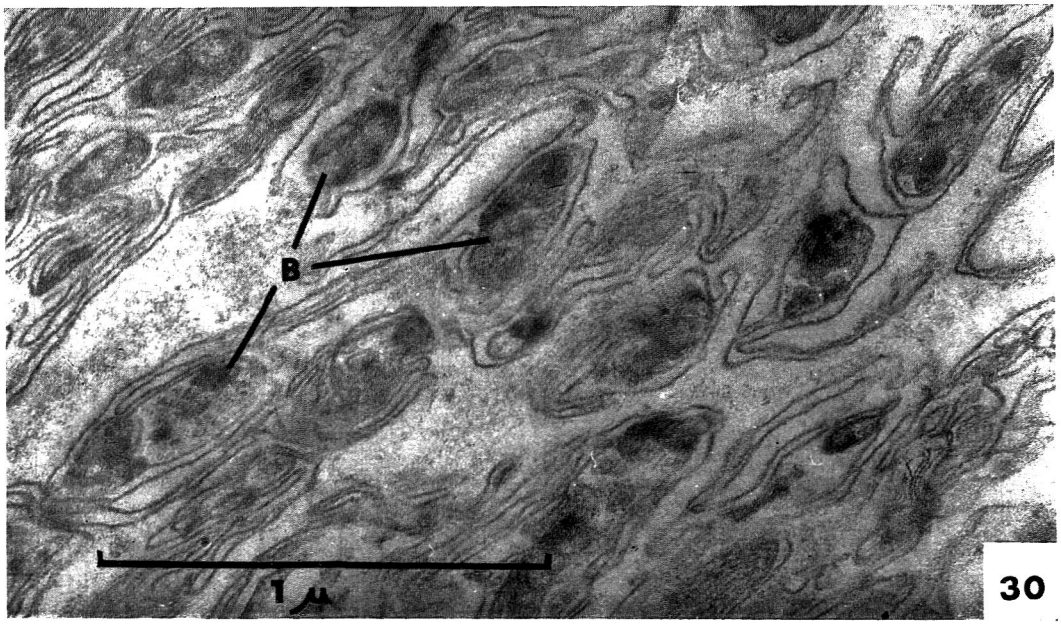

B
1 μ
30

PLATE 6

Fig. 10. Lateral vesicles on an infection thread in region II. (Freezing-microtome section.)

Fig. 11. Section of a vesicle in region II. The bacteria contain transparent vacuoles (sc) interpreted as storage carbohydrates, and dense, approximately polyhedral granules resembling precursor phage particles. The arrow indicates a bacterium about to divide by budding.

PLATE 7

Fig. 12. Emergence of a group of bacteria with their surrounding transparent region from a vesicle in region II.

Fig. 13. Late emergence of bacteria in region III. Groups of bacteria, still surrounded by an electron-transparent zone, are enclosed by a plant membrane. The swollen bacterium (marked by arrow) is distending the enclosing membrane preparatory to emergence. Dense polyhedral particles are confined to bacteria still protected by the transparent zone or only recently emerged from it.

PLATE 8

Fig. 14. Bacteria recently emerged into a meristematic cell in region I. At arrows the continuity of the enclosing membrane with the endoplasmic reticulum of the host cell is clearly shown.

Fig. 15. Recently emerged bacteria in region I of an ineffective nodule (*T. repens* inoculated *R. trifolii* H.K.C.). Characteristically the enclosing membrane retains open connexion with the endoplasmic reticulum. (See also Pl. 16, fig. 30.)

Fig. 16. Rhizobia in the rhizosphere.

PLATE 9

Fig. 17. Division of bacteria in the host cytoplasm (region I). The dense cytoplasm, large nucleus, Golgi body and endoplasmic reticulum are evidence of the active state of the host cell. The nuclear region of the bacteria is much contracted.

Fig. 18. Bacteria is an expanding host cell (region II) beginning their transition to the bacteroid form. Invaginations of the bacterial cytoplasmic membrane are marked by arrows. The endoplasmic reticulum of the host cell is tending to break up into vesicles, and the cytoplasm is becoming more dispersed.

PLATE 10

Fig. 19. Osmium tetroxide fixation. Late stage in the transition to the bacteroid form. The small granules attached to the plant membranes (RR) are interpreted as RNA. The expanded plant membrane around the bacteria is characteristically without granules.

PLATE 11

Fig. 20. Final stage in the transition to the bacteroid form. The space between the much expanded enclosing membrane and the bacterium is filled with a substance resembling vacuolar fluid. Precursors of a carbohydrate storage material laid down as rectangular platelets appear in the peripheral plastids.

PLATE 12

Fig. 21. Bacteroids in region III of a nitrogen-fixing nodule of *T. repens* inoculated *T. trifolii* Cl.F. Each bacteroid has three membranes.

Fig. 22. Detail of bacteroid membranes.

PLATE 13

Fig. 23. An intercellular space and parts of three adjacent cells at the boundary between regions II and III. Fully developed carbohydrate platelets characteristically occur opposite the intercellular space. The swollen bacteria in the distended space lack the enclosing membrane of host origin. Bacteria labelled B are emerging into the host cytoplasm.

Fig. 24. Plastid with chloroplast-like system of parallel lamellae in nodules of *T. repens* inoculated *R. trifolii* (Cl. F.).

PLATE 14

Fig. 25. Disintegration of host cell and bacteroids in region IV. A split cell wall containing young bacteria is situated below a cell (top of photograph) containing clumped remnants of bacteroids ('Bd'). Two disintegrating bacteroids are shown in two cells at the bottom of the photograph. A bacterium containing polyhedral particles is marked by the arrow.

Fig. 26. Early stages of infection in region I of an ineffective (non-fixing) nodule of *T. repens* inoculated *R. trifolii* H.K.C. Note the large vacuoles, fragmentary membranes of the host cell, electron-transparent thread matrix, and extensive transparent zones around emerged bacteria.

Fig. 27. Part of an already vacuolated cell and infection thread from region I of an ineffective nodule of *T. parviflorum* inoculated *R. trifolii* Cl.F. Fibrils between the nucleus and the thread tip are indicated by an arrow.

PLATE 15

Fig. 28. Rupture of the cell wall and release of bacteria into the vacuole in region II of an ineffective nodule of *T. parviflorum* inoculated *R. trifolii* H.K.C. The host cell contains only a thin lining of peripheral cytoplasm.

PLATE 16

Fig. 29. Collapse of bacterial and host structure in region I of an ineffective nodule of *T. repens* inoculated with *R. trifolii* H.K.C. Note the infection thread with electron-transparent matrix (middle bottom) and the appearance of lysis in the released bacteria.

Fig. 30. Extensive system of membranes surrounding emerged bacteria in an ineffective nodule of *T. repens* inoculated *R. trifolii* H.K.C.

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Comparative Studies of the Mineral Nutrition of Three Species of Phytophthora

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AND J. H. CHILD

Department of Chemistry and Applied Chemistry, Royal College of Advanced Technology, Salford

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SUMMARY

Nutritional experiments were carried out dealing with: (1) the utilization of sulphur compounds; (2) the utilization of phosphorus compounds; (3) the action of different mineral salts and their interaction. The results showed that *Phytophthora erythroseptica* grew well at 28° and pH 6·6 incubated for 20 days; *P. parasitica* grew well at 28° and pH 6·6 for 17 days; and *P. infestans* at 20° and pH 4·5 for 16 days. In the adjusted controlled medium pH changes were generally within one pH unit. The best carbon and nitrogen sources are stated. The only satisfactory sulphur sources for *P. infestans* were sodium sulphate and sodium thiosulphate. These compounds were also among the best S sources for *P. erythroseptica* and *P. parasitica*, but a number of other compounds also were utilized equally well, e.g. sodium metabisulphite, sodium sulphite, α-cysteine, sodium sulphide, sodium dithionite and methionine. For all organisms the best phosphorus sources were sodium dihydrogen orthophosphate, sodium metaphosphate and lecithin. Rate of utilization of the phosphorus was an important factor in mycelial yield. Factorial experiments were carried out in which *P. erythroseptica* and *P. parasitica* were incubated at 28° for 17 days. Statistical analysis of the results showed that under the given conditions optimal growth measured as mg. dry wt. was obtained in liquid media containing glucose, 25 g./l.; DL-asparagine, 4·0 g./l.; $FeSO_4.7H_2O$, 0·001 g./l.; thiamine, 0·8 mg./l.; $ZnSO_4.7H_2O$, 1 p.p.m.; H_2MoO_4, $CuSO_4.5H_2O$, 0·02 p.p.m.; with varying amounts of K_2HPO_4, $MgSO_4.7H_2O$ and $CaSO_4.2H_2O$ for the different species specified. There was a balance between all combinations of K_2HPO_4, $MgSO_4.7H_2O$ and $CaSO_4.2H_2O$ for *P. parasitica* but not for *P. erythroseptica*, but there were significant interactions between the salts taken two at a time for both these fungi. There was interaction for all three salts together for both species. *Phytophthora infestans* and *P. parasitica* are more exacting in their nutritional requirements than *P. erythroseptica*.

INTRODUCTION

Previous work at Newcastle-upon-Tyne (see Fothergill & Raine, 1954; Fothergill & Ashcroft, 1955; Fothergill & Yeoman, 1957; Fothergill & Jones, 1958; Fothergill & Hide, 1962) indicated that for some parasitic fungi the balance of the major inorganic salts in a culture medium was more important for the good growth of the mycelium than the concentration of individual salts, but that with some

saprophytic species the balance was much less important. For a wide range of fungi, however, the results varied. The object of the present experiments was to continue these investigations, with three species of the genus *Phytophthora* which are usually regarded as facultative parasites having only a short saprophytic existence in the soil. While a large amount of work has been done on these species, particularly *P. infestans* concerning taxonomy, cytology and host-parasite relationships, little comparative work seems to have been done on their mineral nutrition. Earlier experiments, such as those of Payette & Perrault (1944), were of small value from the nutritional aspect because of the use of undefined media. More exact investigations of these fungi were carried out by Hall (1959), Sakai (1955, 1956) and Mehrotra (1950).

METHODS

The following organisms were used: *Phytophthora infestans* (Mont.) de Bary, *P. erythroseptica* Pethybridge and *P. parasitica* Dastur. All of them were obtained from the Central Bureau voor Schimmelcultures, Baarn. *Phytophthora infestans* is the cause of Late Blight disease of potato; *P. erythroseptica* is a cause of Pink Rot of potatoes, 'shanking' in forced tulips, wilt in *Atropa belladonna* and other diseases; *P. parasitica* attacks *Ricinus communis* and causes 'damping off' and 'Foot Rot' of tomato and other plants. Throughout this paper the following abbreviations are used: *P. infestans* is designated PI; *P. erythroseptica*, PE, and *P. parasitica*, PP.

Initially single spore isolates of each of these species were prepared according to the method of Hall (1959) and stock cultures were maintained in 1 oz. screw-topped bottles each containing 10 ml. French bean oatmeal agar. The stock cultures were stored at 5° and subcultured at 3-monthly intervals, or when required. No alteration of morphological and cultural characteristics of these fungi occurred during the experimental period. In all experiments cultures were grown in 20 ml. medium in 150 ml. Erlenmeyer flasks. To avoid caramelization of media, sterilization was carried out at 115° for 10 min. After the growth period, the mycelia were filtered on to tared Whatman No. 5 filter-papers and washed with water. The mycelium with the filter paper was then dried overnight at 80–85°, cooled in a desiccator and weighed. Results are expressed as mg. dry wt. mycelium/flask, average of 4 or 5 replicates. It was not practicable to separate the mycelium from the filter paper after filtration, and, as the filter papers used lost 5% of their original weight on drying under the above conditions, appropriate allowances were made. For preliminary experiments the followed basal defined liquid medium A was used. This was a modification of a medium successfully used by Lopatecki & Newton (1956) for organisms PE and PP, in which the amounts of glucose and thiamine present were varied as follows: glucose, 25 g./l.; DL-asparagine, 4·0 g./l.; K_2HPO_4, 1·0 g./l.; $MgSO_4.7H_2O$, 0·1 g./l.; $CaSO_4.2H_2O$, 0·1 g./l.; $FeSO_4.7H_2O$, 0·001 g./l.; thiamine, 0·8 mg./l.; $ZnSO_4.7H_2O$, 1 p.p.m.; H_2MoO_4, $CuSO_4.5H_2O$ and $MnSO_4.4H_2O$ at 2 p.p.m. This solution was initially at pH 4·7.

Experiments were made to determine the best method of inoculation. It was found that a minced mycelium technique gave the best and most consistent results, giving a low coefficient of variation between replicate cultures and affording some control over the amount of fungal material added to each experimental flask. In this method, cultures were blended for 10 sec. with 50 ml. sterile distilled water in a

Monel metal container of an 'Atomix' blender. The resulting homogenate was then centrifuged for 5 min. at 3000 rev./min. in tubes with Oxoid metal caps; the supernatant liquid was discarded and the mycelium was taken up in sterile distilled water. This washing process was repeated three times. The mycelium was finally added to a known volume of sterile distilled water, 10 ml. removed and added to an EEL colorimeter tube and the reading recorded. By reference to a previously calibrated graph with a linear scale, the quantity of blended mycelium per ml. could be calculated and the volume of the remainder of the mycelial suspension adjusted to give a concentration of 1 mg. of blended mycelium/ml. water. One ml. of suspension obtained in this way was used as the inoculum in all subsequent experiments.

Preliminary experiments indicated that the optimum temperature for organisms PE and PP was 28°, and 20° for PI. These temperatures were used in subsequent experiments. PE grew faster at all temperatures than either PP or PI which had the slowest growth rate. With medium A maximum dry wt. mycelium was obtained with PE after 20 days of incubation, with PP after 13 days, and after 16 days with PI. These incubation times were used in subsequent experiments unless otherwise stated.

Further experiments with medium A showed that glucose, asparagine, KH_2PO_4 and $MgSO_4.7H_2O$ were essential nutrients for all three fungi. The omission of $CaSO_4.2H_2O$ from the medium decreased the growth of PE by 70 %, of PI by 50 % and of PP by only 7 %. The omission of the trace elements had little effect on growth-yield of PP but seriously affected that of the other two species. Omission of $FeSO_4.7H_2O$ had a very marked effect on the yield of PI and a considerable effect on PP and PE.

The growth of PE and PP in this medium resulted in an increase of acidity as great as 3·5 pH units. The optimum pH for both PP and PE was 6·6, that for PI was 5·0. Medium A was thus subsequently modified by omission of the initial concentration of KH_2PO_4 replacing it with Sorensen's salt adjusted to give pH 6·6 (i.e. with KH_2PO_4, 4·746 g./l. + $Na_2HPO_4.2H_2O$, 5·443 g./l.). This medium is referred to as medium B and was used subsequently for organisms PE and PP. Sorensen's salt at any concentration had a distinctly depressing effect on the growth of PI and medium A at pH 4·7 was used for this fungus.

Tests showed that none of the fungi needed an exogenous supply of biotin, pyridoxine, nicotinamide, folic acid, riboflavin, calcium pantothenate, *p*-aminobenzoic acid, inositol or ascorbic acid; but thiamine was necessary for good growth. These results confirm those of earlier workers (Robbins, 1937; Cantino, 1955). Preliminary experiments with a wide range of structurally different carbon compounds also showed that the best carbon sources for these fungi were those of similar constitution, namely, sucrose, glucose and mannose for organisms PP and PI, and sucrose, glucose and fructose for PE. Similarly the best nitrogen sources in order of mycelial yield were as follows: for PE glycine, calcium nitrate, cystine, ethyl aminoacetate hydrochloride, histidine, asparagine; for PP aspartic acid, glycine, calcium nitrate, asparagine, γ-aminobutyric acid, ethyl aminoacetate hydrochloride; and for PI γ-aminobutyric acid, citrulline, glutamine, arginine and asparagine.

RESULTS

Sulphur requirements

While Mehrotra (1950) made a survey of the sulphur requirements of some species of Phytophthora, the results were criticized by Cantino (1955) because of a lack of pH control during the growth period. The sulphur requirement of the Phytophthora species used in the present work was thus investigated by varying the sulphur sources individually in media A and B. The sulphur compounds were each added in turn to these media to give a final concentration of 0·03 g./l. The basal media were modified by the replacement of $MgSO_4.7H_2O$, $CaSO_4.2H_2O$, $FeSO_4.7H_2O$ and trace elements containing sulphur with $MgCl_2.6H_2O$, $CaCl_2.6H_2O$ and $FeCl_3.6H_2O$ to give final concentrations of 0·1, 0·1 and 0·001 g./l., respectively. Preliminary experiments showed that the addition of chloride ion as KCl did not

Table 1. *The utilization of inorganic and organic sulphur compounds by Phytophthora species*

Highest yields expressed as percentages of yields from $Na_2SO_4.10H_2O$ taken as 100. Initial pH value for *Phytophthora erythroseptica* (PE) and *P. parasitica* (PP) = 6·6; for *P. infestans* (PI) = 4·7. Final pH values recorded in table. (Mg. dry wt. mycelium/flask average of 5 replicates. Incubation period for PI 16 days; those for PE and PP shown in brackets.)

	Species					
Sulphur source	Organism PE (mg. dry wt./flask and incubation time)	Final pH value	Organism PP (mg. dry wt./flask and incubation time)	Final pH value	Organism PI (mg. dry wt./flask at 16 days)	Final pH value
Sodium sulphate	100 (20)	5·8	100 (17)	5·7	100	5·8
Potassium persulphate	75 (20)	5·8	100 (17)	5·5	24	3·9
Sodium metabisulphite	124 (25)	5·7	110 (17)	5·5	29	4·0
Sodium dithionite	100 (25)	5·1	90 (17)	5·3	33	4·2
Sodium thiosulphate	81 (20)	5·8	100 (17)	5·4	100	6·0
Sodium sulphite	124 (25)	5·8	132 (13)	5·3	81	5·8
L-cysteine hydrochloride	115 (25)	5·8	98 (17)	5·3	43	5·9
Cystine	86 (25)	5·7	93 (17)	5·6	95	5·1
Thiourea	27 (20)	5·9	28 (17)	5·4	57	7·3
Sodium sulphide	127 (25)	5·7	103 (17)	5·4	62	5·6
Sulphanilic acid	20 (20)	6·2	15 (13)	5·6	57	6·4
Sulphamic acid	26 (20)	5·8	14 (13)	5·8	43	4·1
Sulphanilamide	10 (25)	6·0	16 (13)	5·5	57	7·4
Sulphosalicylic acid	18 (25)	6·1	22 (9)	5·5	19	3·9
Methionine	110 (25)	5·8	104 (17)	5·4	86	5·7
Thiazamide	20 (20)	6·2	17 (17)	6·0	53	7·5
Control (no sulphur)	17 (20)	6·1	16 (17)	5·5	24	4·7

affect the growth of these fungi. The modified medium A was inoculated with organism PI and the medium B with organisms PE and PP as previously described. Organisms PE and PP were incubated at 28° and PI at 20°. Dry weights of 5 replicate cultures were determined after 9, 13 and 17 days of incubation for PP, after 15, 20 and 25 days for PE, and after 16 days for PI. The final pH values of

the media were also determined. For organisms PE and PI there was a general increase in acidity varying between 0·4 and 1·5 pH units, while for PI the increase in acidity was never greater than 0·8 pH unit, but an increase in alkalinity as high as 2·8 pH units was sometimes recorded. The results are shown in Table 1 which records the highest percentage yields expressed in terms of the yield from $Na_2SO_4.10H_2O$ as 100%. With $Na_2SO_4.10H_2O$ the actual highest mycelial yields were 155 mg. after 17 days of incubation for organism PP, 94 mg. after 20 days for PE, and 21 mg after 16 days for PI.

Since persulphate breaks down to sulphate very easily in solution, the results for potassium persulphate must be treated with reserve. In general, the results indicated that sodium sulphate and sodium thiosulphate were very good sulphur sources for all three fungi, while sodium metabisulphite, sodium dithionite, sodium sulphite, sodium sulphide, cystine and methionine were good sources for organisms PE and PP, but gave only moderate or poor mycelial yields with organism PI. Essentially similar observations were made by Fothergill & Hide (1962) with four species of Pythium. Thus, in general, the oxidation level of the sulphur atom had no consistent significant effect on mycelial growth; a thiosulphate ($S_2O_3^{2-}$), a metabisulphite ($S_2O_5^{2-}$), a sulphite (SO_3^{2-}) and a sulphate (SO_4^{2-}) were all good sulphur sources. The yields from the sulphur-containing methionine and cystine were as high or nearly so, as the yields from the best inorganic sulphur sources in contrast to the findings of Volkonsky (1933) who worked with aquatic Saprolegniales. The other compounds used, particularly those containing a benzene ring (i.e. sulphanilic acid, thiazamide, sulphanilamide, sulphosalicylic acid), were very poor sulphur sources for all three fungi, except that moderate yields of mycelium were obtained with organism PI. These results are similar to those obtained by Steinberg (1941) with *Aspergillus niger*. No further correlation of structure with utilization could be made with the fungi used, thus emphasizing their ability to utilize a wide range of sulphur-containing compounds. Preliminary experiments indicated that the metabolism of organisms PE and PP, as expressed by their nutritional requirements, was very similar and differed considerably from that of organism PI. The results with the sulphur compounds have emphasized this distinction. Organism PI shows a much narrower range of sulphur utilization than organisms PE and PP, but it seems to possess an ability to use compounds which are not used to any great extent by the other two fungi.

The requirement for phosphorus

The importance of phosphorus in the growth of fungi is, of course, well known, but few comparative studies on the use of different phosphates by species of Phytophthora have appeared in the literature. Hence an experiment was done to study the effects of different phosphates on mycelial production. In this experiment medium A was used in which potassium dihydrogen orthophosphate was replaced by K_2SO_4, 0·67 g./l. To this medium were added singly in turn various phosphorus compounds to give a final concentration of phosphorus 0·3 g./l. The medium was otherwise unbuffered at initial pH 4·7 and the final pH value, recorded in each case, showed an increase in alkalinity of 1·0–1·5 pH units. As before, dry weights of 5 replicate cultures were determined after 9, 13 and 17 days of incubation for organism PP, after 15, 20 and 25 days for PE, and after 16 days for PI. The results are shown in Table 2 which records the highest mycelial yields expressed as a percentage

of that for sodium dihydrogen orthophosphate taken as 100 %. With NaH_2PO_4 the actual highest mycelial yields were 148 mg. for organism PE after 25 days of incubation, 178 mg. for PP after 17 days, and 45 mg. for PI after 16 days.

For all organisms sodium dihydrogen orthophosphate, sodium metaphosphate and lecithin were excellent sources of phosphorus, while tetra-sodium pyrophosphate, sodium β-glycerophosphate and nucleic acid gave high yields with organisms PE and PP, but the corresponding yields for organism PI were greatly diminished. The highest comparative yields were obtained with lecithin for organism PE, and nucleic acid for organism PP but, as a whole, the inorganic phosphorus sources seemed to be better than the organic compounds. The remaining compounds gave poor yields of mycelium. There were not great differences in yield with favourable compounds but differences were noted in the rate of utilization of the compounds. For example, the two orthophosphates and sodium β-glycerophosphate were utilized much more rapidly than sodium metaphosphate or tetra-sodium pyrophosphate. Similar results have been recorded for species of Pythium by Hide (1961) and for *Rhizopus stolonifera* by Yeoman (1954). This suggests that orthophosphate is more readily available to these fungi and that metaphosphate and pyrophosphate are first converted in the mycelium to orthophosphate before they are utilized. Casein was a good source of phosphorus and gave visibly good mycelial growth but measurement was difficult because of heavy precipitation in the medium after incubation for 1 or 2 days; this precipitate was insoluble in water, N-HCl or H_2SO_4 and dry weights were not recorded.

Table 2. *The utilization of various phosphorus compounds by Phytophthora species*

Highest yields expressed as percentages of yields from $Na_2H_2PO_4$ taken as 100. Initially all pH 4·7. (Mg. dry wt. mycelium/flask average of 5 replicates.)

Phosphorus source	Organism		
	P. erythroseptica (mg. dry wt./flask and incubation time, days)	*P. parasitica* (mg. dry wt./flask and incubation time, days)	*P. infestans* (mg. dry wt./flask incubation time 16 days)
Sodium dihydrogen orthophosphate	100 (25)	100 (17)	100
Sodium metaphosphate	94 (25)	96 (17)	129
Tetra-sodium pyrophosphate	101 (25)	97 (13)	4
Sodium β-glycerophosphate	100 (20)	87 (13)	7
Sodium phosphite	8 (20)	19 (17)	4
Sodium hypophosphite	11 (20)	14 (13)	13
Nucleic acid	97 (20)	116 (17)	49
Lecithin	119 (20)	77 (13)	81
Casein	+	+	+
Control (no phosphate)	23 (20)	11 (13)	11

+, Good mycelial growth but weight not determinable.

Factorial experiments

Some of the basal physical and chemical requirements of a culture medium for the growth of the organisms PE and PP having been determined, the effect of the balance of the chemicals used in the medium was next investigated. The factorial design and statistical analysis of the results should indicate whether or not a balance

between the salts is necessary for high mycelial yields under the given experimental conditions. The direct effect of the individual salts in the medium and the interaction between them is also determined. Previous experiments showed that medium A was a satisfactory medium for organisms PE and PP but KH_2PO_4 was replaced by K_2HPO_4 at a concentration of 1·046 g./l. to give initial solutions nearer to the pH growth optimum for these fungi. The solution was adjusted to pH 6·6.

Initial factorial experiments showed that the highest concentrations of the salts were possibly limiting growth and this medium A was further modified to give basal concentrations of K_2HPO_4 at 1·568 g./l., of $MgSO_4.7H_2O$ at 0·41 g./l. and of $CaSO_4.2H_2O$ at 0·414 g./l. The new solutions were also adjusted to pH 6·6. In all experiments the final pH values of all replicates were determined; the variation of pH value was never more than one pH unit. The cultures were incubated at 28° for 17 days for organisms PE and PP. The concentrations of the salts were fixed on the basis of halving and doubling those in the basal medium containing K_2HPO_4. Thus K_2HPO_4, $MgSO_4.7H_2O$ and $CaSO_4.2H_2O$ were each used at three concentrations and all possible combinations of them were set up, giving a total of 27 variations. Each combination was done in quintuplicate, thus, with each species of fungus, 135 culture flasks in each experiment were incubated. Each 150 ml. Erlenmeyer flask contained 30 ml. medium. The results given are the average mg. dry wt./mycelium of 5 replicates. The grouped results are shown in Table 3 and the grouped analyses of variance are given in Table 4. For abbreviations of the salt concentrations used in the text below see Table 3.

Table 3. *Growth responses to two species of Phytophthora to different conditions of K_2HPO_4, $MgSO_4.7H_2O$ and $CaSO_4.2H_2O$*

Concentration of K_2HPO_4 : P_1, P_2, P_3, = 0·0045, 0·009, 0·018 g./l.; of $MgSO_4.7H_2O$: Mg_1, Mg_2, Mg_3 = 0·0008, 0·0016, 0·0032 g./l.; of $CaSO_4.2H_2O$: Ca_1, Ca_2, Ca_3 = 0·0012, 0·0024, 0·0048 g./l.

Difference required between means for significance at odds of 99 : 1 for K_2HPO_4, $MgSO_4.7H_2O$ and $CaSO_4.2H_2O$ = 9·547 mg. for *P. crythroseptica* (PE) and 6·518 mg. for *P. parasitica* (PP).

	K_2HPO_4 and $MgSO_4.7H_2O$ conditions																	
	P_1						P_2						P_3					
	Mg_1		Mg_2		Mg_3		Mg_1		Mg_2		Mg_3		Mg_1		Mg_2		Mg_3	
	PE	PP	PE	PP	PE	PP	PE	PP	PE	PP	PE	PP	PE	PP	PE	PP	PE	PP
	Mean dry wt. mycelium/flask																	
Ca_1	60	89	65	83	75	92	118	160	110	172	120	144	212	178	192	174	217	186
Ca_2	56	100	66	90	70	92	109	172	117	149	81	154	177	172	149	191	169	187
Ca_3	80	98	80	186	89	163	123	102	116	173	120	169	237	96	162	167	165	174

From the single salt analysis it is evident that there was no consistent result with all salts for both organisms. This analysis showed that the differences between mycelial dry weight with K_2HPO_4 were significant at all concentrations of the salt for both organisms, and in both cases the highest yield was obtained at the P_1 level of the salt. For $MgSO_4.7H_2O$ with both organisms the differences observed between the Mg_1 and Mg_2 levels of this salt were significant. For organism PE the highest mean weight was obtained at the Mg_1 level, while for organism PP the Mg_2 level

gave the highest yield. For $CaSO_4.2H_2O$ with organism PE there were significant differences between the mean mycelial weights at the Ca_1 and Ca_2 level and also at the Ca_2 and Ca_3 levels, but not between the Ca_1 and Ca_3 levels of this salt. For organism PE these latter levels gave the same mycelial yield but for organism PP the highest mean yield was obtained with the Ca_3 concentration.

Table 4. *Analysis of variance (grouped) for Phytophthora erythroseptica (PE) and P. parasitica (PP)*

Required '*F*' and '*t*' values taken from Snedecor's tables (1946). Sums of squares and mean squares are omitted from table.

		F required odds			
		Found organisms			
Variance	DF	organism PE	organism PP	99:1	19:1
Total	134	—	—	—	—
$CaSO_4.2H_2O$	2	20·12	2·53	4·88	3·11
K_2HPO_4	2	5419·19	325·55	4·88	3·11
$MgSO_4.7H_2O$	2	64·66	60·61	4·88	3·11
$CaSO_4$ x K_2HPO_4	4	25·48	50·99	3·56	2·49
K_2HPO_4 x $MgSO_4$	4	94·25	72·41	3·56	2·49
$CaSO_4$ x $MgSO_4$	4	4·54	36·73	3·56	2·49
K_2HPO_4 x $MgSO_4$ x $CaSO_4$	8	7·13	23·12	2·74	2·06
Residual error	108	—	—	—	—

The grouped analyses of variance showed that there were significant interactions between all the salts in the first order combinations for both organisms. These results show that there was a physiological balance in some cases between these salts under the given conditions. With organism PE the interaction between K_2HPO_4 and $MgSO_4.7H_2O$ showed that at the P_1 and P_2 levels increasing the concentration of $MgSO_4.7H_2O$ had only a small effect. The highest yield was obtained with the highest concentration of K_2HPO_4 and the lowest concentration of $MgSO_4.7H_2O$; no balance is indicated. But with organism PP similar dry weights were obtained at the P_1 and P_3 levels with the Mg_1 level and, in general, increasing the concentration of K_2HPO_4 led to increasing yields relative to an increasing level of $MgSO_4.7H_2O$; a balance between the salts is indicated. The interaction of $MgSO_4.7H_2O$ and $CaSO_4.2H_2O$ is significant but there were only relatively small gross differences between the yields of PE. The highest yields were obtained with the Mg_1 and Ca_3 levels and with Mg_3 and Ca_1 levels. The Ca_2 levels did not show any consistent change in yield with increasing concentration of $MgSO_4.7H_2O$. With organism PP the interaction of these two salts is interesting. At the Ca_1 and Ca_2 levels, increasing the $MgSO_4.7H_2O$ concentration had little effect on yield and there were no significant differences between them, but with the Ca_3 level there appeared to be inhibition of growth with the lowest concentration of $MgSO_4.7H_2O$. Doubling the amount of this salt in the medium gave a great increase in mycelial yield which was maintained, but not improved, by further increase in concentration of $MgSO_4.7H_2O$. A balance between these two salts is necessary and seems to be operative in the ratio of 2 $CaSO_4.2H_2O$:1 $MgSO_4.7H_2O$. With organism PE the interaction between K_2HPO_4 and $CaSO_4.2H_2O$ showed that at the P_1, P_2 and P_3 levels there was increasing yield with increasing concentration of $CaSO_4.2H_2O$, but

at the P_1 level the highest yield was given with the Ca_3 level, while at the P_3 level it was given with the Ca_1 level. With organism PP at the Ca_3 level there was no consistent growth increase with increasing K_2HPO_4 concentration and the highest yield was obtained at the P_3 and Ca_2 levels. The yields were nearly identical at the P_1Ca_3, P_2Ca_3 and P_3Ca_3 levels. Thus a balance is indicated.

The analysis of variance also showed that the interaction of all three salts was significant for both organisms. The concentration of K_2HPO_4 was here the most important factor controlling the yield of the organism PE. The highest yields were obtained at the P_3 level. At the P_1 level increasing the concentration of the other two salts only affected the yield slightly. At the P_3 and Ca_3 levels high yields were only obtained when the $MgSO_4.7H_2O$ concentration was low. With organism PP there was little difference between the mean mycelial yields at the $P_2Mg_1Ca_2$ and $P_3Mg_2Ca_3$ levels. There was a consistently increasing yield as the concentration of the salts was increased proportionately from the $P_1Mg_2Ca_1$, $P_2Mg_2Ca_2$ and the $P_3Mg_3Ca_3$ levels. The highest mean weight of 191 mg. was obtained at the $P_3Mg_2Ca_2$ level, but weights of 186, 187 and 181 mg. were also obtained at the $P_3Mg_3Ca_1$, $P_3Mg_3Ca_2$ and $P_1Mg_2Ca_3$ levels, respectively, suggesting that the concentration is not so important when the other two salts are in a relatively high concentration in the approximate ratio of $2CaSO_4.2H_2O:1MgSO_4.7H_2O$.

Balance between $MgSO_4.7H_2O$ and $CaSO_4.2H_2O$

The previous experiments indicated that there was a physiological balance between K_2HPO_4 and $CaSO_4.2H_2O$. While balance has been shown to exist between various combinations of salts by different workers, this would seem to be the first time a balance has been indicated between these two salts. To confirm this result another experiment was designed to test the change in the amount of growth which might result when the concentrations of the salts were varied while still maintaining the balance between them. This experiment was designed factorially following the method of Talley & Blank (1941) and Fothergill & Ashcroft (1955). Thus the basic concentrations of $MgSO_4.7H_2O$ and $CaSO_4.2H_2O$ were 0·205 and 0·414 g./l., respectively; these were decreased to half in one set of solutions and increased twice and then four times in other solutions. This gave four treatments each having the same balance between the salts but the ratio of their concentrations was 0·5:1:2:4. Each of these four solutions was tested singly with K_2HPO_4 at 2·091, 3·136, 4·181 and 5·227 g./l., respectively, giving 16 solutions in all. The remaining ingredients of the basal medium remained the same. The medium was adjusted initially to pH 6·6 and the cultures incubated in 4-replicate at 28° for 11 days. The results expressed as mg. mean dry wt. mycelium per flask are shown in Table 5 where treatments are numbered 1–16 in parentheses.

The results showed that the mean mycelial yields for K_2HPO_4 at the P_1, P_2, P_3 and P_4 levels were not significantly different from each other but they were different from the yields at the Mg_1 and Ca_1 levels. To show the interaction of $MgSO_4.7H_2O$ and $CaSO_4.2H_2O$ '*t*' tests were performed. There were no significant differences between treatments 2, 3 and 4 at the P_1 level, but there was between treatments 1 and 2. At all other concentrations of K_2HPO_4 a similar pattern was shown, that is, there were no significant differences between treatments 6, 7 and 8, treatments 10, 11 and 12, and treatments 14, 15 and 16; but at the P_2, P_3 and P_4 levels, respec-

tively, there were significant differences between treatments 14, 15 and 16. At the P_2, P_3 and P_4 levels, respectively, there were significant differences between treatments 5 and 6, 9 and 10, 13 and 14 at these levels of K_2HPO_4. These results confirm the conclusion that a balance between $MgSO_4.7H_2O$ and $CaSO_4.2H_2O$ exists. But since significantly different mean dry weights were recorded with the Mg_1 and Ca_1 levels at all levels of K_2HPO_4, it is evident that the effect of the balance between $MgSO_4.7H_2O$ and $CaSO_4.2H_2O$ operated only after a certain concentration of the salts had been reached. Thereafter the concentration of either of these salts could be varied without affecting the mycelial yield of *Phytophthora parasitica*, provided that the concentration of the other salt is varied proportionately.

Table 5. *Mean dry weights and growth responses of Phytophthora parasitica after* 11 *days incubation at* 28° *on media with the same balance but with different concentrations of certain components*

Concentrations of K_2HPO_4: P_1, P_2, P_3, P_4 = 2·091, 3·136, 4·181, 5·227 g./l.; of $MgSO_4.7H_2O$: Mg_1, Mg_2, Mg_4, Mg_4 = 0·102, 0·205, 0·410, 1·620 g./l.; of $CaSO_4.2H_2O$: Ca_1, Ca_2, Ca_3, Ca_4 = 0·202, 0·414, 0·828, 1·656 g./l. 't' = 2·447 for 6 degrees of freedom at P = 0·05; values of 't' for combinations of treatments, 1 × 2 = 4·063, 2 × 3 = 0·390, 3 × 4 = 0·917, 5 × 6 = 7·170, 6 × 7 = 0·780, 7 × 8 = 1·764, 9 × 10 = 4·176, 10 × 11 = 0·258, 11 × 12 = 0·249, 13 × 14 = 17·159, 14 × 15 = 0·599, 15 × 16 = 0·532. Figures in brackets are treatment numbers, i.e. 1–16, referred to in text.

	Mg_1 Ca_1	Mg_2 Ca_2	Mg_3 Ca_3	Mg_4 Ca_4	Mean N
	Mean dry wt. mycelium (mg./flask)				
P_1	114 (1)	152 (2)	154 (3)	140 (4)	140
P_2	97 (5)	173 (6)	162 (7)	143 (8)	143·7
P_3	106 (9)	141 (10)	144 (11)	146 (12)	134·2
P_4	92 (13)	166 (14)	157 (15)	147 (16)	140·5

General mean = 139·6 mg. dry wt. mycelium/flask.

CONCLUSION

In general, the results of the factorial experiments showed that there was a considerable difference between the nutritional requirements of *Phytophthora erythroseptica* (PE) and *P. parasitica* (PP). With the individual salts used in the medium the greatest effect on mycelial growth was produced by varying the concentration of K_2HPO_4 for both organisms. Results for the other two salts were more variable and significant differences in dry weights did not vary in any general way. An absolute balance between the major mineral nutrients studied was not shown for *P. erythroseptica* but was shown for all combinations of salts with *P. parasitica*. The latter fungus seems to have more specific nutritional requirements than the former. The interaction of $CaSO_4.2H_2O$ and $MgSO_4.7H_2O$ at higher concentrations was outstanding for *P. parasitica*. Although the omission of $CaSO_4.2H_2O$ from the original medium A had only a small effect on growth, the increase in acidity of the unbuffered medium during growth was great. Hence the interactions of $CaSO_4.2H_2O$ and $MgSO_4.7H_2O$ may only occur at, or near to, the optimum growth pH for *P. parasitica*. Second-order interactions between three salts were demonstrated.

The results for *Phytophthora erythroseptica* and *P. parasitica* support the findings of other workers. For example, Talley & Blank (1941), Fothergill & Ashcroft (1955) and Fothergill & Hide (1962) found that a physiological balance between mineral salts in the medium was necessary for good growth of the fungal parasites *Phymatotrichum omnivorum* and *Venturia inaequalis*, and the two species *Pythium debaryanum* and *P. ultimum*. No balance, however, was required for *P. afertile* and *P. torulosum*. The highest mycelial yields (237 mg. for organism *P. erythroseptica* and 191 mg. for *P. parasitica*) were obtained with the modified liquid basal medium A containing glucose, 25 g./l.; DL-asparagine, 4·0 g./l.; $FeSO_4.7H_2O$, 0·001 g./l.; thiamine, 0·8 mg./l.; $ZnSO_4.7H_2O$, 1 p.p.m.; H_2MoO_4 and $CuSO_4.5H_2O$, 2 p.p.m. with K_2HPO_4, 0·018 g./l.; $MgSO_4.7H_2O$, 0·0008 g./l.; and $CaSO_4.2H_2O$, 0·0048 g./l. for organism PE and K_2HPO_4, 0·018 g./l.; $MgSO_4.7H_2O$, 0·0016 g./l., and $CaSO_4.2H_2O$, 0·0024 g./l. for *P. parasitica*. *Phytophthora infestans* has the most exacting nutritional requirements but grows satisfactorily on fewer sulphur and phosphorus compounds than either *P. parasitica* or *P. erythroseptica*. Mycelial yields of *P. infestans* were also very much less than those for the other fungi for all variations tried of the two basal media A and B. The highest mycelial yield for *P. infestans* was only 58 mg. and was obtained with medium A above but containing K_2SO_4, 0·67 g./l. and $NaPO_3$, 0·3 g./l. in place of K_2HPO_4.

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Transmission of Tobacco Necrosis Virus by Zoospores of *Olpidium brassicae*

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SUMMARY

Strain D of tobacco necrosis virus (TNV) was transmitted by zoospores of 3 different isolates of *Olpidium brassicae* (Wor.) Dang. to roots of Mung bean and lettuce grown in modified Hoagland's solution diluted 1/20. On Mung bean roots necrotic local lesions formed one day after exposure to virus and zoospores. Virus in lettuce was assayed by inoculation to leaves of French bean. Virus transmission was favoured by decreasing salt concentration and increasing the pH value of the nutrient solution and depended also on the concentrations of virus and zoospores. With 10^5 zoospores/ml. transmission to lettuce was obtained with as little virus as 0·05 μg./l. When the virus concentration was 5 μg./l., 50–100 zoospores/ml. were effective. Fungus infection as measured by the number of zoosporangia in the root was not strictly correlated with virus infection.

Exposure of roots to virus + zoospore mixture for 1 min. sufficed to infect them with virus. More transmission occurred when virus was added before or together with zoospores, than after. Roots, exposed to zoospores for 10 min., then washed, were more readily infected by TNV when virus was introduced during the first hour or two after zoospore attachment to the root cells than later; there was some transmission even when virus was withheld till 4 hr after washing. Immersing roots, inoculated with fungus and virus, in hot water (60°) killed the fungus but not the virus, and varying the interval between inoculation and heating showed that virus became established after 2–3 hr.

Isolates of *Olpidium brassicae* naturally contaminated by strain D or A of TNV were freed from contamination by inoculating lettuce roots with dilute zoospore suspensions. Zoospores mixed or naturally contaminated with TNV were partially separated from it by centrifugation. Virus transmission was prevented by adding concentrated homologous antiserum to zoospores that had already been exposed to virus, or by adding very dilute antiserum to virus before mixing it with zoospores. The extent to which transmission was prevented by antisera to other strains of TNV depended on the degree of their serological relationship to strain D. The present evidence does not support the suggestion that TNV is carried inside the fungus.

INTRODUCTION

Tobacco necrosis virus (TNV) is soil-borne and often occurs in roots of normal-looking plants but becomes systemic and causes an obvious disease in only a few species. Although first described some 30 years ago (Smith & Bald, 1935), little was done until recently to find how it is transmitted. Bawden & Kassanis (1947) suggested that soil micro-organisms might be vectors or reservoir hosts of TNV, but did not gain evidence for the idea from their tests with several soil bacteria and

two soil fungi (*Thielaviopsis basicola*, *Rhizoctonia solani*) parasitic on tobacco. Other parasites in the roots of plants grown in infective soil were not tested and the eventual discovery that TNV is transmitted by the parasitic Chytrid fungus *Olpidium brassicae* (Wor.) Dang. was a by-product of research on 'big-vein' of lettuce (Teakle, 1960).

As an obligate intracellular parasite, *Olpidium brassicae* seems well fitted to transmit virus to plants it infects. The posteriorly uniflagellate zoospore attaches itself to a young root cell, encysts, and the contents of the cyst discharge into the host cell. The young thallus grows to form a zoosporangium, putting out discharge tubes to the exterior. Zoospores are differentiated and released, to repeat the infection process. Sometimes, zoospores function as gametes and the fusion body (perhaps the zygote) invades plant cells and becomes a thick-walled resistant sporangium. In favourable conditions the resistant sporangium releases zoospores to begin the life cycle anew (Meer, 1926; Jacobsen, 1943; Sahtiyanci, 1962).

Teakle (1960, 1962*a*) found that roots of lettuce and Mung bean (*Phaseolus aureus* Roxb.) placed in a suspension of *Olpidium brassicae* zoospores containing TNV became infected with virus but did not do so in a solution of TNV alone. Since *O. brassicae* must be grown on its host plant and so far has not been obtained free from other micro-organisms, doubts must remain as to the identity of the transmitting agent. Teakle (1962*a*) recognized this uncertainty and sought to show indirectly that *O. brassicae* transmitted the virus. He gave the following reasons in support of this conclusion: (1) Virus enters the root at about the same time as the fungus, i.e. 2–3 hr after inoculation. (2) Virus and zoospores enter on the same part of the root. (3) Treatments which decrease or stop the motility of the zoospores also decrease or prevent virus transmission. (4) The number of infections produced by washings from infected lettuce roots depends on their zoospore content. (5) Transmission is achieved by keeping roots in contact with virus and zoospores for only 1 min., after which the encysted zoospores are not removed by vigorous washing. (6) If an agent other than *O. brassicae* is involved, it is always present with *O. brassicae*. This evidence makes it highly probable that *O. brassicae* transmits the virus, and, at present, we accept this conclusion, which our results confirm. We now describe some factors which affect transmission of TNV by *O. brassicae* and results which bear on the mechanism of transmission.

METHODS

Plants. The variety Cheshunt 5B of lettuce (*Lactuca sativa* L.) was used to maintain Olpidium cultures and for the transmission experiments. Seeds were usually surface sterilized with Ca hypochlorite and germinated on filter paper, wet with tap water, at 18–20°. Two days later seedlings were transferred to water-culture vessels. Mung beans (*Phaseolus aureus* Roxb.) free from obvious damage or disease were treated with hypochlorite and germinated aseptically in Petri dishes on wet filter paper, at 20°; 2 days later they were transferred to the culture vessels.

Sand culture and nutrient solution. Sand, in layers 2–3 cm. deep, was autoclaved at 120° for 1 hr. For cultivating Olpidium, plants were grown in small white polyethylene flower pots (rim diameter 5·5 cm.) with the drainage hole plugged with glass wool. After use the pots were sterilized in a solution of sodium hypochlorite

(3 g. Cl/l.) for 1 day, washed with detergent and again treated with hypochlorite. Plants in sand were grown in the glasshouse (10–25°) and in winter were given supplementary artificial light. Hoagland's solution (Hoagland & Snyder, 1933), modified by doubling the standard concentration of KH_2PO_4, was used as a plant nutrient and as an infection medium. Iron was given as tartrate (Arnon, 1938) and micronutrients were as recommended by Arnon (1940).

Virus. Purified preparations of strain D of tobacco necrosis virus (TNVD) were used. The isolation and purification were described by Babos & Kassanis (1963*a*).

Olpidium cultures. Three isolates of *Olpidium brassicae* (Wor.) Dang. were used. Olpidium 1 was isolated from lettuce grown in soil supplied by Dr J. A. Tomlinson from a big-vein site in Cheshire. TNVD and lettuce big-vein virus (Campbell & Grogan, 1963) were associated with this isolate. Olpidium 2 was recultured from dried infected roots sent by Dr D. S. Teakle from California. Olpidium 3 was obtained from lettuce grown in unsterilized sand in unsterilized pots at Rothamsted; it was contaminated with TNVA and TNVD. The contaminated cultures were freed from TNV (see Results) and virus-free Olpidium was used in all transmission experiments unless otherwise mentioned. In referring our Olpidium cultures to the species *O. brassicae* (Wor.) Dang. we follow Sampson (1939); the question of nomenclature has been re-opened by Sahtiyanci (1962) and it might be argued that our cultures should be called *Olpidium* spp. (see Emerson, 1958). For convenience we shall call the fungus we have used simply Olpidium.

To obtain a regular supply of zoospore suspensions lettuces were grown in sand cultures automatically sub-irrigated with nutrient solution every 3 hr. Lettuce seedlings were grown in sand for about 2 weeks and then inoculated with the appropriate zoospore suspension. The intermittent flooding gave opportunities for zoospores to be released and re-infect the roots. Good yields of zoospores were obtained 1–2 weeks after inoculation. To obtain a zoospore suspension, a plant was removed from the irrigation tank and the sand allowed to dry slightly overnight. Next day the roots were quickly washed free from sand and put in a dish of culture solution diluted 1/20. Zoospore release began within a few minutes and was usually allowed to continue for 10–15 min., when the suspension was filtered through stainless steel gauze to remove root fragments. Such a plant typically produced from 5 to 25×10^6 spores; some produced more.

Experimental procedure. Most transmission experiments were done with seedlings growing in culture solution and kept under fluorescent lights (18-hr day) in a basement room at 20°. Newly emerged seedlings of lettuce or Mung bean were transferred to vials 2 in. × 1 in. containing 5 ml. nutrient solution, diluted 1/2. The plants were supported on stainless steel mesh bent in the form of a table and with apertures that could be penetrated by the radicle but not by the hypocotyl of the seedling. The height of the support was such that 5 ml. fluid covered the young root and the vials were covered with a polythene sheet. Lettuce plants were inoculated 4 days later and Mung beans 1 day later. At inoculation the half-strength solution was decanted, replaced by solution diluted 1/20 to which were usually added 1 ml. zoospore suspension in 1/20 solution and 0·5 ml. containing virus. Unless stated otherwise, the final concentration of TNVD in the vials was 1·5 μg./l. Lettuce roots were assayed for virus by infectivity test 3 days later. Each lettuce root was washed under the tap, crushed with a pestle in a small glass mortar, and

inoculated to the 4 primary leaves of 2 French bean plants (*Phaseolus vulgaris*, L., variety 'Prince') which had been dusted with Carborundum 600 mesh. The number of local lesions produced on the bean leaves 2–4 days later was a measure of the virus content of the root. Sap from washed roots primarily kept in solution containing virus alone at concentrations greater than 0·1 mg/l. had some infectivity presumably because virus adhered to the surface of the root. This residual infectivity was diminished by dipping the roots in 'Teepol' before washing them. However, even without this treatment virus alone always gave root extracts much less infective than when virus was mixed with zoospores.

Infection of lettuce roots was detected by assays 2 days after inoculation; the infectivity of root extracts reached a maximum in 6–8 days. Extracts of infected roots of the variety 'Cheshunt 5B' were more infective than those from 'Great Lakes'. 'Cheshunt 5B' may be more susceptible to infection than the other, or produces more virus per infection centre, or both.

Twenty-four hours after inoculation with virus and zoospores, Mung bean roots develop small necrotic local lesions (Teakle, 1962*b*), countable with the aid of a hand lens, and probably reflecting single infection centres. Mung beans probably reflect the number of root infections better than lettuce. The number of lesions obtained in assays of lettuce roots will reflect the amount of virus produced in each infection centre as well as the number of infection centres. A possible advantage of using lettuce is that it may allow a few infections to be detected. Our results are expressed as mean numbers of lesions per Mung bean root or per bean leaf inoculated with a lettuce root extract. The means are based on all plants used in the transmission experiments, usually 4 lettuce or 8 Mung bean roots per treatment.

Table 1. *Virus transmission by Olpidium to lettuce roots at different virus concentrations*

Lettuce roots inoculated with virus alone at 6 μg./l. produced 0·2 lesions/bean leaf/root. Figures for virus and zoospores are the final concentrations in the vial.

Virus inoculum		Infectivity of root extract. Mean number lesions/bean leaf/root	
Concentration (μg./l.)	Infectivity. Mean no. lesions/leaf	Olpidium 1 (5×10^4 zoosp./ml.)	Olpidium 2 (2×10^4 zoosp./ml.)
6	53	325	222
1·2	13	143	186
0·24	1	84	125
0·05	0·2	11	10
0·01	—	1	0

RESULTS

Concentration of virus and zoospores

With inocula containing many zoospores of any of the 3 Olpidia, lettuce roots became infected with TNVD when the virus in the inoculum was as dilute as 0·05 μg./l. (Table 1). This is about the least virus concentration detectable by mechanical inoculation with carborundum to very susceptible French bean leaves.

Roots of young lettuce seedlings were infected by rubbing with a small stiff camel-hair brush dipped in virus and carborundum. This method succeeded only when the inoculum was 6×10^5 times more concentrated than needed for infection

by Olpidium. Lettuce leaves were more easily infected than roots by brush inoculation; the minimal concentration of virus needed was 50 μg./l.

Some infected lettuce roots showed discoloured areas but infectivity tests showed that these were not connected with virus infection. The two lowest lettuce leaves sometimes developed minute necrotic local lesions when inoculated mechanically with TNVD, but at other times leaves became infected without showing lesions.

Experiments to find the fewest zoospores needed to transmit TNVD gave variable results (Table 2). However, with inoculum containing 5 μg. virus/l., 50–100 zoospores/ml. usually gave some virus infections. The number of virus infections produced depends on the concentration of virus (compare experiments 1 and 2, Table 2) and of zoospores, and over a limited range of concentrations decreasing one was compensated by increasing the other.

Table 2. *Virus transmission at different zoospore concentrations*

Olpidium 2 was used for all the experiments except for experiment 3 when Olpidium 3 was taken from lettuce also infected by virus. Virus concentration in vial was: 7, 0·35, 1·6, 4·6 and 46 μg./l. for experiments 1, 2, 4, 5, 6, respectively.

	Exp no.			Exp. no.		
	1	2	3	4	5	6
	Lettuce			Mung bean		
	Infectivity of root extract.			Total number of lesions on 8 roots		
Zoospores/ ml. in vial	Mean number of lesions/bean leaf/root					
9×10^3	318	211	—	—	—	—
3×10^3	—	—	—	134	131	356
9×10^2	132	0	—	—	—	—
6×10^2	—	—	—	—	12	109
3×10^2	—	—	79	38	—	—
9×10	118	—	—	—	1	18
5×10	—	—	—	18	—	—
3×10	—	—	0	—	0	0
1×10	0	—	—	6	—	—

Not all inoculated roots that became infected by Olipidium also contracted TNV infection. To find what correlation there was between fungus and virus infection, 4 roots inoculated 2 days previously were examined microscopically and the position of sporangia along the main root recorded. The roots were then cut into 7 sections of 5 mm., each of which was macerated and inoculated separately to bean leaves. The number of sporangia in a root segment and the number of virus lesions produced in beans was not related. In many different experiments where virus and fungus infection was estimated in the same root no consistent relation was found between the two. It seems that conditions in the cell favourable for one might not always favour the development of the other. A possible conclusion is that virus infection may sometimes result from penetration by zoospores which do not develop further.

Concentration and pH value of the culture solution

At the beginning of this work, zoospores were extracted in, and lettuce seedlings grown and inoculated in, the modified Hoagland's solution at half strength. However, it became apparent that this solution was not suitable for virus transmission by Olpidium. In several experiments when inoculated lettuce roots were washed

in tap water at different times after inoculation, virus was not transmitted even when the interval between inoculation and washing was 3 hr, in contrast with Teakle's experience (Teakle, 1962*a*). Our failure was because of the concentration of the culture solution used; virus transmission by Olpidium improved when the solution was diluted. In one comparison, extracts from plants grown in solution diluted 1/2 gave an average of 69 lesions per bean leaf whereas in solution diluted 1/20 the average was 340.

The effect of increasing salt concentration in decreasing virus transmission was greater when the inoculated roots were also subjected to other conditions unfavourable for transmission, such as acidity (Table 3). Virus transmissions by Olpidium were also tested at values above pH 7. There was considerable transmission at pH 8, some at pH 9, and none at pH 10. Zoospores seemed equally motile in solutions from pH 7 to 9; they stopped swimming 3 hr after extraction. At pH 9·5 they stopped swimming in about 1 hr and at pH 10 disintegrated in 5 min.

Table 3. *Virus transmission to lettuce roots by Olpidium in nutrient solutions of two salt concentrations and three pH values*

Lettuce roots infected with Olpidium were divided into six portions and placed in the six solutions. The solutions in which the young lettuce seedlings were growing were replaced with the spore suspensions with added virus.

Zoospore concentrations varied from $4{\cdot}5 \times 10^4$/ml. to $1{\cdot}8 \times 10^5$/ml. but this variation did not affect virus transmission. The solution as prepared was at pH 4·5; dilute KOH was used to adjust to other values.

	Infectivity of root extract. Mean number of lesions/bean leaf/root	
pH values	Solution 1/2	Solution 1/20
4·5	6	77
6	5	358
7	145	927

Sequence of fungus and virus inoculations

Some experiments were made to find the time roots must be in contact with inoculum for the virus to infect. Lettuce roots were placed in a suspension containing 4×10^4 zoospores/ml. and 10 μg. virus/l., and after 1, 5, 30, and 180 min. were washed in running tap water and placed in fresh nutrient solution. Three days later the roots were tested for virus content and produced on average 64, 69, 60 and 65 lesions/bean leaf, respectively. Tests were made with Mung beans to determine the effectiveness in transmitting virus of (1) virus + zoospore mixture, (2) virus added before or (3) after a brief exposure to zoospores. The experimental treatments were: (1) Roots exposed to virus + zoospore mixture for 5 min., washed and placed in fresh nutrient solution. (2) Roots exposed to virus for 30 min., washed and placed in fresh solution containing zoospores. Five minutes later the roots were washed again and placed in fresh solution. (3) Roots exposed to zoospores for 5 min., washed in running tap water and placed in fresh nutrient solution containing virus. The inocula contained 6×10^4 zoospores/ml. and 0·14 mg. virus/l. The average number of lesions, when the root extracts were inoculated to bean leaves were 80, 90 and 36, respectively, showing more infection when virus was added to the roots before or together with zoospores than when virus was added after zoospores, even

though in the last treatment virus was present in the solution until next day when the lesions were counted. To obtain further information about this, an attempt was made to find how long the introduction of virus can be delayed after the roots are

Table 4. *Virus transmission to Mung bean roots by varying the interval between inoculation with Olpidium and virus*

Olpidium zoospores were added to Mung bean seedlings in vials at concentrations of 3×10^4 to 6×10^4/ml. After 10 min. the roots and vials were washed in tap water and solution replaced in vials. Virus was added at intervals, giving a final concentration of 1·6 μg./l. In experiments 1 and 2 the roots were left with virus until lesions developed. In experiment 3 roots were washed after 10 min. exposure to virus and solution replaced in vials.

Time interval between fungus and virus inoculation	Exp. no. 1 (6 roots)	2 (7 roots)	3 (8 roots)
	Total number of lesions on roots		
None	68	166	77
10 min.	34	—	—
20 min.	46	—	—
30 min.	—	87	75
1 hr	35	43	54
2 hr	—	20	5
3 hr	—	11	6
4 hr	—	5	5
5 hr	—	0	1

Table 5. *Virus transmission to lettuce roots by zoospores heated at* 50° *for different times or when inoculated roots were heated for* 10 *sec at* 50° *at different times after inoculation*

Final virus concentration 1·5 μg./l., and zoospores $1{\cdot}8 \times 10^5$/ml. The zoospores were heated in 1 ml. of suspension in long narrow tubes of 4 mm. inner diameter, sealed at one end. The tubes were dipped in large volumes of water at 50°. Roots to be heated were inoculated by placing them in virus + zoospore mixture for 30 min. before being washed. The roots were dipped in hot water up to the hypocotyls. The times below refer to placing the roots in the inoculum.

Treatment	Infectivity of root extract. Mean number lesions/bean leaf/root
Zoospores heated for	
10 sec.	29
30 sec.	0
60 sec.	0
Unheated spores	160
Virus alone	0
Roots heated	
30 min. after inoculation	0
1 hr after inoculation	0
2 hr after inoculation	172
3 hr after inoculation	237

inoculated with zoospores, and still cause virus infection. Table 4 shows that a delay of 1–2 hr greatly decreased virus transmission but there was some transmission even when virus was withheld for 4 hr.

Teakle (1962*a*), by heating inoculated Mung bean roots for 10 sec. at 50° (which

kills zoospores but not virus), found that the shortest time necessary for the virus to become established in the host cell was between 2 and 3 hr. We confirmed this result with lettuce seedlings, but found that the virus was established within 1–2 hr after inoculation. This is within the period of 2–4 hr observed by Meer (1926) and Jacobsen (1943) to be necessary for zoospore penetration. The results in Table 5 show the effect of heating zoospores in suspension and when attached to roots. Under our conditions zoospores become motionless after being heated for 10 sec. at 50° but were able to transmit some virus.

When the suspension was heated for 30 sec., all ability to transmit virus was lost. However, fungus already attached to or even in the roots was destroyed after 10 sec. at 50° as was shown by heating roots one day after inoculation. This was established by microscopic examination 2 days later to find whether zoosporangia had formed.

Because virus was transmitted when roots were washed after being dipped in virus solution and before inoculation with zoospores, we concluded that some virus became attached to the roots. This was also shown by direct inoculation to bean leaves of extracts of roots that had been left for 2 hr in a solution containing 1 mg. virus/l. and washed. The average number of lesions/leaf was 5 in one experiment and 10 in another. Usually, in transmission experiments with zoospores, the inoculum contained between 10 and 1 μg. virus/l.; hence the residual infectivity of roots exposed to virus alone was very small.

Washing zoospores by centrifugation after exposure to virus

To find whether virus combines closely or loosely with zoospores, virus and zoospores were mixed and then centrifuged. The experimental conditions were such that centrifugation damaged the zoospores so much that several centrifugations were not possible. Nevertheless, even the few centrifugations possible provided some information. Zoospore suspensions in 5 ml. tubes were centrifuged for 5 min. at 7000 *g* (Baird & Tatlock, small angle centrifuge). Suspensions, tubes and buckets were chilled in ice-water beforehand. Only the top half of the supernatant fluid was used, and was carefully pipetted off. The tubes were drained, wiped with absorbent paper and the pellets resuspended in solution to the original volume. In one experiment, as well as centrifuging virus + zoospores, zoospores alone (Olpidium 1, 2×10^5 zoospores/ml.) were centrifuged first and their capacity to transmit virus then tested. The first part of Table 6 shows that the centrifugation removed all the zoospores from the suspension and that they could still transmit virus.

Another sample of the same zoospore suspension was mixed with virus, centrifuged, and the supernatant fluids and resuspended pellets, alone or after mixing with uncentrifuged zoospores or more virus, used to infect lettuce roots. The results obtained with supernatant fluid to which uncentrifuged zoospores were added did not show that virus had been removed by the zoospores during centrifugation. The resuspended pellet alone transmitted poorly but was more infective when mixed with virus to the original concentration (Table 6). Dilution, to the extent of resuspending what virus was left in the pellet and in the drained tube in the original volume of solution, clearly brought the concentration below that at which the virus was readily transmitted by the centrifuged zoospores. The residual virus in the pellet was more clearly shown in another experiment by centrifuging the resuspended

first pellet and testing the consequent supernatant fluid and pellet (Table 7). The resuspended first pellet, when tested alone, did not transmit virus but did so when more virus was added. The second centrifugation gave a supernatant fluid from which uncentrifuged zoospores transmitted virus (showing that the virus present in the first pellet was not transmitted by the once-centrifuged zoospores). The resuspended pellet from the second centrifugation still contained viable zoospores

Table 6. *Virus transmission by zoospore suspensions centrifuged before or after mixing with virus*

Final virus concentration in vials 20 μg./l., and of zoospores 3×10^4/ml. when added after centrifugation. The volume in all treatments was equalized with nutrient solution.

Treatment	Infectivity of root extract. Mean number lesions/ bean leaf/root
Centrifuged before mixing	
Supernatant fluid with virus added	0·3
Resuspended pellet with virus added	237
Centrifuged after mixing	
Supernatant fluid	1
Supernatant with uncentrifuged fungus added	308
Resuspended pellet	16
Resuspended pellet with virus added	170
Virus alone	2

Table 7. *Virus transmission by zoospore suspensions containing virus after one or two successive centrifugations*

Final virus concentration in vials 10 μg./l. when added after centrifugation, initial zoospore concentration of Olpidium 1 was $1{\cdot}4 \times 10^6$/ml., final concentration when added after centrifugation was 2×10^5/ml.

Treatments	Infectivity of root extract. Mean number of lesions/bean leaf/root
1st centrifugation	
Supernatant	4
Supernatant with uncentrifuged zoospores	253
Resuspended pellet	0·5
Resuspended pellet with new virus	21
2nd centrifugation	
Supernatant	0·3
Supernatant with uncentrifuged zoospores	140
Resuspended pellet	0·3
Resuspended pellet with new virus	58
Controls	
Virus alone	2
Uncentrifuged zoospores and virus	290

able to infect roots when mixed with fresh virus. The supernatant fluid from the first and second centrifugations inoculated to bean leaves gave averages of 29 and 0·7 lesions/leaf, respectively. Microscopical observation showed that centrifugation

had damaged many of the zoospores and in this experiment the centrifuged zoospores were clearly unable to transmit the small amount of virus remaining, although uncentrifuged zoospores could. The importance of the interaction between zoospore concentration and virus concentration in virus-transmission experiments has already been noted. Caution is therefore necessary in interpreting the results of the centrifugation experiments lest freedom from virus be overestimated. That said, this experiment, like the previous one, suggests that the centrifuged zoospores, some of which at least were still able to transmit the virus, had not appreciably combined with virus before centrifugation.

Similar results were obtained by centrifuging a zoospore suspension ($2{\cdot}5 \times 10^5$ zoospores/ml.) of Olpidium 3 from a virus infected plant. The first supernatant fluid did not transmit virus except when mixed with uncentrifuged zoospores of Olpidium 2. The resuspended pellet was infective but much more so when mixed with more virus. When the suspended pellet ($1{\cdot}7 \times 10^5$ zoospores/ml.) was centrifuged again, there was still considerable virus in the supernatant fluid; this when mixed with uncentrifuged zoospores of Olpidium 2 infected all 4 lettuce roots. Extracts from these roots produced on average 234 lesions/bean leaf. The resuspended pellet of the second centrifugation when used alone did not transmit virus but did so after being mixed with more virus. These results suggested that centrifugation might be used to free Olpidium from TNV, but our attempt to do so failed.

The extent to which centrifugation damaged zoospores differed at different times and sometimes only occasional zoospores retained their motility. Zoospores survived better when some serum was added to the suspension before centrifugation. How many of the zoospores remained viable is unknown.

Freeing Olpidium cultures from contamination with virus

Further evidence that TNV and Olpidium are only loosely associated was obtained when the isolates which were naturally contaminated with TNV were freed from virus. This was accomplished on three occasions by adding highly diluted (1/1000–1/10,000) spore suspensions to vials each containing two lettuce seedlings. Infectivity tests and microscopical examination of respective roots showed that plants in a few vials were infected with fungus alone whereas those in the other vials had both fungus and virus.

Infectivity tests showed that virus was present in suspensions of zoospores from plants infected with virus-contaminated Olpidium but there was no correlation between numbers of zoospores and amount of virus released as shown by extracts from roots in small lots of culture solution at 5 min. intervals. This may also explain how an Olpidium culture, contaminated with TNV could be freed from virus. In contrast to TNV, lettuce big-vein virus was not separated from Olpidium in this way. Olpidium 1 has been maintained free from TNV for the past 2 years but, although recultured many times, still carries big-vein virus.

Effect of antisera on virus and zoospore mixtures

Teakle & Gold (1963) reported that virus transmission was prevented by an antiserum to TNV diluted 1/50, when mixed with virus before zoospores were added, but not after. They concluded from this 'that the Olpidium zoospore acquires and

harbours TNV in such a manner that antiserum cannot inactivate it. Therefore it is probably not merely a surface carriage.' Our antiserum to TNVD at a dilution of 1/20 stopped virus transmission when added either before or after virus was mixed with zoospores. Table 8 shows that when homologous antiserum was added before virus met zoospores, virus transmission was prevented in all experiments, whereas when added 5 min. after virus and zoospores were mixed transmission was prevented in 5 of 7 experiments and substantially diminished in the other two. Virus transmission was also considerably decreased when antiserum was added to the roots either after contact with virus for 5 min. and before zoospores were added, or

Table 8. *Virus transmission by Olpidium in the presence of homologous antiserum or serum added before or after the virus was mixed with zoospores*

V, virus; Z, zoospores; A/S, homologous antiserum; S, normal serum. In treatments 1–6 virus was mixed in test tubes with nutrient solution, A/S, S or zoospores for 5 min., then appropriate additional components added. All suspensions were brought to the same volume before 2·5 ml. of the final mixture was distributed to each vial. The dilution of A/S or S when it met the virus was always 1/20. Final concentration of virus in the vial was 5 μg./l. and that of zoospores $2{\cdot}6 \times 10^4$ to $8{\cdot}6 \times 10^4$/ml.

Treatment	Lettuce with Olpidium 1. Exp. no.						Mung bean with Olpidium 2.
	1	2	3	4	5	6	7
	Infectivity of root extract. Mean number of lesions/bean leaf/root						Number of lesions on 8 roots
1 V	0	0	0	—	—	0	—
2 V+Z	71	197	—	—	473	195	112
3 [V+A/S] 5 min.+Z	0	0	0	—	2	0	—
4 [V+S] 5 min.+Z.	—	165	313	—	—	—	—
5 [(V+Z) 5 min.+A/S] 5 min.	0	4	0	23	149	2	0
6 [(V+Z) 5 min.+S] 5 min.	—	—	294	559	—	—	144
7 [(V to plant) 5 min.+A/S] 5 min.+Z	0	50	—	—	—	—	—
8 [(V to plant) 5 min.+S] 5 min.+Z	—	191	—	—	—	—	—
9 [V+Z to plant] 5 min.+A/S	—	10	15	—	—	—	112
10 [V+Z to plant] 5 min.+S	—	243	97	—	—	—	224

5 min. after the roots came in contact with the virus + zoospore mixture. Much more dilute antiserum prevented transmission when mixed with virus and the zoospores added later, than when virus and zoospores were mixed first. The top part of Table 9 shows the effect of adding antiserum to virus before adding zoospores. Our antiserum to TNVD completely stopped virus transmission at a dilution of 1/2500 and decreased transmission at 1/12,500. Teakle & Gold (1963), with the same mixing sequence, did not prevent transmission when they diluted their antiserum from 1/50 to 1/100 and doubled the virus concentration. This suggests that their antiserum contained less antibody. The results with the two heterologous antisera (Table 9) show that antiserum to TNVE, which is closely related serologically to TNVD, was nearly as effective as antiserum to TNVD in preventing virus transmission by Olpidium. By contrast, antiserum to TNVA, which is only remotely related serologically to TNVD, had little effect in preventing virus transmission. Teakle &

Gold's results resemble ours with antiserum to TNVA. There is no information that their virus culture contained only one strain of TNV and their antiserum may have been prepared against one strain of virus and used in transmission experiments against another. The second part of Table 9 shows that the three antisera similarly differed when virus and zoospores were mixed first, but that the antiserum then needed to be more concentrated to prevent transmission.

Table 9. *Virus transmission to lettuce or Mung bean roots by Olpidium in the presence of antisera added before or after virus was mixed with zoospores*

The treatments were similar to 3 and 5 in Table 8. Virus was mixed in test tubes with A/S and nutrient solution or zoospores: after 5 min., as appropriate, A/S or zoospores were added so that virus always met A/S at constant volume. The dilutions of A/S given in the table are those at which the A/S met the virus. When an A/S was diluted more than 1/20, the concentration was made up by addition of normal serum. Final concentrations in vial: of virus 1 μg./l. and of zoospores $1{\cdot}2 \times 10^5$/ml.

Treatment		Lettuce with Olpidium 1. Infectivity of root extract Mean number of lesions/bean leaf/root	Mung bean with Olpidium 2. Number of lesions on 8 roots
Antiserum added to virus before zoospores			
A/S to TNVD dil.	1/2500	0	0
	1/12,500	20	0
	1/62,500	260	58
A/S to TNVE dil.	1/2500	1	0
	1/12,500	146	31
	1/62,500	304	120
A/S to TNVA dil.	1/100	27	37
	1/500	128	59
	1/2500	275	111
Antiserum added to virus after zoospores			
A/S to TNVD dil.	1/20	3	8
	1/100	17	19
A/S to TNVE dil.	1/20	22	53
	1/100	41	142
A/S to TNVA dil.	1/20	203	103
	1/100	250	256

The precipitation titres of the three antisera when tested against TNVD at 40 mg./l., were 1/320, 1/80 and 1/5 for antisera to TNVD, TNVE and TNVA, respectively. These values measure the serological relationships already mentioned and the effects on transmission rank in the same order. In addition, the relative effects of the three antisera in stopping transmission of virus by Olpidium, irrespective of the order of mixing, are nearly proportional to their precipitation titres. This suggests that precipitating antibodies may be responsible for the phenomenon.

Table 10 shows that prevention of virus transmission by homologous antiserum, when added 5 min. after virus and zoospores were mixed, depended on the ratio of virus to antiserum and not on a particular antiserum dilution. In a factorial experiment with Mung bean roots a comparison was made of the effects of two concentrations each of antiserum, virus and zoospores and of adding the antiserum

5 or 20 min. after virus and zoospores were mixed. The results showed that the extent to which the antiserum inhibited virus transmission depended not only on virus concentration and number of zoospores but to some extent on the time virus and zoospores were left together before adding antiserum.

Table 10. *Virus transmission to lettuce by Olpidium when homologous antiserum was added to virus + zoospore mixture at two concentrations of virus and antiserum*

Virus (0·5 ml.) and zoospores (5 ml.) were mixed in test tubes and after 5 min. 5 ml. of antiserum 1/10 or 1/30 were added. When antiserum 1/30 was used, concentration was made up with normal serum. Two ml. of mixture was put in each vial. Final concentration in vial of zoospores was $1{\cdot}4 \times 10^5$/ml.

Final virus concentration (μg./l.)	Dilution of antiserum added	Infectivity of root extract Mean number lesions/ bean leaf/root
1·4	1/10	15
1·4	1/30	54
0·07	1/10	0
0·07	1/30	0·5
0·07 Normal serum	1/10	251
1·2 Virus alone	—	0

Sometimes virus transmission was increased when normal serum was added to virus zoospore mixture; Olpidium infection of the inoculated roots was enhanced when normal serum or antiserum was added to the mixture. Consequently, roots inoculated with a virus + zoospore mixture, to which antiserum was added after mixing virus with zoospores, had numerous zoosporangia but little or no virus. The results with antiserum are compatible with the idea that during infection the virus is outside the zoospores, because even when antiserum was added 20 min. after virus and zoospores were mixed, virus transmission was prevented or greatly diminished. Much more antiserum was needed to stop virus transmission when the antiserum was added to the virus after zoospores than before. A possible explanation for this is that less antibody was sufficient to aggregate virus particles in solution but much more was needed to coat and make ineffective virus particles attached to the zoospore surface. The centrifugation experiments suggest that virus did not become closely bound to zoospore.

DISCUSSION

In general our results agree closely with those of Teakle but with some differences in the experiments with antisera. Our results support his conclusion (Teakle, 1962*a*) that Olpidium, and not some other agent present in the zoospore suspensions, was responsible for virus transmission. An agent of virus transmission other than Olpidium cannot be entirely excluded until Olpidium is cultivated free from other micro-organisms. Such doubt might arise from our observation that the number of zoosporangia in roots was not always correlated with the extent of virus infection, and sporangia and virus were not always found in the same root area. An occasional zoosporangium might be over-looked, but this cannot apply when many fungus sporangia were observed but no virus found. The entry of virus simply when Olpidium zoospores are present elsewhere in the same medium must be discounted because,

in experiments not reported here, cell-free filtrates from zoospore suspensions, in which numerous seedling roots were suspended for 1 hr, did not transmit virus. Unless the conclusion that TNV is transmitted by Olpidium is wrong, the most likely explanation for the results is that penetration by the zoospores is sufficient to allow the virus to enter, but the fungus need not develop and produce zoosporangia. The experiment in which inoculated roots were heated to kill the fungus, but in which virus nevertheless multiplied, shows that this is possible. Similarly, virus may have failed to multiply in some cells in which fungus developed.

If Olpidium be accepted as a vector of TNV then it is a very efficient one, especially when the difficulty of transmitting the virus by rubbing the roots of lettuce plants is considered. Natural transmission of TNV through the soil when roots are abraded by soil particles is therefore unlikely to occur, although experimental conditions have been described where this may have happened. Root infection in naturally infected soils can be stopped by adding the fungicide Captan at 2 g./kg. of soil. However, if in an autoclaved soil virus is added then roots can be infected even when Captan is added, and Babos & Kassanis (1963*b*) concluded that virus can be transmitted by abrasion provided there is enough virus in the soil.

Virus transmission by Olpidium depends on the concentration of virus and of zoospores. The effectiveness of zoospores tended to differ considerably from one experiment to another, but there was no evidence that fewer zoospores could cause virus transmission when taken from roots also infected with virus than when taken from virus-free roots and mixed with purified virus. Such a difference might be expected if the virus entered the fungus when the two were multiplying in the same cell, unless the free zoospores take up virus very rapidly.

The experiments with antisera suggested that the virus is on the zoospore surface and if this is so the centrifugation experiments showed that the attachment is loose. The zoospores while in contact with virus in suspension, before and during centrifugation, did not acquire enough virus to infect the roots. Similarly, zoospores from roots also infected with TNV, and perhaps present with the virus in the same cell, were separated from virus by centrifugation. The comparative ease with which virus-free Olpidium cultures were obtained from spore suspensions from virus-infected roots suggests that there is no intimate association between zoospores and virus.

The simplest explanation of transmission is that virus enters the host by the same route as does the fungus. Transmission appears to be most certain when virus is already attached to the area of the root cell wall through which the zoospore will penetrate. Virus particles would then almost inevitably be pushed into the host cell by the advancing front of the zoospore. However, some virus infection occurs when virus is added even 4 hr after zoospores have become attached to the cell wall. If virus particles can penetrate the zoospore cyst they could follow or attach to the penetrating protoplast. The diminishing chances of virus transmission after zoospore attachment might relate to changes in the cyst wall or to an increasing proportion of the attached zoospores having completed penetration when, presumably, the breach in the cell wall is repaired. Alternatively, virus may enter the host cell in the vicinity of the path of the zoospore (where the cell wall must in some way be altered at least temporarily) and the ease with which virus can invade here may decrease with the progress of penetration by the zoospores.

Teakle & Gold (1963) failed to transmit some other viruses which, in size and shape, resemble TNV. This does not necessarily mean that there is some special relationship between TNV and Olpidium. Possibly other viruses morphologically similar to TNV also enter the cell at the entry point of the zoospore but the metabolism of the cell as it is changed by the fungus is suitable only for the multiplication of TNV. Equally, two strains of Olpidium may affect the cell differently and one leave conditions suitable for the multiplication of TNV but the other not. A very similar situation may exist with the transmission by aphids of the non-persistent viruses. It is generally accepted that these viruses are transmitted mechanically on the tips of the aphid stylets. Nevertheless, there is vector specificity. For example, one species of aphid can transmit one particular strain of cucumber mosaic virus but not another (Badami, 1958).

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Chemical Analysis and Inhibition Reactions of the Group and Type Antigens of Group F Streptococci

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SUMMARY

Qualitative and quantitative analyses of formamide extracts of group F streptococci revealed rhamnose, glucose, galactose, glucosamine and galactosamine as main components. The rhamnose content was high. Streptococci, carrying a type antigen in the cell wall, excreted into the medium also a polysaccharide with the same serological activity. These soluble polysaccharides contained no muramic acid, small amounts of rhamnose, but had a high glucosamine and mannose content. Column chromatography on DEAE-cellulose revealed the formamide extracts to be heterogeneous. Over 90 % of the serological activity could be recovered in one of the fractions. Inhibition reactions of the quantitative precipitation with a number of simple sugars gave indications about the composition of the determinant groups of group and type antigens. Rhamnose was inactive in all cases. The determinant group of the group F antigen is at least a disaccharide with a β-glucosidic moiety. The determinants of the type antigens I and II probably contain N-acetyl-galactosamine. There is evidence for a second determinant in type antigen II. The determinants of type antigens III and V contain a β-glucosidic moiety, that of antigen IV a β-galactosidic moiety.

INTRODUCTION

A large number of indifferent (no α- or β-haemolysis) and haemolytic streptococci isolated from dental root canals have been shown to belong to the serological groups F and G of the Lancefield system (Winkler & van Amerongen, 1959). Ottens (1961) and Ottens & Winkler (1962) found that some of the group F strains contained several type antigens apart from the group antigen. These type antigens were called I, II, III, IV and V. Other strains containing merely the group antigen but no known type antigen were indicated as F. O. Strains which contained a type antigen but no group antigen were indicated as O. I, O. II and O. IV. The type antigens III and V were never found separately from the group antigen. Ottens & Winkler (1962) demonstrated that type antigen I occurred in indifferent and haemolytic strains of group G; the type III antigen occurred in indifferent strains of group C. Both the group antigen F and the type antigens were shown to be carbohydrates.

Cummins & Harris (1956) have shown that the cell walls of group F streptococci contain the following sugars and amino sugars: glucose, galactose, rhamnose, glucosamine, galactosamine and muramic acid. In the present paper analyses of cell walls and cell wall antigens, obtained by formamide extraction of the afore-

mentioned strains are presented. During the investigation it appeared that some of the strains containing type antigens in the cell wall also excreted antigen in the medium. These soluble antigens with the same serological activity were also analysed. The results of qualitative and quantitative analysis of the group and type antigens were compared. On an anion exchange column, the formamide extracts proved to be heterogeneous. The consequence of this is discussed. The inhibition by simple sugars of the quantitative precipitation of the antigens F, I, II, III, IV and V with their antibodies provided evidence that the determinant groups of these antigens are different and contain different sugars.

METHODS

Culture conditions. All strains were grown in 4 l. Todd Hewitt Broth enriched with 0·2 % glucose for 24 hr at 37°. To obtain polysaccharides excreted in the medium, the strains were grown without additional glucose to reduce autolysis. Cells were obtained by centrifugation at 4000 rev./min., washed three times with distilled water and finally centrifuged at 17,000 rev./min.

Isolation of cell walls. Cell walls were isolated as described by Michel & Gooder (1962) with minor modifications. Enzyme treatment of the disintegrated bacteria was restricted to trypsin (1 mg./ml. for 2 hr at 37°, followed by another 0·5 mg./ml. for 1 hr).

Formamide extraction. The group- and/or type-specific polysaccharide was extracted from the bacteria with formamide according to Fuller (1938). As a final step the acetone precipitated material was dissolved in 20 ml. of distilled water and dialysed for 48 hr against several changes of distilled water at 4°. A small precipitate which often appeared during dialysis inside the bag was discarded.

Isolation of the polysaccharides excreted in the medium. The polysaccharide excreting strains O. I, O. II, F. II, F. III, O. IV and F. V were grown as indicated. After centrifugation at 4000 rev./min. for 40 min. the supernatant of a culture was sterilized for 20 min. at 120°. The volume of the medium was then reduced to one-twentieth by dialysis against Carbowax (polyethyleneglycol 4000). The proteins were precipitated from the concentrate by addition of two and a half volumes 96 % alcohol containing 1 % concentrated hydrochloric acid and discarded by centrifugation. The crude polysaccharide was precipitated from the supernatant by the addition of an equal volume of acetone. After centrifugation the precipitate was dissolved in distilled water and extracted with an equal volume of 25 % (v/v) trichloracetic acid. Undissolved impurities were centrifuged out and the polysaccharide reprecipitated by adding ten volumes of acetone, redissolved in distilled water and finally dialysed for 48 hr at 4° against several changes of distilled water.

Purification of antigen by the corresponding antiserum. Type V antigen was precipitated from the F. V formamide extract by adding type V antiserum at the optimum quantity, as determined by the quantitative precipitation, described below. The precipitate was centrifuged out after 24 hr, washed three times with cold saline and dissolved in 0·01 N-NaOH. An equal volume of 25 % (v/v) trichloracetic acid was added to precipitate the antibody proteins. The antigen in the supernatant was isolated in the same manner as that described for the isolation of the polysaccharides excreted in the medium.

Hydrolysis conditions. For the qualitative sugar analysis of cell walls (20 mg. of dry material), Fuller extracts (5 ml.) or polysaccharides isolated from the medium (5 ml.), and the material was hydrolysed in a final concentration of 2 N H_2SO_4 at 100° for 2 hr. After cooling, the hydrolysates were neutralized with a saturated solution of $Ba(OH)_2$, centrifuged and the sediment washed once with distilled water. The combined supernatants were dried at 45° *in vacuo* and redissolved in 0·35 ml. distilled water.

For the quantitative analysis of formamide extracts (5 ml.) and polysaccharides isolated from the medium (5 ml.), the material was hydrolysed in a final concentration of 4 N-HCl at 100° for 3 hr. The hydrolysates were then evaporated three times *in vacuo* and dissolved in a small amount of distilled water.

Paper chromatography. Amino sugars and sugars were separated by two dimensional chromatography on Whatman no. 1 paper (40 × 30 cm.). N-Butanol + acetic acid + water (60 + 10 + 20 by vol.) and 2·4–2·5 lutidine redistilled at 154° + water (65 + 35 by vol.) were used as solvent for 42 and 20 hr respectively. After drying the papers were sprayed with silver nitrate reagent as described by Trevelyan, Procter & Harrison (1950). Using as markers all the sugars and amino sugars which could reasonably be expected (Slade & Slamp, 1962) good separation with this system was obtained except for the N-acetyl derivatives of glucosamine and galactosamine.

Determination of sugars and amino sugars. Hydrolysates of formamide extracts and of polysaccharides isolated from the medium were put on a Zeokarb 225 column H^+ form (0·8 × 40 cm.) and a sufficient amount of distilled water was used to eluate the hexoses and rhamnose. Glucosamine and galactosamine were then eluated *and* separated on a fraction collector with 0·33 N-HCl (Gardell, 1953). Amino sugars were determined with the Elson and Morgan reaction as modified by Rondle & Morgan (1955). The hexoses and rhamnose were determined in the water eluate of the Zeokarb column. Glucose was determined with glucose oxidase, as described by Hugett & Nixon (1957), rhamnose with the thioglycollic acid/sulphuric acid reaction according to Gibbons (1955), mannose as described by Dische, Shettles & Osnos (1949). The galactose determination was corrected for the presence of the previously determined amounts of glucose and rhamnose; the mannose determination was corrected for the presence of the previously determined amounts of glucose, galactose and rhamnose.

Anion exchange chromatography. Column chromatography of formamide extracts was carried out on DEAE-cellulose (Serva, Heidelberg), as described by Kündig, Neukom & Deuel (1961) for cereals. The column was eluted with the following solutions: distilled water, phosphate buffer pH 7, 0·0175 M (PB), PB + 0·1 M-NaCl, PB + 0·25 M-NaCl, 0·1 N-NaOH. The dry weight of the eluted fractions was determined and the serological activity was measured by quantitative precipitation.

Quantitative precipitation inhibition. Sera were prepared in rabbits by four weekly subcutaneous injections of heat-killed streptococci followed by twelve intravenous injections on 4 days in each of 3 subsequent weeks. After an interval of 2 months, booster injections were given followed by bleeding 8–10 days later. Quantitative precipitations were carried out as described by McCarty & Lancefield (1955). Apart from the sugars mentioned in Table 4, the following sugars were used in the inhibition reactions: rhamnose, mannose, α-methyl-galactoside, α-methyl-glucoside,

2-desoxy-glucose, phenyl-β-glucoside, 3-o-methyl-glucoside, N-acetyl-glucosamine, maltose, trehalose, turanose, saccharose, raffinose, melezitose, stachyose and galactitol. The reactions were carried out by incubating 8 mg. of the sugars with the diluted antiserum during 1 hr at 37°. Thereafter the antigen was added and the incubation was continued for 1 hr at 37° and overnight at 4°. Of the sugars giving more than 10 % inhibition, inhibition-curves for amounts ranging from 10 to 90 μmole of sugar were determined.

RESULTS

The data resulting from the qualitative analyses of the hydrolysates of the cell walls, the formamide extracts and the polysaccharides excreted in the medium are given in Table 1. Only the cell walls contained muramic acid. Rhamnose was found in the cell walls and in the formamide extracts, but the rhamnose spots were definitely less pronounced in the hydrolysates of the excreted polysaccharides. On the other hand, the last material had a high content of mannose, whereas this sugar was almost absent in both cell walls and formamide extracts. The hydrolysates of the cell wall have a high glucosamine content which is in part derived from the mucopeptide complex. The high glucosamine content of all polysaccharides excreted into the medium distinguishes them from those in the formamide extracts. In the latter case the amount of glucosamine varied with different strains. Most of the hydrolysates contained galactosamine and the hexoses glucose and galactose. It is remarkable that in the case of F. O both the glucosamine and the galactose content are nil or low. Though hydrolysis conditions were kept constant the frequent presence of N-acetyl-hexosamine spots except in the hydrolysates of the cell walls points to differences in acid sensitivity of the materials examined.

Finally all chromatograms showed a varying amount of a number of five rather weak spots named *A* to *D* and *Z*. Noteworthy is the fact that the strongest spot (*A*) corresponded to a substance which was split off from the group antigen F only and was never seen in hydrolysates prepared from type antigens I, II and IV. The unknown Z was substantially different from the other ones giving a reddish brown colour with the silvernitrate spray and having high velocity in both solvents (Rg. butanol–acetic acid 3·00, Rg. lutidine 1·27).

Quantitative analyses are generally in agreement with the qualitative ones, taking in mind that the conditions of hydrolysis were different. As the excreted polysaccharides were not yet sufficiently pure (maximum 25 %, based upon saccharide content), only the formamide extracts were studied (Table 2). Rhamnose was always present in large amounts. In contradistinction to the results of qualitative chromatography mannose was found in all the hydrolysates tested, except in F. O. As the demonstration of small amounts of mannose on the chromatogram is relatively insensitive the results of the quantitative analysis should prevail. As the quantitative determination of mannose is the least reliable one, in view of the corrections for other sugars, low values in Table 2 should be distrusted. In the antigens F, F. III and F. V low glucosamine contents were found. These might be derived from contaminating mucopeptide. The antigen F had by far the simplest composition. It consisted mainly of rhamnose, galactosamine and glucose. In addition a small amount of galactose was found to be present. The composition of

Table 1. *Qualitative composition of cell walls, formamide extracts and polysaccharides excreted in the medium of group F streptococci*

	Cell wall					Formamide extracts of whole cells						Polysaccharide excreted into the medium					
	F. O	F. III	F. V	O. I	O. II	F. O	F. I	F. II	F. III	F. V	O. I	O. II	O. IV	F. II	F. III	F. V	O. I	O. II	O. IV
Muramic acid	+ +	+ +	+	+ +	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Rhamnose	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ +	+ + +	+ + +	+ + +	+ + +	+ + +	+	+	tr	+ +	+ +	+
Mannose	−	−	+	−	−	+	+ + +	−	tr	tr	−	tr	tr	+ +	+ + +	+ + +	+ + +	+ + +	+ +
Glucosamine	+ +	+	+	+	+ +	−	+	+	+	+	+	+ +	+ +	+ +	+ + +	+ + +	+ +	+ +	+ +
Galactosamine	+ + +	+ + +	+ + +	+ + +	+ + +	+ +	+ +	+	+ + +	+	−	+ +	−	+ +	+ +	+ +	+	+	+
Galactose	−	+ + +	+ + +	+ + +	+ + +	−	+	+	+ +	+	+	+ +	+ +	+ +	+ + +	+ +	+ +	+ +	+ +
Glucose	+ +	+ + +	+ + +	+ + +	+ + +	+ +	+ + +	+ +	+ + +	+ +	+ + +	+ + +	+ + +	+ + +	+ + +	+ +	+ + +	+ +	+ +
N-Acetylhexosamine	−	+ +	−	tr	−	+ +	+	+ +	+ +	+	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+	+ +
Unknown *A*	+ +	+ +	+ +	−	−	+ +	+ +	+ +	+ +	+ +	−	−	−	−	−	−	−	−	−
B	+	+	+	+	+	+	+	+	+	+ +	−	+ +	+	+ +	+	+	+	+ +	+
C	+	+	+	−	−	−	+	−	−	−	+	+	−	−	tr	−	−	−	−
D	−	+	−	−	−	+	−	−	+	+	+	+	+	−	−	−	+	−	−
Z	−	+ +	−	+	−	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+	+	−	−	−	−	−

the isolated antigen V was also very simple. It consisted mainly of glucose and galactose and had a low rhamnose and glucosamine content.

Formamide extracts hydrolysed by hydrochloric acid did not contain N-acetylhexosamines, indicating that the hydrolysis was complete under these conditions. The total weight of sugars and amino sugars after hydrolysis of the formamide extracts amounted to only 40% of the dry weight of the unhydrolysed material. The low sugar yield has to be ascribed to impurities of non-saccharidelike nature and to some destruction by acid hydrolysis.

Table 2. *Quantitative composition of formamide extracts of group F streptococci*

The figures give the percentages of the sugars calculated on the total recovery of each strain.

Name of strain	Rhamnose	Mannose	Glucosamine	Galactosamine	Galactose	Glucose
F. O	48·2	0	1	27	3	20·6
F. I	17·3	20·5	18·9	17·3	13	13
F. II	55·3	5·9	4·5	11·9	14·6	4·5
F. III	36·4	12·1	0·8	14·6	24·3	11·7
F. V	50	Not done	1	20	12	17
O. I	33·6	7	13·4	4·2	16·8	25·2
O. II	30·4	15	26	4·3	19	5·2
O. IV	32·8	Not done	21·8	0	35·8	4·5
AgV*	4·6	Not done	4	0	57·2	34·3

* This polysaccharide was obtained by precipitating the V antigen from a F. V formamide extract by anti V serum. The V antigen was isolated from the precipitate.

Table 3. *Fractionation of the formamide extracts of strains O. I and O. II on a DEAE-cellulose column*

The serological activity is determined by a quantitative precipitation reaction. The number of units is the product of the reciprocal of the dilution at the optimum, and the volume of the antigen solution added.

Eluted with	Formamide extract O. I		Formamide extract O. II	
	Weight of the fraction (mg.)	Serological activity (units)	Weight of the fraction (mg.)	Serological activity (units)
Distilled water	342	1600	267	800
PB*	342	< 10	313	< 10
PB + 0·1 M-NaCl	187	< 10	154	< 10
PB + 0·25 M-NaCl	91	< 10	66	< 10
0·1 N-NaOH	124	< 10	162	< 10

* PB, Phosphate buffer 0·0175 M (pH 7).

Column chromatography. The low percentages of monosaccharides recovered raised questions about the purity of the formamide extracts. Fractionation on a DEAE-cellulose column revealed that at least 5 fractions could be obtained. Table 3 shows in a typical experiment the heterogeneity of the formamide extracts of strains O. I and O. II. In both cases most of the serological activity was found in the distilled water eluate.

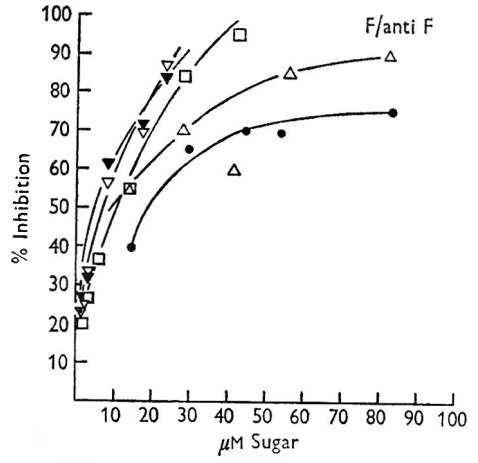

Fig. 1

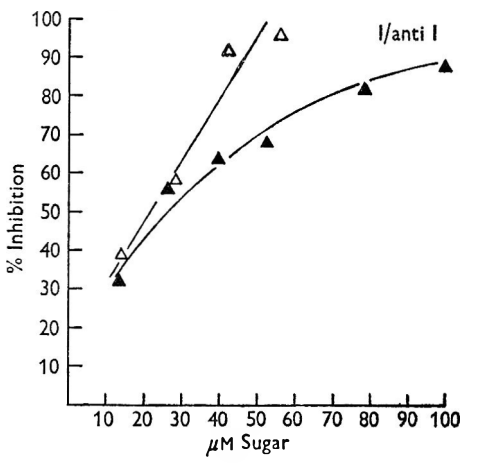

Fig. 2

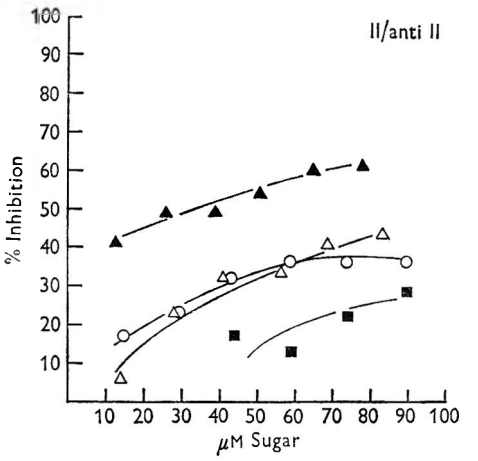

Fig. 3

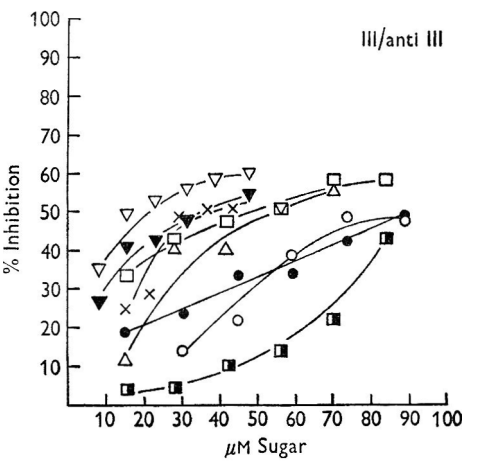

Fig. 4

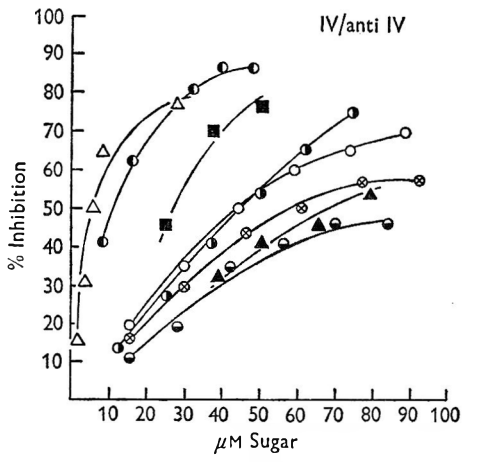

Fig. 5

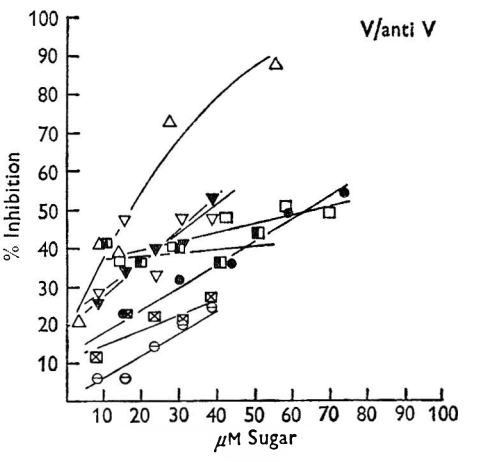

Fig. 6

Figs. 1–6. Inhibition reactions of quantitative precipitations by the following sugars: ●, Glucose; ○, galactose; ◑, glucosamine; ■, galactosamine; ▲, N-acetyl-galactosamine; ◨, α-methyl-glucoside; □, β-methyl-glucoside; ◧, phenyl-β-glucoside; ◒, α-methyl-galactoside; ⊗, 2 desoxy-galactose; △, N-methyl-glucamine; ▼, cellobiose; ▽, gentiobiose; ◐, lactose; ×, melibiose; ⊖, maltose; ⊠, trehalose.

Quantitative precipitation inhibition analysis. As antigens in these reactions, the eluates from the DEAE-cellulose column showing the highest serological activity were used. In the case of formamide extracts of strains F. O, O. I, O. II and F. III the distilled water fractions were used. For the serology of O. IV and F. V the distilled water fractions showed no activity and the next two fractions were used. Figs. 1–6 give the inhibitions of the serological reactions between the antigens F, I, II, III, IV, V and their corresponding antisera by the sugars that were present in the formamide extracts. Several derivatives of the sugars were also tested. The figures give only the inhibitions by sugars which in preliminary experiments inhibited the antigen–antibody reaction more than 10 % at a level of 8 mg. sugar in 0·2 ml. serum or serum dilution. The other sugars tested (see Methods) gave less than 10 % inhibition. The implications of these data for the composition of the determinant groups of the different polysaccharides examined will be discussed in the next section.

Table 4. *Inhibitions by simple sugars of the quantitative precipitation between the F, I, II, III, IV, V antigens and their respective antisera*

The values in the table give the 50 % inhibition expressed in μM sugar.

	Antigen					
	F	I	II	III	IV	V
Glucose	19	.	.	90	.	65
Galactose	.	.	.	.	45	.
Glucosamine	.	.	.	.	45	.
Galactosamine	.	.	.	.	27	.
N-Acetyl-galactosamine	.	27	35	.	67	.
β-Methyl-glucoside	13	.	.	46	.	67
2-Desoxygalactose	.	.	.	.	59	.
N-Methyl-glucamine	10	22	.	54	6	17
Cellobiose	6	.	.	34	.	37
Gentiobiose	7	.	.	18	.	38
Lactose	.	.	.	.	10	.
Melibiose	.	.	.	35	.	.

DISCUSSION

Results of the qualitative analysis of the different materials as given in Table 1 are in general agreement with the results obtained for streptococci by other workers (Cummins & Harris, 1956; Slade & Slamp, 1962).

A peculiar observation was the excretion of a serologically active polysaccharide into the medium by some of the strains. Quantitative analysis of this material and comparison with the data obtained in the analysis of the formamide extracts awaits further purification. Because these polysaccharides are undegraded they may be expected to give additional information as to the structure of the antigens. It is probable that this polysaccharide can be purified with a less violent method than formamide extraction.

The low velocity on paper chromatograms in different solvents of most of the unknown spots pointed to the presence of disaccharides and in fact the unknown *A* proved to be a disaccharide consisting of glucose and galactosamine.

Chromatography on DEAE-cellulose columns shows the formamide extracts to

be heterogeneous, though the serological specificity resided mainly in one fraction. A quantitative analysis of these purified antigens will be necessary.

A quite different approach was followed in the analysis of antigen V, which was purified by precipitation with its homologous antibody. Here only that part of the formamide extract which reacted with antibody was analysed.

The quantitative precipitation inhibition analysis showed appreciable differences in the determinant groups of the group and type antigens.

In the system F/anti F (Fig. 1) only glucose and its β derivatives gave good inhibition. As the inhibitions of gentiobiose (6-β-glucosido-glucose), cellobiose (4-β-glucosido-glucose) and β-methyl-glucoside were of the same level, the determinant group is probably a disaccharide with a β-glucopyranosyl group as the first sugar.

The inhibition of the I/anti I reaction as shown in Fig. 2 demonstrates that only N-acetyl-galactosamine is important in this system. The meaning of the strong inhibition given by N-methyl-glucamine in this and the other systems is not clear.

In the II/anti II reaction the strongest inhibition was given by N-acetyl-galactosamine (Fig. 3). This reaction was also inhibited by N-methyl-glucamine, D-galactose and galactosamine. Here the determinant group can be composed of N-acetyl-galactosamine in the first and galactose in second position, or alternatively there could be two separate determinant groups. By cross absorptions between the I and II systems it was shown that galactose was part of a separate determinant group, specific for the II antigen.

From Fig. 4 it is clear that the determinant group of antigen III is also a β-glucosidic disaccharide. β-Methyl-glucoside gave better inhibitions than the α-form. Gentiobiose and cellobiose gave also strong inhibitions. The second sugar of the III antigen should be different from the F antigen because the III and F systems do not give cross reactions. The possibility exists that a second determinant group containing galactose is present. The good inhibition given by galactose and melibiose (6-α-galactosido-glucose) and not by other galactose containing oligosaccharides could point to a determinant group with an α-galactosido-group in the first position.

The IV/anti IV system gave even more problems (Fig. 5). There was a good inhibition by galactose and its derivatives α-methyl-galactose, 2-desoxy-galactose and lactose. The inhibition given by α-methyl-galactose being less than that given by lactose points to a disaccharide with a β-galactosidic group. There was no evidence for the presence of a second sugar. Glucosamine + galactosamine gave better inhibition than N-acetyl-galactosamine, while N-acetyl-glucosamine gave no inhibition at all. This could be an argument in favour of a compound with a free amino group. The very strong inhibition by N-methyl-glucamine might point in the same direction.

The inhibition reactions of the V/anti V system are given in Fig. 6. The β-glucosido-glucoses gave inhibitions of the same level as glucose. This finding is in contrast with the results obtained in the F/anti F system where a β-glucosido-linkage in the determinant group could be demonstrated. There is no evidence about the linkage of glucose or the presence of a second sugar in the determinant group of the V antigen.

In all the systems tested, rhamnose and mannose were completely inactive serologically. This observation is, particularly in respect with rhamnose, in agreement with the findings of McCarty (1956) and Krause & McCarty (1962). In the

investigations of the group A and C streptococci these workers demonstrated a high rhamnose content of the respective polysaccharides. In their systems rhamnose was also completely inactive, except in the cases where the terminal groups of the side chains were removed (McCarty, 1956; Araujo & Krause, 1963).

The results of the quantitative analysis (Table 2) on the one hand and the values of the 50 % inhibition on the other hand (Table 4) are in broad lines in accordance with each other. However two important discrepancies in the respective results are to be relevated. The strong inhibitory effect of N-acetyl-galactosamine in the I and II systems was an unexpected finding given the fact that galactosamine was only weakly represented in both polysaccharides. It is expected that purification of the antigen might bring substantial alteration in the relative sugar composition of the polysaccharide. This is shown for instance by the remarkable differences in glucose and galactose contents of the V antigen after its purification by means of precipitation by the homologous antibody.

We are indebted to the Netherlands Organization for the Advancement of Pure Research (Z.W.O.) for financial support for part of these studies. The able assistance of Mr J. Bours is gratefully acknowledged.

This is the first paper of a series on the immunochemistry of group F streptococci.

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The Decomposition of Toluene by Soil Bacteria

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SUMMARY

Strains of two bacteria, a Pseudomonas and an Achromobacter, which grow with toluene, benzene or certain other related aromatic compounds as sole carbon source were isolated from soil. The use of aromatic compounds by these bacteria was an induced phenomenon. Toluene-grown organisms oxidized without lag toluene, benzene, catechol, 3-methylcatechol, benzyl alcohol and, more slowly, *o*- and *m*-cresol, but not benzaldehyde or benzoic acid. 3-Methylcatechol, acetic acid, pyruvic acid, and a yellow ether-soluble acidic substance which was colourless in acid solution, were detected in toluene-oxidizing cultures. Acetic and pyruvic acids were also formed during the bacterial oxidation of 3-methylcatechol. 3-Methylcatechol is probably an early stage in the bacterial metabolism of toluene; benzaldehyde and benzoic acid seem not to be intermediates in this metabolism.

INTRODUCTION

Störmer (1908) and Wagner (1914) showed that various aromatic compounds, including toluene, were probably susceptible to bacterial decomposition in soil. Gray & Thornton (1928) isolated from soil two strains of *Mycobacterium agreste* that could use toluene, and Tausson (1929) described four species, *Bacterium toluolicum* a, b, c and d, which grew with toluene and some other benzenoid hydrocarbons as sole carbon source. Tausson also investigated the conditions under which low boiling-point petroleum fractions might be oxidized by bacteria in the petroliferous soils of the Black Sea oilfields and established conditions for the laboratory cultivation of bacteria which decomposed toluene and other liquid hydrocarbons.

Kitagawa (1956) studied the oxidation of toluene and related compounds by a strain of *Pseudomonas aeruginosa* which had been grown in nutrient broth and afterwards exposed for some hours to small concentrations of toluene, benzyl alcohol, benzaldehyde or benzoic acid in phosphate buffer solutions. He concluded that toluene was possibly oxidized through this series of intermediates. Wieland, Griss & Haccius (1958) concluded that benzene was oxidized directly to catechol by a Nocardia strain isolated from manured soil.

Dagley & Patel (1957) showed that *p*-cresol was oxidized by a *Pseudomonas* sp. through *p*-hydroxybenzaldehyde, *p*-hydroxybenzoic acid and protocatechuic acid and that 2,4- and 3,4-xylenols were oxidized analogously, by the same organism, only to 3- and 2-methyl-4-hydroxybenzoic acids, respectively, through the corresponding alcohols and aldehydes. Leibnitz, Behrens, Striegler & Gabert (1962)

found that a *Pseudomonas* sp., isolated from a biological filter for purifying phenol-containing effluents, oxidized 2,4- and 3,4-xylenols under micro-aerophilic conditions to 3- and 2-methyl-4-hydroxybenzoic acids, with 3- and 2-methyl-4-hydroxybenzyl alcohol and 3- and 2-methyl-4-hydroxybenzaldehyde, respectively, as intermediate products. Pankhurst (1959), with buffered suspensions of a mixed population of bacteria obtained from a pilot plant for the biological oxidation of gas liquor, demonstrated the oxidation in Warburg respirometers of phenol, cresols, catechol, 3- and 4-methylcatechol, resorcinol, 2- and 4-methylresorcinol, quinol and phloroglucinol.

From all these results it might be inferred that the pathway of the bacterial metabolism of toluene is → benzyl alcohol → benzaldehyde → benzoic acid → catechol, as suggested by Kitagawa (1956); this process has not yet been unequivocally established with toluene-grown bacteria. We have isolated from soil several strains of bacteria which grow with toluene as sole carbon source and have studied their metabolism. Some preliminary results were reported to the VIIIth International Congress for Microbiology held at Montreal in August 1962.

METHODS

Media and culture methods. The following basal inorganic media were used, with the addition of the appropriate organic compound and, when required, of agar (2%). Medium A was a slight modification of Tausson's (1929) medium and compounded from solution *a*: $(NH_4)_2SO_4$, 1·2 g.; $CaCl_2.2H_2O$, 0·1 g.; $MgSO_4.7H_2O$, 0·1 g.; Fe (as ferric citrate), 0·002 g.; distilled water, 1 l., and solution *b*: K_2HPO_4, 0·2 g.; KH_2PO_4, 0·1 g.; distilled water, 200 ml., sterilized separately and mixed aseptically in the proportion of 5 vol. *a* to 1 vol. *b*. Medium B contained: K_2HPO_4, 0·8 g.; KH_2PO_4, 0·2 g.; $CaSO_4.2H_2O$, 0·05 g.; $MgSO_4.7H_2O$, 0·5 g.; $FeSO_4.7H_2O$, 0·01 g.; $(NH_4)_2SO_4$, 1·0 g.; distilled water, 1 l. Because of marked changes in pH value which occurred with different substrates, 1 g. $CaCO_3$/l. was added to this medium for the cultures to be used in oxygen-uptake experiments.

Cultural conditions for growing bacteria on toluene or other liquid aromatic hydrocarbon made use of Tausson's 'Methode des Diffusionszuflusses'. Agar plate cultures were incubated in a closed desiccator, over a saturated aqueous solution of toluene kept saturated by contact with liquid toluene contained in an inverted glass dish. For shaken-flask cultures, a small test tube with a hole in its wall and containing a little toluene was suspended inside the flask; or an open glass tube (5–6 mm. bore) was inserted through the cottonwool plug, dipped into the medium in the flask and a few drops of toluene were introduced into this tube. Toluene thus diffused as vapour or by solution into the medium without liquid toluene itself coming into direct contact with the bacteria. Toluene was supplied to larger cultures in flasks (5 or 10 l.) or in the continuous culture apparatus (described by Skinner & Walker, 1961) by aerating them with air previously saturated with water and toluene vapours.

Chemicals. Benzene, toluene and benzaldehyde were analytically pure reagents. Benzyl alcohol was of B.P. grade; *o*-, *m*- and *p*-cresol were laboratory grade chemicals from British Drug Houses Ltd. or from Light and Co. *o*-, *m*- and *p*-xylene, ethyl benzene, catechol, 4-methylcatechol and 2,3-dihydroxybenzoic acid were pure

laboratory reagents from Light and Co., the other dihydroxytoluenes, mono- and di-hydroxybenzoic acids were from Fluka (Switzerland). 3-Methylcatechol was prepared by reduction of *o*-vanillin by Clemmensen's method to 3-methylcatechol monomethyl ether, followed by demethylation of the latter in boiling 48 % aqueous hydrobromic acid. The reaction mixture was diluted with water and extracted with diethyl ether. The ether extract was washed with water, dried over anhydrous sodium sulphate, evaporated and the residue, after removal of ether, distilled under reduced pressure to yield a colourless distillate of 3-methylcatechol. We are also grateful to Dr I. Wender (Bureau of Mines, U.S. Department of the Interior, Pittsburgh) for a gift of 3-methylcatechol.

Manometric methods. Micro-organisms were washed twice by centrifuging in 0·02 M-phosphate buffer solution (pH 6·9) and re-suspended in the same solution. Different batches of Achromobacter suspensions had total N contents ranging from 0·1 to 0·4 mg. N/ml. and contained from 2 to 5 mg. dry matter/ml. Suspensions of Pseudomonas organisms were standardized by means of a photometer showing an optical density reading of 1·0 for a suspension containing about 0·2 mg. N/ml. and 2 mg. dry matter/ml. The oxygen uptakes by 0·5 ml. Achromobacter suspension or 0·3 ml. Pseudomonas suspension were determined in Warburg manometers at 30° in the usual way. Achromobacter suspension and enough phosphate buffer solution to give a final volume of 3 ml. were placed in the main cup of the Warburg flask and substrates in the side bulb. The centre cup contained 0·2 ml. 20 % (w/v) aqueous potassium hydroxide solution. Pseudomonas suspensions were added from the side bulb, the main cup in this case containing the substrate in 2 ml. phosphate buffer solution. When required, chloramphenicol (1 mg. in 0·1 ml. water) was added from the side bulb. Usually 2 μmole substrate were used except when otherwise stated.

Detection of intermediate products. In experiments with washed organisms 0·02 M-phosphate buffer (pH 6·8; 1000 ml.) containing substrate (5 μmole/ml. toluene, 3-methylcatechol, benzyl alcohol) with the addition of bacterial suspension (15 ml., optical density reading 1·0) was used as the reaction mixture. To detect phenolic compounds, the reaction mixture was shaken at 28° for 45 min. in 150 ml. portions, cooled to 0°, centrifuged and the supernatant liquid extracted with ether at once or after acidifying to pH 2·5 and concentrating *in vacuo*. Organic acids were detected in reaction mixtures that had been shaken at 28° for 2 hr; volatile acids were separated by steam distillation and keto acids determined as semicarbazones by the method of MacGee & Doudoroff (1954), with pyruvate for comparison purposes. 2,4-Dinitrophenylhydrazones were prepared by the method of Hulme (1961). Other organic acids were detected by paper chromatography after concentrating supernatant fluid at pH 4·0 or pH 9·0 *in vacuo* and extracting the acidified concentrates with ether for 36 or 48 hr. Acetate was estimated enzymatically with acetate kinase from *Escherichia coli* by the procedure of Rose (1955) and also by titration.

Paper chromatography. Phenols were chromatographed on Schleicher and Schüll (SS) paper 2043*a* with the following solvent systems: CCl_4 + *n*-butanol (19 + 1 by vol.), the paper saturated over water for 5 hr (Mráz, 1950); water-saturated *n*-butanol, the paper impregnated with 0·1 M-borate buffer (pH 8·7; Wachtmeister, 1952). Cresols were detected by Hudeček's (1955) method, using cyclohexane +

$CHCl_3$ + ethanol (27 + 3 + 0·6, by vol.), between glass plates. The mixture of isopropanol + conc. ammonia, sp.gr. 0·88 + water (8 + 1 + 1, by vol.) (Armstrong, Shaw & Wall, 1956) was used to separate hydroxybenzoic acids. Aqueous $FeCl_3$ (2%), Folin–Ciocalteau's reagent or diazotized *p*-nitroaniline (Procházka, 1958) were used as detecting sprays. Organic acids on paper SS 2043*a* were chromatographed with either *n*-propanol + conc. ammonia, sp.gr. 0·88 + water (6 + 3 + 1, by vol.), isobutanol + formic acid + water (6 + 1 + 2, by vol., Metzner, 1962) or ethanol + conc. ammonia, sp.gr. 0·88 (100 + 1, by vol., Kennedy & Barker, 1951). For detection sprays, aniline + xylose (Hulme, 1961) or bromophenol blue was used, or, in the case of keto acids, *o*-phenylenediamine (Wieland & Fischer, 1949). To separate dinitrophenylhydrazones, paper SS 2043*b* and *n*-butanol + ethanol + water (5 + 1 + 4, by vol.), *n*-butanol saturated with 3% ammonia solution or *t*-amyl alcohol + ethanol + water (5 + 1 + 4, by vol.) were used (Ranson, 1955). The spots were eluted with 0·2M-$NaHCO_3$ to determine absorption maxima.

RESULTS

Isolation and characterization of organisms

Toluene-decomposing bacteria were isolated from enrichment cultures obtained by inoculating medium A or B with a small quantity of moist soil previously treated with toluene vapour for several days, or with fresh soil. Pure cultures were obtained by the usual plating method, and plates were incubated in a toluene-containing atmosphere. In all, five isolates were obtained. The first isolate differed in several respects from the other four; it formed Gram-negative non-motile rods, 0·8 to 1·0μ × 1·5 to 1·7μ, usually occurring in pairs arranged end to end and did not form spores. It did not form indole in peptone water, reduce nitrate to nitrite, or liquefy gelatin; it was catalase-positive but oxidase-negative. It grew in nutrient broth or peptone water with a diffuse turbidity. Colonies on mineral salts agar with toluene as carbon and energy source were creamy white, smooth, shiny and entire, about 1–2 mm. in diameter. It did not ferment glucose, galactose, arabinose, sucrose, maltose, lactose, mannose, mannitol, glycerol, salicin, starch, fructose or xylose. The optimum temperature for growth was about 30°. It did not affect the pH value of arginine-containing peptone water or grow at the surface of a deep layer of glucose + peptone dilute agar medium, as would be expected of a typical pseudomonad. (See Thornley, 1960; Hugh & Leifson, 1953.) We consider this organism, therefore, to belong to the genus *Achromobacter*, but have failed to identify it with any species of this genus described in *Bergey's Manual of Determinative Bacteriology* (7th ed.). (Brisou & Prévot, 1954, suggested the separation of *Achromobacter* species into two genera, *Achromobacter* for motile species and a new genus *Acinetobacter* for non-motile ones. This suggestion, however, has not yet been generally adopted.)

The other four isolates were very much alike, physiologically and morphologically, and perhaps represented the same species. Isolate 4, for example, had the following characteristics: Gram-negative rods, 0·3 to 0·6μ × 1·0 to 1·7μ, single or in pairs, motile with one or occasionally two polar flagella, strictly aerobic. On nutrient agar, colonies were round or irregular, flat to convex, glistening cream-coloured. A diffuse turbidity with a pellicle was produced in nutrient broth cultures after 24 hr. There was only oxidative acid production in carbohydrate + peptone or carbohydrate +

mineral salts medium from glucose, fructose, galactose, xylose, sucrose, glycerol or ethanol, but no change with lactose, mannitol and sorbitol. The organism was oxidase-positive and catalase-positive; gave no hydrolysis of gelatin; nitrate was reduced to nitrite without gas formation. An alkaline reaction was produced anaerobically in arginine-containing peptone water. The optimum growth temperature was between 28° and 30°; the organism grew at 37° but not at 42°. A diffusible, yellowish-green pigment was produced on peptone glycerol agar but not on nutrient agar. We consider these four isolates, therefore, to belong to the genus *Pseudomonas*.

On spectroscopic examination of a thick paste of toluene-grown isolate 4 organisms, two intense broad bands at 550–558 mμ and 560–565 mμ were seen; there was a much fainter band at 520–528 mμ. This suggested the presence of a cytochrome *b* and a cytochrome *c* component. A similar observation of the Achromobacter organisms revealed only one intense band at 560–565 mμ, indicating that the main component was a cytochrome *b*.

Toluene metabolism was studied in one of these Pseudomonas isolates (isolate 4) and in the Achromobacter isolate.

Growth experiments

The use of various compounds for growth was tested in shaken flask cultures at 25° or 28°. The compounds examined or their solutions, sterilized by heat or by filtration, were added to medium B at different concentrations. The Pseudomonas isolate grew on toluene, benzene, benzyl alcohol (0·01 %; feebly at 0·1 %), benzaldehyde (0·01 %), benzoate (0·1 %), *o*-cresol (0·01 %, and 0·005 % with only feeble growth after 64 hr), *m*-cresol (0·01 %), phenol (0·01 %), catechol (0·01 %), 3-methylcatechol (0·005 %). Growth was not observed on *p*-cresol (0·01 %, 0·005 %), 4-methylcatechol (0·01 %, 0·005 %) or naphthalene.

The Achromobacter isolate grew on toluene, benzene, ethylbenzene, benzyl alcohol (0·05 %, 0·1 %), benzaldehyde (0·02 %), benzoate (0·1 %), catechol (0·01 %), 3-methylcatechol (0·002 %), *m*-cresol (0·02 %) but not on *cis*- or *trans*-stilbene, 4-methylcatechol or naphthalene.

Oxygen uptake experiments with bacteria grown on different compounds

To test compounds that might be intermediates in toluene catabolism, we examined several substances by the sequential induction method of Stanier (1947) by using washed organisms from cultures grown on different substrates.

Glucose-grown organisms were used to show that toluene metabolism by bacteria was an induced phenomenon. The results for Pseudomonas organisms showed that only glucose was oxidized without lag. The low rate of oxidation of the aromatic compounds other than benzyl alcohol increased gradually during the experiment, attaining a maximum after about 60 min. for catechol and benzaldehyde, 80 min. for benzoic acid, 150 min. for toluene and 190 min. for benzene. With benzyl alcohol the initial low rate of oxygen uptake did not increase with time.

Peptone-grown organisms of the Achromobacter isolate showed no immediate oxidation of any of the aromatic compounds tested (only catechol was oxidized after a short lag period), so confirming that toluene oxidation was an induced phenomenon.

Toluene-grown organisms. When cultures became too acid, the organisms obtained were much less active enzymatically than from neutral or slightly acid cultures. It was necessary, therefore, to grow cultures for only 24 hr or to add $CaCO_3$ to the medium. The Achromobacter isolate oxidized toluene rapidly, benzyl alcohol a little less rapidly, while benzaldehyde and benzoic acid were not oxidized. In another experiment, benzene and catechol were oxidized fairly rapidly after a short lag. The failure to oxidize benzaldehyde, benzoic acid and catechol without lag casts doubt on whether these compounds are true intermediates in the dissimilation of toluene.

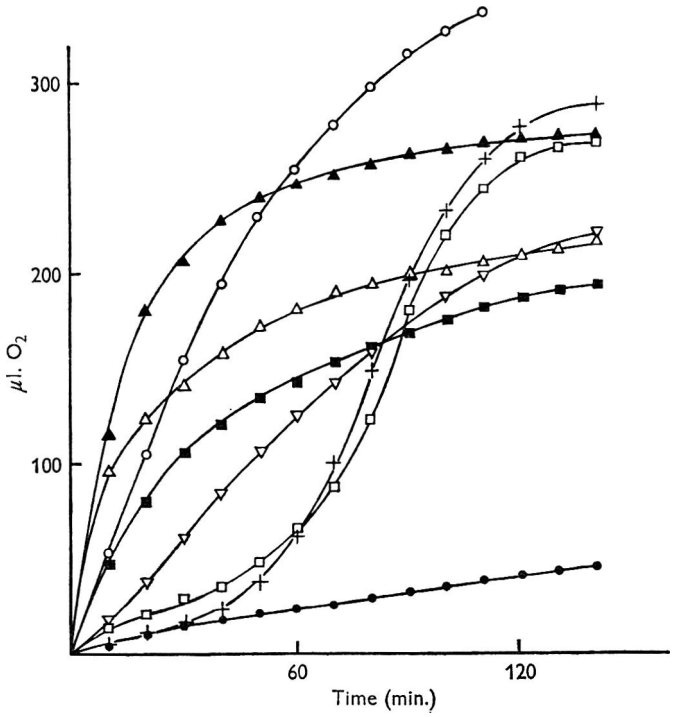

Fig. 1. Rate of oxygen uptake by washed, benzene-grown Pseudomonas organisms (●——●) and in the presence of benzene (▲——▲), toluene (△——△), benzyl alcohol (○——○), benzaldehyde (□——□), benzoic acid (+——+), catechol (■——■) and phenol (▽——▽).

Toluene and benzyl alcohol were oxidized immediately by the Pseudomonas suspensions; benzaldehyde and benzoic acid were oxidized only after an induction period. No lag was observed with benzene or catechol, although these were oxidized more slowly than toluene. Both Achromobacter and Pseudomonas organisms oxidized *o*- and *m*-cresol at a moderate rate but not *p*-cresol. Other compounds not oxidized by either organism included resorcinol, hydroquinone, 2,5-, 2,6- and 3,5-dihydroxytoluene, *o*-, *m*- and *p*-hydroxybenzoic acid and 2,3-, 2,4-, 2,5-, 3,4- and 3,5 dihydroxybenzoic acids.

Benzene-grown organisms. With Pseudomonas, benzene, toluene and catechol were all oxidized immediately, but benzaldehyde and benzoic acid only after a lag; benzyl alcohol was also oxidized fairly quickly and phenol more slowly. *o*- and *m*-Cresol

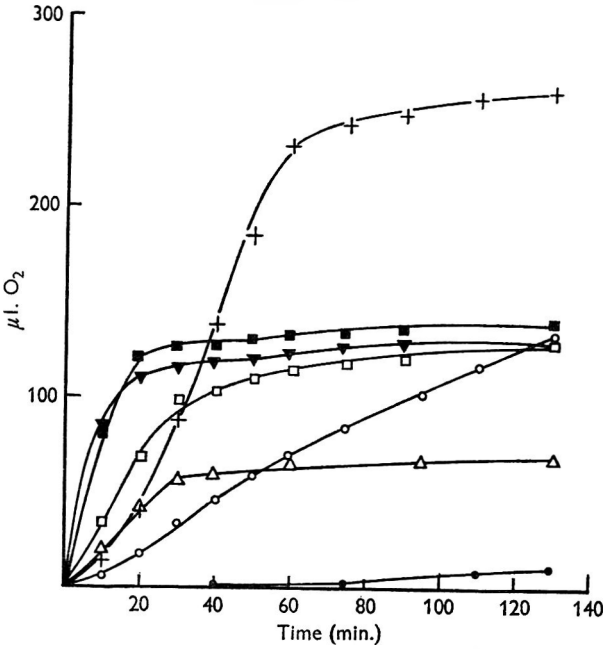

Fig. 2. Rate of oxygen uptake by washed, benzyl alcohol-grown Achromobacter organisms alone (●——●) and in the presence of toluene (△——△), benzyl alcohol (○——○), benzaldehyde (□——□), benzoic acid (+——+), catechol (■——■) and 3-methylcatechol (▼——▼).

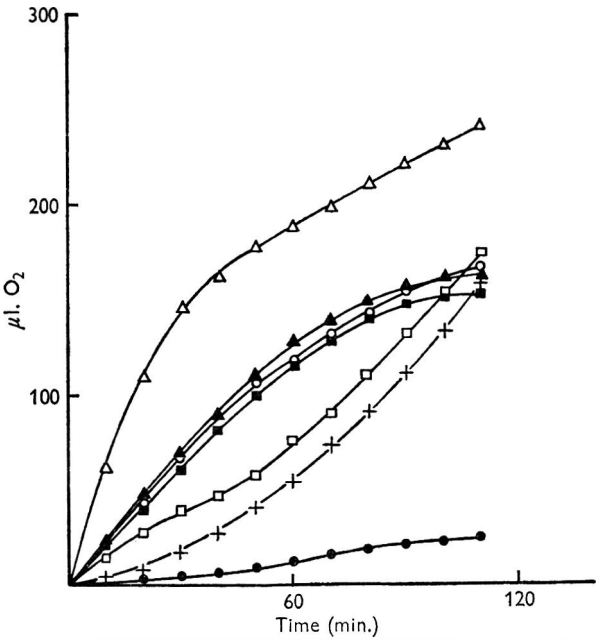

Fig. 3. Rate of oxygen uptake by washed, benzyl alcohol-grown Pseudomonas organisms alone (●——●) and in the presence of toluene (△——△), benzyl alcohol (○——○), benzaldehyde (□——□), benzoic acid (+——+), catechol (■——■) and benzene (▲——▲).

were oxidized at a similar rate to phenol, so the rates of oxygen uptake with cresols have been omitted from Fig. 1. The Achromobacter isolate behaved similarly.

Benzyl alcohol-grown organisms. The Achromobacter isolate oxidized catechol and 3-methylcatechol rapidly, benzyl alcohol, benzaldehyde and toluene at a moderate rate and, after a very short lag, benzoic acid. Similar results were obtained with

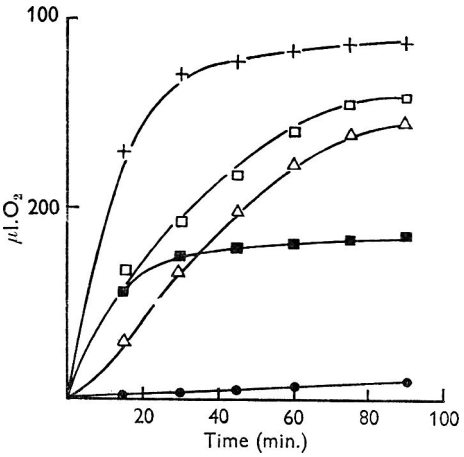

Fig. 4. Rate of oxygen uptake by washed, benzaldehyde-grown Achromobacter organisms alone (●——●) and in the presence of toluene (△——△), benzaldehyde (□——□), benzoic acid (+——+) and catechol (■——■).

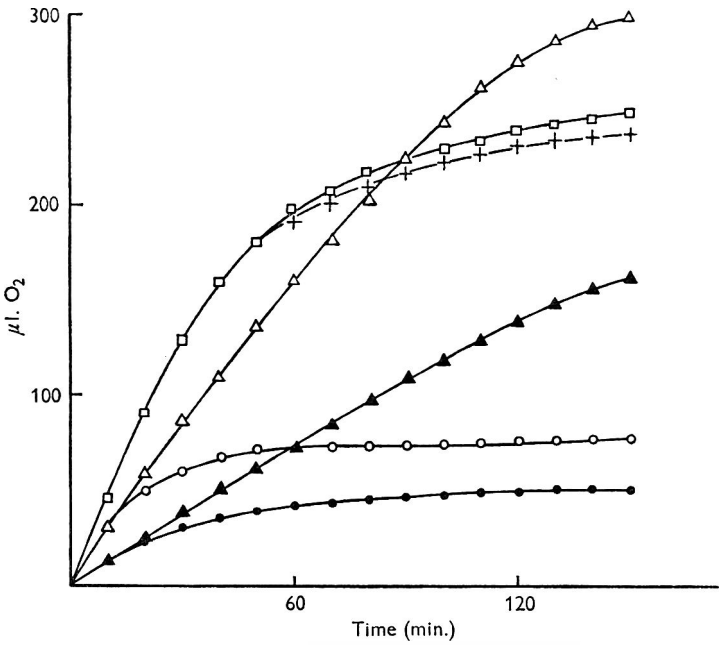

Fig. 5. Rate of oxygen uptake by washed, benzaldehyde-grown Pseudomonas organisms alone (●——●) and in the presence of toluene (△——△), benzene (▲——▲), benzyl alcohol (○——○), benzaldehyde (□——□) and benzoic acid (+——+).

the Pseudomonas isolate, toluene being oxidized more rapidly than benzyl alcohol. There was a lag before benzoate was oxidized. With benzaldehyde, uptake of oxygen began at once, but after 30 min. the rate decreased slightly, to be followed by an increase to the rate of benzoate oxidation. This second rate of oxygen uptake seemed to reflect a true induction process (see Fig. 9; chloramphenicol experiments). Catechol and benzene were oxidized as readily as benzyl alcohol; the phenol and cresols were not oxidized (Figs. 2 and 3).

Benzaldehyde-grown organisms. Oxygen uptake by the Achromobacter isolate from 24 hr cultures was immediate and rapid with benzoic acid, rather slower with benzaldehyde or catechol, slower still with toluene after a short lag; curiously, there was no uptake with benzyl alcohol (Fig. 4). Organisms from 72 hr cultures, however, oxidized benzyl alcohol slowly. Pseudomonas organisms oxidized without lag benzaldehyde, benzoic acid, toluene and, more slowly, benzene; benzyl alcohol was oxidized immediately but the amount of oxygen uptake was small. Phenol and cresols were not oxidized (Fig. 5).

Benzoate-grown organisms. The Achromobacter suspensions oxidized benzoate, benzaldehyde and catechol rapidly, benzyl alcohol much more slowly and toluene scarcely at all. The Pseudomonas isolate oxidized rapidly and without lag benzoate, benzaldehyde and catechol; benzyl alcohol and toluene were only feebly oxidized, the rate of oxygen uptake with toluene increasing very slightly after a long delay. A yellow colour was not formed consistently with catechol as substrate, whereas toluene-grown organisms always produced a yellow colour from both benzene and catechol substrates.

Catechol-grown organisms. The Pseudomonas organisms gave an oxygen uptake immediately with catechol, after a short lag with benzoate and after longer lags with toluene or benzene. The Achromobacter isolate showed a rapid oxygen uptake with catechol immediately, a very slow uptake with benzoate and none with toluene or benzene.

Oxidation of methylcatechols

The immediate oxidation of catechol by toluene-grown organisms and their failure to oxidize benzoate without a lag led us to study the methylcatechols in similar oxygen uptake experiments. 3-Methylcatechol was oxidized immediately and rapidly by toluene-grown Pseudomonas organisms, and in addition by organisms grown on benzyl alcohol, benzaldehyde or benzene. Benzoate-grown organisms oxidized 3-methylcatechol only slowly and catechol-grown organisms not at all (Fig. 6). Indeed, 3-methylcatechol decreased the rate of oxidation of catechol by catechol-grown organisms. Catechol-grown Achromobacter organisms also oxidized 3-methylcatechol very feebly. 4-Methylcatechol was oxidized slightly or not at all by the Pseudomonas organisms, irrespective of the carbon source on which they had been grown.

As there was no growth on 4-methylcatechol, only organisms grown on 3-methylcatechol were tested; these oxidized toluene, catechol and 3-methylcatechol rapidly and without lag (Fig. 7).

The oxygen uptake with *o*- or *m*-cresol by toluene-grown Pseudomonas organisms was greatly inhibited by 4-methylcatechol. The apparently feeble oxidation of 4-methylcatechol itself, therefore, might have been caused by its toxicity and its

oxidation in sequential induction experiments could not be appraised. 4-Methylcatechol only slightly depressed the rate of oxidation of toluene (Fig. 8). If toluene were oxidized through 4-methylcatechol, then the rate of toluene oxidation in presence of 4-methylcatechol should run parallel to that of 4-methylcatechol after the uptake of 1 mole O_2/ μ mole toluene, which is not the case. 3-Methylcatechol, *o*- or *m*-cresol had no effect on toluene oxidation.

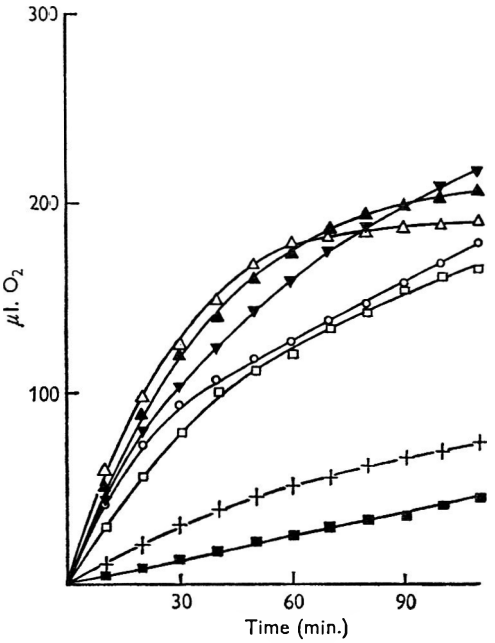

Fig. 6. Rates of oxygen uptake in the presence of 2 μmole 3-methylcatechol by washed Pseudomonas organisms grown on either benzene (▲——▲), toluene (△——△), benzyl alcohol (○——○), benzaldehyde (□——□), benzoic acid (+——+), catechol (■——■) or 3-methylcatechol (▼——▼).

Effect of chloramphenicol on oxidations by organisms adapted to different substrates

In some experiments the lag or induction phase in the oxidation of a substrate by non-adapted organisms was not always clearly shown. For this reason and also to detect very rapid enzyme inductions, some experiments were made in the presence of chloramphenicol, which prevents neo-enzyme formation by blocking protein synthesis (see Brock, 1961). Table 1 shows results of these experiments with the Pseudomonas isolate. The oxidation of benzaldehyde by benzyl alcohol-grown organisms, where two different processes were apparently taking place, deserves special note (Fig. 9). The immediate oxygen uptake not affected by chloramphenicol was about 0·5 μmole O_2/μmole benzaldehyde; in contrast, benzoate was not oxidized immediately. This may be an unspecific oxidation, because the phenomenon occurred also, though less distinctly, with toluene- or benzene-grown organisms.

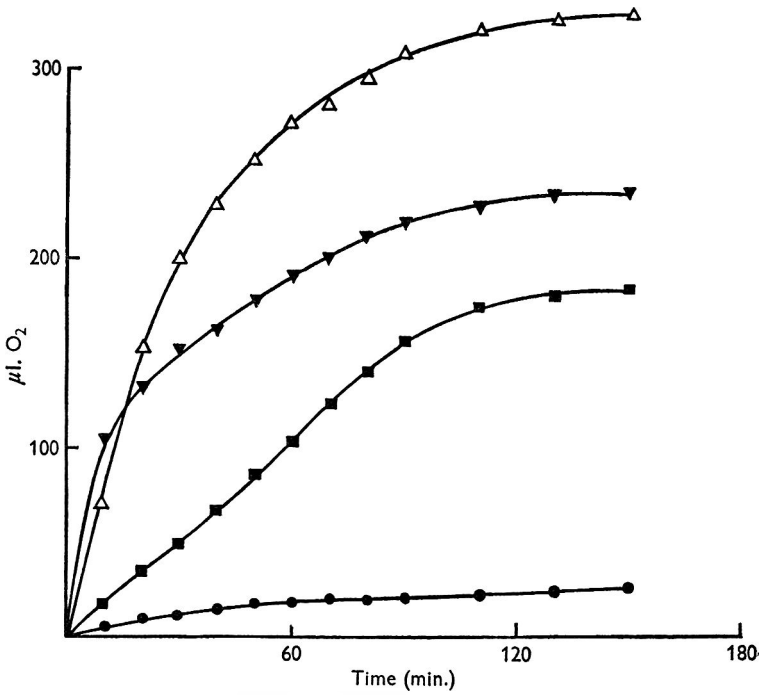

Fig. 7. Rate of oxygen uptake by washed, 3-methylcatechol-grown Pseudomonas organisms alone (●——●) and in the presence of toluene (△——△), catechol (■——■) and 3-methylcatechol (▼——▼).

Table 1. *Effect of chloramphenicol on oxidation of different substrates by washed Pseudomonas organisms grown on different compounds*

Rates of oxygen uptake were measured in Warburg manometers as described under 'manometric methods'. +, Immediate oxygen uptake, without lag, chloramphenicol without effect; −, complete inhibition of oxygen uptake by chloramphenicol; o, no oxygen uptake even in absence of chloramphenicol; ×, partial inhibition of oxygen uptake by chloramphenicol; /, not tested.

	Substrates									
Organisms grown on	Toluene	Benzene	Benzyl alcohol	Benzal-dehyde	Benzoate	Catechol	3-Methyl-catechol	Phenol	*o*-Cresol	*m*-Cresol
Toluene	+	+	+	−	−	+	+	+	+	+
Benzene	+	+	+	−	−	+	+	+	/	/
Benzyl alcohol	+	+	+	×	−	+	+	o	o	o
Benzaldehyde	+	+	+	+	+	+	+	o	o	o
Benzoate	−	/	−	/	+	+	/	o	o	o
Catechol	−	−	o	/	−	+	o	/	/	/
3-Methylcatechol	+	/	/	/	/	+	+	/	/	/
Glucose	−	−	−	−	−	−	/	/	/	/

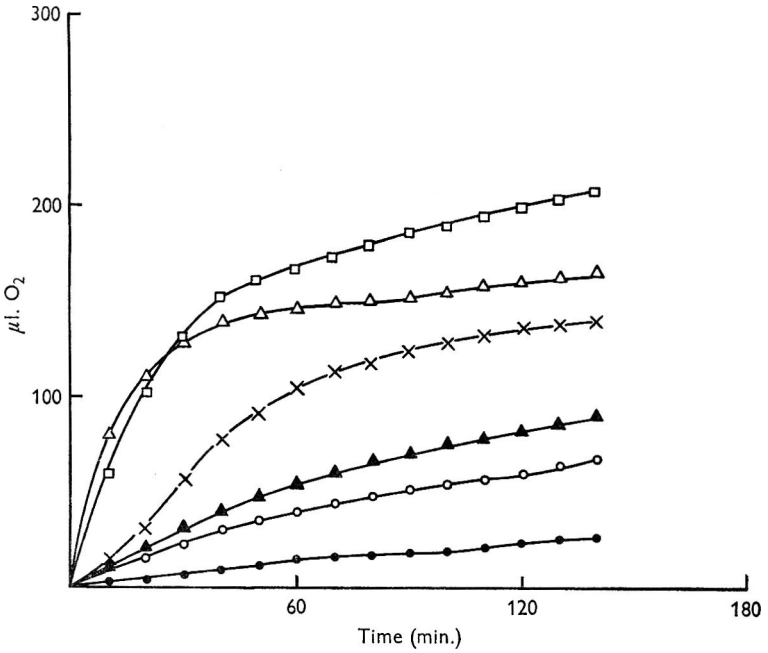

Fig. 8. Effect of 4-methylcatechol (1 µmole/3 ml.) on the oxidation of toluene or *o*-cresol by washed, toluene-grown Pseudomonas organisms. Rates of oxygen uptake by washed organisms alone (●——●) and in the presence of toluene (1 µmole) (△——△), o-cresol (1 µmole) (×——×), 4-methylcatechol (1 µmole) (○——○), a mixture of toluene (1 µmole) and 4-methylcatechol (1 µmole) (□——□) or a mixture of *o*-cresol (1 µmole) and 4-methylcatechol (1 µmole) (▲——▲).

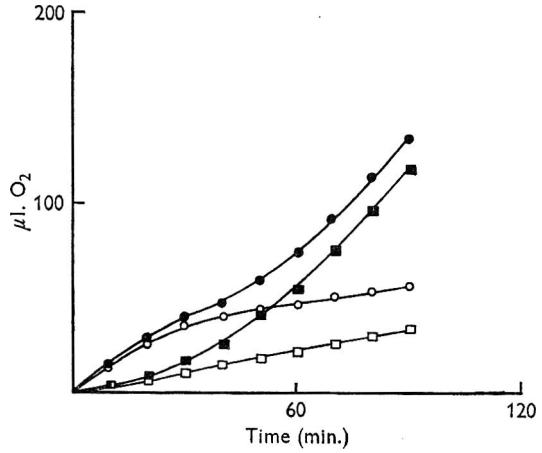

Fig. 9. Rates of oxygen uptake by washed, benzyl alcohol-grown Pseudomonas organisms in the presence of benzaldehyde (●——●) or benzoic acid (■——■) and with the addition of chloramphenicol; benzaldehyde + chloramphenicol (○——○): benzoic acid + chloramphenicol (□——□).

Detection of intermediate products of toluene metabolism

To obtain more direct evidence of the pathway of toluene dissimilation, we tried to detect possible intermediates accumulating in cultures or in buffer suspensions of washed organisms after added toluene was oxidized. Hydroxylated compounds might be expected in the early stages of toluene metabolism and the lowering of the pH value of growing cultures must be caused by acidic substances.

Phenolic compounds. Manometric experiments showed that 3-methylcatechol was oxidized more slowly than toluene and, if it were an intermediate, it might therefore accumulate in the medium. 3-Methylcatechol was indeed detected by paper chromatography at small concentrations in ether extracts of toluene-oxidizing Pseudomonas suspensions. Material from the ether extracts showed the same colour reaction with $FeCl_3$ as an authentic specimen and the same R_F value in two different solvent mixtures, one on borate-treated paper; the borate-treated paper was used since it retains *o*-diphenols more strongly than other phenols, because of complex formation. Another substance, not identical with any mono- or di-hydroxytoluene but reacting with the Folin–Ciocalteau reagent, was regularly detected (R_F 0·51, water-saturated *n*-butanol; ratio of movement to that of 3-methylcatechol, 0·50, in CCl_4 + *n*-butanol where the solvent ran off the paper). Occasionally α- and β-resorcylic acids were detected, but only in traces; these acids were not oxidized by toluene-grown organisms. Cresols were not detected.

Organic acids. Cultures of Pseudomonas isolates or the Achromobacter isolate grown in medium A with toluene as carbon source became acid (pH 4·2) after 36–48 hr. The cultures became yellow after 20–24 hr; the colour disappeared later as the medium became more acid, but was restored by adding sodium carbonate. After acidification with dilute sulphuric acid to pH 2, ether extraction of such cultures (12 l.) afforded about 5 mg. of a yellow product not yet identified. It behaved as an acid, being extracted from ethereal solution by aqueous sodium hydrogen carbonate; it was deep yellow in neutral or alkaline solution and colourless in acid. A similar pigment was produced when toluene or benzene were oxidized by washed suspensions of organisms but in amounts too small for further analysis.

When toluene (5 m-mole/1000 ml.) was oxidized by the Pseudomonas organisms, acids were formed and the pH of the mixture fell from 6·8 to 4·5 in 2 hr. Distillation of the reaction mixture with steam gave 4·5 m-mole volatile acid that behaved like acetic acid on paper chromatography. After admixture with pure acetic acid a uniform peak was obtained by chromatography on Celite. Determination of acetic acid in the steam distillate by the acetate kinase method gave values in agreement with those obtained by titration. Assay for keto acids indicated 3·0 m-mole/1000 ml. keto acid in parallel experiments. By conversion to its 2,4-dinitrophenylhydrazone followed by paper chromatography, the same R_F value as for authentic pyruvic acid 2,4-dinitrophenylhydrazone was obtained; elution of the spots with 0·2 N-$NaHCO_3$ gave eluates showing the same absorption maxima. In addition, there was one other fainter unidentified spot (R_F 0·32, *t*-amyl alcohol + ethanol + water; R_F 0·09, *n*-butanol + 3 % NH_3 in water) on the paper chromatogram.

Separation of the total acids from concentrated solutions or ether extracts by column chromatography on Celite was not achieved. Gas formation within the

column during elution, perhaps due to decarboxylation of some keto acid, prevented a precise separation.

The oxidation of 5 m-mole 3-methylcatechol by toluene-grown organisms gave 4·9 m-mole volatile acid and 3·8 m-mole keto acids. Similar results (4·3 m-mole volatile acid and 3·8 m-mole keto acids) were obtained in the oxidation of toluene (5 m-mole/1000 ml.) by benzene-grown organisms. These acids or their 2,4-dinitrophenylhydrazones behaved on paper chromatography like those obtained when toluene was oxidized by toluene-grown organisms. Estimation with acetate kinase also showed that the volatile acid was acetic acid.

In the ether extracts of the different concentrated cell-free solutions, we detected by paper chromatography, besides acetic acid, only one other acid, namely pyruvic acid. Other organic acids were not found in the above experiments; the course of the oxidation of toluene by benzene-grown organisms was not affected by the addition of chloramphenicol.

When benzyl alcohol was oxidized by toluene-grown Pseudomonas organisms, distillation of the reaction mixture with steam afforded a very small quantity of glycollic acid in addition to acetic acid.

DISCUSSION

A feature of our experiments on sequential induction is that various substrates acted as inducers for compounds that cannot be metabolic intermediates of the inducing substrate. Toluene, for example, was oxidized by organisms grown with benzene, benzyl alcohol, benzaldehyde or 3-methylcatechol. This low degree of substrate specificity complicates the elucidation of the pathway of toluene metabolism by the method of sequential induction and additional evidence obtained by detecting intermediates or from enzyme studies is desirable. Some variations in amounts of oxygen consumed in different experiments were unavoidable; the gradual acidification of cultures caused a decrease in the enzymic activity of the organisms and a slight loss of benzene or toluene from aqueous solutions could occur through volatilization.

Our isolates seem not to oxidize toluene or benzyl alcohol via the intermediates benzaldehyde, benzoic acid and catechol, as proposed by Kitagawa (1956) for *Pseudomonas aeruginosa*. Benzoate was oxidized only after a distinct lag, the duration of which scarcely differed with the organisms grown on glucose, catechol, toluene or benzene. Chloramphenicol completely prevented this oxidation. Further, with more than twenty other uncharacterized bacterial strains from toluene or cresol enrichments, the same picture was found as with Pseudomonas and Achromobacter isolates, namely, immediate oxidation of 3-methylcatechol but of benzoate only after a lag.

Benzyl alcohol, *o*- and *m*-cresol and 3-methylcatechol have to be considered as possible initial metabolites of toluene because they were all oxidized by the toluene-grown organisms without any lag. The cresols, however, were oxidized only slowly and the analogous oxidation of phenol by benzene-grown organisms must be borne in mind, since phenol was not oxidized by other benzene-decomposing bacteria studied by Wieland *et al.* (1958) and by Marr & Stone (1961). We do not consider these cresols to be intermediates, because, whereas 4-methylcatechol did not affect

toluene oxidation, it greatly inhibited oxidation of the cresols. Benzyl alcohol-grown organisms rapidly oxidized toluene, but not the cresols. Perhaps the monohydroxy compounds are at first oxidized non-specifically by phenol oxidases to the corresponding *o*-dihydric phenols. The results of O_2-uptake experiments alone do not permit a decision whether 3-methylcatechol or benzyl alcohol is the first oxidation product.

The mutual adaptations to use benzene and toluene suggest that enzymes with similar activities may be involved in the metabolism of the two compounds; the presence of a methyl group in the molecule may be without decisive influence on the specificity of the enzymes. Benzyl alcohol oxidation, induced by toluene or benzene, and the oxidation of these hydrocarbons induced by benzyl alcohol, may be a parallel phenomenon. Analogous observations were made by Rogoff & Wender (1959) with naphthalene- and monomethylnaphthalene-grown pseudomonads which oxidized catechol, 3- and 4-methylcatechol at very similar rates.

These considerations, therefore, point to a degradation of toluene via 3-methylcatechol, comparable with the bacterial oxidation of benzene via catechol (Marr & Stone, 1961; Wieland *et al.* 1958). There are some difficulties in this explanation. 3-Methylcatechol-grown organisms are adapted to oxidize toluene, benzene and catechol, whereas catechol-grown organisms do not metabolize 3-methylcatechol, or only feebly. Conversion of 3-methylcatechol to catechol through the formation of *o*-hydroxybenzoic acid seems most unlikely. We have no explanation for the induction effect of 3-methylcatechol towards toluene; preliminary experiments with cell-free preparations suggest that the toluene-oxidizing enzyme differs from that which oxidizes 3-methylcatechol.

3-Methylcatechol was detected by paper chromatography in toluene cultures, which is further evidence that it is an intermediate. Moreover, oxidation of toluene or 3-methylcatechol by toluene-grown organisms gave the same oxidation products in about the same amounts. The formation of glycollic acid from benzyl alcohol but not from toluene seems to be caused by the presence of the $CH_2OH.C$ grouping, whereas in toluene the corresponding $CH_3.C$ results in acetic acid. A study of toluene metabolism by benzene-grown organisms has not proved helpful because these organisms metabolize toluene even in presence of chloramphenicol without accumulation of any methyl-substituted products. Further knowledge of the mechanism of ring fission in toluene metabolism must await experiments with cell-free enzyme preparations.

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Studies on the Mechanism of Potassium-stimulated Respiration in *Escherichia coli*

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SUMMARY

The inter-relationship between the intracellular concentration of potassium ions and the rate of oxidation of glutamate was investigated in washed *Escherichia coli*. The time-curve of glutamate oxidation by the potassium-depleted coli showed a marked lag phase and the rate of oxygen uptake increased concurrently with the accumulation of potassium ions. After a constant intracellular potassium concentration was reached, the rate of oxidation remained constant. Carbonyl cyanide *m*-chlorophenylhydrazone (*m*-Cl-CCP) and methylene blue inhibited the respiration when added to the reaction mixture during the initial phase of K^+ accumulation. The extent of inhibition induced by these compounds was inversely related to the rate of oxidation prevailing at the time of their addition. No inhibition resulted when the substances were added after the K^+ accumulation and respiratory rate had reached the steady state values. Pre-incubation with glucose and KCl abolished the initial lag of glutamate oxidation as well as the inhibitory action of *m*-Cl-CCP. It is concluded that the intracellular concentration rather than the flux of potassium ion governs the control of respiration in *E. coli*. The possible relation of the mode of respiratory inhibition induced by *m*-Cl-CCP and methylene blue to the known ability of these compounds to uncouple oxidative phosphorylation, is discussed in terms of the presumed energy requirements of the system mediating the K^+ transport.

INTRODUCTION

An enhancing effect of potassium ions on bacterial respiration has been reported by several investigators (Quastel & Webley, 1942; Krebs, Whittam & Hems, 1957; Miller & Avi-Dor, 1958). The potassium-stimulated respiration was found to be attended by an accumulation of potassium ions in the bacteria (Krebs *et al.* 1957). However, the question of whether the rate of oxidation of the various substrates is governed by the flux of ions (see Whittam, 1961) or by the size of the intracellular K^+ pool (see Krebs *et al.* 1957; Hems & Krebs, 1962) remains as yet unsettled. The present paper is concerned with some aspects of the mechanism of potassium accumulation and the relationship between the intracellular concentration of potassium and the respiratory activity of the bacteria.

METHODS

Chemicals. The chemicals were obtained from the following sources. L-glutamic acid (Merck A. G., Darmstadt); succinic acid (Hopkin & Williams Ltd.); the solutions of the free acids were adjusted to pH 7·2 by adding suitable amounts of tris buffer. Carbonyl cyanide *m*-chlorophenylhydrazone (*m*-Cl-CCP) was generously supplied by Dr P. G. Heytler (E. I. Du Pont de Nemours and Co.). Uniformly labelled (^{14}C) glutamic acid (specific activity: 24 μc./mg.) from the Radiochemical Centre, Amersham, Buckinghamshire. Cab-O-Sil gelatinizing agent was obtained from the Packard Instrument Co. (LaGrange, Illinois, U.S.A.).

Bacterial suspensions. Escherichia coli strain B was used throughout. Unless otherwise specified, the organism was grown on Difco nutrient agar. In some experiments the organism was grown on the minimal medium of Davis & Mingioli (1950) in which, however, glucose was replaced by succinate or glutamate (0·4%, w/v) as carbon source. Following incubation at 37° for 24 hr the bacteria were harvested and washed three times with ice-cold redistilled water. The centrifugations were carried out for 10 min. at 12,000 *g* at a temperature near 0°. The washed organisms were suspended in 0·25 M-sucrose solution to a final concentration equivalent to 50 mg. wet wt./ml.

Analytical methods. To determine the concentration of intracellular potassium a sample of the bacterial suspension was centrifuged for 15 min. at 12,000 *g* and the organisms washed once with the original volume of 0·25 M-sucrose. Acid-digestion and flame photometric determination of potassium were then performed as described by Krebs *et al.* (1957).

Oxygen uptake was measured by the conventional manometric method. For measuring (^{14}C) CO_2 production from labelled substrates, the respiratory CO_2 was trapped by KOH-moistened filter paper placed in the centre well of the Warburg flask and radioactivity was counted in a toluene-ethanol scintillator solution according to Buhler (1962). For measuring the radioactivity incorporated into the bacteria, a sample of the suspension was washed as described above for the determination of potassium. The bacteria were then resuspended in 0·5 ml. water and added to 10 ml. of Bray's scintillator solution (Bray, 1960) gelatinized with Cab-O-Sil thixotropic gel (4%, w/v). Radioactivity was measured in a Tri-Carb Model 314 F liquid scintillation counter of the Packard Instrument Company.

RESULTS

Stimulatory effect of potassium ions on respiration

In a previous communication (Miller & Avi-Dor, 1958) potassium ions were found to stimulate the oxidation of glutamate and glucose by washed *Escherichia coli*. It may be seen from Table 1 that the oxidation rate of several other substrates was also substantially accelerated by K^+. The magnitude of the effect, however, varied with the different substrates, when tested under standard conditions involving three successive washings of the bacteria. While this treatment was usually sufficient to render the oxidation of glutamate strictly dependent on added K^+, the oxidation of other substrates, in particular of succinate, proceeded at an appreciable rate without added K^+. However, on increasing the number of washings the

effect of K^+ on succinate oxidation became increasingly prominent. Since the K^+ requirement for glutamate oxidation was the most readily shown, the latter substrate was used in most of the experiments to be described.

Table 1. *Stimulation by potassium chloride of the oxidation of various substrates by Escherichia coli*

Each Warburg flask contained 50 mM-tris buffer (pH 7·2), 3·3 mM of the respective substrate, 15 mg. wet wt. washed *E. coli*, and 3·3 mM KCl when indicated. Acetate and pyruvate were added as sodium salts. The solutions of succinic and glutamic acid were adjusted to pH 7·2 by adding suitable amounts of tris buffer (free base). Total volume: 3 ml. Incubation temperature: 28°.

Substrate	Oxygen uptake (μl./hr)	
	KCl omitted	KCl added
None	0	6
L-Glutamate	12	160
Succinate	90	120
Acetate	60	160
Pyruvate	40	140
D-Glucose	80	170

To explore the correlation between K^+ accumulation and respiratory activity, washed *Escherichia coli* was incubated in Warburg flasks in the presence of glutamate and graded amounts of KCl. The rate of oxygen uptake was recorded and at the end of a 60 min. incubation period the intracellular K^+ concentration was determined. As shown in Fig. 1, both the intracellular K^+ and the oxidation rate increased in a nearly parallel fashion with increasing concentrations of KCl in the medium. At 3 mM-KCl the rate of oxygen uptake reached its maximum value and the curve representing intracellular K^+ started to level off.

Figure 2 illustrates a kinetic experiment in which the time-curves of K^+ accumulation and of oxygen uptake were compared. It can be seen that, apart from a small amount of K^+ which entered the organisms instantaneously, there was very little accumulation of K^+ in the organisms during the first 10 min. of incubation. This initial lag was followed by a phase of rapid increase in intracellular K^+, approaching a constant value after 30 min. of incubation. The oxygen uptake exhibited a similar course of gradual increase until a steady state was attained which closely coincided with the time the intracellular K^+ concentration reached a plateau.

The observed correlation between the intracellular K^+ concentration and the oxidation rate was further corroborated by the results summarized in Table 2. In this experiment washed *Escherichia coli* organisms were enriched in K^+ by previous incubation in the presence of glutamate + KCl, followed by washing with 0·25 M-sucrose solution. The organisms so treated, when transferred to fresh medium containing no KCl, continued to oxidize glutamate at a rate amounting to about 70% of that observed in the presence of added K^+. Control organisms which had been pre-incubated with KCl but without glutamate showed no detectable respiration. Furthermore, when the potassium-enriched organisms were incubated in the absence of an energy-yielding substrate, their intracellular K^+ pool rapidly became exhausted by diffusion into the surrounding medium.

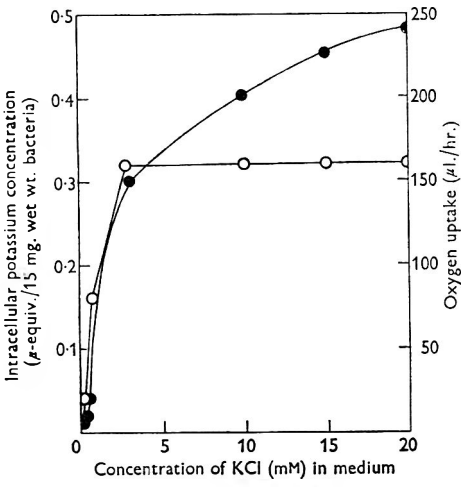

Fig. 1

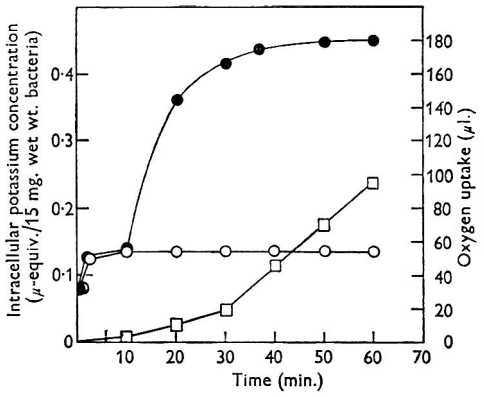

Fig. 2

Fig. 1. Effect of KCl concentration in the medium on the intracellular accumulation of potassium and glutamate oxidation. Each Warburg flask contained 50 mM-tris buffer chloride (pH 7·2), 3·3 mM-glutamate, 15 mg. wet wt. washed bacteria and KCl as indicated. Total volume: 3·0 ml. Incubation: 60 min. at 28°. At the end of incubation intracellular potassium was determined as described under Methods. ○, Rate of oxygen uptake; ●, concentration of intracellular potassium.

Fig. 2. Time-course of oxygen uptake and of intracellular accumulation of potassium. Each Warburg flask contained 50 mM-tris buffer (pH 7·2), 3·3 mM-KCl, 15 mg. wet wt. washed bacteria and 3·3 mM-glutamate when indicated. Total volume 3·0 ml. Other conditions as in Fig. 1. ○, Intracellular potassium in the absence of glutamate; ●, intracellular potassium in the presence of glutamate; □, oxygen uptake.

Table 2. *Effect of the intracellular potassium concentration on the rate of glutamate oxidation by Escherichia coli*

Pre-treatments. Washed bacteria were incubated with shaking at 28° for 60 min. with KCl alone (A) or with 3·3 mM-glutamate + KCl (B). The pretreated bacteria were separated by centrifugation (20 min. at 12,000 *g*), and washed once with 0·25 M-sucrose. The final resuspension was made up in 0·25 M-sucrose. At the end of the 'pretreatment' the bacterial suspensions A and B contained 0·1 and 0·4 μ-equiv. potassium, respectively, per 15 mg. bacterial wet wt.

Assay. To each Warburg flask containing 50 mM-tris buffer (pH 7·2) and 3·3 mM-KCl and/or 3·3 mM-glutamate as indicated, 15 mg. wet wt. bacteria of the suspension (A) or (B) were added; total volume 3·0 ml. Incubation was carried out for 60 min. at 28°. Oxygen uptake was recorded every 10 min. At the end of the incubation the potassium content of the bacteria was determined as described under Methods.

Bacterial suspension	Additions to the reaction mixture		Oxygen uptake (μl./hr)	Potassium content at the end of the assay (μ-equiv./15 mg. wet. wt.)
	Glutamate	KCl		
A	None	None	0	0·06
	+	None	0	0·12
	+	+	90	0·35
B	None	None	0	0·04
	+	None	90	0·40
	+	+	120	0·44

It seemed of interest to test whether oxidation of glutamate would start without any lag in K^+-rich organisms. For this purpose organisms were pre-incubated with KCl + glucose (the latter compound served as energy source for K^+ accumulation). Organisms incubated with KCl alone served as controls. Following pre-incubation, (^{14}C)-labelled glutamate was added and the time-course of (^{14}C) CO_2 liberation and of glutamate uptake (in terms of radioactivity incorporated into the cell mass) was followed. The organisms which respired in the presence of glucose + KCl were found to contain 0·40 μ-equiv. K^+/15 mg. wet wt. at the time glutamate was added and showed practically no delay in the liberation of (^{14}C) CO_2. This behaviour con-

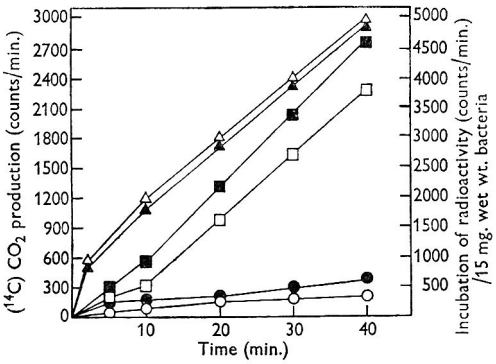

Fig. 3

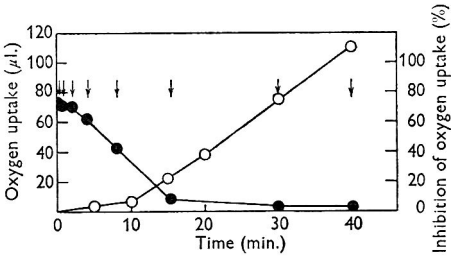

Fig. 4

Fig. 3. Effect of pre-incubation with glucose on the rate of oxidation of (^{14}C) glutamate and incorporation of radioactivity into the bacterial cells. Each Warburg flask contained 50 mM-tris buffer (pH 7·2), 15 mg. wet wt. washed bacteria and 3·3 mM-KCl or/and 3·3 mM-glucose when indicated. Total volume: 3·0 ml. After 10 min. pre-incubation at 28° 10 μmole of (^{14}C) glutamate (specific activity 0·025 μc./μmole) were added from the side-arm and the incubation was continued. The amount of (^{14}C) CO_2 liberated and of the radioactivity taken up by the bacteria was estimated as described under Methods. ○, ●, □, ■, (^{14}C) CO_2 production; △ ▲, incorporation of radioactivity into the cells organism. Shaded marks: glucose added. In the experiments represented by the curves ○ and ●, KCl was omitted from the reaction mixture.

Fig. 4. Time-dependence of the *m*-Cl-CCP-induced inhibition of glutamate oxidation. Each Warburg flask contained 50 mM-tris buffer (pH 7·2), 3·3 mM-KCl, 3·3 mM-glutamate and 15 mg. wet wt. washed bactera. Total volume: 3·0 ml. Incubation temperature: 28°. After various times of incubation *m*-Cl-CCP (to 1 μM final concentration) was added from the side-arm to the respiring bacteria. ○, Time-course of oxygen uptake; ●, % inhibition of respiration with *m*-Cl-CCP added at the times indicated by the arrows.

trasted with the prolonged lag of oxygen uptake displayed by the cells pre-incubated in the absence of glucose and which at the time of glutamate addition contained only 0·08 μ-equiv. K^+/15 mg. wet wt. In contradistinction to the time-course of CO_2 output the incorporation of radioactivity into the cell mass was practically unaffected by the omission of glucose (Fig. 3).

The above findings indicated therefore that the initial lag observed in the oxygen uptake by the K^+-depleted organisms reflected the time required for the intracellular accumulation of K^+.

Effect of metabolic inhibitors

The current notion that ion transport across biological membranes is an energy-dependent process suggested that blockage of the synthesis of ATP by suitable

metabolic inhibitors might abolish the initial phase of K^+ accumulation and the apparently correlated increase in the respiration rate. Consequently, substances known for their ability to uncouple oxidative phosphorylations, such as 2,4-dinitrophenol, methylene blue (Lehninger, 1949) and *m*-Cl-CCP (Heytler, 1963) were added at various stages of incubation and their effect on oxygen uptake was noted. While 2,4-dinitrophenol up to 0·3 mM showed no measurable effect on the respiration, methylene blue and *m*-Cl-CCP gave strong inhibition of glutamate oxidation when added at the start of incubation. The extent of this inhibitory effect, however, decreased in a striking fashion when the addition of the inhibitor was postponed for 30 min. following the onset of incubation, i.e. until the respiration has reached the steady-state value (Table 3).

The correlation between the time of addition of the inhibitor and its effect on the respiration was also studied in the kinetic experiment depicted in Fig. 4. As seen from the figure, the inhibition induced by *m*-Cl-CCP was inversely related to the rate of oxidation prevailing at the time of its addition.

Table 3. *Effect of inhibitors*

Each Warburg flask contained 50 mM-tris buffer (pH 7·2), 3·3 mM-glutamate, 3·3 mM-KCl and washed *E. coli* (15 mg. wet wt.); total volume 3·0 ml. The inhibitors were added at the time indicated. Incubation was at 28°.

		Time of addition			
Inhibitor		Zero time	30 min. after substrate	Oxygen uptake* (μl./hr)	Inhibition (%)
None		−	−	165	None
m-Cl-CCP	1·0 μM	+	−	34	80
		−	+	170	None
	0·5 μM	+	−	75	65
		−	+	168	None
Methylene blue	0·3 mM	+	−	20	88
		−	+	135	18
	0·6 mM	+	−	15	91
		−	+	50	70
2,4-Dinitrophenol	0·3 mM	+	−	163	None
		−	+	168	None

* In the 60 min. following the addition of the inhibitor.

It has been previously reported that *m*-Cl-CCP inhibits the glucose-dependent respiration to a much lesser degree than the oxidation of other substrates (Avi-Dor, 1963). It was reasoned, therefore, that if *m*-Cl-CCP inhibits glutamate oxidation by interfering with the process of energy generation required for furthering the K^+ transport, then addition of glucose would restore the normal rate of both K^+ accumulation and glutamate oxidation. This prediction was fully borne out by the results presented in Table 4.

Effect of growth conditions

In some experiments *Escherichia coli* was grown on the minimal medium of Davis & Mingioli (1950) in which glucose was replaced by succinate or glutamate

as a carbon source. As seen from Table 5, organisms grown on succinate oxidized glutamate at a lower rate than those which were grown on glutamate or on nutrient agar. Nevertheless, this difference in the rate of oxidation was not accompanied by a change in the dependence on potassium ion or an alteration in the pattern of response to *m*-Cl-CCP.

Table 4. *Abolition by glucose of m-Cl-CCP-induced inhibition of glutamate oxidation by Escherichia coli*

Each Warburg flask contained 50 mM-tris buffer (pH 7·2), 3·3 mM-KCl, 3·3 mM-glutamate containing 0·025 μc. uniformly labelled (^{14}C) glutamate, 15 mg. wet wt. washed bacteria and where indicated, 3·3 mM-glucose and 1 μM *m*-Cl-CCP. Total volume 3·0 ml. Oxidation of glutamate is expressed in terms of (^{14}C) CO_2 evolution. Incubation: 60 min. at 28°. The K^+ content determined at the start of incubation was 0·08 μ-equiv./15 mg. bacterial wet wt.

Additions to the basal reaction mixture		Intracellular potassium concentration after incubation (μ-equiv./15 mg. wet wt.)	(^{14}C) CO_2 production in 60 min. (counts/min.)
m-Cl-CCP	Glucose		
None (control)	None	0·48	2650
+	None	0·10	540
None	+	0·49	2600
+	+	0·40	2320

Table 5. *Identical patterns of response to K^+ and m-Cl-CCP of Escherichia coli cells grown on various media*

The growth conditions were as described under Methods. Each Warburg flask contained 50 mM-tris chloride (pH 7·2), 3·3 mM-KCl, 3·3 mM-glutamate and 15 mg. wet wt. of *Escherichia coli* cell, grown on the various solid media indicated in the Table. Total volume: 3 ml. 'Lag' denotes the time required for attainment of a constant rate of oxygen uptake.

	Growth medium					
	Nutrient agar		Minimal glutamate		Minimal succinate	
Reaction mixture	Oxygen uptake (μl./hr)	Lag (min.)	Oxygen uptake (μl./hr)	Lag (min.)	Oxygen uptake (μl./hr)	Lag (min.)
Complete	150	30	160	30	73	30
KCl omitted	20	—	20	—	20	—
m-Cl-CCP (1 μM) added at the start of incubation	30	—	30	—	15	—
m-Cl-CCP (1 μM) added after 30 min. incubation	145	—	150	—	70	—

DISCUSSION

Respiratory and glycolytic energy is known to drive ion uptake and accumulation in various biological systems (see Rothstein, 1959; Robertson, 1960). The converse situation is that the rate of respiration is determined by the rate of active transport. Anion transport is thought to regulate respiration in plants (Lundegaardh, 1954) and the respiratory activity of brain slices seems to depend on the flux of sodium and

potassium ions (Whittam, 1961). Oxidative processes in tightly coupled systems are governed by the availability of ADP and inorganic phosphate (Lardy & Wellman, 1952) and an ATP-splitting mechanism appears to underly the transport of potassium and sodium across biological membranes (see Hokin & Hokin, 1963).

The results of the present kinetic studies indicate that the rate of glutamate oxidation in *Escherichia coli* is determined by the intracellular concentration of K^+ rather than by the flux of this ion. This conclusion is also supported by the characteristic pattern of respiratory inhibition induced by methylene blue and even more strikingly by *m*-Cl-CPP, a potent uncoupling agent of oxidative phosphorylation recently described by Heytler (1963). These compounds interfered with glutamate oxidation only when added in the initial phase of incubation, i.e. before a sufficient pool of K^+ accumulated in the organism. Assuming that the *m*-Cl-CCP action in bacteria is also attributable to inhibition of oxidative phosphorylation (see Avi-Dor, 1963), the following explanation seems to account for the above observation. The accumulation of K^+ which takes place in the depleted bacteria during the lag-phase of glutamate oxidation requires that the limited amount of energy generated by the initial low rate of glutamate oxidation should be entirely available for ATP synthesis. Therefore, uncoupling of oxidative phosphorylation by eliminating the major source of ATP supply inhibits both K^+ transport and the potassium-dependent oxidation of glutamate; the resulting vicious circle will ultimately lead to the complete interruption of the two interdependent processes. On the other hand, once the requisite amount of K^+ has accumulated in the bacteria, the substrate-level phosphorylation (which is presumably insensitive to the blocking effect of *m*-Cl-CCP and methylene blue) may be adequate to meet the energy requirement of the system for maintaining a constant level of intracellular K^+. This interpretation is also in line with the observation that the *m*-Cl-CCP inhibition was largely abolished by adding glucose, presumably owing to the capacity of the latter substrate to insure a copious supply of ATP via the glycolytic pathway. It is noteworthy that depletion of intracellular K^+ which resulted in a practically complete suppression of glutamate oxidation did not impair the rate of succinate oxidation. Since succinoxidase is a particle-bound enzyme in *Escherichia coli* (Hughes, 1962; Kashket & Brodie, 1963) it would be of interest to see whether the lower potassium requirement is a distinctive feature shared also by other particulate oxidative enzymes.

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Micrococcus violagabriellae Castellani

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SUMMARY

The biochemical and cultural characters of *Micrococcus violagabriellae* Castellani were examined, and the cultural conditions necessary for pigment production investigated; iron and manganese were essential for pigmentation. Some pigment-containing material was isolated and its simple properties were examined. Taxonomically, the organism might warrant sub-specific rank as *Staphylococcus epidermidis* (or *S. saprophyticus*) var. *violagabriellae*.

INTRODUCTION

A staphylococcus which under certain circumstances produced purple colonies was described by Castellani (1955), who named it *Micrococcus violagabriellae*. The organism was first isolated from an erythematous disease of the axilla in American sailors who had apparently contracted the infection in the West Indies and Central America (Castellani, 1956). It has since been isolated occasionally from Portuguese patients who had never been abroad (Professor A. Castellani, personal communication to the National Collection of Type Cultures, 1957). Pigment production was constant only on potato; it occurred occasionally on glucose agar or in gelatin media when these were prepared with American but not English ingredients, but never on nutrient agar. Castellani's original isolate has been investigated and the results are reported here.

METHODS

Organism. The strain of *Micrococcus violagabriellae* deposited in the National Collection of Type Cultures by Professor A. Castellani in 1956 as NCTC 9865 was used. The strain had been maintained in the freeze-dried state since shortly after its deposition. A strain of *M. violagabriellae* received from Professor Castellani in December 1963 behaved in a manner identical with that to be described for organism NCTC 9865, apart from its failure to produce acid from glycerol.

Bacteriology. The morphological, cultural and biochemical tests were the routine ones used in this Collection, some of which were listed by Shaw, Stitt & Cowan (1951).

Pigment production. The optimal conditions were determined by growing the organism on 22 different media at four temperatures (37°, 30°, 22°, and ambient room temperature, about 15–20°), in light and in darkness, in air, in an atmosphere of CO_2 and anaerobically.

Nature of the pigment. Attempts to isolate the pigment were made by solvent treatment of the bacterial growth and of aqueous extracts of the pigmented organisms.

RESULTS

Biochemically and culturally, the organism corresponded with the description of *Staphylococcus saprophyticus* (Shaw *et al.* 1951). In some tests the results were at variance with those of other workers, and these discrepancies are shown in Table 1.

Some pigmentation occurred on mannitol yeast-extract agar, yeast-extract agar, potato slopes and in nutrient gelatin, but was constant and clearly evident only on casein-yeast-lactate-glucose (CYLG) agar (Marshall & Kelsey, 1960). Growth and pigmentation were best after 48 hr at 30° on CYLG agar aerobically. There was little difference in pigment production whether incubation was in light or darkness. Pigment was not produced when the cultures were incubated anaerobically and less well than in air when incubated in the presence of CO_2 (about 10 %, v/v). By omitting one ingredient in turn from CYLG agar, it was found that the sodium glycerophosphate was not essential for growth or pigmentation. Pigment was not produced in the absence of the inorganic salts solution; this contained iron, magnesium and manganese. Pigment production occurred only in the presence of iron + manganese; magnesium was not required.

Table 1. *Biochemical characteristics of Micrococcus violagabriellae according to different workers*

Test	Castellani (1955, 1956)	Stocchi (1956*a*, *b*)	Tripodi (1958)	Cassingena *et al.* (1960)	Kocur & Martinec (1963)	This paper
Methyl red	+	0	0	+	−	+
Voges-Proskauer	−	−	−	0	+	+
Gelatin liquefaction	+	+	+	−	−	+
Milk coagulation	After 8–10 days	0	0	+	−	Rennet clot
Urease	0	−	−	−	+	+
Acid from						
Xylose	0	−	+	−	−	−
Fructose	+	d	−	+	+	+
Galactose	−	0	+	−	+	−
Lactose	−	−	−	−	+	−
Sucrose	+	−	+	+	+	+
Glycerol	0	−	−	0	+	+
Mannitol	−	−	−	−	+	−

Key: +, positive; −, negative; d, variable, some strains +, others −; 0, information not recorded.

Pigment was not produced by *Micrococcus violagabriellae* on nutrient agar with or without glucose (1 %); the addition of Marshall & Kelsey's (1960) inorganic salts solution to these media resulted in some pigmentation. The differences in pigment production noted by Castellani (1956), as between media prepared from English and American ingredients, might be due to variations in their content of trace metals. During observation of this organism for 2 years, non-pigmented variants were not seen on media which induced pigmentation, and organisms from media which did not elicit pigment formation produced pigmented colonies on suitable media. This purple pigmentation was not produced by biochemically similar strains of *Staphylococcus saprophyticus* or by strains of other staphylococci and micrococci

examined, when grown under conditions identical with those for *M. violagabriellae*. A greenish-grey pigment was produced by a strain (NCTC 7011) of *S. flavo-cyaneus* (Knaysi, 1942) but this was not investigated.

Nature of the pigment

The purple pigment produced by *Micrococcus violagabriellae* grown on CYLG agar diffused into the medium immediately below the colonies, but not around them. After the bacterial growth had been scraped from the surface of the plates, the brown-purple colour in the medium became pink on the addition of N-HCl and yellow with N-NaOH. The pigment thus acted as a pH indicator, the colour of which was reversible up to 18 hr after adding acid or alkali to the plates. Neither the colonies nor the pigmented medium exhibited fluorescence under ultraviolet radiation. It was not possible to extract any pigmented material from the organisms by treating the harvested growth with the following solvents: water, NH_4OH and NaOH solutions, HCl, acetic, formic or lactic acids, ether, methanol, ethanol, *n*-butanol, *sec*-butanol, *n*-amyl alcohol, *tert*-amyl alcohol, capryl alcohol, ethyl acetate, acetone, benzene, toluene, xylene, chloroform, carbon tetrachloride, carbon disulphide, pyridine.

Pigmented material was obtained by the following methods.

(1) The growth from CYLG agar incubated at 30° for 48 hr was harvested and suspended in distilled water. The suspension was shaken with glass beads for 1 hr on a Kahn shaker and then kept at 4° overnight. The organisms were removed by gentle centrifugation, and the supernatant fluid treated with 0·1 N-HCl. This resulted in the formation of a flocculent pink-red precipitate; formic acid, lactic acid and 5 % acetic acid in water were also effective.

(2) As in (1), but shaking the supernatant fluid after centrifugation with chloroform, *tert*-amyl alcohol, or *n*-butanol, when red material collected at the interface of the two liquids.

(3) By storing the harvested suspension of organisms at 37° for 24 hr considerable autolysis occurred, and small quantities of dark purple liquid could be collected by centrifugation.

(4) Prolonged centrifugation of the suspension of organisms gave a deposit composed of two layers, a thick lower layer of bacteria and a thin upper layer of pigmented material. After centrifugation, pigment could not be demonstrated in the supernatant fluid.

(5) The organism was grown on circles of filter-paper laid on the surface of CYLG agar and incubated at 30° for 48 hr. By this method, pigment did not appear in the medium. The papers were removed and macerated with water, and the suspension was centrifuged and treated with acid as in (1) above.

Method (1) was the most convenient, and pigmented material was obtained by washing the precipitate until the washings were neutral in reaction and chloride-free; the aqueous suspension was finally freeze-dried and a red-purple amorphous solid obtained. This was insoluble in water and the common organic solvents but soluble in concentrated mineral acids. It was insoluble in $NaHCO_3$ solution but dissolved in N-NaOH to a yellow solution from which it could be reprecipitated by the addition of acid. A solution in 10 N-NaOH deposited a colourless flocculent precipitate after 18 hr at room temperature; after this, the red pigment could not be reprecipitated

by acid. Qualitative elemental analysis showed the presence of nitrogen, and the absence of halogens, sulphur and phosphorus. Manganese was not detected by the tests used. Iron was detected in the filtrate after gentle ignition of the material and digestion of the residue with dilute HCl. Millon's reaction was negative and reducing sugars were not found.

DISCUSSION

After its description by Castellani (1955), *Micrococcus violagabriellae* remained a bacteriological curiosity until Canadian workers found it a valuable organism in studying the effects of ultraviolet irradiation (Payne & Campbell, 1962, 1963). These workers (1962) found that excess iron was necessary for pigment production and that mutation or selection were not involved. Nichols & Campbell (1963) reported their preliminary findings on the nature of the pigment of *M. violagabriellae*. In some of its characters it was typical of a melanin but it also contained a pentose and peptide fraction. Iron was present but was removed quantitatively by alkali treatment; it was not thought to be part of the chromophore.

Bacterial pigments may be divided into carotenoids, melanins, phenazines, pyrroles and quinones. The pigments of micrococci and staphylococci are generally thought to be carotenoids and are non-diffusible in aqueous phases but soluble in fat solvents. Castellani (1956) considered that the pigment of *Micrococcus violagabriellae* was not diffusible; my results appear to oppose this view but it is possible that the pigment is formed extra-cellularly. In its colour-response to pH change the pigment resembles the red pyrrole derivative prodigiosin produced by *Serratia marcescens*. The pigment might be called violagabriellin but this has been resisted on the grounds that a trivial name would lose its significance if the same pigment was later found in other bacteria or micro-organisms. It seems better to withhold a name until its chemical structure has been elucidated. Canale-Parola (1963) drew attention to the possible similarity of the pigment of *M. violagabriellae* to pulcherrimin, a red pigment produced by *Candida pulcherrima*.

Among Gram-positive organisms, red or purple pigments are found in species of *Bacillus*, *Clostridium*, *Corynebacterium*, *Micrococcus*, *Mycobacterium* and *Streptomyces*. A large number of pigmented cocci have been described; those which produce red pigments were discussed by Hucker (1924, 1928), and those with purple pigments by Sneath (1960). The human axilla is a rich source of bacteria and many pigmented cocci have been isolated from this site, for example *Micrococcus haematodes* (Zopf, 1885), *M. chromidrogenus ruber* (Trommsdorff, 1904) and *M. castellanii* (Chalmers & O'Farrell, 1913). The name *M. haematodes* was given by Zopf (1885) to an organism described by Babes (1882) as the causative organism of 'red sweat'. Babes noted that the pigment of the organism had properties similar to that of *Monas prodigiosus* (*Serratia marcescens*). Unfortunately, cultures of *M. haematodes* are no longer extant and the original description did not give sufficient information to enable it to be re-identified with certainty. The descriptions given by Flügge (1890), who spelled the specific epithet *haematoides*, and by Migula (1900), added little fresh information.

Castellani's observation (1955), that pigmentation of *Micrococcus violagabriellae* varied with the origin of the media constituents, may explain in part the isolated reports of the occurrence of such pigmented organisms. It is possible that the

organism Castellani described had been described by earlier workers. A review of the literature has not revealed the description of similar organisms other than *M. moricolor*. This was isolated from open wounds of three different patients by Holmes & Wilson (1945). The description shows a similarity with *M. violagabriellae* but differences are that *M. moricolor* produced acid from lactose but not from maltose or glycerol, acid and coagulation occurred in litmus milk, and non-pigmented variants were often produced after cold storage.

Although Castellani (1955, 1956) named his isolate *Micrococcus violagabriellae*, he thought that if further study showed it to be a variety of *M. pyogenes* then the name should be *M. pyogenes* var. *violagabriellae*. Stocchi (1956*b*) also considered that the organism should be either an autonomous species or a variety of *M. pyogenes*. At that time staphylococci were included in the genus *Micrococcus* in *Bergey's Manual* (1948). In a later publication, Castellani (1957) used the binomial *Staphylococcus violagabriellae* for his isolate. It is certainly a staphylococcus rather than a micrococcus, by the criteria of Baird-Parker (1963) and Jones, Deibel & Niven (1963); Baird-Parker (personal communication) would include it in his staphylococcus subgroup II. Kocur & Martinec (1963) were unable to demonstrate pigment production by Castellani's strain, which they considered to belong to the species *S. epidermidis*.

Micrococcus violagabriellae does not seem to have been isolated by workers other than Castellani and it is doubtful whether the apparent rarity of this organism justifies the creation of a separate taxon. It might be considered as a variety of *Staphylococcus saprophyticus* (Shaw *et al.* 1951), in which instance the name would be *S. saprophyticus* var. *violagabriellae*; on the criteria given in *Bergey's Manual* (1957) the name could be *S. epidermidis* var. *violagabriellae*. The classification of micrococci and staphylococci is, however, in a state of flux and it would be preferable not to add to the confusion by using new names, until the international subcommittee set up to study the problem has produced a report.

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Relation between Energy Production and Growth of *Aerobacter aerogenes*

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SUMMARY

Molar growth yields were measured for *Aerobacter aerogenes* growing with a number of substrates as sole carbon and energy source in a minimal medium. Under anaerobic conditions the molar growth yield for glucose was 26·1 g. This amount of dry weight is produced at the expense of 2·55 mole of ATP (1·71 mole from glycolysis and 0·84 mole from acetate produced from pyruvate by the thioclastic reaction). The yield per mole ATP is thus 10·2 g., which value is very close to the one found for other micro-organisms. Under aerobic conditions the molar growth yield for glucose is 72·7 g. During growth 1·14 mole of O_2 are taken up. The yield per atom oxygen is thus 31·9 g. By dividing the yield per atom oxygen by the yield per mole ATP we find the number of ATP mole formed per atom O. The values found are very close to 3, indicating that the efficiency of oxidative phosphorylation in this organism is the same as that in mitochondria. During the experiments it was observed that growth was maximal before the maximal O_2 uptake was reached. The explanation is that during aerobic growth acetate accumulates, which is oxidized after maximal growth. Acetate oxidation after glucose consumption does not contribute to the dry weight.

INTRODUCTION

It has been shown that the dry weight of various micro-organisms is proportional to the amount of energy source added (Monod, 1942) when the energy source in an otherwise complete medium is the growth limiting factor. The experiments were performed with *Bacillus subtilis*, *Escherichia coli* and *Salmonella typhimurium*, growing aerobically in a mineral medium. Under these circumstances, a considerable part of the energy source is used for the synthesis of cell material. De Moss, Bard & Gunsalus (1951) repeated this type of experiment with *Streptococcus faecalis* and *Leuconostoc mesenteroides* under anaerobic conditions. Both organisms have very complex nutritional requirements and almost the total amount of glucose is therefore used as energy source. They found that with *S. faecalis* the dry weight of organisms per mole glucose fermented was significantly greater than with *L. mesenteroides*. De Moss *et al.* (1951) concluded from these experiments that *S. faecalis* obtained more energy from the fermented glucose than did *L. mesenteroides*, a conclusion confirmed by later work. (For a review on the fermentation patterns in these organisms see Wood (1961).) Bauchop & Elsden (1960) studied the growth of *S. faecalis*, *Saccharomyces cerevisiae*, *Pseudomonas lindneri* and *Propionibacterium*

pentosaceum growing anaerobically in complex media with limiting amounts of glucose or other compounds as energy sources. These organisms ferment glucose by different pathways, yielding different amounts of ATP per mole glucose fermented. From experiments with various energy sources these authors concluded that the four organisms formed about the same dry weight of organisms per mole ATP produced during the fermentation of the energy source ($Y^{ATP} = 10{\cdot}5$). Recently Twarog & Wolfe (1963) found the same value for Y^{ATP} with *Clostridium tetanomorphum*. The amount of growth per mole ATP found by these authors may be regarded as a constant which may be used to calculate the ATP production from the growth yields obtained with other micro-organisms. In a recent review on the energetics of bacterial growth Gunsalus & Shuster (1961) used this constant to calculate the ATP production from previous aerobic growth experiments with *E. coli* (Monod, 1942). The constant was also used to calculate the ATP yield from growth experiments with *Desulfovibrio desulfuricans* (Senez, 1962) and with *Gluconobacter liquefaciens* (=*Acetobacter aceti*) (Stouthamer, 1962). In the latter case a good agreement was found between the ATP production calculated from growth experiments and from direct measurements of P/O ratios in oxidative phosphorylation by cell-free extracts. By dividing the difference in the molar growth yields ($= Y$) of ethanol and acetate by $2 \times$ the P/O ratio for the conversion of ethanol to acetate it was found that Y^{ATP} for *G. liquefaciens* is also about the same as that found by Bauchop & Elsden (1960). In most cases the P/O ratios found in cell-free extracts of micro-organisms are small and it is not clear whether the energy of oxidation is really used inefficiently in micro-organisms or that it is used efficiently but that the P/O ratios are low because the phosphorylative system is damaged during the preparations of the cell-free extract. In *G. liquefaciens* there are indications (low molar growth yields and extensive heat production during growth) that the energy of oxidation is used inefficiently indeed.

To have an indication of the energy production in other bacteria these experiments have now been repeated with *Aerobacter aerogenes*, since this organism is known to give much higher growth yields than *Gluconobacter liquefaciens* (Pichinoty, 1960). Because this organism can be grown under anaerobic conditions it is possible to obtain an independent estimation of Y^{ATP}, which avoids the use of Y^{ATP} obtained from other micro-organisms to calculate the aerobic ATP yield for *A. aerogenes*. As pointed out before, a large fraction of the carbon source is converted into cell material in aerobic experiments with bacteria growing in minimal medium. All kinds of carbon compounds are synthesized during growth, some compounds with a net yield of ATP and others with a consumption of ATP. We therefore decided to determine the oxygen consumption during growth as a measure of that part of the glucose which is completely oxidized, and to relate this oxygen uptake to the molar growth yield, as proposed by Whitaker & Elsden (1963). The oxygen uptake (if all the oxygen participates in oxidative phosphorylation) and Y was used to measure the efficiency of oxidative phosphorylation in this organism.

METHODS

Aerobacter aerogenes was kept on yeast extract agar slants at 4°. For the measurement of the molar growth yield the organism was grown in minimal medium. The salt solution of the minimal medium ($4 \times$ concentrated) contained K_2HPO_4,

3·0 %; KH_2PO_4, 1·8 %; NH_4Cl, 0·8 %; $MgSO_4.7H_2O$, 0·02 % and $FeSO_4$, 0·002 %; adjusted to pH 6·8. The solutions of carbon and energy sources were sterilized and stored at −20° or freshly prepared. Extinctometer tubes (diam. 2·5 cm.; length 8·5 cm.) containing concentrated salt solution, carbon source and distilled water to give a final volume of 10 ml., were inoculated with 0·1 ml. of an overnight culture in minimal medium, with the same substrate as carbon source. For every substrate 5 tubes were used with 5 different concentrations ranging from 0·25 to 6 μmole/ml., depending on the nature of the substrate. The tubes were incubated in a shaking machine at 37°.

To obtain anaerobic conditions tubes closed with rubber stoppers with inlet- and outlet-tubes, were gassed for 30 min. with nitrogen and then closed. In a number of experiments a small tube containing 1 ml. of a mixture of equal volumes of 15 % (w/v) pyrogallol and of 10 % (w/v) Na_2CO_3 was inserted in the extinctometer tubes. This did not influence the results however and therefore these precautions to ascertain absolute anaerobic conditions were not taken in later experiments. In the anaerobic experiments 2–10 μmole substrate/ml. were used and the tubes were inoculated from a pre-culture grown under anaerobic conditions on the same substrate.

The turbidity of the cultures in the extinctometer tubes was measured at regular intervals in a Moll extinctometer (built in this laboratory; see Buyze, 1949) until the turbidity became constant. Then the extinction of the culture was measured in a Unicam SP 600 spectrophotometer at 660 mμ. Dry weight of bacteria was determined directly by centrifugation and washing the pellet three times with distilled water. The bacteria were then dried at 105° to constant weight.

Oxygen consumption of growing cultures was measured in sterile Warburg flasks, using conventional Warburg apparatus and methods. In a number of experiments the O_2 uptake was measured in the presence of CO_2 by the diethanolamine method of Pardee (1949), with 0·3 % (v/v) CO_2 in the gas-phase.

Glucose and maltose were determined by the method of Dische, Shettles & Osnos (1949); acetate was determined as described by Rose, Grunberg-Manago, Korey & Ochoa (1954).

In experiments with radioactive glucose the radioactivity of the bacteria, the supernatant fluid and the CO_2 was measured after conversion to $BaCO_3$. The bacteria and the supernatant fluid were therefore oxidized with the van Slyke reagent in the apparatus for static wet combustion as described by Aronoff (1960) and the CO_2 collected as $BaCO_3$. Carbon dioxide liberated from the growing cultures in Warburg vessels was collected in KOH in the centre well and converted to $BaCO_3$. The $BaCO_3$ was handled as described by Aronoff and the radioactivity determined on a layer of infinite thickness, by using an end-window GM tube.

The following abbreviations will be used: ATP, adenosine triphosphate; CoA, coenzyme A and NADH, reduced nicotinamide-adenine dinucleotide.

RESULTS

Determination of the constant relating extinction to dry weight

The extinction and the number of viable cells of a culture grown aerobically with a limiting amount of maltose is shown in Fig. 1. It may be seen from Fig. 1 that the extinction of the culture decreases after a maximum. The same result was found in

similar experiments with *Bacillus subtilis* (Monod, 1942). In the case of *B. subtilis* this effect was found to be due to lysis of part of the bacteria (Hadjipetrou & Stouthamer, 1963). This is not the explanation for the decrease in extinction after the maximum with *Aerobacter aerogenes*, however, because the number of viable organisms remains constant after the maximum (Fig. 1). Moreover the dry weight bacteria in this and other experiments decreased only slightly on prolonged incubation (Table 1). It is thus evident, that the decrease in extinction after the maximum is mostly due to a difference in the light-scattering properties of the bacteria. For bacteria taken after the maximum the constant, which relates extinction to dry weight of bacteria, is higher than for bacteria at the maximum (Table 1). Therefore the constant which relates extinction to dry weight was determined with bacteria taken at the maximum extinction. From a number of experiments we found the

Table 1. *Relation between optical density and dry weight in cultures of Aerobacter aerogenes*

The conditions in this experiment are the same as those in the experiment of Fig. 1. At the time indicated the bacteria from 1 l. culture were harvested, washed and dried as described under Methods.

Time of incubation (hr)	Extinction (660 mμ)	Bacterial dry weight (mg./l.)	Dry weight / extinction
3	0·284	108·6	382
7	0·256	105·4	412

relation: dry weight (μg./ml.) = 380 × extinction at 660 mμ. This relation was found to be valid for extinctions smaller than 0·9. The same relation was found when the bacteria were grown with glucose, maltose, citrate, galactose or ribose as growth-limiting carbon source. Thus the nature of the carbon source had no influence on the light-scattering properties of the bacteria. In all experiments for the determination of Y the extinction was followed until a maximum was reached. From the maximum extinction the dry weight was calculated with the constant given above.

Molar growth yields under anaerobic conditions

It is known that at neutral pH value the fermentation of glucose by *Aerobacter aerogenes* closely resembles that of *Escherichia coli* (Mickelson & Werkman, 1938). The main fermentation products are then acetate, ethanol, formate and lactate. In this type of fermentation, pyruvate is decomposed by a thioclastic reaction to acetyl-CoA and formate. Acetyl-CoA is converted to acetate by means of phosphate acetyltransferase and acetatekinase. Thus above the net gain of 2 mole of ATP from the conversion of 1 mole of glucose to 2 mole of pyruvate, an additional mole of ATP is generated for each mole of acetate formed. Ethanol which is also formed in this fermentation is produced by reduction of acetyl-CoA (Dawes & Foster, 1956) and the energy-rich bond present in acetyl-CoA is lost during its conversion to ethanol. Therefore the net gain of ATP can only be calculated when the amount of acetate produced during the fermentation is known. The amount of acetate formed during the fermentation of the substrate was determined in the supernatant when the maximal optical density was reached.

The molar growth yield and the amount of acetate formed for glucose and fructose are given in Table 2. From the results of Table 2 we can calculate Y^{ATP} (see Discussion). The value obtained in this calculation is 10·2.

Table 2. *Molar growth yield and acetate production for Aerobacter aerogenes growing under anaerobic conditions in minimal medium with glucose or fructose as carbon source*

Substrate	Molar growth yield (g./mole)	Acetate production (mole/mole substrate)
Glucose	26·1 ± 0·5 (17)*	0·84 ± 0·04 (15)
Fructose	26·7 ± 0·4 (7)	0·8 (3)

* The number of determinations is given in parentheses.

Molar growth yields under aerobic conditions

A linear relationship was found between the glucose content in the medium and the dry weight of bacteria produced, with glucose concentrations between 0 and 2·5 μmole/ml. (Fig. 2). At higher concentrations a lower Y was found. Similar results were found with other substrates. Therefore the molar growth yields were always determined at low substrate concentrations. The results are given in Table 3. To facilitate the comparison of the molar growth yields with different substrates the number of mole O_2 needed for complete oxidation is included in Table 3 for all substrates. It is evident from Table 3 that in most cases the molar growth yields for a group of compounds which need the same amount of O_2 for complete oxidation are very similar. For instance, mannitol and sorbitol form one group; the results for glucose, galactose, mannose and fructose are also similar. The results for the compounds which need 5·5 mole O_2 for complete oxidation are near each other and

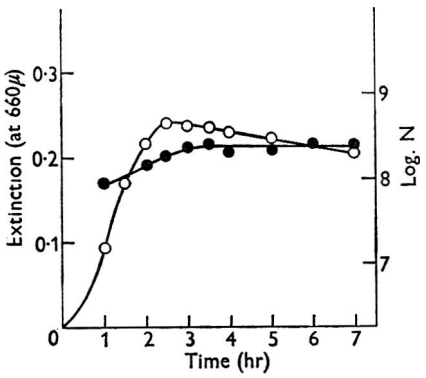

Fig. 1

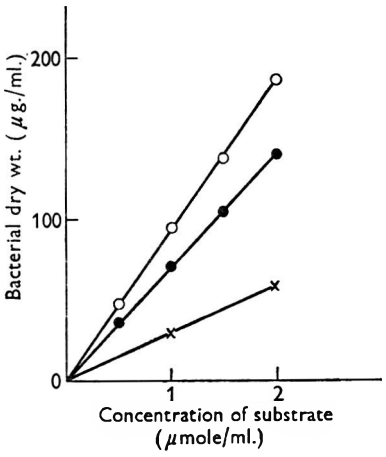

Fig. 2

Fig. 1. Extinction and viable count of a culture of *Aerobacter aerogenes* growing aerobically with 0·5 μmole maltose/ml. in a minimal medium: ○——○, optical density; ●——●, viable count.

Fig. 2. Growth yields with *Aerobacter aerogenes* growing aerobically with the indicated substrates as sole carbon source in a minimal medium: ○——○, sorbitol; ●——●, glucose; ×——×. succinate.

the same applies (with the exception of L-arabinose) to the compounds which need 5 mole of O_2. For a number of substrates (rhamnose, inositol, DL-lactate, DL-malate, L-glutamate) the molar growth yields are low in comparison with substrates which need the same amount of O_2 for complete oxidation. On the other hand the growth yields with L-arabinose and citrate are unexpectedly high. Most of these irregularities are explained by the later observation that the O_2 uptakes during growth on

Table 3. *Molar growth yields and O_2 consumption during aerobic growth for Aerobacter aerogenes with the indicated substrates as sole carbon source in a mineral medium*

Y was determined for cultures growing in extinctometer tubes. The O_2 uptakes were measured for separate cultures growing in Warburg vessels. For the substrates marked with an asterisk the O_2 consumption is given at the moment the curve for the O_2 uptake showed a sharp bend. For the remaining substrates the maximal O_2 uptake is given. The O_2 uptake during growth is the mean of at least 4 determinations. The number of determinations is given in parentheses. No growth was obtained with α-ketoglutarate, D-arabinose and erythritol.

Substrate	Mole O_2 for complete oxidation	Y	O_2 consumption (mole/mole)	Y°	Y°/Y^{ATP}
Maltose*	12	149·2 ± 1·7 (8)	2·55	29·2	2·9
Sucrose*	12	172·7 ± 1·8 (12)	2·68	32·2	3·2
D-Mannitol	6·5	95·5 ± 1·1 (15)	2·51	19·0	1·9
D-Sorbitol*	6·5	97·4 ± 2·3 (5)	1·80	27·1	2·7
L-Rhamnose*	6·5	49·0 ± 1·1 (8)	1·24	19·7	1·9
D-Glucose	6·0	72·7 ± 1·0 (21)	2·05 ± 0·05 (14)	17·7	1·7
D-Glucose*	6·0	72·7 ± 1·0 (21)	1·14 ± 0·05 (11)	31·9	3·1
D-Fructose*	6·0	76·1 ± 1·3 (15)	1·34	28·4	2·8
D-Galactose	6·0	73·5 ± 1·9 (11)	1·93	19·0	1·9
D-Mannose	6·0	69·4 ± 0·8 (13)	1·77	19·6	1·9
Myo-inositol*	6·0	52·2 ± 1·4 (9)	1·15	22·7	2·2
D-Gluconate*	5·5	62·2 ± 1·3 (21)	1·13	27·5	2·7
D-Galactonate*	5·5	66·3 ± 1·8 (6)	1·29	25·7	2·5
Ribitol	5·5	61·8 ± 1·5 (6)	1·97	15·7	1·5
D-Ribose	5·0	53·2 ± 0·6 (13)	1·71	15·6	1·5
L-Arabinose*	5·0	65·2 ± 0·9 (17)	1·40	23·3	2·3
D-Xylose	5·0	52·2 ± 0·9 (9)	1·86	14·0	1·4
D-Glucuronate*	5·0	55·3 ± 1·5 (6)	1·25	22·1	2·2
D-Galacturonate*	5·0	55·6 ± 1·5 (5)	1·11	25·3	2·5
Citrate	4·5	61·8 ± 0·7 (12)	1·73	18·0	1·8
L-Glutamate	4·5	27·1 ± 0·8 (17)	1·86	7·3	0·7
Succinate	3·5	29·7 ± 0·9 (10)	1·48	10·0	1·0
Glycerol	3·5	41·8 ± 0·6 (14)	1·35	15·5	1·5
Dihydroxyacetone	3·0	31·9 ± 0·9 (12)	0·90	17·7	1·7
Fumarate	3·0	26·3 ± 0·7 (15)	1·24	10·6	1·0
L-Aspartate	3·0	28·3 ± 1·4 (3)	1·45	9·8	1·0
DL-Lactate*	3·0	16·6 ± 0·4 (14)	1·41	5·9	0·6
DL-Malate	3·0	14·8 ± 0·4 (12)	1·16	6·4	0·6
Pyruvate	2·5	17·9 ± 0·4 (16)	1·18	7·6	0·7
Acetate	2·0	10·5 ± 0·3 (8)	1·06	5·0	0·5

these substrates are different from those obtained with corresponding compounds which need the same theoretical amount of O_2. Thus in most cases the Y° values (Y° = yield per atom oxygen taken up during growth) are normal.

The most important fact, which emerges from the results of Table 3 is that Y_{mannitol} and Y_{sorbitol} are much larger than Y_{fructose}. This indicates that the O-atom

needed for the conversion of the hexitols to fructose supplies energy for the assimilation of a larger part of the substrate. Similar conclusions can be reached by comparison of the results with ribitol/ribose and glycerol/dihydroxyacetone.

Oxygen consumption during growth

As described in the introduction we followed the method of Whitaker & Elsden (1963) to relate the oxygen uptake during growth to the Y. In the first experiments the molar growth yields in Warburg flasks were much lower than those found in extinctometer tubes. Originally we supposed that the absence of CO_2, due to the presence of KOH in the centre well of the Warburg vessel was the cause of the low yields. In some experiments the O_2 uptake and the molar growth yield were measured in the presence of CO_2 by the method of Pardee (1949). The presence or absence of CO_2 had no influence on Y. Finally, we found that the low yields in Warburg flasks were caused by a difference in the efficiency of shaking of the extinctometer tubes and the Warburg vessels. By varying the rate of shaking we found that Y for glucose under these circumstances varied from 55 with inefficient shaking (25 complete 2·5 cm. strokes/min.) to 71 with good shaking (125 complete 5 cm. strokes/min.); although the O_2 uptake during growth was always the same. A further increase of the shaking rate did not influence the results. A similar influence of the oxygen supply on the growth yield of *Aerobacter cloacae* was found by Pirt (1957). From this result we may conclude that O_2 consumption and molar growth yield may vary independently. Even with efficient shaking Y found in the Warburg experiments was somewhat smaller than the value found in the extinctometer tubes.

After we had found the influence of the shaking rate on Y we measured the O_2 uptake with the same (efficient) shaking for a number of substrates. In these experiments we noticed that maximal O_2 uptake in the Warburg flasks was reached at a later time than maximal growth in extinctometer tubes. This suggested that maximal growth in Warburg flasks was obtained before the O_2 uptake was maximal. This possibility was tested by growing the bacteria in several Warburg flasks with a growth limiting concentration of substrate. At intervals the content of a Warburg flask was taken for the measurement of the extinction at 660 mμ. In this experiment the rate of oxygen uptake increases in a logarithmic fashion until growth stops and thereafter the rate becomes linear (Fig. 3). The results indicate that indeed maximal growth is obtained much earlier than maximal O_2 uptake. We must assume that during growth a compound is formed, which is oxidized during the second stage of the curve. The oxidation of this compound does not seem to be accompanied by an increase in the dry weight of bacteria. Dagley, Dawes & Morrison (1951) found that pyruvate accumulated during glucose oxidation by *Aerobacter aerogenes* and that aeration had a strong influence on this accumulation. We found, however, that in our case pyruvate accumulation was very unimportant. Instead of pyruvate significant amounts of acetate were found in the supernatant fluid. The kinetics of acetate accumulation during glucose oxidation are shown in Fig. 4. Acetate accumulation during aerobic growth was also found by Pirt (1957) for *A. cloacae* when the oxygen supply was insufficient. It is evident from Fig. 4 that during the second stage of the O_2 consumption curve acetate was oxidized. We must thus conclude that acetate oxidation after glucose disappearance does not

lead to an increase in the dry weight of bacteria. The curves for the O_2 uptakes during growth do not show a bend for a number of substrates. It is not known whether there is acetate accumulation in these cases or whether acetate is immediately oxidized. In this aspect it is important to note that acetate oxidation in the presence of glucose seems to contribute to the dry weight of bacteria (Table 4). Under these circumstances the energy of oxidation of acetate seems to be utilized for the assimilation of part of the glucose. The same may happen with acetate oxidation during growth with the substrates that do not show the bend in the curve for the O_2 uptake during growth.

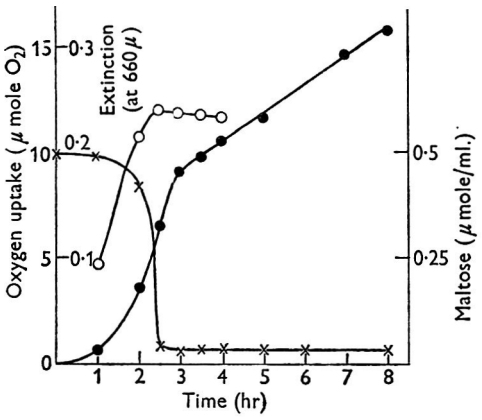

Fig. 3

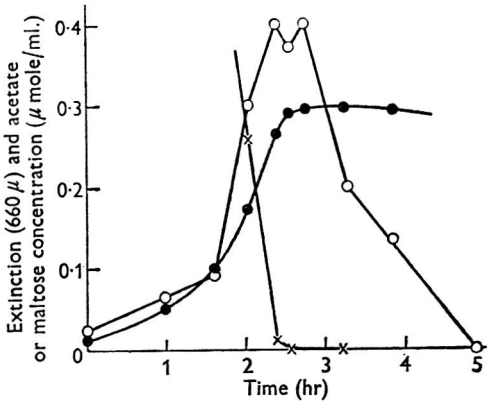

Fig. 4

Fig. 3. Extinction, O_2 consumption and maltose concentration in a culture of *Aerobacter aerogenes* growing in minimal medium: ○——○, extinction; ●——●, O_2 consumption; × —— ×, maltose concentration.

Fig. 4. Extinction, glucose concentration and acetate accumulation in a culture of *Aerobacter aerogenes* growing with 1 μmole glucose/ml.: ●——●, extinction; ○——○, acetate concentration ; × —— ×, glucose concentration.

Table 4. *Growth yield of Aerobacter aerogenes growing aerobically in minimal medium with glucose or glucose + acetate as sole carbon source*

The acetate concentration in these experiments was unimportant as the same growth yield was obtained when 0·5 or 1·0 μmole/ml. was added.

Glucose (μmole/ml.)	Acetate (μmole/ml.)	Growth yield per mole glucose (mg. dry weight)
1·5	0	72·7 ± 1·0 (21)
1·5	0·5 or 1·0	78·0 ± 0·5 (6)

The results are given in Table 3. For the substrates, which are oxidized with a sharp bend in the curve for the O_2 uptake (as in Fig. 3), the O_2 uptake at the moment of the bend is given (substrates marked with an asterisk). For the substrates which are oxidized without a bend in the curve the final O_2 consumption is given. For comparison both values for glucose are given. When we take the maximal O_2 uptake for glucose, we find a similar value for Y° as for galactose and mannose, which do not give a bend in the curve for the O_2 uptake. It is very likely that acetate is

oxidized during growth on these substrates, but that the oxidation of acetate does not lead to an increase or only a very small increase in the dry weight of bacteria. In this respect we note that the increase in dry weight in the experiments with glucose and acetate is very low (Table 4). It is evident that Y° for the substrates, which do not show the bend in the O_2 consumption curves, is too low and that it should be regarded as a minimal value. For the substrates which show the bend in the curve for the O_2 uptake, the Y° values are much larger. When we divide Y° by Y^{ATP} we find the number of ATP molecules per atom O taken up. The results with these substrates are sometimes in the neighbourhood of 3.

From the results of Table 3 it is evident that the Y° values for the carbohydrates, or the compounds which are easily converted into carbohydrates, are much higher than for the substrates which are more difficult to convert into carbohydrates. When we take only the maximal O_2 uptakes into account, Y° varies from 14·0 (D-xylose) to 19·6 (D-mannose) for the first class of compounds and from 5·0 (acetate) to 10·6 (fumarate) for the second class. The only exception is citrate, which gives an Y° of 18·0. No explanation can be given for this result.

Table 5. *Assimilation of glucose by growing Aerobacter aerogenes*

In this experiment the glucose concentration was 1 μmole/ml. and the Y value obtained was 70–71. The radioactivity was determined after combustion to CO_2 and counted as $BaCO_3$ as described under Methods.

		Radioactivity (counts/min.)	% ^{14}C added
	Glucose	12·370	100
Maximal growth	Bacteria	4·885	39·5
	Supernatant	1·444	11·7
	CO_2	3·817	30·8
	Recovery	10·146	82·0
Maximal O_2 uptake	Bacteria	4·544	36·7
	Supernatant	1·469	11·9
	CO_2	5·365	43·4
	Recovery	11·378	92·0

Assimilation of glucose

The assimilation of glucose was studied by measuring the incorporation of ^{14}C from uniformly labelled glucose into cell material. In these experiments the radioactivity was measured in the bacteria, in the CO_2 and in the supernatant fluid at the moment of maximal growth and maximal O_2 uptake. The results are shown in Table 5. It is evident that the recovery of the radioactivity at the point of maximal growth is low. This is due to the fact that acetate is not converted to CO_2 by the combustion method used. At the point of maximal O_2 uptake the recovery is much better and the radioactivity in the CO_2 is much larger. This confirms our earlier observation that during this stage acetate is oxidized to CO_2. The radioactivity in the bacteria decreased slightly during acetate oxidation which confirms our earlier observation (Table 1) that the dry weight decreases somewhat on prolonged incubation. The nature of the compound (or compounds) in which the radioactivity in the supernatant fluid is present is unknown. It is not glucose remaining in the medium,

because chemical estimation showed that no glucose was present after maximal growth (Fig. 4).

From the result of Table 5 (39·5 % of ^{14}C in the bacteria and 70·5 g. dry weight) we find that the C-content of the bacteria is 40·0 %.

DISCUSSION

From the anaerobic experiments (Table 2) we can calculate Y^{ATP}. The molar growth yield for glucose is 26·1. The result of the radioactive experiment shows that the carbon content of the bacteria is 40·0 %. The C-content of the bacteria is the same as that of glucose. This means that in the anaerobic experiment 26·1 g. glucose have been converted to cell material and $180-26{\cdot}1 = 153{\cdot}9$ g. have been fermented. As the net gain of ATP per mole glucose transformed into pyruvate is 2 mole and as one extra ATP is formed per mole of acetate produced, the amount of ATP formed from 153·9 g. glucose is $2\times(153{\cdot}9/180)+0{\cdot}84 = 2{\cdot}55$ mole of ATP. From these results we can calculate Y^{ATP} as 10·2. From the molar growth yield and the acetate production with fructose we find a value of 10·7 for Y^{ATP}. It is thus evident, that we find a similar value for Y^{ATP} as Bauchop & Elsden found (1960) for *Streptococcus faecalis*, *Saccharomyces cerevisiae* and *Pseudomonas lindneri*, as Stouthamer (1962) found for *Gluconobacter liquefaciens* and as Twarog & Wolfe (1963) found for *Clostridium tetanomorphum*. Because Y^{ATP} is the same for so many organisms it is very likely that it may be used to calculate the ATP yield from growth experiments with all micro-organisms.

In the aerobic experiments we found with some minor exceptions that Y was about the same for each class of substrate (disaccharides, hexitols, hexoses, hexonates, pentoses, etc.). Y for mannitol and sorbitol was much higher than Y for fructose indicating that the oxygen atom needed for the conversion of mannitol to fructose allowed the assimilation of a larger part of the substrate. Some of the irregularities in the values for Y (Table 3) are eliminated when we take the Y° values. For instance the low Y values for rhamnose, inositol and glutamate lead to normal Y° values when divided by the O_2 uptake. In contrast to the results of Whitaker & Elsden (1963) we found that Y° for a given substrate is not a constant. By varying the shaking rate of the Warburg apparatus we found that Y° for glucose varied from 31·9 with efficient shaking to 25·7 with inefficient shaking. It should be pointed out, however, that under identical conditions Y° is a constant. These results cannot be ascribed to a difference in acetate accumulation because similar amounts of acetate were found at the moment of maximal growth under both conditions. With inefficient shaking both the growth rate and Y° are low. This may be explained by supposing that there exists some mechanism either bye passing ATP generation or permitting the dissipation of ATP in the cell.

At high glucose concentrations the molar growth yield is lower. Under these circumstances we must also assume that less ATP is formed per mole of O_2 taken up or that the normal amount of ATP is formed, but that part of the ATP is wasted.

Acetate accumulation under the conditions of our experiments is not caused by an insufficient rate of oxygenation. In none of these experiments (with small or high glucose concentrations) was the formation of any of the fermentation products (formic acid, butanediol or ethanol) from glucose observed (Hadjipetrou &

Stouthamer, unpublished experiments). Even with very vigorous aeration acetate accumulation was obtained. We must suppose, therefore, that the ability of *Aerobacter aerogenes* to oxidize acetate is insufficient to prevent its accumulation during glucose oxidation.

Acetate oxidation in the absence of glucose does not lead to an increase in dry weight of bacteria. This can be explained by the fact that during growth on acetate two adaptive enzymes, which constitute the glyoxylate by-pass, must be induced (for reviews see Kornberg, 1959; Kornberg & Elsden, 1961). Before the induction in the culture has taken place the acetate may already have been oxidized by the bacteria. How the energy of oxidation during acetate oxidation is used is not known.

The Y° values obtained by dividing the Y values by the maximal O_2 uptakes fall in two classes. The compounds which are intermediates of the citric acid cycle or which are easily fed into the citric acid cycle give (with the exception of citrate) low Y° values. This is very clear when we compare the results with glycerol and succinate, which need both 3·5 mole O_2 per mole of substrate for complete oxidation. For glycerol we found $Y = 41{\cdot}8$ and for succinate $Y = 29{\cdot}7$; the O_2 uptakes during growth for these substrates were 1·35 mole and 1·48 mole per mole substrate respectively. The Y° for glycerol is 15·5 and that for succinate is 10·0. This may be explained by the hypothesis that the assimilation of succinate to cell material needs more energy than assimilation of glycerol. The conversion of each mole of succinate to glyceraldehyde-3-phosphate, which is necessary for the synthesis of all carbohydrate components of the cell needs two ATP. Besides NADH is used for this conversion, which limits the amount of NADH available for oxidation and ATP production.

The Y°/Y^{ATP} values of Table 3 are the same as the phosphorus/oxygen (P/O) ratios. Some of the values of Table 3 are minimal values because we found that maximal O_2 uptake was reached much later than maximal growth (Fig. 3). This was found to be due to acetate oxidation. For a number of substrates the Y° values could be corrected by dividing Y by the O_2 uptake at the moment the curve for the O_2 uptake showed a sharp bend. The Y°/Y^{ATP} values obtained with these O_2 uptakes are very close to 3. This indicates that in the living bacterial cell oxidative phosphorylation is as efficient as in mitochondria. This is in contrast with the low P/O ratios found in cell-free extracts of *Aerobacter aerogenes* (Nossal, Keech & Morton, 1956; Hadjipetrou & Stouthamer, unpublished results). Thus the low P/O ratios in cell-free extracts are most probably caused by the damage in the phosphorylative system, which occurs during the preparation of the cell-free extract.

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Serological Types of *Streptococcus faecalis* and its Varieties and their Cell Wall Type Antigen

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SUMMARY

When serological type strains of *Streptococcus faecalis* from different workers were compared with those of Sharpe & Shattock (1952) by using precipitin and reciprocal absorption tests, several of these types were found to possess the same type antigens. The location of these type antigens was in the cell wall and they were polysaccharide in nature. Some strains had two of these antigens. The distribution of *S. faecalis* serotypes is discussed and a typing scheme applicable to all recognized types of group D streptococci is suggested.

INTRODUCTION

Shattock (1962) showed that the Lancefield group D streptococci could be divided on the basis of their physiological characteristics into three subdivisions consisting of: (i) *Streptococcus faecalis* and its varieties *zymogenes* and *liquefaciens*; (ii) *S. faecium* and *S. durans*; (iii) *S. bovis* and *S. equinus*. These broad divisions were also supported by serological evidence, as type antigens occurring in the strains within each subdivision are distinct from those found in strains within the other subdivisions (Skadhauge, 1950; Sharpe & Shattock, 1952; Sharpe & Fewins, 1960; Medrek & Barnes, 1962).

Within the subdivision *Streptococcus faecalis* (this term is used here to include the varieties *S. faecalis* var. *zymogenes* and *S. faecalis* var. *liquefaciens*) of group D streptococci a number of serological types have been defined. Lancefield's original group D streptococci included 3 strains of *S. faecalis* each having a different type antigen (Lancefield, 1941). Grumbach (1943) used agglutination and precipitin typing to define 21 serological types of enterococci, but did not include sufficient physiological tests to subdivide these organisms further. Later Skadhauge (1950) divided *S. faecalis* strains into 6 groups, based on the presence of thermostable 'O' agglutinins; Sharpe & Shattock (1952) differentiated by agglutination and precipitin tests 9 serological types of *S. faecalis*, 2 of which appeared to be homologous with 2 of the original Lancefield strains, and Elliott (1957) and Elliott & Barnes (1959) have distinguished by precipitin typing 2 further serological types, one of which they thought to be the same as one of the Sharpe & Shattock types. They also found that the third Lancefield type, which had not previously been available for comparison, was the same as another of the Sharpe & Shattock types.

Since group D streptococci are of importance as indicators of faecal pollution, as agents of food spoilage, as possible pathogens, and as components of the normal intestinal flora concerned in nutrition, their serological identification may sometimes

be necessary. It has been suggested that in order to prevent replication of types and to provide a common reference, a serological typing scheme should be recommended for use by workers in this field. As Elliott (1960) found the type specific antigens of 3 strains of *Streptococcus faecalis* and 1 of *S. durans* to be cell wall polysaccharide, it has been suggested by Lancefield, quoted in the report of the International Sub-committee on Streptococci and Pneumococci (1963) that the serological subdivision of group D should be based on the precipitin reactions of numbered antigens, these antigens being cell wall polysaccharides. The work reported here describes a comparison of the *S. faecalis* serological types of different workers, and the location of their specific cell wall type antigens. The ability of periodate to inactivate cell wall antigens was used as an indication of their carbohydrate nature. Similar work has been undertaken with the type antigens of *S. faecium* by Dr E. M. Barnes, Low Temperature Research Station, Cambridge.

METHODS

Strains of Streptococcus faecalis examined. The three original Lancefield (1941) strains, the 9 type strains of Sharpe & Shattock (1952), the 2 Elliott (1957) types and 18 strains from Skadhauge, representing his 6 'O' groups, were investigated. In addition all of the 21 Grumbach (1943) type strains of enterococci were obtained from the Streptococcal Reference Laboratory (Colindale, London, N.W.9), and their physiological characteristics examined to amplify the previous description, by using most of the tests described by Shattock (1962). A sufficient number of physiological characteristics of the other strains used in this work were examined to confirm their identity as *S. faecalis*. Strains were cultured in 1·0 % (w/v) glucose nutrient broth and were maintained in glucose litmus milk + chalk. Lyophilized cultures grown in GLB were used for the production of antisera.

Preparation of group antisera. The method of Shattock (1949) was used, the cells being disrupted in the Mickle tissue disintegrator.

Preparation of type antisera. Type antisera were prepared against the 3 Lancefield types, the 9 Sharpe & Shattock types and the 2 Elliott types, and were absorbed if necessary to make them specific (Sharpe & Fewins, 1960).

HCl extracts of whole organisms. HCl extracts (Lancefield, 1933) were prepared as described by Sharpe & Shattock (1952) using the growth from 40 ml. glucose nutrient broth, except that cultures were incubated for 18 hr at 22° instead of at 30° (Sharpe & Fewins, 1960).

Precipitin ring tests. Jones & Shattock (1960). Extracts were layered over sera and examined at intervals up to 30 min.

Crude cell walls. Using the method of Elliott (1960) strains were grown for 24 hr at 22° in 500 ml. of 0·5 % (w/v) glucose nutrient broth, washed twice in sterile distilled water, suspended in 6 ml. distilled water + 4 g. Ballotini beads No. 12 and treated on the Mickle tissue disintegrator in the cold for 1½ hr or until a microscopic examination revealed that all the cocci had been disrupted. The beads and any undisrupted cocci were removed by slow centrifugation, and the cell walls separated from the cell contents by centrifuging at 4800 rev./min. and washed with distilled water.

HCl extracts of cell walls. Extracts were prepared by the same method as used for whole organisms.

Lysozyme extracts. Cell walls were suspended in 3 ml. of 0·05 M-phosphate buffer (pH 6·2) and incubated with crystalline egg white lysozyme (Armour) in a concentration of 1·0 mg./ml. for 3 hr at 37°, and then overnight if no clearing had occurred. The suspension was then adjusted to pH 7·6 with N-NaOH and the preparation centrifuged to removed undigested cell debris.

Trypsin digestion. Crystalline trypsin (1 mg./ml.) was added to the lysed cell walls, and digestion allowed to proceed for 3 hr at 37° at pH 6·2; then adjusted to pH 7·0.

Further purification of antigens (Elliott, 1960). By ethanol precipitation (2 vol. antigen preparation:5 vol. ethanol), an ethanol-soluble fraction and an ethanol-insoluble fraction were separated. The ethanol-soluble fraction was taken up in acetone (5 times the original volume) and then redissolved in water.

Periodate test for carbohydrate on partially purified cell wall preparations. (*a*) By the method of Bobbitt (1956). To 1·0 ml. of the acetone precipitated fraction of cell walls, 1·0 ml. of 0·04 M-sodium periodate in 0·4 M-Na acetate buffer (pH 4·0) was added. After leaving for 1–1½ hr in the dark at room temperature 0·1 ml. of 5·0 % (v/v) ethylene glycol in water was added to destroy the periodate and the solution was adjusted with N-NaOH to pH 7·0. Appropriate controls with distilled water instead of antigen, and also with antigen added after the periodate had been destroyed and adjusted to pH 7·0, were also included. (*b*) By the method of Elliott (1960). One ml. of antigen + 1·0 ml. of 0·002 M-sodium periodate solution were incubated at 37° for 4 hr. Periodate-treated cell wall extracts were then tested against the homologous type antisera.

RESULTS

Serological types

By using specific type antisera and HCl extracts of whole organisms, it was confirmed by reciprocal absorption tests that the three Lancefield types were homologous with 3 of the 9 Sharpe & Shattock types, namely 1, 3 and 4, whilst the 2 Elliott types did not correspond to any types previously described. Eleven serologically distinct precipitin antigens were thus differentiated (Table 1). When HCl extracts of the Skadhauge strains were tested against these 11 antisera, it was found that the Skadhauge 'O' groups, defined by agglutination tests, did not correspond exactly to the serological types defined by other workers using the precipitin test. However, the different Skadhauge strains representing each group are identified by a strain number (Table 1) so that the specific reactions of these numbered strains can be related to the serological types of other workers and Skadhauge's results correlated with theirs. Of the 21 Grumbach type strains 5 were *Streptococcus faecalis*. Skadhauge's Group II contained two of the Sharpe & Shattock types, whilst some of his strains were shown by absorption to possess two of the type precipitin antigens recognized by these workers (Table 2). The remainder were identified as *S. faecium*, *S. durans* or were unclassified. Of the 5 *S. faecalis* strains the proteolytic properties of 2 strains and the haemolytic activity of 2 other strains did not correspond to those of the original description. In addition 3 strains identified here as *S. faecium* had been identified previously by Skadhauge (1950) as *S. faecalis*. The serological reactions of extracts of the Grumbach strains with the Sharpe & Shattock

type antisera were as follows: 1 and 4 reacted with antiserum type 6, 3 reacted with antiserum type 9, 24 with antiserum type 3, and 5 did not react with any of the Sharpe & Shattock type antisera. In view of the uncertainty of the identity of the strains with those originally described, no further work was done with them.

Table 1. *Serological types of Streptococcus faecalis designated by different workers and found to possess homologous type antigens by reciprocal absorption tests*

Sharpe & Shattock (1952) Serological type	Lancefield (1941)	Elliott (1957)	Skadhauge (1950)
1	D 76	.	Group I (503)*
3	H 69 D 5	.	Group VI (120)
4	C 1	.	.
5	.	.	Group II (50)
6	.	.	Group IV (782)
8	.	.	.
9	.	.	Group III (3)
19	.	.	Group II (783)
20	.	.	.
.	.	D 10	.
.	.	D 15	.

* ( ) Skadhauge strain number.

Table 2. *Precipitin reactions of HCl extracts of Skadhauge's strains of group D streptococci, previously differentiated into groups by their 'O' agglutinins*

Skadhauge 'O' group	Strains (Skadhauge numbers)	Reaction with specific antisera type							
		1	5	19	9	D 15	3	6	4, 5, 8, D 10
I	28, 44, 46, 52, 438, 503, 520	+	−	−	−	−	−	−	−
II	50, 53,	−	+	+	−	−	−	−	−
	335	−	−	+	−	−	−	−	−
	377	−	+	−	−	−	−	−	−
III	3, 75	−	−	−	+	−	−	−	−
	58	−	−	−	+	+	−	−	−
IV	782	−	−	−	−	−	−	+	−
V	567	−	−	−	−	−	−	−	−
VI	120	−	−	−	−	−	+	−	−

Cell wall antigens

Cell wall preparations of the 3 Lancefield serological type strains, the 9 Sharpe & Shattock types, 2 Elliott types and 8 of the Skadhauge strains were examined.

HCl extracts of cell walls. When HCl extracts of cell walls of several different type strains were made, they gave no type reactions although HCl extracts of whole organisms and lysozyme extracts of cell walls of the same strains gave strong type precipitin reactions. It was found that at 100° the antigen in the cell wall was more sensitive to pH 1·5 (produced by the addition of 0·05 N-HCl) than when it was in the whole cell. When adjusted to pH 2·0 (Table 3) the antigen was not destroyed. A specific reaction then occurred between cell wall extract and antiserum, each

type strain reacting with its homologous antiserum. Elliott (1960) found that the type antigen was destroyed by heating to 100° for 10 min. at pH 1·0, but not at pH 2·0.

Table 3. *Precipitin reactions of HCl extracts of whole cells and cell walls of strain* N37 (*type* 1) *heated to* 100° *at different pHs for different times*

Material	Length of time extracted heated to 100° (min.)	pH during heat treatment	Precipitin reaction of extract with homologous type antiserum
Cell walls	3	1·3	++
	5	1·5	++
	10	1·5	+
	20	1·45	−
Whole cocci	20	1·4	++
Cell walls	3	2·0	++
	5	2·05	++
	10	2·0	++
	20	2·0	++
Whole cocci	20	2·0	++

++, Strong precipitin reaction within 1 min.; +, moderate precipitin reaction within 5 min.; −, no precipitin reaction within 20 min.

Lysozyme extracts of cell walls and further treatment and purification

Table 4 shows the precipitin reactions of cell walls, after treatment with lysozyme and with trypsin, and of the ethanol- and acetone-precipitated fractions. In some cases no serological reaction was observed with the acetone-precipitated fraction although a very strong reaction occurred with the ethanol-precipitated one. On reprecipitating the latter fraction with acetone the precipitate obtained was serologically active and it was assumed, as suggested by Dr S. D. Elliott (personal communication) that the type specific material must all have become entrained in the first instance with the original very heavy ethanol precipitate. The lysed cell walls of each of the 22 strains reacted strongly with the specific antiserum, confirming Elliott's (1960) observations with 3 strains of *Streptococcus faecalis* that the type antigen of this organism was situated in the cell wall. Cell contents, separated from the cell walls by centrifugation, in addition to reacting with group D antiserum also reacted with the type antisera, probably because some parts of the cell walls had been disrupted into very small fragments which had not been removed by centrifugation.

Treatment with trypsin did not destroy the type antigens, suggesting that they are unlikely to be protein. Treatment of the cell walls with lysozyme followed by the addition of dilute NaOH drop by drop did not always result in clearing. Sometimes the preparation remained cloudy, at others it was cleared completely. This was found to be associated with the age of the culture and temperature of incubation. When a comparison was made between cultures of strain N37 grown in glucose nutrient broth and incubated for 18 or 48 hr at 37° with cultures incubated at 22°, the cultures grown at 37° remained cloudy, whilst those at 22° were completely cleared; an 8-hr culture incubated at 37° also showed complete clearing. However,

precipitin tests, using 10-fold serial dilutions of antigens, showed no difference in the reacting titre of the type antigen released (Table 5). Cell wall components not lysed by lysozyme are present after incubation for 18 hr at 37° but not after 8 hr, whilst even after incubation for 48 hr at 22° lysozyme-resistant components were not present. This was not due to the state of growth of the organisms either in the logarithmic or stationary state; counts of viable organisms at intervals up to 72 hr showed that with cultures incubated at 37° the logarithmic phase of growth occurred

Table 4. *Precipitin reactions of cell wall extracts of serological types of group D streptococci*

Type and strain	Precipitin reactions against type antisera of cell wall extracts after treatment: (1) digested with lysozyme =(1)	(1) digested with trypsin =(2)	EtOH precipitate of (2) =(3)	Acetone precipitate after removal of (3) =(4)	Type antisera tested against	Precipitin reaction of (4) against group antiserum
1, N37	+	+	+	+	1	–
D76	+	+	+	+	1	–
3, H69 D5	+	+	+	+	3	–
S161	+	+	+	+	3	–
4, C1	+	+	+	+	4	–
GB122	+	+	+	+	4	–
5, N83	+	+	+	+	5	–
6, B65	+	+	+	+	6	–
8, 1AS	+	+	+	+*	8	–
9, N97	+	+	+	+	9	–
19, D36	+	+	+	+	19	–
20, N161	+	+	+	+	20	–
Skadhauge strains						
Group I, 28	+	+	+	+	1	–
Group II, 50	+	+	±	±	5 and 19	–
335	+	+	+	±	19	–
783	+	+	+	+	5 and 19	–
Group III, 3	+	+	+	+*	9	–
58	+	+	+	+*	9 and D15	–
Group IV, 782	+	+	+	+*	3	–
Group VI, 120	+	+	+	+*	6	–

+, Reaction to the full titre that had been observed with extract (1); ±, reaction to the 1/10 titre that had been observed with extract (1).

* No reaction with acetone precipitated fraction but on reprecipitating the EtOH-insoluble fraction with acetone a precipitin reaction was obtained.

within the first 6 to 12 hr, but after this time the numbers were the same at both incubation temperatures, remaining stationary for 60 hr and then decreasing. Smith (1963) observed that with whole organisms of group D streptococci lysis was usually complete in less than 3 hr, and that it was more rapid in the log phase than in the stationary phase; Douglas & Parker (1958) found that vegetative cells of *Bacillus megaterium* were more susceptible to lysozyme when in the exponential phase, than were older cultures.

Effect of periodate treatment on type antigens

When the acetone-precipitated cell wall preparations of the 22 strains of *Streptococcus faecalis* listed in Table 4 were treated with periodate, complete inactivation of the specific type reaction resulted in each case when Bobbitt's (1956) method (*a*) above, was used. With the Skadhauge strains which appeared to contain two type antigens, both antigens were inactivated by this treatment. By using Elliott's (1960) method (*b*) above, similar results were obtained except that with the preparations from 5 strains (type 9, type 19, Skadhauge 3, 28, 335) the type precipitin reaction was greatly diminished but not completely destroyed.

Table 5. *The effect of time and temperature of incubation of a culture of* N37 (*type* 1) *on the lytic effect of lysozyme on the cell walls*

Experiment no.	Conditions of growth in glucose nutrient broth		Effect of lysozyme on the cell wall suspension	Precipitin reaction of lysozyme-treated cell walls or controls* with homologous type antiserum. Dilution of cell wall preparation			
	Time (hr)	Temp. (°C)		10^{-1}	10^{-2}	10^{-3}	10^{-4}
1	18	37	Slight lysis	+ +	+	±	−
	18	22	Complete lysis	+ +	+	±	−
2	48	37	No lysis	+ +	+	±	−
	48	22	Complete lysis	+ +	+	±	−
3	8	37	Complete lysis	+ +	+	−	−
4	24	37	Slight lysis	+ +	+ +	+	−
Control*	24	37	—	+ +	−	−	−
5	48	37	No lysis	+ +	+ +	+	−
Control*	48	37	—	+ +	+	−	−
	48	22	Complete lysis	+ +	+ +	+	−
Control*	22	22	—	+ +	−	−	−

* Control = cell walls suspended in phosphate buffer at pH 6·2, without added lysozyme, and incubated for the same time and at the same temperature as the lysozyme treated cell walls. No lysis occurred on any occasion.

+ +, Strong precipitin reaction in 1 min.; +, moderate precipitin reaction in 5 min.; ±, weak precipitin reaction in 20 min.

Antisera to cell walls

During this work an attempt was made to prepare a type antiserum against cell walls. Inoculation of a rabbit with a crude cell wall preparation of strain D76 resulted in a non-type-specific serum which reacted with HCl extracts of other types of *Streptococcus faecalis* and with only a moderately strong type reaction. On continued immunization of the rabbit the type reaction became even weaker.

DISCUSSION

Table 6 shows the distribution of serological types of *Streptococcus faecalis*, group D, isolated from different materials. In order to compare the data, the type numbers of Sharpe & Shattock have been used throughout, although both Lancefield (1941) and Wheeler & Foley (1945*a*, *b*) used the Lancefield strains to prepare their antisera and Skadhauge used his own strains. The same serological types have been reported over a period of years, from different habitats, both animal and

human, in healthy and pathological material and in different countries. In most of the work quoted here only a small number of strains were not typed, and the evidence strongly suggests that only a limited number of serological types of *S. faecalis* are likely to occur. There is at least one more serological type, as Skadhauge's group V strain did not react with any of the type antisera and therefore must possess a different type antigen.

Table 6. *Serological types of Streptococcus faecalis, and its varieties isolated by different workers*

Reference	Source of strains	Serological types identified
Lancefield (1941)	Vagina, U.K. Cheese, human faeces, U.S.A.	1, 3, 4
Grumbach (1943)	Human faeces, vagina, Germany	3, 6, 9
Wheeler & Foley (1945*a*)	Rectum and throat, healthy and sick infants Adult throat, urine, tonsillitis Post-mortem blood, lungs } U.S.A.	1, 3, 4
Wheeler & Foley (1945*b*)	Subacute endocarditis, U.S.A.	1, 3, 4
Skadhauge (1950)	Appendices, blood, faeces, urine, Denmark	1, 3, 5, 6, 9, 19
Sharpe & Shattock (1952)	Faeces, healthy and sick infants, U.K.	1, 3, 4, 5, 6, 8, 9, 19, 20
Elliott (1957)	Bovine endocarditis, U.K.	40 (strain D10) 41 (strain D15)
Barnes, Sharpe & Fewins (1958)	Healthy adult faeces, U.K.	1, 4, 40, 41
Elliott & Barnes (1959)	Chicken intestine, U.K.	1, 3, 4, 41
Sharpe*	Human endocarditis, U.K.	1
Franklin & Sharpe*	Milk, U.K.	1, 9, 41
Maxted & Frazer (private communication)	Urine, wounds, dental specimens, blood culture, faeces, U.K.	1, 3, 4, 5, 6, 9, 19

* Unpublished data.

Examination of 20 strains of *Streptococcus faecalis* belonging to 11 different serological types shows that the type antigen is situated in the cell wall. It is unaffected by trypsin but inactivated by periodate; this suggests that the antigenic determinants contain sugars and/or sugar alcohols and are unlikely to be protein, although they might be teichoic acids. This confirms Elliott's findings with 3 strains of this species belonging to different serological types. These specific cell wall antigens can thus be used to designate the different serological types. It has been agreed by the International Committee on Bacteriological Nomenclature (1963) to retain the numbers of Sharpe & Shattock as the cell wall types and to number new types of group D streptococci serially as they arise, following those already designated. It was stressed by Lancefield in the report of the International Subcommittee on Streptococci and Pneumococci (1963) that the serological system of classifying strains of group D streptococci should be independent of their physiological properties and should be kept on a serological basis with chemically defined

type-specific antigens. This would allow for the possibility of other kinds of type-specific substances which might be discovered later and be differently located.

Table 7 shows the numbering of the cell wall polysaccharide type antigens of *Streptococcus faecalis* so far designated. The Lancefield strains D76, H69 D5 and C1 replace the Sharpe & Shattock type strains N37, S161 and GB122 in types 1, 3 and 4, respectively, as they have prior designation. The Elliott type strains D10 and D15 are also incorporated in this scheme. They are numbered cell wall types 40 and 41 as the Sharpe & Fewins types of *S. faecium* which are consecutive to the numbers of the Sharpe & Shattock types extended to type 39. Further cell wall types based on cell wall polysaccharide antigens will continue from this.

Table 7. *Numerical series of cell wall polysaccharide type antigens of Streptococcus faecalis and the representative type strains containing these antigens*

Cell wall type no.	Type strain	Cell wall type no.	Type strain
1	D76	9	N97
3	H69 D5	19	D36
4	C1	20	N161
5	N83	40	D10
6	B65	41	D15
8	1AS		

In certain strains examined two cell wall type-antigens were found (Table 2), both being polysaccharides and each being common to a different serological type. These can be designated very conveniently by the above system. Skadhauge strains 50 and 53 will be cell wall type 5/19, and strain 58 will be cell wall type 9/41. In this way the antigenic components of these strains or any others which have more than one such type antigen are readily described. It is possible that some of the type strains designated here may possess more than one cell wall polysaccharide antigen which would only become evident if strains having only the single different antigens were found. This system of numbering provides for this contingency.

The destruction of the active antigenic groups of the cell wall type antigens by periodate observed here are in agreement with Elliott's (1960) findings. Cummins & Slade (1962) however, by using agglutination tests, found that whilst the cell wall of a strain of *Streptococcus faecalis* was not agglutinated by its homologous antiserum after periodate treatment, cell walls of a strain of *S. liquefaciens* and of *S. zymogenes* were still agglutinated by their homologous antisera after such treatment. These two varieties of *S. faecalis* are closely related to *S. faecalis* on the basis of their physiological characteristics (Shattock, 1962), differing from the latter only in their proteolytic activity and in the ability of *S. zymogenes* to produce β-haemolysis on horse blood agar. As the type antigens of strains within this species are shared among the 3 varieties, it is somewhat unexpected to find that one strain possesses a periodate-sensitive antigen whilst two possess antigens insensitive to the oxidative effect of periodate. However, the antigens revealed by agglutination tests may not be the same as those type antigens participating in the precipitin test, which are oxidized by periodate.

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