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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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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.

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Characterization of Brucellaphages

By J. G. CALDERONE and M. J. PICKETT
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(*Received* 9 *September* 1964)

SUMMARY

Eleven brucellaphages of Russian or Polish origin were examined to determine their biological, biochemical and physical characteristics. All the phages gave clear plaques of variable size (0·5–3·5 mm.) on the host *Brucella abortus* strain 19. Plaque formation occurred with all smooth cultures of *B. abortus* and *B. neotomae*. *Brucella suis* cultures were incompletely lysed or inhibited by high-titre (about 1×10^9 plaque-forming units/ml.) phage. This was shown to be true phage lysis since small (0·5 mm.) turbid plaques were later observed with this host. A partial inhibition or lysis of cultures of *Brucella melitensis* by high-titre phage was noted and could be associated with the presence of intact phage. Replication of phage on this host without plaque formation was observed by means of an elution technique. The phages reacted serologically as a group and comparative studies by electron microscopy indicated a very similar if not identical morphology. The average dimensions of the phage head were 59×66 mμ and it presented an outline consistent with that of a regular icosahedron. Attached to the phage head was a short (10×23 mμ) tail having a somewhat wedge-shaped terminal end. The % guanine + cytosine of the DNA from *B. abortus*, *B. melitensis*, *B. suis* and the eleven brucellaphages was determined from the thermal denaturation temperature of their nucleic acids. These were 57·3 % for *B. abortus*, 57·9 % for *B. melitensis*, and 58·5 % for *B. suis*. The base compositions of the phage nucleic acids were 45·3–46·7 % guanine + cytosine. On the basis of their similar fundamental characteristics these phages may be considered as closely related if not identical.

INTRODUCTION

Bacteriophages active upon bacteria of the genus *Brucella* have been isolated during the last two decades by several investigators (Sergienko, Shultz & Natovich, 1940; Pickett & Nelson, 1950; Drozhevkina, 1951; Mamatsashvili, 1957; Parnas, Feltynowski & Bulikowski, 1958). However, many of the fundamental properties of this group of phages have yet to be unequivocally determined. The reason for this may include such factors as problems in the correct speciation of some Brucella cultures (Meyer & Morgan, 1962; Pickett & Calderone, 1963), the existence of a number of different phages, or an absence of uniformity in the methods of analysis. To gain a more precise understanding of the brucellaphages and to determine the degree of homology which exists among them, a study of their biological, biochemical and physical characteristics was made.

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Since phage-typing techniques have been shown to be reliable and practical in identifying bacteria at the generic (e.g. Salmonella; Cherry, Davis, Edwards & Hogan, 1954) and species levels (e.g. Mycobacterium species; Redmond, Cater & Ward, 1963), a phage-typing scheme for Brucella species was developed after the generic specificity of the brucellaphages had been evaluated. This evaluation was particularly significant in view of the reported serological relationships between Brucella species and the other members of the family Brucellaceae (Francis & Evans, 1926; Parfentjev, Goodline & Viron, 1947).

METHODS

Phage sources. The eleven phages (F1 to F11) used in this work were originally isolated in Russia or Poland. Phage F1 was received from Dr E. L. Nelson as 'polyvalent brucellaphage, Russian series 3'. Phage F2 was obtained through the courtesy of Dr R. W. I. Kessel as 'Russian *Brucella* phage type *abortus*, strain 3'. Phages F3 to F11 were received from Dr J. Parnas (of Lublin, Poland), respectively as A-1, A-2, A-3, A-4, A-5, 10/I, 24/II, 212/XV and 371/XXIX.

Preparation of phage stocks. Albimi's brucella broth supplemented (BHB medium) with 0·0001% (w/v) nicotinamide, 0·0001% (w/v) thiamine hydrochloride, and 0·0004% (w/v) haemin (Liberman & Pickett, 1952) was used in the propagation and dilution of phages. This medium solidified with 2·0% (w/v) Albimi's agar (BH agar) was used for the growth of the host *Brucella abortus* strain 19. Working stocks of each phage were prepared by seeding each of several 250 ml. Erlenmeyer flasks, containing 40·0 ml. of BHB medium, with 0·2 ml. of a suspension (5×10^9 bacteria/ml.) of the host culture harvested from a 48 hr BH agar slope into sterile distilled water. Each flask then received that volume of phage suspension (originally derived from a single plaque) which would give a 0·04 multiplicity of infection. After incubation for 20 hr at 35° on a rotary shaker (set for 120 rev./min.) the cultures were removed and centrifuged at 1700 *g* for 30 min. The supernatant fluid was then sterilized by passage through a membrane filter (type HA, 0·45 μ, Millipore Filter Corp., Bedford, Mass., U.S.A.) and stored at 4°. Titres of these phage suspensions ranged from 10^7 to 10^{10} plaque-forming units (p.f.u.)/ml. and were stable at 4° for at least 1 year.

Phage plaque morphology. A small volume (0·1 ml.) of each phage, diluted in BHB medium, was mixed with an equal volume of a 48 hr suspension (5×10^9 bacteria/ml.) of *Brucella abortus* strain 19 and incorporated into 3·0 ml. of semi-solid agar (0·7%, w/v) at 45°. The mixture was then poured over the surface of a BH agar plate and incubated at 35° for 48 hr. After incubation, those plates with well-isolated plaques were selected for further study. Plaques from each plate were picked to tubes containing 1·0 ml. volumes of BHB medium. The contents of the tubes were membrane filtered and assayed for phage in the manner described above. The size and appearance of the plaques which developed on these plates were recorded.

Phage sensitivity of heterologous species. Undiluted preparations of phages F1 to F11 were spotted on the surface of agar layer plates (Adams, 1959) of *Pasteurella tularensis*, *P. novicida*, *P. multocida*, *P. haemolytica*, *P. pseudotuberculosis*, *P. pestis*, *Haemophilus influenzae*, *Bordetella parapertussis*, *B. pertussis*, and *B. bronchiseptica*. After the spots had dried the plates were incubated at 35° and the results noted.

Phage sensitivity of Brucella species. Agar layer plates (BH agar) of 12 strains

of *Brucella abortus* (both CO_2 dependent and CO_2 independent), 12 strains of *B. suis*, 12 strains of *B. melitensis*, 7 strains of *B. neotomae*, and 2 strains of *B. ovis* were prepared from 48 hr cultures and spotted with undiluted phages F1 to F11. Each culture had previously been examined for smoothness by the acriflavine test (Wilson & Miles, 1955). After the spots had dried the plates were incubated for 48 hr at 35°.

Phage replication on Brucella suis. Since formation of very small and turbid plaques by diluted brucellaphage on *Brucella suis* might occur but be impossible to see against the relatively opaque background of BH agar plates, a search for a more transparent medium for preparing agar layer plates of this host was made. Baltimore Biological Laboratory's brucella agar (BBL-BA) was eventually selected and used for this work. A small volume (0·1 ml.) of a suspension of *B. suis* s-100 (5×10^9 organisms/ml.), prepared from a 48 hr slope of the culture, was incorporated into 3·0 ml. semisolid agar (0·7 %, w/v) at 45° and poured over the surface of a BBL-BA plate. This plate contained about 22·0 ml. of medium and had been stored at room temperature (about 25°) for 48 hr before use. The agar layer plate was then spotted with broth dilutions of phage F2 and incubated at 35° for 48 hr. Plaques which appeared were picked to 1·0 ml. volumes of BHB medium. The resulting suspensions were sterilized by membrane filtration and spotted on agar layer plates of *B. suis* s-100 prepared as above. These plates were incubated at 35° for 48 hr and examined for phage plaques.

Phage replication on Brucella melitensis. Undiluted phage F2 ($3·7 \times 10^{10}$ p.f.u./ml.) was spotted (0·01 ml.) on the surface of BBL-BA layer plates of six strains of *Brucella melitensis* and the plates incubated at 35° for 48 hr. A partial lysis or inhibition of the bacterial lawns occurred in the areas of phage application. From one of these plates (*B. melitensis* m-42) a block (1 cm.^{2}) of agar was removed from the area of phage action, placed in a tube containing 2·0 ml. of BHB medium and cooled (4°) for 24 hr. The medium was centrifuged at 1935*g* for 20 min. and the supernatant fluid membrane-filtered. This filtrate at 10^{-0}, 10^{-1}, and 10^{-2} dilutions was spotted (0·01 ml.) on agar layer plates of *B. melitensis* m-42 and *B. abortus* strain 19. The undiluted filtrate was also titrated for phage activity on *B. abortus* strain 19. The plates were incubated at 35° for 48 hr and then examined for evidence of inhibition or phage plaques. An agar block was again removed from the *B. melitensis* m-42 plate in the area on which the undiluted filtrate had been placed and the process outlined above repeated; six such passages were made.

Phage typing of Brucella cultures. BBL-BA layer plates of 12 strains of *Brucella abortus*, 12 strains of *B. suis*, and 14 strains of *B. melitensis* were prepared from 48 hr BH agar slopes. These cultures were shown to be smooth by the acriflavine test and conform to the characteristic given for typical *Brucella* cultures (Pickett & Calderone, 1963). The agar layer plates were spotted with 0·01 ml. of a BHB medium dilution of phage F2 which under identical conditions just failed to give confluent lysis of *B. abortus* strain 19, i.e. the routine test dilution (RTD). Each plate was also spotted with 0·01 ml. of the phage at 100 × RTD and 10,000 × RTD. After the plates had remained at room temperature for 30 min. they were incubated at 35° for 48 hr and then read.

Antigenic characterization of the phages. Antiphage serum was prepared against each phage by intraperitoneal inoculations of young rabbits with stock phage.

Serum inactivation rates of homologous and heterologous phages were determined by the standard method (Adams, 1959). Velocity constants (K), representing the fractional rate of phage neutralization per minute, were based on 90–95% inactivation values.

Electron microscopy. Phage lysates were treated with deoxyribonuclease (2 μg./ml.) and ribonuclease (5 μg./ml.) for 1 hr at 37°. The lysates were purified and concentrated by differential centrifugation (alternate cycles at 34,408 *g* for 60 min. and 1935 *g* for 20 min.). The phage pellets were resuspended in 0·05 M-tris buffer (pH 7·5) which also contained 0·012% (w/v) $MgSO_4$, 0·04% (w/v) NaCl, and 0·005% (w/v) gelatin. Purified phage F1 was applied to Formvar nets (Sjöstrand, 1958) which had been coated with a thin layer of carbon. The specimens were negatively stained with a neutral solution of 2% (w/v) phosphotungstic acid containing 0·4% (w/v) sucrose (Brenner & Horne, 1959). The specimens were then examined with a Siemens Elmiskop I at ×80,000. Purified phages F1 to F11 were applied to carbon-coated Formvar grids, negatively stained, and examined with the Hitachi No. 9 electron microscope at ×21,000.

Estimation of phage and host DNA base composition by the thermal denaturation method. Deoxyribonucleic acid (DNA) from *Brucella abortus* 8a-89, *B. melitensis* m-42, *B. suis* s-100 and *Escherichia coli* K-12 was extracted (by a modification of the method of Marmur, 1961) from cell pastes of the organisms, which had been partially disrupted by passage through a Hughes type (Sagers, 1962) pressure vessel. The modification consisted of the omission of the perchlorate and lysozyme treatments and the addition of a phenol extraction step. An equal volume of a phenol versene solution (phenol saturated with 0·001 M-EDTA) was added to the sodium lauryl sulphate treated cells, the mixture shaken for 5 min. and then centrifuged at 10,000 *g* for 10 min. The nucleic acids were then precipitated from the aqueous phase by 95% (v/v) ethanol in water in the usual way and the Marmur method then used without further change.

Nucleic acid from preparations of purified and concentrated phages F1 to F11 (prepared as for electron microscopy) was extracted (3 times) by the phenol method of Gierer & Schramm (1956). The aqueous phase of the phenol extracts was mixed with an equal volume of cold 2-ethoxyethanol (Tokunaga & Sellers, 1964) which precipitated the nucleic acid. The precipitate was collected, dissolved in 2·0 ml. of a neutral solution of 0·015 M-NaCl + 0·0015 M-trisodium citrate, then dialysed against the solvent at 4° for 24 hr, a few drops of chloroform added and the solutions stored at 4°.

Thermal denaturation temperatures (T_m) of bacterial, phage and calf thymus (Worthington Biochemical Corp., Freehold, New Jersey, U.S.A.) DNA were determined by a modification of the Marmur & Doty (1962) technique. Test samples were diluted (solution of 0·15 M-NaCl + 0·015 M-trisodium citrate) and adjusted to contain about 20 μg. DNA/ml. They were then placed in quartz cuvettes having a 1 cm. light path and placed in a Beckman DU spectrophotometer. The chamber temperature of the instrument was regulated by a Gilford heat exchanger (Gilford Instrument Laboratories, Oberlin, Ohio, U.S.A.) which was coupled to a Haake circulator (Model F). Ethyleneglycol (25%, v/v) was used as the circulating fluid. The temperature and extinction (260 mμ) of the samples were traced by a Gilford multiple sample recorder (Model 2000). A temperature increase of about 1·5°/min.

was maintained during the period of denaturation, by adjustment of the voltage input to the circulator. Duplicate determinations were made on all samples. The T_m value of each sample was determined from recorded temperature and absorbance data.

RESULTS

Plaque morphology. Brucellaphages F1 to F11 all formed clear plaques on *Brucella abortus* strain 19. The diameter of the plaques varied from 0·5 to 3·5 mm. and this variation was a consistent characteristic. Phage particles isolated from either small or large plaques gave rise to a mixture of both sizes upon infection of the host culture. This phenomenon may be due to a slow rate of phage adsorption (Adams, 1959).

Phage sensitivity of heterologous species. Strains of *Pasteurella tularensis*, *P. novicida*, *P. multocida*, *P. haemolytica*, *P. pseudotuberculosis*, *P. pestis*, *Haemophilus influenzae*, *Bordetella parapertussis*, *B. pertussis* and *B. bronchiseptica* were not susceptible to lysis by any of the eleven phages. These results indicate that the brucellaphages are generically specific with regard to the family Brucellaceae.

Phage sensitivity of Brucella species. All the Brucella cultures except both strains of *Brucella ovis* were smooth as shown by negative acriflavine tests. *Brucella abortus* and *B. neotomae* were uniformly sensitive to each of the phages. Plaque formation was observed when these cultures were spotted with diluted preparations of the phages. None of the *B. melitensis* or *B. ovis* cultures was susceptible to phage lysis. The inclusion of *B. ovis* in the genus *Brucella* has been seriously questioned (Biberstein & Cameron, 1961). *Brucella suis* cultures were subject to a degree of lysis (incomplete lysis) by those phages which had a titre of at least 1×10^9 p.f.u./ml. Since plaque formation was not observed on these cultures with dilute phage preparations, phage replication on this host was open to question and was further investigated.

Phage replication on Brucella suis. Small (0·5 mm.), turbid plaques were observed on BBL-BA plates of *B. suis* s-100 which had been spotted with phage F2. These plaques were transferable from the original plate to new culture plates of *B. suis* and therefore gave evidence of replication of brucellaphage on this host. A mutant of this phage has been isolated (Calderone & Pickett, 1963) which gives rise to small (0·5–1·5 mm.), clear plaques on *B. suis*.

Phage replication on Brucella melitensis. The inhibitory or lytic action of high-titre phage on *B. melitensis* m-42 was dependent on the presence of viable virus. This was shown by passing the phage preparation through a membrane filter (Millipore, 0·30 μ) which retained this virus and examining the filtrate. This filtrate had no lytic or inhibitory effect upon cultures of *B. melitensis* or *B. abortus* strain 19. Thus it seems reasonable to assume that the phenomenon was not mediated by a toxic bacterial residue. Table 1 indicates the results obtained when elutions were made from the areas of inhibition or lysis of *B. melitensis* m-42 lawns by phage F2. If a complete recovery of the applied phage ($3{\cdot}7 \times 10^9$ p.f.u.) had been accomplished at each elution step and no new phage had been produced, the final elution fluid would have contained few if any phage particles, since a dilution of 1/200 occurred at each of the six elution steps. The fact that the final elution fluid had a titre of $8{\cdot}3 \times 10^6$ p.f.u./ml. supports the view that phage replication had occurred in *B.*

melitensis m-42 in the absence of visible plaque formation, and that the phenomenon observed was referable to true phage lysis of the host.

Phage typing of Brucella cultures. Phage-typing patterns of typical cultures of *Brucella abortus*, *B. suis* and *B. melitensis* are given in Table 2. Both CO_2-dependent and CO_2-independent *B. abortus* cultures were uniformly lysed at the three concentrations of phage used. All the *B. suis* cultures were partially lysed at 10,000 × RTD, and about 75% of these cultures gave many small turbid plaques in the areas spotted with 100 × RTD of the phage. A few cultures formed these plaques at the

Table 1. *Serially passaged elutions from lawns of Brucella melitensis m-42 spotted with brucellaphage F2*

Elution no.	*B. abortus* strain 19	*B. melitensis* m-42	Titre on *B. abortus* strain 19 in p.f.u./ml.
0	4+	2+	$3{\cdot}7 \times 10^{10}$
1	4+	1+	$2{\cdot}1 \times 10^{8}$
2	4+	1+	$9{\cdot}6 \times 10^{8}$
3	4+	1+	$4{\cdot}7 \times 10^{8}$
4	4+	1+	$4{\cdot}5 \times 10^{8}$
5	4+	−	$2{\cdot}2 \times 10^{8}$
6	4+	−	$8{\cdot}3 \times 10^{6}$

4+ = complete inhibition or lysis; 2+ = about 50% inhibition or lysis; 1+ = about 25% inhibition or lysis; − = no inhibition or lysis.

Table 2. *Phage-typing patterns of typical Brucella cultures*

	No. of strains tested	Brucellaphage F2 RTD × 10,000	RTD × 100	RTD × 1
Brucella abortus	12	4+	4+	SCL
Brucella suis	2	3+	MP*	FP*
	7	2+	MP*	.
	3	1+	.	.
Brucella melitensis	4	2+	MP**	.
	1	1+	.	.
	3	±	.	.
	6	.	.	.

4+, Complete lysis; 3+, 75% lysis; 2+, 50% lysis; 1+, 25% lysis. SCL, semi-confluent lysis, MP, many plaques; FP, few plaques; *, turbid; **, very turbid; RTD, routine test dilution.

RTD level. About half of the *B. melitensis* cultures were resistant to lysis by the concentrations of phage used. The remaining cultures gave a partial lysis reaction (± to 2+) with 10,000 × RTD. Small (0·5 mm.) very turbid plaques appeared on about 25% of the cultures with 100 × RTD of the phage. Failure to observe plaques on *B. melitensis* in earlier experiments probably reflects the use, at that time, of a less clear medium (BA agar). The observation of plaques on *B. melitensis* cultures offers additional evidence that phage replication occurred on this species.

Antigenic studies. The homologous and heterologous K values of each antiphage serum are given in Table 3. The degree of variation in heterologous K values

obtained with this group of phages is similar to that reported by Seto, Kaesberg & Wilson (1956) in their serological study of staphylococcal phages. With a few anti-brucellaphage sera, the homologous K values were lower than some heterologous values. The reason for this is not entirely clear but may be associated with impurities in the phage preparation (Morgan, 1963). It is apparent, however, that antibody formed against any one of the eleven phages neutralized to a marked degree any of the other phages. These results indicate that the phages may be placed in the same serological group.

Table 3. *Homologous and heterologous K values of anti-brucellaphage sera*

	Brucellaphages										
Anti-sera	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11
	K values*										
S-F1	82	75	58	46	59	48	52	56	66	58	74
S-F2	116	165	160	129	133	110	146	105	186	154	137
S-F3	98	79	122	125	80	86	127	73	53	86	55
S-F4	193	132	140	145	128	102	154	116	93	143	108
S-F5	122	118	46	45	110	129	60	150	133	132	152
S-F6	65	50	41	32	69	80	48	106	152	145	145
S-F7	48	32	38	36	35	29	42	35	39	29	27
S-F8	88	77	57	77	89	86	78	93	64	69	82
S-F9	87	50	46	44	82	106	51	110	107	83	77
S-F10	124	107	100	112	145	127	127	143	132	143	132
S-F11	60	38	47	47	65	69	41	78	68	73	74

* Based on 90–95 % inactivation levels.

Electron microscopy. The morphology of negatively stained phage F1 is presented in Pl. 1. Intact phage heads (average dimensions 59×66 mμ) are in the form of regular polygons and give outlines consistent with that of regular icosahedra. Many of the phages are attached to particulate material by a short tail (average length 23 mμ) which, in some instances, has a somewhat wedge-shaped terminal end. These measurements agree essentially with those reported by Parnas & Chiozzotto (1962) for brucellaphage morphology. Comparative studies of phages F1 to F11 by electron microscopy revealed no evidence of morphological differences among them.

Base compositions of DNA from brucellaphages and their hosts. The linear relationship of thermal denaturation temperature and the base composition of DNA was established by plotting the experimentally obtained average T_m values of the nucleic acids (*Escherichia coli* K-12, calf thymus, and *Brucella abortus* 3a-89) as a function of their published (Chargaff, 1955; Belozersky & Spirin, 1960), chemically determined, guanine + cytosine content (% G + C) expressed as a percentage of the total base. By means of this standard curve, an estimate of the % G + C of the DNA from *B. melitensis* m-42 and *B. suis* s-100 was obtained based upon the average T_m value of the nucleic acid. These values were respectively 57·9 % G + C for *B. melitensis* and 58·5 % G + C for *B. suis*. Since *B. abortus* DNA has a chemically determined base composition of 57·9 % G + C, the base compositions of these three Brucella species appear to be almost identical. These results further substantiate the taxonomically based relatedness of these organisms.

The identification of phage nucleic acid as DNA was based on enzymic evidence. The extinction at 260 mμ of deoxyribonuclease-treated phage nucleic acid increased, while no change was observed when it was treated by ribonuclease. Table 4 gives the average T_m value and % G+C (based on the T_m value) of the DNA isolated from each of the brucellaphages. The similarity of the base content of their nucleic acid (45·3–46·7 % G+C ± 1 %) indicates that these phages are closely related.

Table 4. *Average thermal denaturation temperatures (T_m) and % guanine+cytosine (% G+C) of DNA isolated from brucellaphages*

Brucellaphage DNA	Average T_m (°C.)	% G+C from standard curve
F1	89·0	46·1
F2	89·2	46·5
F3	88·9	45·9
F4	89·3	46·7
F5	89·2	46·5
F6	88·6	45·3
F7	88·8	45·7
F8	88·7	45·5
F9	88·9	45·9
F10	88·9	45·9
F11	89·1	46·3

DISCUSSION

Accurate determination of phage sensitivity of Brucella cultures is dependent upon a number of factors. An important one is that of the antigenic state of the culture, since our studies and those of others (Stinebring & Braun, 1959) have shown rough cultures to be resistant to phage action. As in the phage typing of Staphylococcus (Blair & Williams, 1961) a standardized phage suspension should be used in any test procedure. Perhaps one of the most significant considerations is the selection of a clear growth medium. When such a medium was used, it was possible to detect the small turbid plaques which were formed on *Brucella suis* cultures by brucellaphage. The hitherto unsuccessful efforts (Morgan, 1963) to show replication of phage on *B. suis* may have been due in part to the opalescence of the medium or to the use of an unstandardized bacterial inoculum.

The phage-typing scheme developed in the present work may be fruitfully applied as an aid in speciation of smooth Brucella cultures. However, the most rewarding application of the method will be in the area of the correct speciation of atypical cultures. By using phage-typing techniques in conjunction with the usual criteria, some of these troublesome atypical cultures have been speciated (Meyer & Morgan, 1962; Pickett & Calderone, 1963).

Although Bradley & Kay (1960) originally placed the brucellaphages in the same morphological group as coliphage T3, more recent studies of these phages indicate that they may belong to separate groups. Coliphage T3 has been reported (Bradley, 1963) to have an octahedral head, in contrast to the icosahedral shape of the brucellaphages. The tail of brucellaphages (23 mμ) appears also to be significantly longer than that of coliphage T3 (14 mμ).

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EXPLANATION OF PLATE

Pl. 1. Electron micrograph of brucellaphage F1 negatively stained with 2% (w/v) neutral phosphotungstic acid. × 320 000.

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Heterotrophy and Nitrogen Fixation in *Chlorogloea fritschii*

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SUMMARY

The blue-green alga *Chlorogloea fritschii* fixed elementary nitrogen to a limited extent in the dark in a strictly inorganic medium. Growth and nitrogen fixation continued in the dark when a suitable organic substrate was present in the medium. Among the organic substrates tested, sucrose (0·01 M) was the most readily utilized and was outstanding in supporting nitrogen fixation in the dark. The alga was adapted to heterotrophic conditions after continuous incubation and repeated subcultivation in the dark. Inter-relations between carbon assimilation and nitrogen fixation under heterotrophic conditions were observed. Sucrose assimilation proceeded more vigorously in the light and resulted in a fourfold increase in the rate of growth and nitrogen fixation. Sucrose assimilation was increased in the light in the absence of carbon dioxide from the gas phase, but nitrogen fixation was greatest when the alga was supplied with sucrose and carbon dioxide. Carbon dioxide was inhibitory to sucrose assimilation and slightly to nitrogen fixation in the dark.

INTRODUCTION

The frequent occurrence of blue-green algae in soils and in habitats rich in organic matter has for long suggested that they might grow in the dark at the expense of organic nutrients (see Fogg, 1956). Although Allison, Hoover & Morris (1937) reported a strain of *Nostoc muscorum* able to grow and fix nitrogen on glucose in the dark, no detailed studies were given. Investigations of the heterotrophic growth of *Tolypothrix tenuis* by Kiyohara, Fujita, Hattori & Watanabe (1960, 1962), another blue-green alga known for its ability to fix molecular nitrogen, were made in the presence of combined nitrogen but no account was given about attempts to grow the alga in nitrogen-free medium in the dark.

When Fay & Fogg (1962) found that the blue-green alga known as *Chlorogloea fritschii* (but see Fay, Kumar & Fogg, 1964) was able to fix nitrogen not only in the light but also in the dark, it was thought desirable to examine the conditions of heterotrophic growth and nitrogen fixation by this alga.

METHODS

Organism, culture and general analytical methods. The strain of alga, culture methods, growth conditions and general methods for the estimation of growth and nitrogen fixation used were as described previously (Fay & Fogg, 1962; Fay *et al.* 1964).

Media containing organic substrates were autoclaved at 115° for 15 min. Bacteriological filters or exposure to ultraviolet radiation were used to sterilize volatile or

thermolabile compounds. The concentration of reducing sugar in autoclaved medium containing sucrose was below 1% of the total sugar content of the medium. Phosphate solutions were sterilized separately and added after cooling to the sterile medium.

Sugar determinations were carried out according to the Hagedorn-Jensen method as modified by Hulme & Narain (1931).

Nitrogen starvation. For certain experiments nitrogen-starved organisms, which showed an increased response when exposed to elementary nitrogen, were used. Organisms from exponentially growing cultures were separated by centrifugation, washed with distilled water, centrifuged down again, and resuspended in fresh standard medium. The suspension was then flushed with argon containing about 2% (v/v) CO_2 for 1 hr and incubated overnight under the same gas phase at 30° and illuminated with about 500 foot-candles light intensity.

^{15}N technique for the determination of nitrogen fixation. This was as described by Burris & Wilson (1957). The algal suspensions were placed in reaction flasks and attached to a closed culture apparatus which was provided with magnetic stirring or shaking mechanism and held under controlled conditions of temperature. Then the vessels were evacuated and filled to atmospheric pressure with a gas mixture containing $^{15}N_2$-enriched nitrogen gas. When a constant 0·2% (v/v) CO_2 concentration in the gas phase was required a 'CO_2 buffer' (Pardee, 1949) was added to the side arm of the reaction flask. After exposure to $^{15}N_2$ the samples were digested by the Kjeldahl method, the ammonia distilled into standard acid and the ammonium-nitrogen converted to elementary nitrogen with alkaline hypobromite. The $^{14}N:^{15}N$ ratio was determined with a MS 3 type (Associated Electrical Industries Ltd., Manchester) mass-spectrometer.

Radioactivity assays. To follow sucrose assimilation, (U)^{14}C-sucrose (supplied by the Radiochemical Centre, Amersham, Buckinghamshire) was added to the algal suspension and the incorporation of ^{14}C into cell material and the radioactivity of the respiratory CO_2 was measured after incubation. To measure incorporation of ^{14}C-sucrose, samples of the algal suspension were filtered through membrane filters (0·5–1·0 μ pore size) which were then washed with distilled water and dried over silica gel and soda lime in a desiccator. Radioactivity was measured with a Tracerlab SC-50 B (Tracerlab Inc., Massachussets) automatic gas-flow proportional counter. The concentration of algal samples taken was chosen so that a thin uniform deposit of the particulate matter was obtained, in a range in which self absorption was found insignificant and thus corrections for self absorption could be omitted.

Respiratory CO_2 was trapped in alkali (10% NaOH or 12% KOH) placed in the side arm of the reaction flasks and converted to barium carbonate. The barium carbonate precipitate was collected on a membrane filter, rinsed with hot distilled water and fixed with a dilute glue solution. The samples were then dried and the radioactivity measured. The dried barium carbonate precipitate was weighed and corrections were applied for self absorption by using a self-absorption curve.

RESULTS

Search for organic substrates supporting growth and nitrogen fixation in the dark

Various compounds were tested for their ability to support growth of the alga in the dark, in the presence and in the absence of combined nitrogen (Table 1). On first transfer from the light, growth in the dark was in general very slow. No significant changes were observed during the first 4 weeks. However, growth proceeded slowly on some of the substrates during the second and third month in the dark. Slight growth occurred on mannitol + nitrate, on glucose + nitrate, on glycine + and − nitrate, and on glutamine + and − nitrate. There was appreciable growth on maltose with nitrate but not without. Best growth was on sucrose in the presence and in absence of combined nitrogen. Sucrose was outstanding among the substrates tested in allowing a substantial fixation of elementary nitrogen in the dark.

Table 1. *Utilization of organic substrates by Chlorogloea fritschii incubated at* 30° *in the dark for* 3 *months in the presence and absence of combined nitrogen in the basic medium*

Substrate concentration: 0·01 M; +, + +, + + + = growth; 0 = no growth.

	Medium	
Substrate	$+NO_3$	N-free
Mannitol	+	0
Glucose	+	0
Sucrose	+ + +	+ +
Maltose	+ +	+
Glycine	+	+
Glutamine	+	+

Growth with acetate, pyruvate, citrate, α-ketoglutarate, succinate, fumarate, malate, glycollate, arabinose, fructose, glutamate and aspartate was nil.

The effect of substrate concentration upon growth and nitrogen fixation in the dark

The effect of sucrose on growth after 10 weeks incubation at 35° in the dark paralleled its effect on nitrogen fixation (Fig. 1); 0·01 M-sucrose was best for both. Attempts to grow the alga with an identical molar range of glucose, fructose, glucose + fructose in equimolar mixture or of fructose-1,6-diphosphate in the dark had little or no success. Only glucose (0·1 M) supported appreciable growth and nitrogen fixation in the dark.

Assimilation of ammonium-nitrogen, nitrate-nitrogen and elementary nitrogen during growth on glucose and on sucrose in the dark

Considerable differences occurred in growth and nitrogen assimilation of the alga in the dark, according to the organic substrate and the source of nitrogen supplied (Table 2). Growth and nitrogen assimilation were best with sucrose (0·01 M) + nitrate, being 4 to 5 times the values obtained with ammonium or elementary

nitrogen; % cell-N, however, was highest with ammonium and lowest in nitrogen-free medium. Glucose (0·1 M) supported an approximately equal growth with all three sources of nitrogen but appreciable nitrogen assimilation occurred only in the presence of ammonium-nitrogen. Ammonium-nitrogen was assimilated by the alga to a similar extent with sucrose or glucose. Nitrate reduction and nitrogen fixation, however, were active in the presence of sucrose but hardly at all with glucose.

Table 2. *Growth and nitrogen assimilation of Chlorogloea fritschii after 6 weeks at 30° in the dark on sucrose and on glucose with various sources of inorganic nitrogen*

NH_4^+, 1·0 g. NH_4Cl/l.; NO_3^-, 2·0 g. KNO_3/l. Values referred to 100 ml. medium. Means in parentheses.

Organic substrate	Source of N	Dry wt. of alga (mg.)	Nitrogen content of alga: mg.	Nitrogen content of alga: % of dry wt.
0·01 M-Sucrose	NH_4^+	6·6, 7·3 (7·0)	0·412, 0·438 (0·425)	6·1
	NO_3^-	56·2, 53·8 (55·0)	2·975, 2·175 (2·575)	4·7
	N_2	13·3, 13·5 (13·4)	0·355, 0·414 (0·385)	2·9
0·1 M-Glucose	NH_4^+	6·0, 5·9 (6·0)	0·327, 0·305 (0·316)	5·3
	NO_3^-	6·8, 6·2 (6·5)	0·049, 0·039 (0·044)	0·7
	N_2	3·7, 5·2 (4·5)	0·054, 0·068 (0·061)	1·3

Physiological adaptation to heterotrophic conditions

It seemed that by continuous incubation and repeated subculture in the dark in the presence of 0·01 M-sucrose, the alga gradually adapted to heterotrophic conditions; this was perceptible by the significant decrease of the duration of lag phase and by the better growth of the alga. After subcultivation for one year (six subcultures) in the dark, the lag was about 1 week (Fig. 2). The yield of organism (110 mg. algal dry weight/100 ml. medium) after 8 weeks was 50 % greater than that obtained with the non-adapted strain (76 mg./100 ml.) after 10 weeks in the dark.

There seemed to be no lag in nitrogen fixation. The amount of nitrogen fixed (6 mg./100 ml.) was 3 times that of the non-adapted strain. The greater nitrogen-fixing capacity of the alga which followed adaptation to heterotrophic conditions was also apparent from the higher (5–8 %) nitrogen content of the organism, in contrast to the 2–3 % nitrogen content of the non-adapted strain grown in the dark. Extracellular nitrogen was high (35 % total-N), as usual, after inoculation but decreased rapidly to 4 % and remained at this value. This was in contrast to the 12–15 % extracellular nitrogen found in light-grown cultures.

Effect of light upon sucrose utilization

The utilization of sucrose in light by the alga resulted in an increase of the growth rate and a prolongation of the period of exponential growth (Fig. 3). After 5 weeks

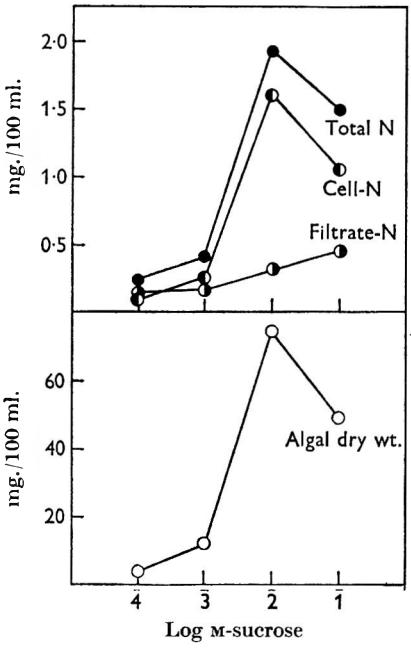


Fig. 1

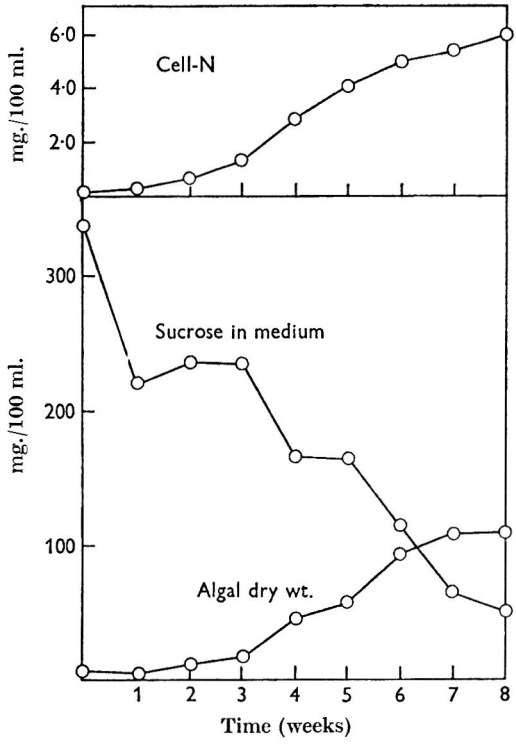


Fig. 2

Fig. 1. Effect of sucrose concentration of the medium on growth (algal dry wt.) and nitrogen fixation (total-N) of *Chlorogloea fritschii* incubated at 35° in the dark for 10 weeks.

Fig. 2. Time course of sucrose uptake, growth (algal dry wt.) and nitrogen fixation (cell-N) by a dark-adapted strain of *Chlorogloea fritschii* incubated in the dark at 30°.

Table 3. *Effect of sucrose on $^{15}N_2$ uptake by the light-grown and dark-adapted strains of Chlorogloea fritschii*

N-starved cells were exposed to N_2 enriched with 8 % $^{15}N_2$ at 30° in the light (200 foot-candles) and in the dark, under aerobic and anaerobic conditions, for 3 days. Means in parentheses.

Strain of alga	Carbon source	O_2	Atom % $^{15}N_2$ excess: In the light	In the dark
Light-grown	6×10^{-4} M-$NaHCO_3$	+	0·498, 0·462 (0·480)	0·154, 0·142 (0·148)
Dark-adapted	6×10^{-4} M-$NaHCO_3$	+	0·306, 0·336 (0·324)	0·052, 0·063 (0·058)
Light-grown	10^{-2} M-sucrose	+	2·394, 2·364 (2·379)	1·099, 1·110 (1·105)
Dark-adapted	10^{-2} M-sucrose	+	2·280, 2·340 (2·310)	0·387, 0·453 (0·415)
Light-grown	10^{-2} M-sucrose	−	2·274, 2·212 (2·243)	0·313, 0·320 (0·317)
Dark-adapted	10^{-2} M-sucrose	−	2·584, 2·653 (2·618)	0·346, 0·364 (0·355)

the yield of organism in cultures grown on sucrose was about 4 times that of control cultures; the sucrose concentration decreased by about 90 %. The percentage cell-N was not appreciably different in the two culture series.

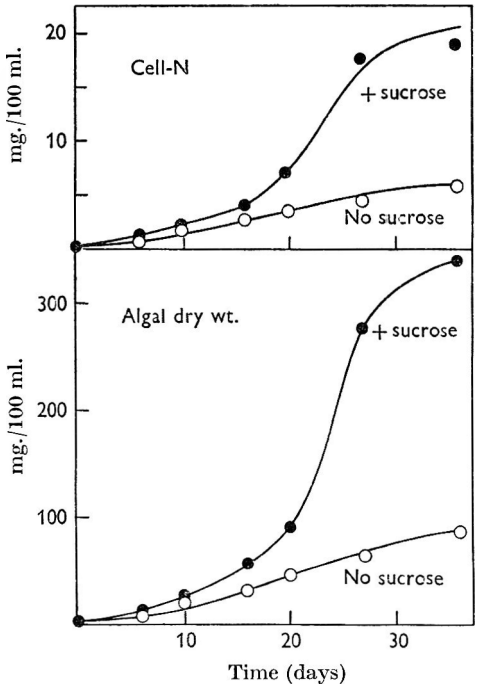


Fig. 3

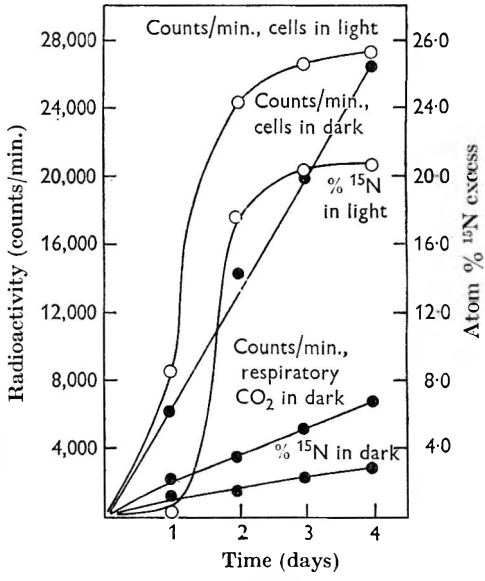


Fig. 4

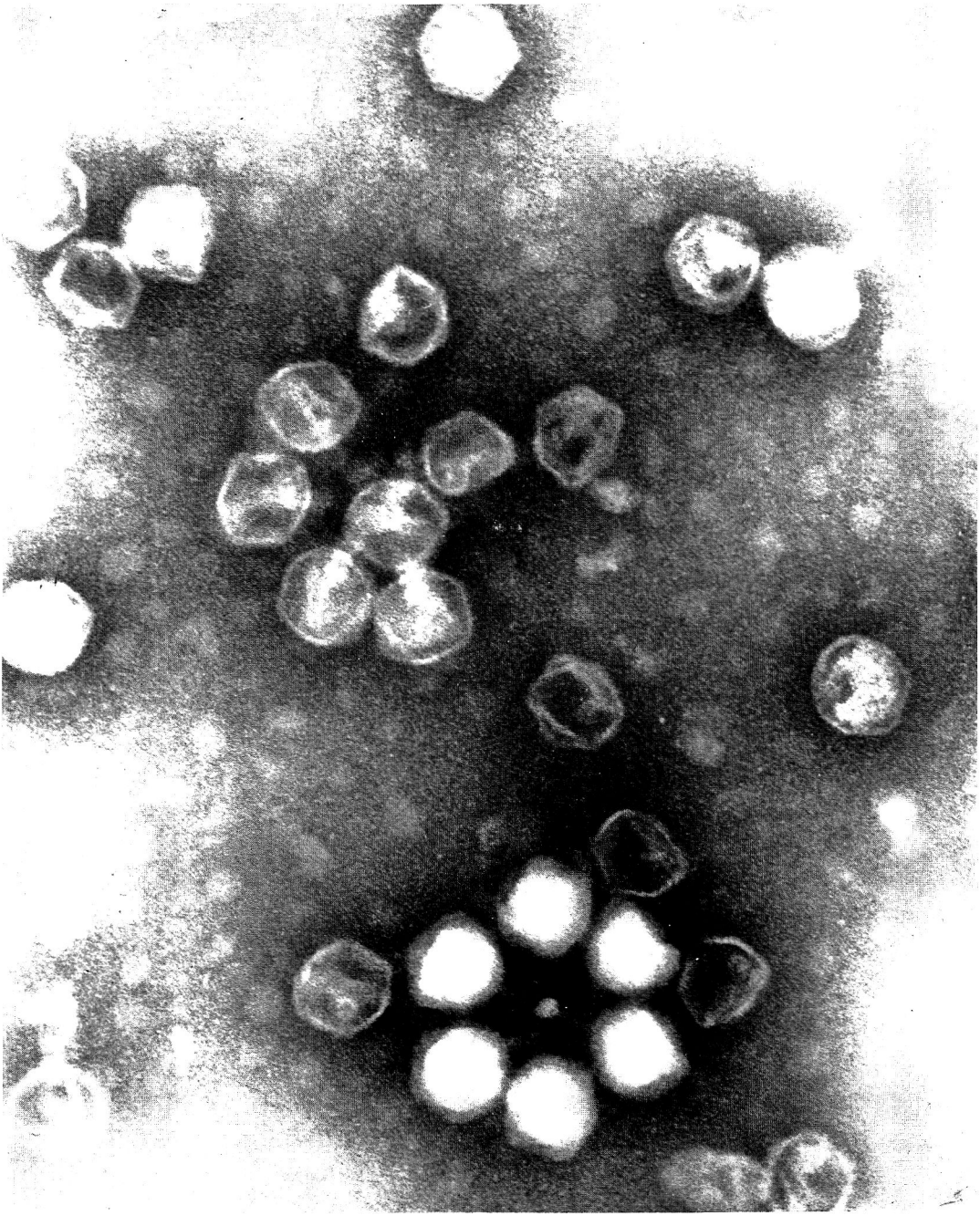
Fig. 3. Effect of 0·01 M-sucrose in the medium upon growth (algal dry wt.) and nitrogen fixation (cell-N) of *Chlorogloea fritschii* incubated in the light (300 foot-candles) at 25° with continuous shaking.

Fig. 4. Relations of ^{14}C-sucrose assimilation and $^{15}N_2$ fixation in *Chlorogloea fritschii*. 4 ml. algal suspensions were incubated in Warburg flasks at 30° in the light (650 foot-candles) and in the dark. Sucrose added at 0·001 M concentration and labelled with 0·5 μc (U)^{14}C-suerose per flask. Pardee buffer provided 0·2 % CO_2 in side arms of light incubated flasks. Respiratory CO_2 trapped in 10 % NaOH in the side arms of dark incubated flasks. Gas phase: 0·2 atm. N_2 enriched with 20 % $^{15}N_2$, 0·2 atm. O_2 and the rest argon. Open circles: incubated in light; solid circles: incubated in dark.

Effects of sucrose upon $^{15}N_2$ uptake in the light and in the dark

Table 3 illustrates the effect of various experimental conditions on the rate of $^{15}N_2$ incorporation by both strains of the alga. It was highest in the light and in the presence of sucrose, and was then not significantly affected by the supply or omission of external oxygen; like growth it exceeded more than 4 times the rate observed in the light without sucrose.

$^{15}N_2$ uptake continued to an appreciable extent in the absence of sucrose, i.e. in the purely inorganic medium in the dark. Though nitrogen fixation in the dark with sucrose was greater under aerobic conditions, it proceeded at a considerable rate also under anaerobic conditions. The light-grown strain fixed more nitrogen than the dark-adapted strain in the dark under aerobic but not under anaerobic conditions.



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Relations between sucrose assimilation and nitrogen fixation

Figure 4 represents a time course of ^{14}C-sucrose assimilation and $^{15}N_2$ fixation in the light and in the dark. ^{14}C-incorporation in the light proceeded exponentially at first but slowed down after 2 days and became stationary after 4 days as a result of sucrose exhaustion from the medium. ^{14}C-incorporation in the dark showed a linear course and reached finally a similar value to that in the light at the end of the incubation period. $^{15}N_2$ fixation in the light, after a slow start, continued at an exponential rate and then followed a similar course to ^{14}C-incorporation in the light. $^{15}N_2$ was taken up at a much lower rate in the dark and this corresponded to the rate of respiratory $^{14}CO_2$ output in the dark.

Table 4. *^{14}C-sucrose assimilation and $^{15}N_2$ fixation in Chlorogloea fritschii in the light (600 foot-candles) and in the dark, with and without CO_2 in the gas phase*

5 ml. algal suspensions, taken from a N-starved culture, were incubated in Warburg flasks for 6 hr. at 35° with continuous agitation. Sucrose added at 0·001 M concentration and labelled with 0·5 μc (U)^{14}C-sucrose per flask. Pardee buffer in side arms provided 0·2 % CO_2 for the light-incubated flasks. Respiratory CO_2 trapped by 12 % KOH in the side arms of the dark-incubated flasks. Gas phase: 0·2 atm. N_2 enriched with 20 % $^{15}N_2$, 0·2 atm. O_2 and the rest argon. Means in parentheses.

Treatment	Radioactivity: Of cells (counts/min.)	Radioactivity: Of respiratory CO_2 (counts/min.)	Atom % $^{15}N_2$ excess
Light + CO_2	18,060 17,770 (17,915)	—	0·685 0·665 (0·675)
Dark + CO_2	9,430 11,350 (10,390)	—	0·095 0·090 (0·093)
Light − CO_2	29,550 30,930 (30,240)	135 55 (95)	0·415 0·340 (0·378)
Dark − CO_2	15,500 15,770 (15,635)	4,410 4,870 (4,640)	0·100 0·112 (0·106)

The results of a short term experiment, in which the effects of CO_2 on ^{14}C-sucrose assimilation and $^{15}N_2$ fixation were investigated, are presented in Table 4. Omission of CO_2 from the gas phase resulted in a marked increase of ^{14}C-incorporation into cell material in the light, and also in the dark. About 25 % of the recovered radioactivity was found in the respiratory CO_2 in the dark. No appreciable radioactivity was, however, recovered as respiratory CO_2 in the light. $^{15}N_2$ fixation was greatest in the light in the presence of CO_2 + sucrose. $^{15}N_2$ fixation decreased when sucrose was the sole source of carbon for the organisms in the light and was still less in the dark. $^{15}N_2$ fixation in the dark was slightly lower in the presence of CO_2 than in its absence.

DISCUSSION

The finding that sucrose was outstanding among the substrates tested in allowing a considerable growth of *Chlorogloea fritschii* in the dark and also in supporting a reasonable rate of nitrogen fixation under heterotrophic conditions, contrasts with

the reports about other blue-green algae (Allison *et al.* 1937; Allen, 1952; Kiyohara *et al.* 1960, 1962) for which glucose was the best substrate for growth in the dark. The utilization of other sugars by *C. fritschii* was either limited, as with glucose, or was dependent on the presence of combined nitrogen in the medium, as with maltose. The failure to grow *C. fritschii* on Krebs cycle intermediates, other sugars and organic compounds, all of which are probably intermediates or products of metabolic cycles, can best be explained by an inability to penetrate the cell membrane (Danforth, 1962).

On the first transfer from photosynthesizing conditions, growth of *Chlorogloea fritschii* in the dark proceeded rather slowly and only after a relatively long period of adaptation to heterotrophic conditions. Similar observations were reported by Allison *et al.* (1937) for *Nostoc muscorum* and by Allen (1952) for several other blue-green algae. With *C. fritschii* the duration of this lag phase decreased markedly after adapting the alga to heterotrophic conditions by repeated subcultivation in the dark. Successful adaptation to these conditions was indicated also by the higher rate of growth and nitrogen fixation and by the more effective utilization of sucrose. $^{15}N_2$ uptake was inhibited in the dark under anaerobic conditions indicating that nitrogen fixation depended upon the oxidative assimilation of sucrose, though it continued, at a lower rate, also under conditions of anaerobic respiration.

The stimulation by light of sucrose assimilation in *Chlorogloea fritschii* may be connected with the abundant supply of high-energy phosphate generated in the photochemical reaction which could be used for the phosphorylation of sucrose, in a similar way as for the phosphorylation of glucose in green algae (Kandler, 1954; Simonis, 1956). Photoassimilation of sucrose in *C. fritschii* is indicated by the increased ^{14}C-sucrose incorporation into cellular material when CO_2 was absent from the gas phase. This shows that CO_2 assimilation plays no part in the uptake of sucrose but a higher proportion of the photochemically generated high-energy phosphates could be available for the phosphorylation of sucrose. In the presence of both CO_2 and sucrose, photoreduction of CO_2 and photoassimilation of sucrose could occur simultaneously, as is indicated by the greatest $^{15}N_2$ fixation observed under these conditions. The incorporation of the fixed nitrogen was apparently enhanced by the ample supply of carbon skeletons.

Inter-relations between carbon assimilation and nitrogen fixation in *Chlorogloea fritschii* observed under photosynthesizing conditions (Fay & Fogg, 1962) were also evident when growing the alga in the dark and comparing the rates of carbon and nitrogen assimilation. Hydrogen carriers produced in respiration were presumably available for the reduction of elementary nitrogen and the ammonia thus formed could be incorporated into compounds derived from the conversion of sucrose. When ^{14}C-sucrose assimilation was inhibited by CO_2 in the dark, this was reflected by the simultaneous decrease of $^{15}N_2$ fixation.

Metabolic inter-relations between photosynthesis and nitrogen fixation in *Anabaena cylindrica* were reported by Fogg & Than-Tun (1960), Cox, Fay & Fogg (1964) and Cobb & Myers (1964). It is, however, not clear how nitrogen fixation is stimulated by the photochemical reaction and how much the observed interrelations are the manifestation of an inter-dependence on the products of photosynthesis and nitrogen fixation, respectively. Nitrogen fixation in *Chlorogloea fritschii* can certainly continue in the dark in the absence of an external substrate and its extent

will apparently depend upon the endogenous sources present. The higher nitrogen-fixing activity of the light-grown strain, observed in a short-term experiment in the dark, may be attributed to a greater store of carbon skeletons and energy accumulated during the preceding period of illumination.

The results presented here support the suggestion of a functional linkage between photosynthesis and respiration in blue-green algae (Stanier & van Niel, 1962; Jones & Myers, 1963). Studies on the relations of nitrogen fixation and photosynthesis in cell-free preparations of *Anabaena cylindrica* (Cox *et al.* 1964) indicate that nitrogen fixation in this alga is located in the photosynthetic lamellae extending throughout the peripheral chromatoplasm. Experiments with cell-free preparations of *Chlorogloea fritschii* confirmed these results and have shown that photosynthesis, respiration and nitrogen fixation was greatest in the same particulate fraction (unpublished data). The chromatoplasm thus may be regarded as a single metabolic unit in the cells of blue-green algae, in which interactions of photosynthesis, respiration and nitrogen fixation are most likely.

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Methionine as an antimutagen in *Schizosaccharomyces pombe*

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(*Received 7 October* 1964)

SUMMARY

Methionine inhibition of the phenotypic expression of $adn^- \rightarrow adn^+$ reverse mutations in the fission yeast *Schizosaccharomyces pombe* was investigated. This inhibition occurred solely with L-methionine, and not with a variety of other growth factors. The inhibitory effect on revertant expression did not apply to a number of reverse and forward mutations other than $adn^- \rightarrow adn^-$. Alteration of the adenine supply to adn^- organisms before and after mutagen treatment had no effect. Methionine was shown to have additional inhibitory effects on the leakiness of an adn^- mutant, on complementation between *adn*-1 mutants, and on the growth of certain adn^- strains in the presence of the analogue 6-methylaminopurine. It is suggested that S-adenosylmethionine may be implicated in these methionine effects.

INTRODUCTION

In earlier papers the presence of methionine in the minimal plating medium was shown to inhibit the phenotypic expression of newly arising reverse mutations to adenine-independence in *Schizosaccharomyces pombe* (Clarke, 1962, 1963). In doubly auxotrophic adenine- and methionine-dependent (adn^- *met*$^-$) strains this methionine effect led to cases of an apparent influence of the methionine allele upon the reverse mutability of the adn^- allele. In these earlier experiments methionine in the plating medium inhibited the phenotypic expression of revertants of spontaneous, nitrous acid-, and ultraviolet radiation-induced origin. There was no effect on the viability of adn^- organisms which had been treated with a mutagen and then plated on a medium containing a high concentration of adenine (50 μg./ml.). Residual divisions of adn^- organisms were decreased on a minimal medium supplemented with methionine. Methionine was without effect on adenine-independent (adn^+) reversions once these had been phenotypically expressed.

The present paper describes the results of experiments designed to give a better understanding of the mode of action of methionine in *Schizosaccharomyces pombe*. In particular it was sought to learn whether methionine alone had this antimutagenic effect, or whether other growth factors acted similarly. Experiments were made to see whether there was a critical dependence upon the methionine concentration in the minimal medium, and whether the methionine effect was in reality merely a delay in the appearance of adn^+ revertants and which would not be

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apparent if revertant colonies were scored later than the routine 7-day period. The possibility that methionine might act only during a critical period before the actual phenotypic expression of *adn*$^+$ reversions was examined by experiments in which there was a delayed removal of methionine from populations of HNO_2-treated organisms on minimal medium. Witkin (1956) and Clavilier, Luzzati & Slonimski (1960) had been able to show by similar experiments that there were critical periods for the action of amino acids in ultraviolet radiation-mutagenesis in bacteria, and the histidine inhibition of revertants arising by a gene conversion process in the ad_3 system of *Saccharomyces cerevisiae*.

Since the earlier experiments had suggested that methionine inhibition of revertant expression might perhaps be the result of decreased residual divisions on minimal medium, attempts were made to test this. Conditions which were tested included alteration of the growth conditions for the organisms before mutagen treatment, starvation for adenine and other growth factors before mutagen treatment, and allowing limited supply or synthesis of adenine after treatment with the mutagen. Although, as mentioned, no influence of methionine had been found upon the viability of mutagen-treated organisms upon minimal medium with a high concentration of adenine (50 μg./ml.), further tests were made on medium containing only a low concentration of adenine. The specificity of the methionine effect was examined in a variety of reverse and forward mutation systems, as was also the influence of methionine upon other aspects of cell physiology: complementation of adenine mutants, leakiness, and growth on the purine analogue 6-methylaminopurine.

METHODS

Details of media, *adenine*-1 mutants of *Schizosaccharomyces pombe* used, and the routine procedures for nitrous acid and ultraviolet radiation reverse mutation experiments have all been given previously (Leupold, 1955; Clarke, 1962, 1963). The suspensions of organisms treated usually contained initially $2–5 \times 10^7$ viable organisms/ml. The source of ultraviolet radiation delivered about 90% of its ouput at 2537 Å; a dose rate of approximately 14·7 ergs/mm^2/sec. was used. Because of supply difficulties 'Oxoid' Agar No. 3 had to be used for some experiments in place of the usual 'Oxoid' Ionagar No. 2.

Abbreviations used: *adn*$^-$ = adenine-requiring; *adn*$^+$ = adenine-independent; Adn = adenine, routinely used at 50 μg./ml.; Met = L-methionine, routinely used at 40 μg./ml.; MMA = minimal medium agar; YEA = yeast extract agar; GSVB = Giese's salts vitamins buffer; h^+ and h^- are the two heterothallic mating-types of *Schizosaccharomyces pombe*.

RESULTS

The earlier experiments revealed the effects of L-methionine on the phenotypic expression of *adn*$^+$ reversions, on the residual divisions of *adn*$^-$ organisms on adenine-deficient medium, on the differential loss of viability of *adn*$^-$ organisms on minimal medium with and without methionine, and on the result of delayed addition of methionine to minimal medium plates bearing mutagen-treated *adn*$^-$ organisms.

The unique activity of L-*methionine.* In the earlier experiments the methionine

had been sterilized, often in minimal medium, by autoclaving (121°, 16 min.). Experiments in which filter-sterilized methionine was added to MMA plates showed that a product formed from methionine during autoclaving was not responsible for the inhibitory action on revertant expression. Experiments in which filter-sterilized L-, DL-, and D-methionine, DL-homocysteine, L-cysteine, DL-cystathionine, mercaptoethylamine (cysteamine) and choline were tested separately showed that the inhibition of *adn*$^+$ mutation expression was obtained uniquely with L-methionine. DL-methionine (at 80 μg./ml.) had the same action as L-methionine at 40 μg./ml. Experiments in which the pools of amino acids, purines and pyrimidines used by Lederberg (1950) were added to MMA gave no indication that any other of these growth factors could, at comparable concentrations, mimic the inhibitory action of L-methionine on *adn*$^+$ revertant expression.

The influence of L-*methionine concentration on the inhibition of revertant expression.* Figure 1 shows the result of experiments in which nitrous acid-treated organisms of mutant *adn*-1, 25 were plated on MMA containing graded concentrations of L-methionine in the range 1·25–80 μg./ml. Concentrations of 20μ g./ml. and above exerted maximal inhibitory activity on *adn*$^+$ revertant expression; concentrations of 1·25 and 2·5 μg./ml. gave no definite inhibition; concentrations between 5 and 20 μg./ml. gave intermediate degrees of inhibition.

The time course of appearance of adn$^+$ *revertant colonies in the presence and absence of* L-*methionine. Adn*$^+$ revertant colonies were scored routinely after incubation for 7 days at 30°. Figure 2 shows the time course of appearance of *adn*$^+$ revertants of mutant *adn*-1, 25 induced by HNO_2 treatment, on minimal medium with and without methionine. On minimal medium most of the *adn*$^+$ revertants formed colonies which could be scored after 7 days at 30°, and only a few additional revertants appeared when incubation was extended to 15 days. In the experiment shown in Fig. 2, no further *adn*$^+$ colonies appeared on MMA during the second week of incubation.

On minimal medium + L-methionine (40 μg./ml.), however, there was a slow continual rise in the number of revertant colonies which appeared on the plates over the 2-week period. Incubation of the plates for longer periods led to the appearance of very many minute colonies, some of which were revertants with an extremely slow growth rate, and others of which resulted from *adn*$^-$ organisms able to grow into minute colonies on the adenine released by dead organisms or *adn*$^+$ revertant colonies.

The transient nature of methionine inhibitory activity. Experiments were made in which *adn*$^-$ mutants were treated with HNO_2 and then spread on cellophan disks placed on the surface of methionine-supplemented minimal medium plates. In this way it was possible to study the effect of temporary incubation in the presence of methionine, followed by continued incubation on methionine-free minimal medium. Control experiments with a methionine auxotroph showed that there was no detectable carry-over of methionine by the cellophan disks. Figure 3 shows the results of two such delayed methionine removal experiments, with HNO_2-treated mutant *adn*-1, 25. There was a very slow loss of potential revertants, expressed on subsequent transfer and incubation on minimal medium, over extended periods of incubation in the presence of methionine. This was most probably a consequence of the loss of viability of *adn*$^-$ organisms on methionine-supplemented minimal

medium (Clarke, 1963). Otherwise potential *adn*$^+$ revertants were not permanently inhibited in their subsequent phenotypic expression by transient exposure to methionine.

The influence of pre-treatment growth conditions. Organisms for *adn*$^-$ → *adn*$^+$ reverse mutation experiments were grown routinely in aerated liquid yeast extract medium + adenine (YE + Adn). It was thus of interest to see whether methionine inhibition of *adn*$^+$ phenotypic expression could be modified or abolished by growing *adn*$^-$ organisms under different conditions before mutagen treatment. Mutant *adn*-1, 25 was grown in liquid aerated media of the following types: minimal +

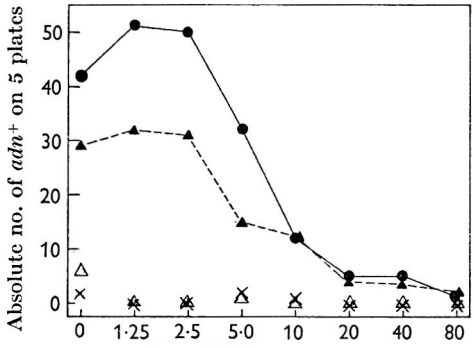

Fig. 1

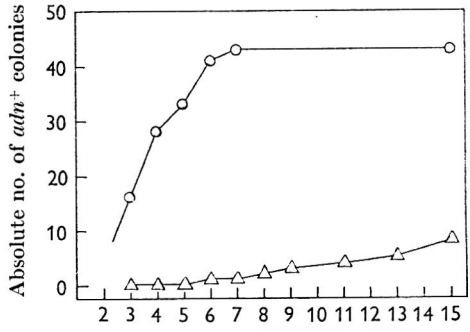

Fig. 2

Fig. 1. The influence of *L*-methionine concentration on inhibition of phenotypic expression of HNO_2-induced revertants of *adn*-1, 25 mutant of *Schizosaccharomyces pombe*. Experiment A: × = untreated controls; ● = HNO_2-treated samples (76·6 % survival). Experiment B: ▲ = controls; △ = HNO_2-treated series (95 % survival).

Fig. 2. Time course of appearance of revertant colonies of HNO_2-treated *adn*-1, 25 mutant of *Schizosaccharomyces pombe* (90·5 % survival). ○ = minimal medium; △ = minimal + L-methionine (40 μg./ml.).

adenine, minimal + hypoxanthine, and minimal + inosinic acid (di-sodium salt). In all cases methionine inhibition of *adn*$^+$ expression occurred, regardless of how the *adn*$^-$ organisms had been grown before nitrous acid treatment.

The influence of pre-treatment starvation. Adenine-1 mutants 25 and 51 were grown as usual in liquid aerated YE + Adn medium, harvested by centrifugation and incubated for 24 hr at 30° in aerated liquid media deficient in a nitrogen source (0·1 %, w/v, KH_2PO_4, glucose, vitamins, adenine), or deficient in adenine but containing amino acids (minimal medium + casein hydrolysate), or deficient in adenine (minimal medium). Organisms pre-incubated under these three sets of conditions were then tested for methionine inhibition of the expression of *adn*$^+$ revertants induced by HNO_2 treatment. In no case was there found an abolition or obvious diminution of the methionine effect. However, *adn*$^-$ organisms pre-incubated in liquid minimal medium appeared to be much more sensitive to the lethal action of nitrous acid than did organisms not so pre-starved.

The influence of trace adenine on the methionine effect. Samples of agars from different sources contain different concentrations of contaminating adenine (Dr M. Luzzati, personal communication), and thus allow different degrees of residual

growth of *adn*⁻ organisms when used for the preparation of minimal medium. Experiments were made in which *adn*⁻ organisms were treated with HNO_2, then plated for *adn*⁺ reversions on minimal medium and on minimal medium + methionine, and containing 1·6 % (w/v) of different specimens of agar. Those used were 'Difco' Purified Agar, 'Difco' Bacto Agar, 'Oxoid' Lonagar No. 2, and 'Oxoid' Agar No. 3. The first-named of these agars contains very little adenine indeed (Dr M. Luzzati, personal communication) while the last-named gives a final concentration of the order of 0·1 μg. adenine/ml. when used in the preparation of minimal medium. The use of these different agars did not affect the degree of

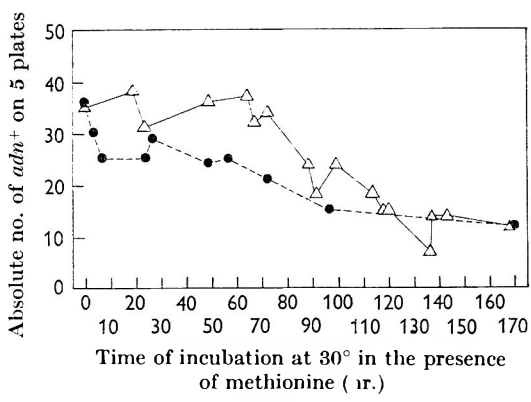

Fig. 3

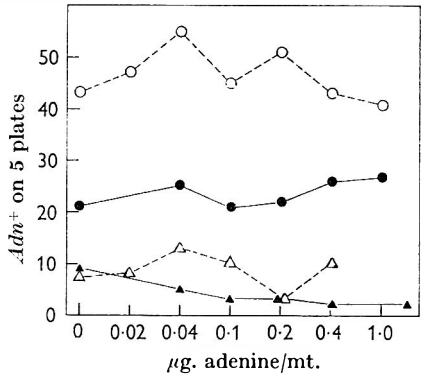

Fig. 4

Fig. 3. Delayed methionine removal experiments with HNO_2-treated *adn*-1, 25 mutant of *Schizosaccharomyces pombe*. △ = experiment A; ● = experiment B.

Fig. 4. The influence of low concentration adenine supplementation of minimal medium ± methionine on the expression of HNO_2-induced *adn*-1 revertants of *Schizosaccharomyces pombe*. ○ = minimal medium agar (MMA), mutant *adn*-1, 25; ● = MMA, mutant *adn*-1, 51; △ = MMA + methionine (Met), mutant *adn*-1, 25; ▲ = MMA + Met, mutant *adn*-1, 51. Mutant *adn*-1, 25, 32·6 % survival; mutant *adn*-1, 51, 21·1 % survival.

methionine inhibition detectably, even when pre-starvation for adenine (see above) was combined with plating on minimal medium, with and without methionine, containing the very pure 'Difco' Purified Agar.

Figure 4 shows the influence of low degrees of adenine supplementation of minimal medium, with and without methionine, upon the yield of *adn*⁺ revertants obtained on treating mutants *adn*-1, 25 and *adn*-1, 51 with HNO_2. Within the range used (0·02–1·0 μg. adenine/ml.) there was no noticeable influence of trace adenine upon the expression of HNO_2-induced revertants. It is not possible to work much beyond the upper value of adenine supplementation used here because of the increasingly heavy background growth of the unreverted *adn*⁻ organisms.

Temporary incubation on high concentration adenine medium. Experiments were made in which *adn*⁻ organisms, after treatment with HNO_2, were spread on cellophan disks and incubated on minimal medium + adenine, at 50 μg./ml. After known periods of incubation sets of membranes were transferred to minimal medium or minimal medium + methionine plates. The usable period of incubation on adenine was limited by the development of heavy background growth of the *adn*⁻ organisms. In all experiments the methionine effect on revertant expression was not abolished

or obviously decreased by periods of post-treatment incubation on high-concentration adenine medium up to 10 hr at 30°.

Use of a temperature-sensitive auxotroph. Mutant *adn*-1, 199 is temperature sensitive, being *adn*$^-$ at 30° but *adn*$^+$ at 25°. Organisms of this mutant were treated with HNO_2 or ultraviolet radiation and then incubated on minimal medium at 25° for limited periods. In this way it was hoped to provide conditions for temporary intracellular production of adenine, rather than mere extracellular supply as in the experiments described above. The effect of this temporary incubation at 25° was tested upon subsequent expression of *adn*$^+$ reversions expressed at 30° in the absence and presence of methionine. Methionine inhibition, weak in the case of HNO_2-induced *adn*$^+$, strong for ultraviolet-induced revertants, was not abolished by incubation up to 11 hr at 25°.

Viable count assays on low-concentration adenine medium. It had been shown earlier (Clarke, 1963) that addition of methionine did not affect estimates of viable counts made with untreated or mutagen-treated organisms when these were plated on minimal media supplemented with adenine 50 μg./ml. Because low degrees of adenine supplementation did not abolish the effect of methionine on *adn*$^+$ expression, it was decided to test the effect of methionine upon viability as assayed at low concentrations of adenine. Organisms of an *adn*$^-$ mutant were treated with HNO_2 and colony counts made on minimal medium + adenine 0·5 μg./ml., in the presence and absence of methionine. The colonies, which were very small, were scored after 7 days at 30°; the counts were the same on both media. These counts were of necessity made at much lower plating densities than was used for estimates of *adn*$^+$ revertants. On the reversion plates the amount of adenine available for each *adn*$^-$ organism was less by a factor of about 10^5 than was the case on the viability plates.

Growth rate of adn$^+$ *revertants.* Samples of 10^{-5} dilution of a culture of two HNO_2-induced revertants of mutant *adn*-1, 51 were sprayed at daily intervals on the surface of minimal medium, and minimal medium + methionine plates, bearing a background of HNO_2-treated *adn*-1, 51. The added *adn*$^+$ organisms formed colonies, on the methionine-free and the methionine-containing minimal medium, which were identical in size after 7 days at 30°. Earlier examination of the plates showed that colonies formed by the added *adn*$^+$ organisms reached full size about 1 day earlier on minimal medium than on minimal medium + methionine. Thus there was a weak inhibition of the growth of *adn*$^+$ organisms but this was certainly not the explanation for the methionine effect on revertant expression.

Influence of methionine on other reverse mutations. In addition to the mutants for which methionine inhibition of revertant expression has been reported earlier (Clarke, 1963), Table 1 and Table 2 show other reverse mutation systems which were tested. Methionine inhibition seems to be general for *adn*$^-$ → *adn*$^+$ reversions, and there seems to be an effect on at least those HNO_2-induced revertants of leucine auxotroph *leu*-3, 241 which formed scorable colonies after incubation for 7 days. Methionine had no obvious inhibitory effect on the expression of HNO_2-induced reversions of the following arginine and lysine auxotrophs: *arg*-D14, *arg*-G2, *lys*-3I, *lys*-7A, *lys*-4B, *lys*-6B, and *lys*-6, 2. The results with di-auxotrophic *adn*-1, 25.*lys*-7A and *adn*-1, 25.*arg*-G2 strains show that methionine inhibition was definitely restricted to the *adn*$^+$ reversions.

Table 1. *Methionine inhibition of revertant expression in Schizosaccharomyces pombe*

		Reversions scored after 7 days at 30° on							
		Minimal medium agar				Minimal medium agar + methionine			
S. pombe mutant	Mutagen	No. of estimates	Mean survival (%)	Actual no.	Reversions/ 10^7 viable survivors	No. of estimates	Mean survival (%)	Actual no.	Reversions/ 10^7 viable survivors
adn-1, 25	Spontaneous	18	—	122	0·79	17	—	21	0·24
adn-1, 51	HNO_2	32	53	1678	25·1	26	47·3	547	8·06
adn-6, D2	HNO_2	14	53·7	150	3·13	14	53·7	49	0·88
adn-8, 321	HNO_2	12	52·2	176	17·6	12	52·2	109	11·5
leu-3, 241	HNO_2	12	78·8	147	2·99	12	78·8	13	0·14

Table 2. *Methionine inhibition of HNO_2-induced adn$^+$ revertant expression in Schizosaccharomyces pombe*

	Mutants											
	adn-2, SP5		*adn*-7, 84		*adn*-8, 177		*adn*-8, 313		*adn*-8, 333		*adn*-1, 199	
	Media											
Time of HNO_2 treatment (min.)	MMA*	Met†	MMA	Met	MMA	Met	MMA	Met	MMA	Met	MMA	Met
	No. revertants											
0 (control)	30	23	23	1	2	0	2	2	1	0	1	0
10	93	69	26	18	24	16	6	6	26	9	—	—
20	134	100	40	18	38	11	5	5	42	17	—	—
30	176	148‡	57	21	52	24	12	6	35	17	731	357
40	231	195	66	29	61	32	10	7	22‡	4	—	—
50	265	222	89	49	55	30	11	5	7	6	—	—
60	345	276	106	50	48	18	25	5	5	2	—	—

Mutagenic treatment with 0·05 or 0·1 M-$NaNO_2$ in Giese's salts vitamins buffer (GSVB) at 25°. Absolute numbers of revertants on sets of 5 or 10 minimal (MMA) and minimal + methionine (Met) plates.

* MMA = minimal medium agar; † Met = minimal medium agar + methionine; ‡ no. estimated from colonies on incomplete sets of plates.

Forward mutation systems. Nitrous acid-induced mutations of the wild-type of *Schizosaccharomyces pombe* from sensitivity to resistance to 5-fluorouracil 50 μg./ml. were not affected by the addition of methionine to the minimal medium + 5-fluorouracil upon which these mutations were scored. Forward mutations at the *adn*-1, -3, -4, -5 and perhaps also other loci (Heslot, 1960; Clarke & Anwar Nasim, 1964) can be scored as white colonies or sectors among the red colonies of an unmutated *adn*-7 strain, on a medium containing limiting adenine. Mutant *adn*-7, 407, which has a very low spontaneous and HNO_2-induced rate of reversion to *adn*$^+$, was treated with HNO_2 and plated on minimal medium containing adenine at 20 μg./ml., with or without methionine. The results obtained indicated no inhibition of the expression of induced *adn*$^-$ forward mutations, though methionine did intensify the red colour of the *adn*-7 colonies.

Leakiness. Mutant *adn*-7, 486 is leaky when incubated on minimal medium at 30°, so that a heavy film of background growth is formed. Supplementation of the minimal medium with methionine resulted in a drastic inhibition of this background, as shown in Pl. 1, fig. 1.

6-*Methylaminopurine.* Certain double adenine mutants of the type *adn*-7, *adn*-1, -3, -4, -5 or -9 can grow slowly on 6-methylaminopurine as an alternative to adenine though the *adn*-7 mutant alone cannot do so (Dr H. Heslot, personal communication). Plating a large number of *adn*-7 organisms on minimal medium containing 6-methylaminopurine thus selected at least some of the spontaneous double *adn*-7, *adn*$^-$ mutants present in the population. Minute white, pink and red colonies appeared after 10–15 days of incubation at 30°. Addition of methionine to the medium prevented the appearance of most of these colonies, and the growth of most isolated *adn*-7, *adn*$^-$ strains on 6-methylaminopurine. This effect is shown in Pl. 1, fig. 2.

Complementation. Plating of large numbers of ascospores from a cross of the type *adn*-1, *a*, $h^+ \times$ *adn*-1, *b*, h^-, between two non-identical *adn*-1 mutants which complement, results in the growth on minimal medium of *adn*$^+$ colonies which are of two types. There are *adn*$^+$ wild-type recombinants (Leupold, 1957) and also complementing diploids and aneuploids, many of which are unstable and undergo meiosis when heterozygous for mating-type (Professor U. Leupold, personal communication). Plating on minimal medium + methionine can, at least in some cases, result in a marked inhibition of the growth of the *adn*$^+$ complementing colonies, though not of the *adn*$^+$ recombinants. This effect is shown in Pl. 2.

Reverse mutations in bacteria. Salmonella typhimurium auxotroph *ath*-A 2 (Yura, 1956) was derived, by transduction with phage PLT 22 grown on wild-type bacteria, from the di-auxotrophic *met*-F 31, *ath*-A 2 (kindly supplied by Dr S. W. Glover). Mutant *ath*-A 2 has a simultaneous requirement for adenine and thiamine. Ultraviolet irradiation of this mutant induces *ath*$^+$ revertants (Yura, 1956). In the present experiments *S. typhimurium ath*-A 2 organisms, grown in aerated nutrient broth, were irradiated in saline with doses of ultraviolet radiation of about 140, 290, and 540 ergs/mm^2, and plated for *ath*$^+$ revertants. Four media were used: minimal medium A (Lederberg, 1950) with and without methionine, and with and without a supplement of 1 % (v/v) nutrient broth. *Ath*$^+$ revertants were scored after 3 days at 30°. There was a very strong effect of the broth supplement in increasing the revertant yield (Witkin, 1956), but no methionine inhibition was seen.

DISCUSSION

The action of L-methionine in inhibiting the phenotypic expression of newly arising *adn*$^+$ reverse mutations in *Schizosaccharomyces pombe* appears to be restricted to L-methionine, and mainly to *adn*$^+$ reversions. An apparent inhibition of HNO_2-induced reversions of the leucine auxotroph *leu*-3, 241 requires explanation and further study. It is possible that there is an effect of methionine upon the viability of *leu*$^-$ organisms which have been treated with nitrous acid; this inhibition applies only to those *leu*$^+$ reversions scored after 7-day incubation. Many of the induced *leu*$^+$ reversions appeared later on the minimal agar plates (up to 2 weeks of incubation) and were most probably of a suppressor type, like the slowly growing revertants studied in the *S. pombe arg*-1, 230 system by Heslot (1962). The *leu*$^-$ mutant has a very high leucine requirement for full growth on minimal medium, and until the biochemical genetics of the *leu*$^+$ revertants are studied it would be premature to suggest possible mechanisms by which methionine could inhibit *leu*$^+$ expression or growth rate.

That methionine has inhibitory activity on leakiness and complementation of adenine auxotrophs and on their growth on a purine analogue indicates that methionine probably affects a biochemical step common to several metabolic processes. An underlying and unifying biochemical mechanism for the varied effects of methionine described here and previously (Clarke, 1963) may lie in the formation of S-adenosylmethionine (Gawel, Turner & Parks, 1962). In a turnip slice system Dr D. D. Davies (1964, and personal communication) found that methionine inhibited respiration, and that its action was annulled by adenine. Formation of S-adenosylmethionine, from ATP and methionine, which can accumulate in the vacuoles of plant and yeast cells, might well lead to a low availability of ADP and ATP in the cell. In this way a 'resting cell' condition might be produced, in which such processes as synthesis of new enzymes in newly mutated or complementing diploid cells or residual cell divisions would not occur. S-adenosylmethionine does not accumulate in bacteria (Gawel *et al.* 1962) so that the absence of an inhibitory effect on *ath*$^+$ reversions in *Salmonella typhimurium* might be expected. One would expect the inhibitory activities of L-methionine in *Schizosaccharomyces pombe* to be counteracted by high concentrations of adenine, but this is, of course, impossible to test in an *adn*$^- \rightarrow$ *adn*$^+$ reverse mutation system.

The experiments described in this paper show that the methionine inhibition of *adn*$^+$ expression in *Schizosaccharomyces pombe* is not readily modified by a variety of adenine removal or addition treatments applied before or after mutagen treatment. The experiments in which methionine was removed from populations of HNO_2-treated organisms incubated on minimal medium showed that there was no critical period for methionine action. Potential *adn*$^+$ revertants remained expressible on subsequent incubation in the absence of methionine, even after quite long periods on methionine-containing medium. High concentrations of methionine, of the order of 400 μg./ml., have been shown by Dr M. Luzzati (personal communication) to simulate the effect of low concentrations of histidine in inhibiting the appearance of *adn*$^+$ *his*$^+$ revertants, formed by a gene conversion process, in heteroallelic diploid *adn*-3 (*adn*$^-$*his*$^-$) strains of *Saccharomyces cerevisiae* (Clavilier *et al.* 1960). It has been shown in the present work that methionine inhibition of *adn*$^+$

revertant expression in *S. pombe* is not restricted either to those reversions due to repair at the original adenine locus, or to suppressor mutations. Both types of revertants occur (Clarke, unpublished results) and methionine inhibits the phenotypic expression of each. It is possible that adenine might under certain conditions inhibit the expression of *met*$^+$ reversions. No evidence has been found for this up till now, in *adn*$^-$*met*$^-$ diauxotrophic strains, but the use of a monoauxotrophic *met*$^-$ strain which is completely blocked, together with tests of revertants induced by a variety of mutagens, would allow this question to be investigated.

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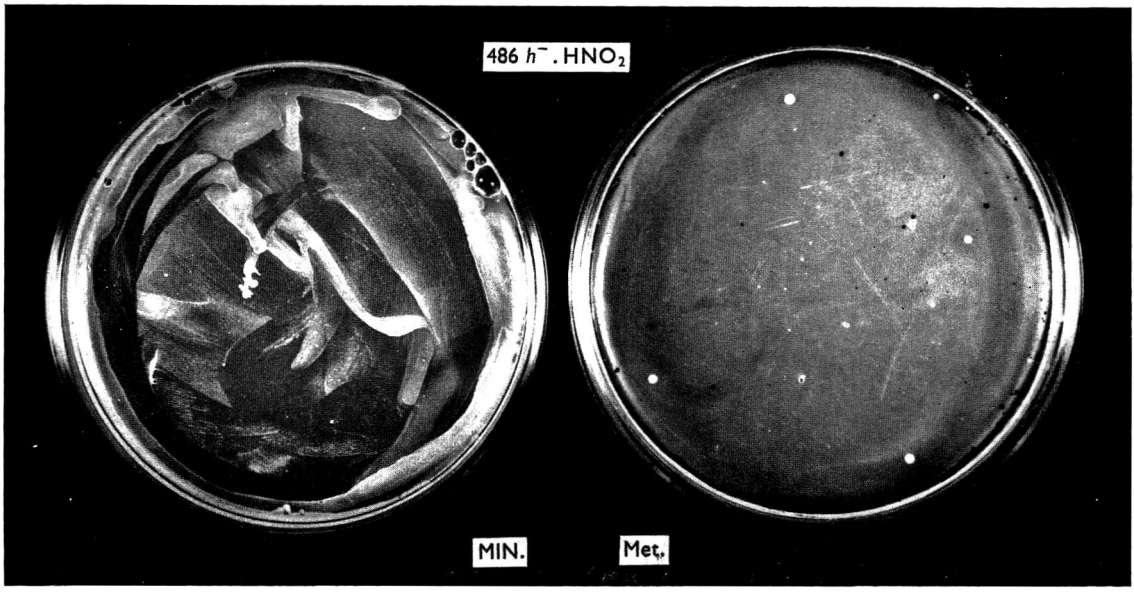


Fig. 1

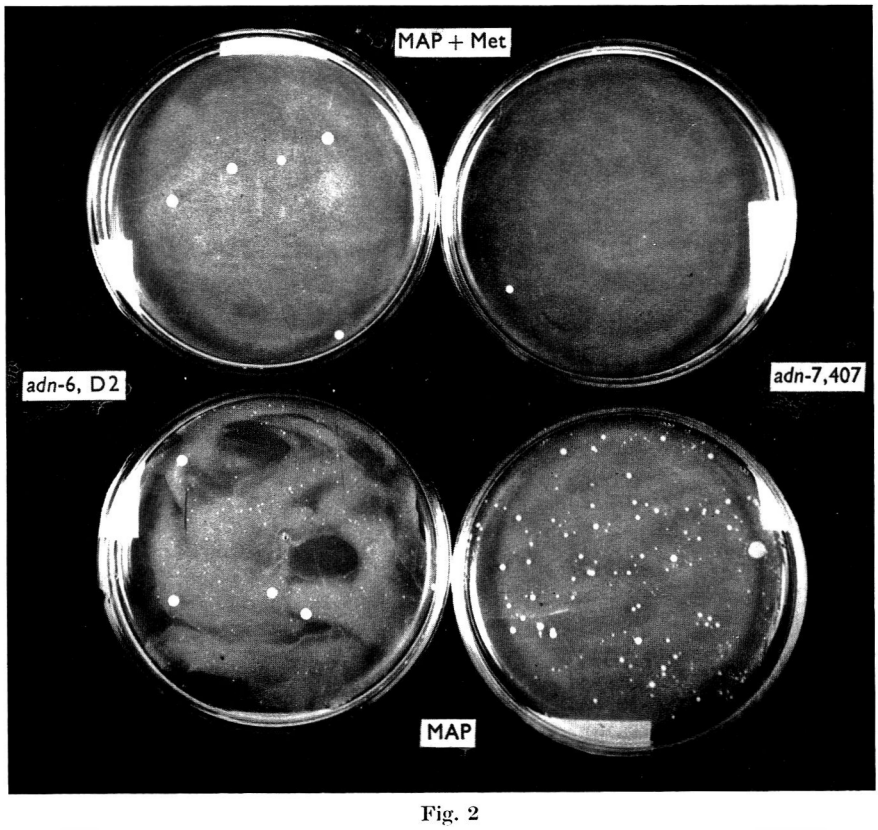
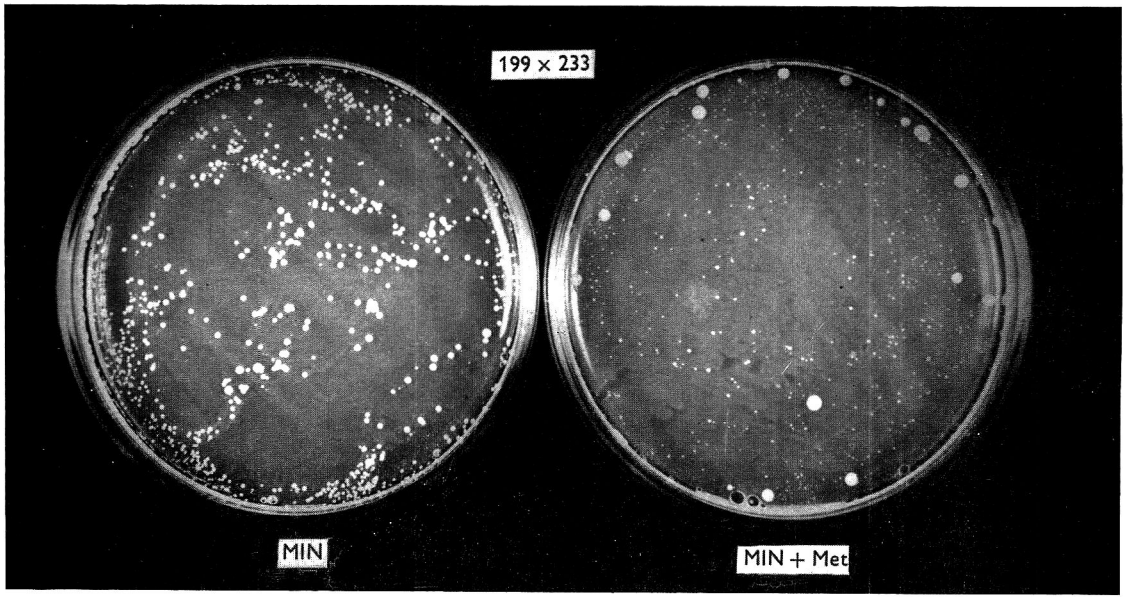


Fig. 2


199 × 233
MIN
MIN + Met

EXPLANATION OF PLATES

PLATE 1

Fig. 1. Residual growth of HNO_2-treated organisms of *Schizosaccharomyces pombe* mutant *adn*-7,486 h^- on minimal medium (MIN) and minimal + methionine (Met). Heavy film of residual growth on the minimal medium; very light background growth with several *adn*$^+$ revertant colonies on the minimal + methionine medium.

Fig. 2. Methionine inhibition of the growth of adenine double mutants of *S. pombe* on 6-methylaminopurine. The two plates on the left have been spread with organisms of mutant *adn*-6, D2; those on the right with mutant *adn*-7, 407. The two lower plates contain minimal medium + 6-methylaminopurine (MAP); the upper two plates minimal medium + 6-methylaminopurine + methionine (MAP + Met). On the MAP plates colonies of *adn*$^+$ revertants and smaller colonies of *adn*$^-$ *adn*$^-$ double mutants are visible; on the MAP + Met plates only *adn*$^+$ revertant colonies are visible.

PLATE 2

Methionine inhibition of complementation between two *adn*-1 mutants of *S. pombe*. Large numbers of ascospores from the cross *adn*-1, 199 × *adn*-1, 233 were plated on minimal medium (MIN) and on minimal + methionine (MIN + Met). On the minimal medium colonies of *adn*$^+$ recombinants and of complementing diploids and aneuploids cannot be distinguished by size. On the minimal medium + methionine *adn*$^+$ recombinants form larger colonies; the complementing diploids and aneuploids form small colonies.

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The Effect of Temperature on the Production of Perithecia by *Neurospora crassa*

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SUMMARY

The wild-type strains of *Neurospora crassa* first isolated by Dodge, Abbott & Chilton were found to differ in the range of temperatures over which protoperithecia were produced; similar results were obtained with two recently isolated wild strains of a species of Neurospora. The ability to develop protoperithecia at 30° was found to be controlled by at least two genes in reciprocal crosses between two wild types differing in this character. A correlation of these observations with the reported features of tyrosinase production is discussed. Certain mutant strains backcrossed repeatedly to a wild-type strain gave exceptional results at 30°. Preliminary observations suggest that the part played by nicotinamide in the sexual cycle may be connected with some of these exceptions. Temperature did not obviously affect ascus development in outbred crosses between Lindegren and Abbott wild-type strains. With inbred crosses ascus development was controlled by several factors which were temperature sensitive and differed in degree of effect in reciprocal crosses. In most strains tested 20° was the optimum temperature for normal ascus development. At higher temperatures gross abnormalities were observed including asci with more than eight spores.

INTRODUCTION

During studies on the effect of temperature on sexual reproduction in *Neurospora crassa*, the production of fertile perithecia at the extremes of temperature at which sexual reproduction will occur was found to vary with the strain used as maternal parent. Evidence indicating that the original wild-type strains involved in the ancestry of most mutant strains were the source of this variability prompted an examination of these wild-type isolates for ability to produce fertile perithecia over a range of temperatures. The production of fertile perithecia may be divided into three stages. (1) Differentiation to form protoperithecia. The formation of protoperithecia in a number of wild-type isolates was studied in the present work. Two recently isolated strains of a Neurospora species were included to determine whether the observed characters might have been present in these wild-type strains when originally isolated or are a result of continuous laboratory culture. An examination of the inheritance of the observed character variation was undertaken and the effect of certain mutations on the expression of this character studied. (2) Fertilization. Observations throughout the present work indicated that the process of

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fertilization was not affected by temperature. (3) Subsequent development, involving meiosis and the delimitation and maturation of ascospores. These stages were studied over the temperature range 15–30° in crosses between a Lindegren and an Abbott wild-type strain (equivalent to outbreeding) and between two Lindegren or two Abbott wild-type strains (equivalent to inbreeding).

METHODS

In all the crosses listed below, the first-named strain was used as protoperithecial parent and is considered to have provided the main source of cytoplasmic inheritance (Srb, 1958). The following strains were used. *Neurospora crassa*: Lindegren 1*A* (L1*A*), wild type; Lindegren 25*a* (L25*a*), wild type; Lindegren 1*a* (L1*a*), a reisolate from the cross L1*A* × L25*a* backcrossed to L1*A* six times and then selfed twice; Abbott 4*A* (A4*A*), wild type; Abbott 12*a* (A12*a*), wild type; Abbott 4*a* (A4*a*), a reisolate from the cross A4*A* × L25*a* backcrossed to A4*A* six times. The derived strains L1*a* and A4*a* resembled closely the strains to which they had been backcrossed and were used as opposite mating-type of those strains. Mutant strains of *N. crassa*: *cr* (F945), crisp, morphological mutant; *lys-3* (4545), lysine requiring; *nic-1* (3416), nicotinamide requiring. Other Neurospora species: Costa Rica CU5, Costa Rica CU8, single ascospore isolates of opposite mating-type from material recently isolated from the bark of a *Musa* cultivar. Taxonomic status not yet verified.

Stock cultures grown on bactopeptone medium (Frost, 1962) and stored at 4° were used as the source of conidial inoculum. Crosses and tests for protoperithecial production were made on minimal reproductive medium (Westergaard & Mitchell, 1947) adjusted to pH 6·7 and dried for 5 days at about 18°. This drying resulted in more consistent protoperithecial production with all the strains used. The protoperithecial parent was grown for 7 days at the required temperature before the fertilizing strain was added as a suspension of conidia in sterile water; the culture was then returned to the same temperature.

The testing of progeny for protoperithecial production was done by using a standardized procedure of medium preparation and inoculation. Protoperithecial strains of one mating-type in each experiment were fertilized with samples of the same conidial suspension.

The production of protoperithecia was estimated by looking at the cultures immediately before the addition of the fertilizing strain and then recording the presence of perithecia 1–4 days later. This allowed, to a considerable extent, the detection of protoperithecia which were embedded in hyphal masses or in the medium, or which were not fertilized although produced with a low or a fair frequency.

Ascus structure was examined in fresh material mounted in 2 M-sucrose solution. This solution made manipulation easier and prevented excessive bursting of mature asci which occurred in water alone.

RESULTS

The effect of temperature on protoperithecial production

Under the conditions of medium and culture used, the wild-type strains showed a marked difference in the range of temperatures over which protoperithecia were produced (Table 1). The Costa Rican strains also showed this variation, suggesting

that the differences observed between the Lindegren and Abbott strains had not arisen during continuous vegetative culture. The observations made on the Lindegren and Abbott strains were confirmed in several experiments and were made from crosses known to be very fertile at 25° and to produce a high frequency of eight-spored asci. The crosses were duplicated and at temperatures borderline for sexual reproduction up to four replicates were made.

Table 1. *The range of temperatures over which protoperithecia were produced by several wild-type strains of Neurospora*

	Temperature					
	10°	15°	20°	25°	30°	35°
Wild-type strain	Protoperithecial production					
Lindegren 1*A*	−	+	+	+	+	−
Lindegren 25*a*	−	+	+	+	+	−
Abbott 4*A*	−	+	+	+	−	−
Abbott 12*a*	−	−	(+)	+	+	−
Chilton *a*	nt	+	+	+	+	−
Costa Rica CU5	nt	+	+	+	+	−
Costa Rica CU8	nt	−	−	+	+	−

+, Protoperithecia produced; −, no protoperithecia produced; (+), abortive protoperithecia occasionally produced; nt, not tested.

More detailed observations were made on the Lindegren and Abbott crosses, including cytological examination of ascogenous hyphae and developing asci in material fixed at intervals after conidiation and stained with acetocarmine. Asci were found to develop with time in three (sometimes four) waves with the oldest asci at the periphery. The results are given in Table 2. Observations of L25*a* ♀, conidiated by A4*A*, gave results similar to L1*A* × A12*a* (Table 2), except that the rates of development of protoperithecia, perithecia, asci and spores were about 1 day longer than in L1*A*. In both sets of these Lindegren crosses, broad asci with eight overlapping spores, tending to be two-ranked in arrangement, were observed only with continuous incubation at 30° (Pl. 1, fig. 2). In all four strains, protoperithecial formation at 25° transferred to 30° after conidiation gave similar results to continuous incubation at 25°. Throughout these crosses at all temperatures at which asci with spores were produced, the frequency of abortive or abnormal asci was nil or very low with spore abortion varying from 1 to 5% (cf. Table 4).

Other characteristics were observed to differ between the Abbott and Lindegren strains. Linear growth rate measurements (made by the method of Ryan, Beadle & Tatum, 1943) indicated a more rapid conidial germination and higher growth rate for the Abbott strains, particularly A4*A*, than for the Lindegren strains. A characteristic, perhaps associated with a higher metabolic rate in A4*A*, was the more rapid pigmentation of two of the four spore pairs in each ascus in crosses involving A4*A* as either the maternal or paternal parent (Pl. 1, fig 3). This character was rather difficult to score owing to its transient nature, and the pattern of segregation in each ascus was not always visually obvious. Nevertheless, it appeared from the segregation patterns in clearly scorable asci that a single gene might control this character. Of 225 asci scored from the cross A4*A* × L1*a*, 63 showed second division

Table 2. *The effect of temperature on the production of protoperithecia, perithecia, asci and ascospores in certain Neurospora wild-type strains*

Cross ♀ × ♂	Temperature: 10°	15°	20°	25°	30°
	Production of protoperithecia, perithecia, asci and ascospores				
L1*A* (× A12*a*)	Loose hyphal clumps; no perithecia develop	Protoperithecia form in 6 days, very numerous and large, normal in colour, many sterile; ascogenous hyphae develop in 4 days; asci slow to mature; spore discharge normal	Protoperithecia form in 3 days, numerous, normal in size, pale in colour, some sterile; ascogenous hyphae develop in 5–6 days; spores not discharged	Protoperithecia form in 3 days, less numerous, normal in size and colour, few sterile; spores in 1st wave of asci delimited in 5 days, spore discharge normal	Protoperithecia form in 6 days, fewer in number, normal in size and colour; fewer perithecia develop rapidly, necks short; fewer asci; spores mature rapidly, normal discharge
A4*A* (× L25*a*)	Loose hyphal clumps; no perithecia develop	Protoperithecia form in 5 days, very numerous and large, normal in colour, many sterile; perithecia and asci less slow to mature; spore discharge normal	Protoperithecia form in 2 to 3 days, numerous, normal in size and colour, some sterile; perithecia and asci develop rapidly; spore discharge normal and early	Protoperithecia form in 2 to 3 days, fewer in number, normal in size and colour; spores in 1st and 2nd waves of asci delimited in 5 days, spore discharge normal and early	Loose hyphal clumps or protoperithecia not produced
A12*a* (× L1*A*)	Very loose hyphal clumps; no perithecia develop	Loose hyphal clumps; no perithecia develop	Protoperithecia numerous, small, normal in colour; perithecia and aseogenous hyphae develop, abort, no spores formed	Protoperithecia numerous, normal in size and colour; rate of development intermediate between L1*A* and A4*A*; spore discharge normal	Protoperithecia fairly numerous, large; a few perithecia devoid of spores; spores mature rapidly, normal discharge

segregation for this spore-maturing factor giving a centromere distance of 14·0. A very low frequency of asci with 5:3, 6:2 or 7:1 ratios were also observed.

Inheritance of the ability to form protoperithecia at 30°

The progeny of reciprocal crosses between L25*a* and A4*A* were analysed for the ability to produce protoperithecia at 30°. Parallel crosses were then made between a single isolate, 44*a* (from the cross L25*a* × A4*A*), which produced abundant protoperithecia at 30°, and either L1*A* or A4*A*, the latter strains acting as protoperithecial parent. The progeny of these crosses were tested for the ability to produce protoperithecia at 30°. Three replicates were made at 30° and one in the controls at 25°. A few strains which showed a marked decrease in protoperithecial production at 25° were rejected from the analysis; the numbers rejected are given in Table 3.

Subsequent to the addition of the fertilizing strain, an assessment of perithecial frequency was made by scoring on an arbitrary scale: 3, 2, 1, rare, very rare, absent; from this an indirect assessment of protoperithecial frequency was made.

Two features emerged showing good agreement between replicates. First, the progeny may be divided primarily into two classes: protoperithecia present or protoperithecia absent. Secondly, among strains in which protoperithecia were produced there was a less distinct subdivision into the presence of a high frequency or a low frequency of perithecia. The results are presented in this form in Table 3.

The results obtained from the progeny of the reciprocal crosses between L25*a* and A4*A* showed marked agreement, indicating the absence of a cytoplasmic effect. A good fit to a 3:1 ratio between the two classes, perithecia present to perithecia absent, was observed although the sample sizes were small (Table 3).

In early experiments to test the progeny of these reciprocal crosses, L1*A* and L25*a* were used as the fertilizing strains since they were known to give fertile crosses even when inbred. However, in some cases with these strains there was the possibility of overgrowth and subsequent protoperithecial production by the conidiating parent so that young perithecia, although absent at 4 days, appeared 7 days after addition of the fertilizing parent. To prevent this, A4*A* and A4*a* were used as the fertilizing strains in subsequent experiments since these strains do not form protoperithecia at 30°. However, as suggested by Barbesgaard & Wagner (1959), a bisexual heterocaryon may have formed after conidiation in which the two component strains were blocked at different stages in sexual differentiation. Complementation in such a heterocaryon would result in an eventual formation of perithecia. The possibility of delayed protoperithecial production by the maternal strains was tested by conidiating two replicates after 7 days and two after 14 days. In no case did cultures in which no perithecia were present in 7 days produce them after 14 days.

The results obtained from the reciprocal crosses between L25*a* and A4*A* suggested that backcrossing a strain which showed a high frequency of protoperithecia at 30° to A4*A* would result in a higher proportion of progeny unable to form protoperithecia at 30° than were able to form them, while the same strain backcrossed to L1*A* would result in the converse. Strain 44*a* (from the cross L25*a* × A4*A*) was used for these backcrosses and one replicate was made at each temperature when testing the progeny. The results from these backcrosses are presented in Table 3

Table 3. *The frequency of perithecial production at* 30° *among the progeny of Neurospora wild-type parents differing in this character*

Wild-type parents crossed at 25°	Number of progeny rejected*	Progeny crossed to tester strains at 30° Perithecia					χ^2 for a 3:1 ratio present: absent	P (D.F. = 1)
		Frequent	Rare	Absent	Present	Absent		
L25*a* × A4*A*	11	33	23	18	56	18	0·018	0·80–0·90
A4*A* × L25*a*	9	14	11	6	25	6	0·527	0·30–0·50
L1*A* × 44*a*†	6	35	20	24	55	24	1·219	0·20–0·30
A4*A* × 44*a*	18	3	0	12	3	12	nt	nt

* Progeny which showed a marked decrease in protoperithecial production at 25° were rejected from the analysis; † strain 44*a* was a single spore isolate from the cross L25*a* × A4*A* and showed a high frequency of protoperithecia at 30°; nt, not tested as the sample size was small.

The predicted trend appears to hold in the ratio of perithecia present to absent obtained.

Strain 44*a* had been chosen because it had produced a high frequency of protoperithecia at 30° in previous experiments. However, when used as a control during the testing of the progeny of the above backcrosses, no protoperithecia were produced at 30°. It was then realized that conidia of 44*a* stored at 4° for 8 months had been used, whereas in all previous experiments conidia stored at 4° only for a short while had been used. Horowitz & Fling (1953) during the investigation of the inheritance of tyrosinase production in *Neurospora crassa* at 35° observed that 'the initial classification of a culture changed when the test was repeated at a later date suggesting that uncontrolled physiological factors are important in the expression of this character in some strains'. The possible correlation between tyrosinase activity and protoperithecial production to be discussed later suggests that the anomalous results from 44*a* may have been due to the operation of similar physiological factors.

The effect of certain mutant genes on the production of protoperithecia at 30°

A series of mutant and non-mutant progeny from the fourth or fifth backcross of three mutant strains to either L1*A* or A4*A* were tested for protoperithecial production at 30°. The mutant strains used were: *cr* (F945), *lys-3* (4545), *nic-1* (3416). In the case of the biochemical mutants, the specific growth requirement was added to the medium for both mutant and non-mutant progeny.

In the case of the crisp strain, both mutant and non-mutant progeny from a sixth backcross of A4*A* showed absence of protoperithecia at 30° as expected. However, from a sixth backcross to L1*A*, while non-mutant progeny (with one exception) produced protoperithecia at 30°, twenty mutant progeny produced protoperithecia in ten cases but none in the other ten cases. After conidiation of the former cultures, small perithecia were produced devoid of ascospores. Similar results were obtained with crisp progeny incubated at 15°.

Non-mutant progeny from backcrosses of the lysine strain behaved as expected with few exceptions. However, none of the mutant progeny from a fifth backcross to either L1*A* or A4*A* would produce protoperithecia at 30° and at this temperature vegetative growth was sparse with the production of a yellowish coloration which rapidly permeated the medium.

In contrast, both mutant and non-mutant progeny of the nicotinic strain from a fifth backcross to L1*A* and a fourth backcross to A4*A* showed high fertility at 30°. It is suggested that the addition of nicotinamide to the medium (at 60 mg./l.) overcame the block in protoperithecial production at 30° present in A4*A*. The effect of nicotinamide on several wild-type strains, including wild strains of *Neurospora sitophila*, was tested; in each case protoperithecial production was enhanced.

The effect of temperature on the production of asci and ascospores in inbred crosses of wild-type strains

In contrast to crosses between an Abbott and a Lindegren strain, crosses of two Abbott or two Lindegren strains showed marked effects of temperature on ascus and ascospore development. The results are presented in Table 4 and illustrated in Pls. 1 and 2.

The crosses of the Abbott strains (Table 4 and Pl. 1) showed a high frequency of abortive asci, asci with less than eight spores and the occurrence of misshapen ascospores at all temperatures tested. Such abnormalities were at a minimum at 20°.

Table 4. *The effect of temperature on the frequency of production of abnormal asci and ascospores in crosses between two Abbott or two Lindegren wild-type strains*

Cross ♀ × ♂	Abnormal condition of asci	Temperature: 15°	20°	25°	25→30°	30°
		Frequency of abnormal asci				
A4*A* × A12*a*	Abortive	a	f	a	a	*
	< 8 black, normal spores	All	All	All	All	*
	Misshapen spores	f	o	f	f	*
A4*A* × A4*a*	Abortive	f	o	f	a	*
	< 8 black, normal spores	f	o	All	All	*
	Misshapen spores	f	r	r	r	*
L1*A* × L25*a*	Abortive	*	o	f	a	va
	< 8 black, normal spores	*	f	f	a	All
	> 8 spores	*	Nil	Nil	f	f
	Broad; overlapping spores	*	Nil	Nil	f	a
	Misshapen spores	*	vr	vr	f	a
L25*a* × L1*A*	Abortive	a	Nil	o	a	a
	< 8 black, normal spores	va	Nil	f	a	va
	> 8 spores	Nil	Nil	Nil	Nil	o
	Broad; overlapping spores	Nil	Nil	Nil	f	a
	Misshapen spores	r	Nil	r	f	a

*, No fertile perithecia produced; 25 → 30°, protoperithecia formed at 25° transferred to 30° after conidiation; a, abundant; f, frequent; o, occasional; r, rare; v, very.

Reciprocal crosses of L1*A* and L25*a* (Table 4 and Pl. 2) showed differences at 20° indicating cytoplasmic control of some stage in ascus development. Crosses of L1*A* × L1*a* were also made and these gave similar results to L1*A* × L25*a* (Table 4). In each set of these Lindegren crosses, asci with more than eight spores were observed with continuous incubation at 27·5° or 30° and, in the case of L1*A* only, with protoperithecial formation at 25° transferred to 30° on conidiation. It was at these three particular temperature régimes too that all the Lindegren crosses showed some asci which were broader than normal with overlapping spores which tended to be two-ranked in arrangement. Again, abnormalities were at a minimum or absent at 20°.

Crosses of Costa Rica CU5 with Costa Rica CU8 were fertile with normal eight-spored asci at 25°. At 30° almost total abortion was observed with short distorted asci containing misshapen thin-walled ascospores.

DISCUSSION

It is now well established that the different wild-type strains of Neurospora show considerable biochemical and genetical variability. In the present work, the differing ability of the wild-type strains to produce protoperithecia over the range of temperature 15° to 30°, and the differing effects of temperature on ascus and

ascospore development in inbred and outbred crosses of the wild types, provide further evidence of variation between the wild strains.

The non-production of protoperithecia at 30° by *Neurospora crassa* A4*A* in contrast to A12*a* and the Lindegren wild-type strains parallels the report by Horowitz & Fling (1953) that A4*A* did not produce tyrosinase at 35° whereas L25*a* and A12*a* did. The report by Hirsch (1954) showing a correlation between the appearance of tyrosinase and protoperithecial formation suggests that the correlation above may have some significance. Markert (1950) and Horowitz, Fling, Macleod & Sueoka (1960) reported an association of female sterility with inability to produce tyrosinase. The enzyme was induced by addition to the medium of an aromatic amino acid, which, however, did not affect the female sterility. These results suggest that the two characters may be related through a common biochemical defect, and that it is some stage in the production of this precursor that is temperature sensitive in A4*A* but not in A12*a* or the Lindegren wild-type strains. Thus the presence of the enzyme may be used as an indication of ability to reach a specific, but as yet unidentified, stage in sexual reproduction. The production of protoperithecia at 30° by strains repeatedly backcrossed to A4*A* and tested on minimal medium + nicotinamide suggests that nicotinamide might be involved in the pathway which leads to protoperithecial formation and that A4*A* is deficient in this compound when incubated at 30°.

The observations reported here on sexual differentiation at the higher temperatures suggest a possible explanation of the anomalous results with the mutant *al-2* (15300) reported by Fox & Gray (1950) and by Horowitz & Shen (1952). The former workers reported the presence of tyrosinase at 30° in *al-2a* but not in *al-2A*. The latter workers found the enzyme present in both strains at 25°. Possibly different re-isolates were used by the two sets of workers such that the 'A' strain of Fox & Gray was derived from A4*A* whereas the other strains were not.

The 3:1 ratios observed in the L25*a* × A4*A* reciprocal crosses suggest that the production of protoperithecia at 30° is controlled by two genes. However, one of the progeny from these crosses, 44*a*, which showed a high production of protoperithecia at 30°, when crossed to L1*A*, which also showed a high production of protoperithecia at 30°, gave in the progeny a 3:1 ratio of perithecia present to absent at 30°. These results suggest that 3 or more genes may be involved. This apparent multifactorial inheritance of protoperithecial production at 30° correlates with the observations of a number of workers on the genetic control of tyrosinase synthesis (Markert, 1950; Fox & Burnett, 1959; Horowitz & Fling, 1953; Lewis & Lewis, 1961).

The phenomenon of asci with more than eight spores was first observed in Neurospora in crosses between strains of unknown ancestry by Frost & Greenhill (1963), who called such asci 'multi-spored'. The present work indicates that the production of multi-spored asci occurs at temperatures of 27·5° or above and is associated only with crosses between two Lindegren strains. Furthermore, the size and distribution of the spores in multi-spored asci suggest meiosis is irregular with spore walls deposited around nuclear material which in some cases may represent only a single chromosome. Similar observations were made in Neurospora by Perkins (1962) in crosses of *bis* × *bis* (morphological mutant, biscuit, B6). Rizet & Engelmann (1949) reported that in *Podospora anserina* asci with more than the normal number of spores were produced in crosses particularly at higher temperatures.

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. Asci produced by the cross Abbott 4*A* × Abbott 12*a* incubated at different temperatures: (1*a*) removed from 25° to 30° at the time of conidiation, (1*b*) 25°, (1*c*) 20°, (1*d*) 15°.

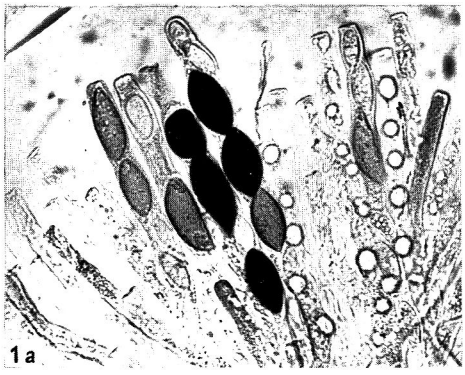
Fig. 2. Overlapping spores in asci from the cross Lindegren 25*a* × Abbott 4*A* incubated at 30°.

Fig. 3. The slow maturation of four spores in an ascus from the cross Abbott 4*A* × Lindegren 25*a* incubated at 25°.

PLATE 2

Fig. 4. Asci produced by the cross Lindegren 1*A* × Lindegren 25*a* incubated at different temperatures: (4*a*) removed from 25° to 30° at the time of conidiation. A multi-spored ascus containing nineteen spores not all of which are visible here (phase-contrast illumination). (4*b*) as in 4*a* but ascus containing nine spores. (4*c*) 25°, (4*d*) 20°.

Fig. 5. Asci produced by the cross Lindegren 25*a* × Lindegren 1*A* incubated at different temperatures: (5*a*) 30°. A multi-spored ascus is indicated by the arrow (phase-contrast illumination). (5*b*) 25°, (5*c*) 20°, (5*d*) 15°.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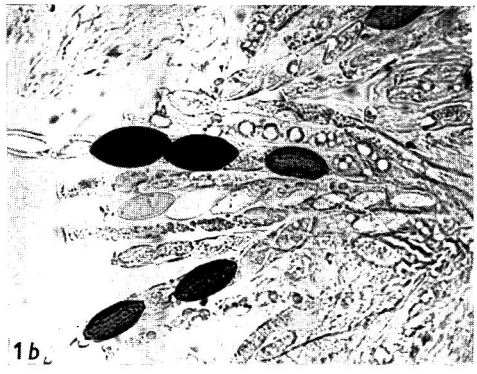
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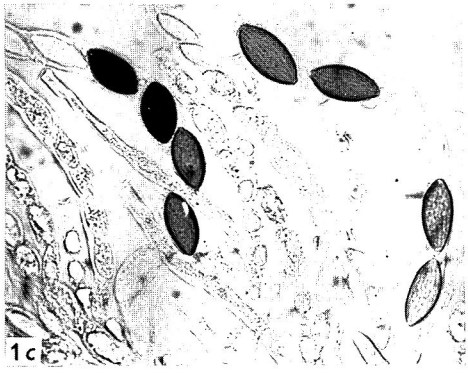
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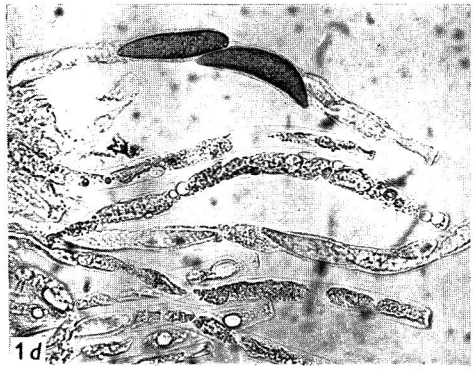
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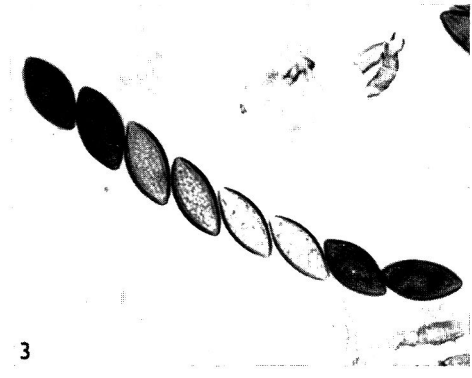
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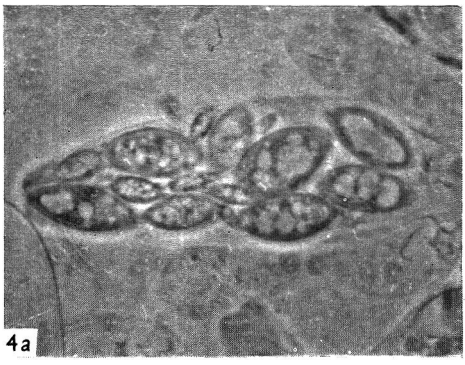
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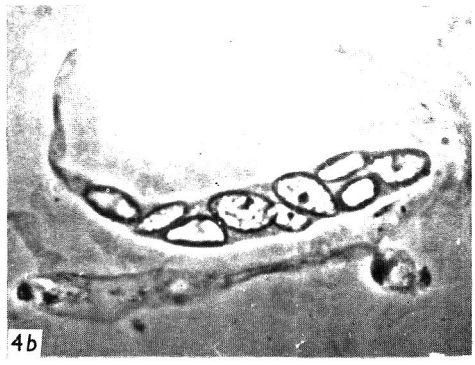
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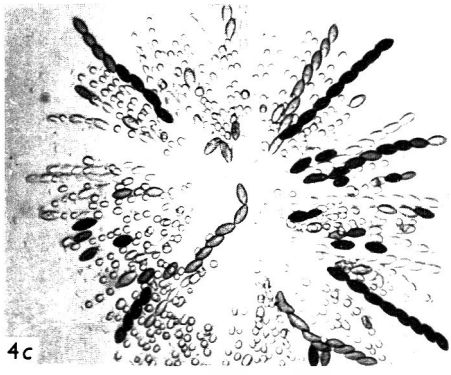
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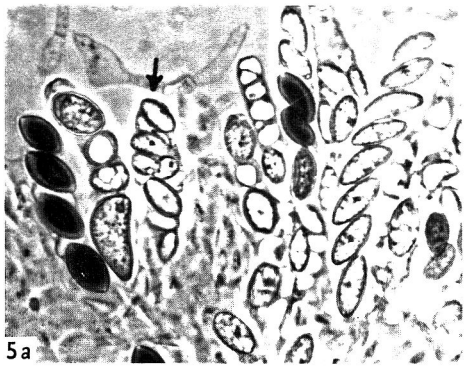
4d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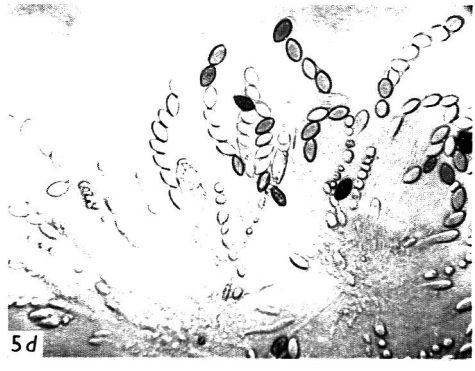
5b


5c


5d

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Interrelations Between Two Pathways of Methionine Biosynthesis in *Aerobacter aerogenes*

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SUMMARY

Two pathways for methionine methyl formation, one cobalamin-dependent and one cobalamin-independent, corresponding to those observed in *Escherichia coli* PA 15 have been found in *Aerobacter aerogenes*. An initial difficulty in showing the cobalamin-dependent pathway in cell-free extracts proved to be due to the presence of enzymes which caused the removal of adenosylmethionine, a cofactor required for this system. In contrast to *E. coli* PA 15, *A. aerogenes* contains holocobalaminmethyltransferase even when grown in the absence of cobalamin. When cobalamin is added to the growth medium, the cobalamin-independent pathway is repressed.

INTRODUCTION

Escherichia coli has two enzymic mechanisms of methyl group transfer from 5-methyltetrahydropteroylglutamate derivatives to homocysteine to form methionine (Woods, Foster & Guest, 1964). A major difference in these two pathways is that one shows a requirement for a cobalamin derivative and the other does not (Kisliuk & Woods, 1960; Kisliuk, 1961). The cobalamin-requiring system also requires reduced flavin adenine dinucleotide and adenosylmethionine as cofactors (Rosenthal & Buchanan, 1963; Buchanan *et al.* 1964). Either 5-methyltetrahydropteroyltriglutamate or 5 methyltetrahydropteroylglutamate can serve as methyl donor. The cobalamin-independent pathway does not require adenosylmethionine or reduced flavin adenine dinucleotide and will utilize only 5-methyltetrahydropteroyltriglutamate as methyl donor (Guest, Friedman & Foster, 1962).

To demonstrate the cobalamin-dependent system the vitamin must be added to the growth medium or to enzymic extracts. Lacking a cobalamin supplement, *Escherichia coli* PA 15 does not synthesize detectable holocobalaminmethyltransferase (Kisliuk, 1961) although apoenzyme is readily demonstrable (Guest, Friedman, Dilworth & Woods, 1964). A role for cobalamin has not as yet been established for any reaction in *E. coli* other than methionine biosynthesis. *Aerobacter aerogenes* was selected for further study because this organism has been shown to require a cobalamin derivative for the dioldehydrase reaction (Lee & Abeles, 1963). The relative amounts of methionine synthesis were determined under various conditions of growth.

METHODS

Organisms. The strain of *Aerobacter aerogenes* (ATCC 8724) used in these studies was the same organism as used to study the cobamide coenzyme requiring dioldehydrase reaction (Lee & Abeles, 1963). *Escherichia coli* PA 15 is an auxotroph which requires serine or glycine for growth.

Growth conditions. The glucose inorganic salts medium of Davis & Mingioli (1950) was used for the growth of *Aerobacter aerogenes*. This medium was supplemented with 4 μg. cobalamin/l. when cobalamin-grown organisms were required. Growth was aerobic for 17 hr at 37°. The inoculum was 10 ml. of a 24 hr culture per 15 l. medium. The organisms were harvested in a refrigerated Sharples centrifuge.

Escherichia coli was grown in the same way except that the medium was supplemented with glycine (0·8 g./l.).

The organisms were maintained on agar slopes prepared from the media indicated.

Preparation of extracts. Acetone-dried organisms were prepared and extracted as described by Kisliuk & Woods (1960) except that 1 g. dried organisms were extracted with 20 ml. water and the extracts were not dialysed. Sonic extracts were prepared and fractionated as described by Kisliuk (1961).

The *Escherichia coli* holocobalaminmethyltransferase used in the present work was an ammonium sulphate fraction (0–35% saturation) of a sonic extract of cobalamin-grown organisms (Kisliuk, 1961) containing 16 mμg. cobalamin/mg. protein. An amount of material containing 22 mμg. cobalamin was added to each incubation mixture where indicated.

Conditions for methionine synthesis in enzyme extracts. The standard reaction mixture used to study the overall formation of methionine from serine and homocysteine via 5-methyltetrahydrofolate consists of diphosphopyridine nucleotide 5×10^{-4} M, adenosine triphosphate 5×10^{-3} M, pyridoxal phosphate 5×10^{-4} M, fructose-1,6-diphosphate 5×10^{-3} M, $MgSO_4$ 5×10^{-3} M, L-serine 5×10^{-3} M, DL-homocysteine 10^{-2} M, potassium phosphate (pH 7·8) $7{\cdot}5 \times 10^{-2}$ M.

The reaction mixtures (2 ml. final volume) were incubated for 4 hr at 37° in Thunberg tubes filled with hydrogen. The reaction was stopped by placing the tubes in a boiling water bath for 3 min. After centrifuging down the precipitate, the supernatant fluid was assayed for methionine

Assays. L-Methionine was determined microbiologically with *Leuconostoc mesenteroides* (ATCC 8042) by using Difco methionine assay medium. Cobalamin was determined with *Lactobacillus leichmannii* (ATCC 7830) by using Difco B_{12} assay medium. The thymidine content of all samples was negligible as determined after destroying cobalamin by autoclaving samples in 0·2 N-NaOH for 30 min.

Protein was determined spectrophotometrically (Layne, 1957).

Materials. Adenosine triphosphate, diphosphopyridine nucleotide, flavin adenine dinucleotide and fructose-1,6-diphosphate were products of the Sigma Chemical Company. S-Adenosyl-L-methionine iodide was obtained from the California Corporation for Biochemical Research. Pteroyltriglutamic acid was supplied by Lederle Laboratories through the courtesy of Dr T. H. Jukes. Tetrahydropteroylglutamic acid was prepared by catalytic reduction (Kisliuk, 1957). Tetrahydropteroyltriglutamate was prepared similarly except that 10 mg. were reduced with

10 mg. catalyst in 10 ml. glacial acetic acid. After removal of the catalyst by filtration under hydrogen the acetic acid was removed by lyophilization.

RESULTS

Methionine synthesis by bacterial suspensions

Suspensions of *Aerobacter aerogenes* and *Escherichia coli* PA 15 were tested for their ability to synthesize methionine from homocysteine, with serine as the precursor of the methyl group under conditions similar to those described by Gibson & Woods (1960). These workers observed that cobalamin added to suspensions of organisms grown in the absence of cobalamin stimulated methionine synthesis. When cobalamin was included in the growth medium, the harvested organisms no longer were stimulated by its addition (Table 1). Suspensions of organisms grown in the absence of cobalamin synthesized considerably more methionine when cobalamin was added to the suspending medium than did the corresponding cobalamin-grown organisms. This suggests that when the cobalamin-dependent pathway operates maximally during growth the cobalamin-independent pathway is repressed. When cobalamin was added to suspensions of organisms grown in its absence, it probably combined with apocobalaminmethyltransferase present in the organisms, thus enabling them to synthesize methionine by both pathways simultaneously. The results given in Table 1 are in agreement with the conclusion of Rowbury & Woods (1961) that substances which enhance methionine synthesis during growth decrease the ability of suspended organisms to synthesize this amino acid.

Table 1. *Methionine synthesis by suspensions of Aerobacter aerogenes and Escherichia coli*

The suspending medium consisted of potassium phosphate, (pH 7·4), 0·1 M; DL-homoeysteine, 0·01 M; D-glucose, 0·02 M; and where indicated L-serine, 0·005 M; cobalamin 7×10^{-8} M. The organisms added were equivalent to 7 mg. dry weight/ml. Incubated at 37° for 3 hr.

Addition to suspension	L-Methionine (μmmoles/mg. dry wt. organism)	
	Growth without cobalamin	Growth with cobalamin
(1) *Aerobacter aerogenes*		
None	6·2	10·2
L-serine	7·4	25·4
L-serine + cobalamin	36·0	22·6
(2) *Escherichia coli*		
None	2·4	2·9
L-serine	11·7	13·1
L-serine + cobalamin	20·3	14·4

Methionine synthesis by extracts of Aerobacter aerogenes

With extracts of acetone-dried *Aerobacter aerogenes* grown in the absence of cobalamin, substantial synthesis of methionine occurred, this synthesis being inhibited by the addition of tetrahydropteroylglutamate (Table 2). However, this

inhibition was not annulled by the addition of cobalaminmethyltransferase as it is in the corresponding *Escherichia coli* PA 15 system (Kisliuk, 1961). With extracts of cobalamin-grown *Aerobacter aerogenes*, methionine synthesis was very small and was further suppressed by tetrahydropteroylglutamate. This again contrasts with the situation in *Escherichia coli* PA 15 where methionine synthesis in extracts of cobalamin-grown organisms was not inhibited by tetrahydropteroylglutamate (Kisliuk & Woods, 1960). These results are consistent with the suggestion above that the presence of cobalamin in the growth medium repressed the cobalamin-independent pathway. Earlier evidence obtained with *E. coli* PA 15 (Kisliuk & Woods, 1960) on the effect of cobalamin in the growth medium in depressing methionine synthesis in extracts may be interpreted in the same manner. Cobalamin-methyltransferase, although present, was not active in extracts of *Aerobacter aerogenes* under the conditions used for Table 2 (see below).

Table 2. *Inhibition of methionine synthesis by tetrahydropteroylglutamate*

The standard reaction mixture was incubated with extracts of *Aerobacter aerogenes* prepared from acetone-dried organisms grown without or with added cobalamin (3·5 mg. protein/incubation in each case). Tetrahydropteroylglutamate $1{\cdot}8 \times 10^{-4}$ M.

Extract of	Additions to reaction mixture	L-Methionine (μmmoles)
(1) *A. aerogenes* grown without cobalamin	None	300
	+ tetrahydropteroylglutamate	0
	+ tetrahydropteroylglutamate and *E. coli* cobalaminmethyltransferase	0
(2) *A. aerogenes* grown with cobalamin	None	20
	+ tetrahydropteroylglutamate	0

Table 3. *Requirements for methionine synthesis by Aerobacter aerogenes extracts*

The complete system consisted of the standard reaction mixture supplemented with flavin adenine dinucleotide and tetrahydropteroyltriglutamate (both 1×10^{-4} M). An extract of acetone-dried *A. aerogenes* (grown in the absence of cobalamin) treated with Sephadex G-50 was used as a source of enzyme (3·8 mg. protein added).

	L-Methionine (μmmoles)
Complete system	160
Minus serine	10
Minus homocysteine	0
Minus tetrahydropteroyltriglutamate	55
Minus DPN	115
Minus ATP	160
Minus fructose-1,6-diphosphate	35
Minus pyridoxal phosphate	40
Minus Mg^{2+}	60
Minus flavin adenine dinucleotide	145

Requirements for methionine synthesis in Aerobacter aerogenes

Extracts of acetone-dried *Aerobacter aerogenes* (grown in the absence of cobalamin) when treated with Sephadex G-50 (Kisliuk, 1960) and supplemented with all the required cofactors except a folate derivative, showed a negligible methionine

synthesis (Fig. 1). As observed with *Escherichia coli* PA 15 (Kisliuk & Woods, 1960) tetrahydropteroylglutamate did not promote methionine synthesis but tetrahydropteroyltriglutamate did reactivate the system (Jones, Guest & Woods, 1961). The additional requirements for the *A. aerogenes* system are shown in Table 3. In this experiment an absolute requirement was observed only for the substrates serine and homocysteine. The extract was only partially depleted of tetrahydropteroyltriglutamate, diphosphopyridine nucleotide, fructose-1,6-diphosphate, pyri-

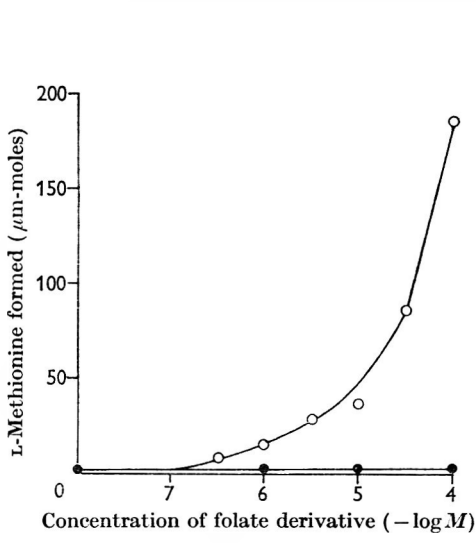

Fig. 1

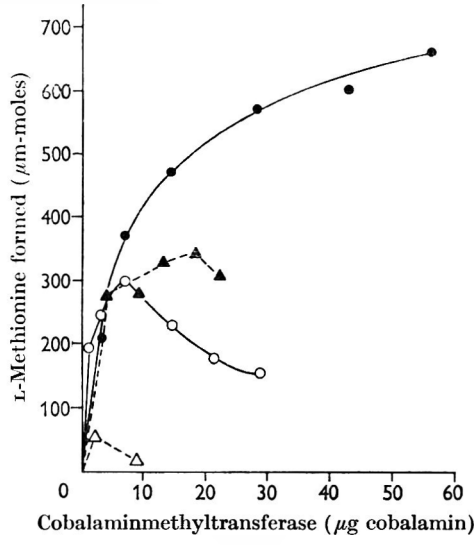

Fig. 2

Fig. 1. The effect of reduced folate derivatives on methionine synthesis in Sephadex-treated extracts of *Aerobacter aerogenes*. The standard reaction mixture was incubated with Sephadex-treated extracts of acetone-dried *A. aerogenes* grown in the absence of cobalamin (3·8 mg. protein) for 4 hr at 37° in a total volume of 2 ml. ●—●, tetrahydropteroylglutamate; ○—○, tetrahydropteroyltriglutamate.

Fig. 2. Assay of fractions of *Aerobacter aerogenes* grown in the presence of cobalamin for cobalaminmethyltransferase activity. Assay conditions are the same as described in Table 5 with *Escherichia coli* cobalamin enzyme omitted. ○—○, fraction 14; ●—●, fraction 14 plus $7{\cdot}5 \times 10^{-5}$ M. adenosylmethionine; △—△, fraction 15; ▲—▲, fraction 15 plus $7{\cdot}5 \times 10^{-5}$ M adenosylmethionine.

doxal phosphate, Mg^{2+} and flavin adenine dinucleotide. A requirement for adenosine triphosphate would not be expected since the cobalamin-independent pathway proceeds without this cofactor (Guest *et al.* 1962).

Inhibition of the cobalaminmethyltransferase reaction by extracts of Aerobacter aerogenes

When extracts of *Aerobacter aerogenes* and *Escherichia coli* PA 15 were mixed under conditions appropriate for the expression of the cobalamin-independent pathway (Table 4, part (*a*)) methionine synthesis occurred. When cobalaminmethyltransferase was added (Table 4, part (*b*)) to *E. coli* extracts, methionine synthesis was greatly stimulated, probably because both pathways were operating simultaneously. There was no corresponding stimulation in *A. aerogenes* extracts nor in a

mixture of *A. aerogenes* and *E. coli* PA 15 extracts. The cobalaminmethyltransferase was apparently inactive in the presence of *A. aerogenes* extracts. This suggestion is substantiated by the results obtained under conditions where the cobalamin-independent pathway was inhibited and only the cobalamin dependent pathway operates, i.e. in the presence of tetrahydropteroylglutamate (Table 4, part (*c*)). The addition of *A. aerogenes* extract under these conditions greatly decreased methionine synthesis.

Table 4. *The effects of extracts of Aerobacter aerogenes on methionine synthesis by Escherichia coli extracts*

The standard reaction mixture was incubated with extracts of acetone-dried *A. aerogenes* on *E. coli* (grown in the absence of cobalamin) 5·3 and 4·2 mg. protein per incubation, respectively. When the extracts were combined half of each amount was added. Tetrahydropteroylglutamate $1{\cdot}8 \times 10^{-4}$ M where indicated.

Type of extract	Additions	L-Methionine (μmmoles)
(*a*) *E. coli*	None	450
A. aerogenes	None	300
E. coli + *A. aerogenes*	None	500
(*b*) *E. coli*	*E. coli* cobalaminmethyltransferase	1850
A. aerogenes	*E. coli* cobalaminmethyltransferase	300
E. coli + *A. aerogenes*	*E. coli* cobalaminmethyltransferase	500
(*c*) *E. coli*	Tetrahydropteroylglutamate	30
E. coli	Tetrahydropteroylglutamate + *E. coli* cobalaminmethyltransferase	700
E. coli + *A. aerogenes*	Tetrahydropteroylglutamate + *E. coli* cobalaminmethyltransferase	100

Table 5. *Annulment of the inhibition of cobalaminmethyltransferase by addition of adenosylmethionine*

The standard reaction mixture was incubated with extracts of acetone-dried *Escherichia coli* PA 15 (6 mg. protein), *E. coli* PA 15 cobalaminmethyltransferase, tetrahydropteroylglutamate $1{\cdot}8 \times 10^{-4}$ M and, where indicated, a sonic extract of *Aerobacter aerogenes* (0·66 mg. protein), adenosylmethionine $7{\cdot}5 \times 10^{-5}$ M (150 μmmoles), L-methionine $2{\cdot}5 \times 10^{-4}$ M (500 μmmoles).

Addition	L-Methionine (μmmoles)
None	250
Adenosylmethionine	250
A. aerogenes extract	30
Adenosylmethionine + *A. aerogenes* extract	200
Adenosylmethionine + *A. aerogenes* extract minus L-serine	30
A. aerogenes extract + L-methionine	550

The nature of the inhibitor in Aerobacter aerogenes extracts

When extracts of *Aerobacter aerogenes* were heated for 5 min. at 100° they were no longer inhibitory, suggesting that the inhibitor is an enzyme. It was considered that the inhibition might be due to enzymic removal of methionine; however, this appeared not to be the case since added methionine was recovered at the end of the incubation (Table 5). It was then observed that the inhibition was annulled by the addition of adenosylmethionine (Table 5). It seems likely therefore that the inhibition of cobalaminmethyltransferase by *A. aerogenes* extracts was due to the ability of these extracts to metabolize adenosylmethionine which is required for this

system in catalytic amounts. Shapiro (1962) observed that extracts of this organism metabolized adenosylmethionine by (1) methylating homocysteine to methionine and adenosylhomocysteine, and (2) decomposition to adenine, methylthioribose and homoserine. The results of Table 5 indicate that the former reaction might account for 20 % of the breakdown of adenosylmethionine (30 of 150 μmmoles). The remainder was presumably destroyed by the latter reaction.

Isolation of cobalaminmethyltransferase from Aerobacter aerogenes grown in the absence and presence of cobalamin

To show conclusively the presence of cobalaminmethyltransferase in extracts of *Aerobacter aerogenes*, sonic extracts were fractionated with ammonium sulphate and calcium phosphate gel by the method of Kisliuk (1961). The distribution of

Table 6. *Fractionation of sonic extracts of Aerobacter aerogenes*

Fraction no.		Protein (mg.)	Cobalamin (μmg./mg.) protein)	Protein (mg.) for 50 % inhibition*
	(*a*) Organisms grown without cobalamin			
	$(NH_4)_2SO_4$ fractionation:			
	Sonic extract (45 mg. protein/ml.)	7050	2·4	0·28
1	0–30 % saturation	240	5·5	0·12
2	30–40 % saturation	1560	3·3	0·18
3	40–50 % saturation	1136	3·5	0·15
4	50–60 % saturation	140	2·8	0·25
	$Ca_3(PO_4)_2$ gel:			
	$(NH_4)_2SO_4$, 30–40 % saturation	1190	3·3	0·18
5	Buffer supernatant, 0·01 M	35	1·6	0·24
6	Buffer supernatant, 0·05 M	122	10·4	—†
7	Buffer supernatant, 0·10 M	171	2·3	—†
8	Buffer supernatant, 1·0 M	343	2·2	0·35
	(*b*) Organisms grown with cobalamin			
	$(NH_4)_2SO_4$ fractionation:			
	Sonic extract (41 mg. protein/ml.)	6953	15·2	0·25
9	0–30 % saturation	432	9·1	0·19
10	30–40 % saturation	1134	21·0	0·11
11	40–50 % saturation	1280	22·0	0·10
12	50–60 % saturation	304	15·3	0·16
	$Ca_3(PO_4)_2$ gel:			
	$(NH_4)_2SO_4$, 30–40 % saturation	1134	21·0	0·11
13	Buffer supernatant, 0·01 M	46	34·6	0·18
14	Buffer supernatant, 0·05 M	158	43·8	—‡
15	Buffer supernatant, 0·10 M	110	15·5	—‡
16	Buffer supernatant, 1·0 M	384	2·7	0·40

* To determine this value, the fraction was added at various concentrations to an assay system containing the standard reaction mixture + tetrahydropteroylglutamate ($1{\cdot}8 \times 10^{-4}$ M), an extract of acetone-dried *E. coli* PA 15 (7 mg. protein) and *E. coli* PA 15 cobalaminmethyltransferase. In each case the inhibition could be annulled with adenosylmethionine.

† Results with these fractions are shown in Table 7.

‡ Results with these fractions are shown in Fig. 2.

cobalamin in these fractions (Table 6) was similar to that obtained with cobalamin-grown *Escherichia coli* (Kisliuk, 1961). Only fraction 6 (Table 6) appeared to be entirely free from inhibitor (Table 7). Fractions 7, 14, 15 showed inhibition which

was annulled with adenosylmethionine (Table 7, Fig. 2). In subsequent trials fractions equivalent to fraction 14 were also obtained free from inhibitor.

Two fractions (Table 7) obtained from organisms grown in the absence of cobalamin were tested for cobalaminmethyltransferase activity (adenosylmethionine added) separately. Activity was demonstrable in both fractions, showing that holocobalaminmethyltransferase was endogenously synthesized. In other organisms so far studied, *Escherichia coli* (Kisliuk, 1961), pigs (Loughlin, Elford & Buchanan, 1964) and chickens (Dickerman, Redfield, Bieri, & Weissbach, 1964), it is formed from exogenous cobalamin.

Table 7. *Assay of fractions of Aerobacter aerogenes grown in the absence of cobalamin for cobalaminmethyltransferase activity*

Assay conditions are the same as described in Table 5. Incubations 1 to 5 contain adenosylmethionine at $7{\cdot}5 \times 10^{-5}$ M.

	L-Methionine (μmmoles)
(1) *E. coli* PA 15 cobalaminmethyltransferase omitted	0
(2) + fraction 6 (6·6 μmg. cobalamin)	460
(3) + fraction 6 (13·2 μmg. cobalamin)	570
(4) + fraction 7 (2·4 μmg. cobalamin)	165
(5) + fraction 7 (4·8 μmg. cobalamin)	280
(6) *E. coli* PA 15 cobalaminmethyltransferase included	270
(7) + fraction 6 (6·6 μmg. cobalamin)	410
(8) + fraction 6 (13·2 μmg. cobalamin)	590
(9) + fraction 7 (2·4 μmg. cobalamin)	310
(10) + fraction 7 (4·8 μmg. cobalamin)	180

The cobalamin content of the various fractions was considerably enhanced by growth on cobalamin. However, the specific activity did not change. The mμmoles methionine formed per mμg cobalamin when 5 mμg cobalamin was added to the assay system was 61 for the material prepared from cobalamin grown organisms (Fig. 2) and 57 for the corresponding preparations from organisms grown in the absence of cobalamin (Table 7). The activity of the *Escherichia coli* preparation used in the present work (12 mμmoles/mμg.) was somewhat lower because of deterioration in storage.

DISCUSSION

During this work, the late Professor D. D. Woods kindly informed us of experiments made in his laboratory in which methylcobalamin served as a methyl donor to homocysteine when incubated with extracts of *Aerobacter aerogenes*. This provides further evidence that cobalaminmethyltransferase is present in this organism. Although the cobalamin-dependent pathway was inactive in extracts because of removal of adenosylmethionine it apparently does function *in vivo*, otherwise one would not expect the observed repression of the cobalamin-independent pathway due to increased synthesis of methionine in cobalamin-grown organisms.

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Inducible Lytic Systems in the Genus *Bacillus*

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SUMMARY

Many different strains of *Bacillus cereus* and members of other species of the genus *Bacillus* were induced to lyse by exposure to the proper concentration of mitomycin C, a radiomimetic drug. For some bacilli, induction of lysis occurred only in Casamino acids containing medium but not in nutrient broth + yeast extract medium, whereas other bacilli showed the opposite behaviour. The efficiency of induction was a function of the growth phase of the culture and of the concentration of mitomycin C. Induction of lysis in *B. thuringiensis* resulted in the release of oedema factor and phospholipase into the medium. Mitomycin C-induced lysis of *B. cereus* was prevented by adding chloramphenicol, actinomycin D, or 5-fluorouracil deoxyriboside up to 40 min. later. There appeared to be no sequential loss of susceptibility to inhibition by these compounds as expected.

INTRODUCTION

The close relationship between lysis induced by ultraviolet (u.v.) radiation and the production of the oedema-producing toxin by a strain of *Bacillus cereus* (6464) was reported by Altenbern (1962). Further work showed that either u.v.-radiation or mitomycin C, a radiomimetic agent, could be used as an inducing agent. Many other strains of *B. cereus* tested gave mitomycin C-induced lysis like that observed for strain 6464. The study was then expanded to include many species of the genus *Bacillus* and sometimes several strains of a species. The present paper is mainly a survey of the action of mitomycin C on members of the genus *Bacillus*, including the effect of medium on ease of induction, the rate of induced lysis, the production of phospholipase and toxin by strains of *B. thuringiensis* and *B. anthracis*, and the effects of chloramphenicol, actinomycin D and 5-fluorouracil deoxyriboside on the course of mitomycin C-induced lysis in certain species.

METHODS

The various organisms used in this work were maintained as spore stocks in soil. Regular spore suspensions were prepared from potato-extract agar slopes as described by Thorne (1962). Two media were routinely used: (1) the Casamino acids medium; (2) the nutrient broth + yeast extract-medium (NBY) described by Thorne (1962).

Cultures of the various organisms in logarithmic growth were prepared by inoculation of tubes with 5 ml. of either of the liquid media with enough spores to give finally 10^6 spores/ml. These tubes were then incubated on a shaker at 37° until the

extinction (Coleman, Model 14) at 650 mμ (E_{650}) attained a value of 0·200–0·250. At this time mitomycin C was added to the desired final concentration, the extinction recorded and the tubes returned to the shaker. Extinction readings were taken at 30 min. intervals until the end of the experiment. On occasion, replicate tubes were prepared and at 30 min. intervals one culture was filtered through a sintered glass filter and the filtrate assayed for toxin (oedema factor) and phospholipase by the methods described by Molnar (1962).

Stock solutions of mitomycin C (0·1 mg./ml.), chloramphenicol (1 mg./ml.), actinomycin D (1 mg./ml.), and 5-fluorouracil deoxyriboside (5-FUDR; 1 mg./ml.) were prepared in 0·1 M-phosphate buffer (pH 7·4) and sterilized by filtration through a sintered glass filter. These solutions were stored at 4° until used.

RESULTS

Effect of concentration of mitomycin C on induction of lysis of Bacillus cereus strains 6464 *and* 317

Logarithmically growing cultures of the two strains in Casamino acids medium received additions of mitomycin C in a range from 0·1 to 1·0 μg./ml. Extinction measurements were made as described under Methods and the results are presented in Fig. 1. It is clear that under these conditions 0·1–0·5 μg. mitomycin C/ml. caused maximum lysis, and both growth and lysis were prevented by 1·0 μg./ml. There was a rather critical concentration of organism equivalent to an extinction of E_{650}, 0·300 at which the mitomycin C was an effective inducer of lysis, but above this value, little induction by mitomycin C was obtained. Mitomycin C induction of *B. cereus* strain 6464 resulted in a pronounced release of mature phage particles and seemed to be at least as effective as u.v.-radiation as an inducing agent.

Inducibility of Bacillus cereus and other species of Bacillus

Many strains of *Bacillus cereus* and other species of the genus were examined for lysis caused by mitomycin C. *B. cereus* strains W, 7004, 7064, 9139 and B-569 were all readily inducible by mitomycin C as they were with u.v.-radiation. Figure 2 gives some results obtained with four other species (*B. subtilis*, *brevis*, *vulgatus*, *thuringiensis* no. 98) for comparison with *B. cereus* strain 6464. In each case, there seemed to be an individual response of the organism to mitomycin C, in that for some organisms relatively high concentrations of mitomycin C (1·0 μg./ml.) produced some lysis (*B. subtilis*, *B. brevis*), whereas with *B. thuringiensis* this concentration of mitomycin C prevented lysis. Other dissimilarities such as the time at which lysis began as a function of concentration of inducer and the rate of lysis once initiated, are difficult to interpret.

The effect of composition of the culture medium on inducibility

Reich, Shatkin & Tatum (1961) reported that the medium exerted considerable influence on the sensitivity to mitomycin C of lysogenic and non-lysogenic bacteria. We found that some species of *Bacillus* show excellent mitomycin C-induced lysis in the Casamino acids medium but not in the nutrient broth + yeast-extract medium, and other species showed the opposite effect. In Fig. 3 are presented results

obtained with *B. subtilis* and *B. anthracis* Sterne strain, to illustrate this behaviour. Also *B. brevis*, *B. vulgatus* and *B. licheniformis* showed good lysis in the Casamino acids medium but not in the nutrient broth + yeast-extract medium, in contrast to *B. anthracis* Sterne strain and *B. cereus* strain 7004, which gave good lysis in nutrient broth + yeast-extract medium but not in the Casamino acids medium. Reports of

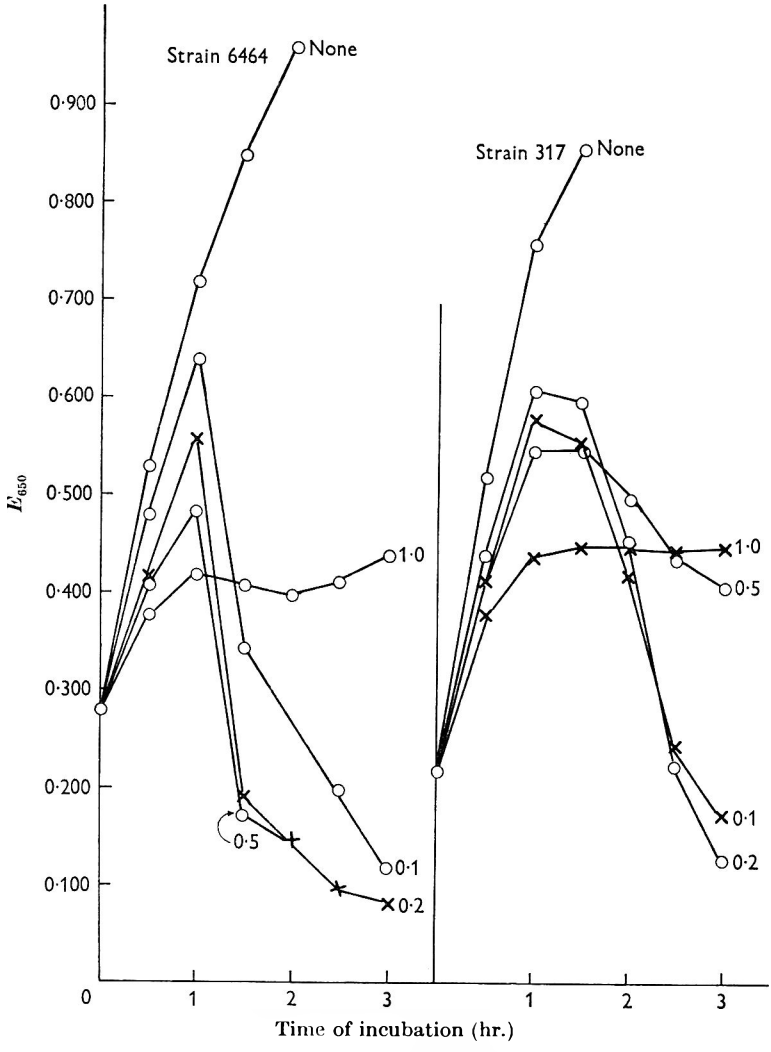

Fig. 1. The induction of lysis of two strains of *Bacillus cereus* by mitomycin C. Organisms grown in Casamino acids medium. Numbers attached to curves refer to mitomycin C μg./ml.

such sensitivity of the lytic response to the composition of the culture medium are not common. Faguet (1962) showed the marked effect of adenosine triphosphate or glycerol on the lysis of induced lysogenic *Escherichia coli*. In contrast to the strains of *B. cereus*, induced lysis of *B. anthracis* Sterne strain in nutrient broth + yeast extract medium, as noted above, did not release large amounts of phospholipase,

oedema factor or protective antigen into the medium. However, mitomycin C-induced lysis of *B. thuringiensis* was accompanied by release of oedema factor and phospholipase into the medium (see Table 1). In this regard, *B. thuringiensis* resembles the various strains of *B. cereus*, yet it is of interest since spores of this organism are used rather indiscriminately in insect control in the U.S.A.

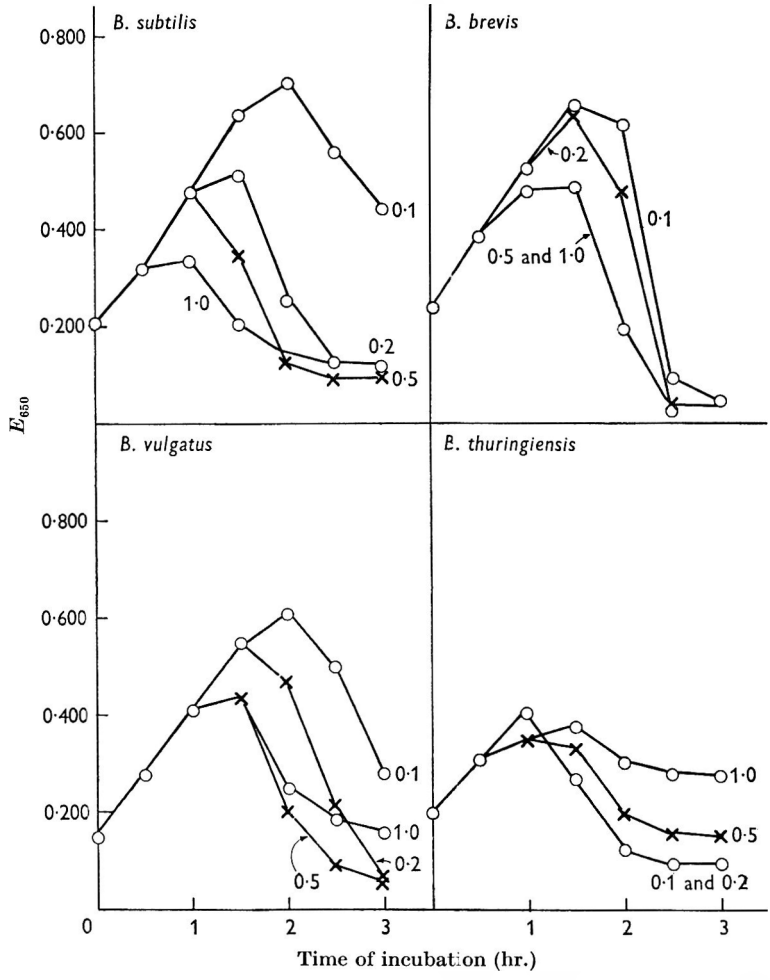

Fig. 2. The induction of four species of Bacillus with varying amounts of mitomycin C. Strains grown in Casamino acids medium. Numbers attached to curves refer to mitomycin C μg./ml.

Action of inhibitors of macromolecular syntheses on mitomycin C-induced lysis

To examine the nature of lysis caused by mitomycin C, the effect of various inhibitors on induced lysis was determined. Chloramphenicol, an inhibitor of protein synthesis, was a potent inhibitor of the mitomycin C-induced lysis of many species of *Bacillus*. Figure 4 shows results obtained with four species of *Bacillus*, and illustrates the prevention of induced lysis. Other experiments with *B. cereus* strains 6464 and 317 showed that chloramphenicol inhibited lysis when added

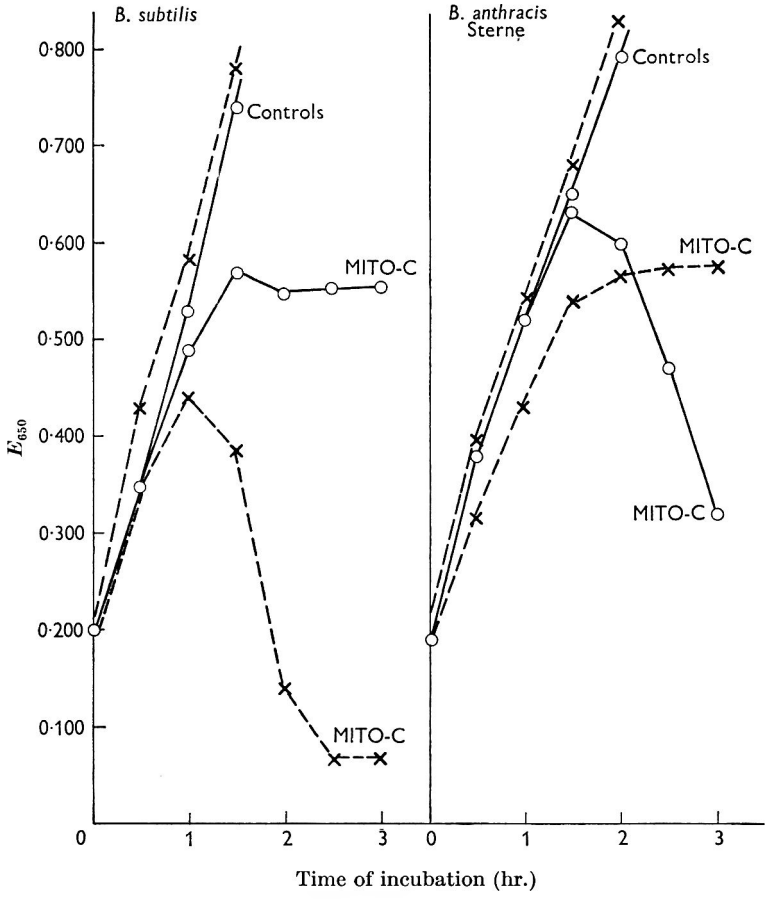

Fig. 3. Induction by mitomycin C of two species of Bacillus as influenced by growth medium. All inductions performed with mitomycin C in a final concentration of 0·2 μg./ml. ○—○, Nutrient broth + yeast extract medium; × - - - ×, Casamino acids medium.

Table 1. *Production of phospholipase and toxin during mitomycin C induced lysis of Bacillus thuringiensis*

Mitomycin C addition: 0·2 μg./ml. Organism grown in nutrient broth + yeast-extract medium.

Time after adding mitomycin C (min.)	Phospholipase (units/ml.)	Toxin (units/ml.)	E_{650}
0	0	2	0·240
30	2	4	0·470
60	4	8	0·565
90	8	32	0·580
120	16	16	0·500
150	8	64	0·420
180	8	—	0·340
210	8	64	0·290

20 min. after adding mitomycin C but not after 40 min. (Fig. 5). However, the time required to yield a detectable degree of lysis when chloramphenicol was added 40 min. after adding mitomycin C was considerably extended and indicates that the comparatively lesser amount of protein produced (lytic enzyme?) took longer to

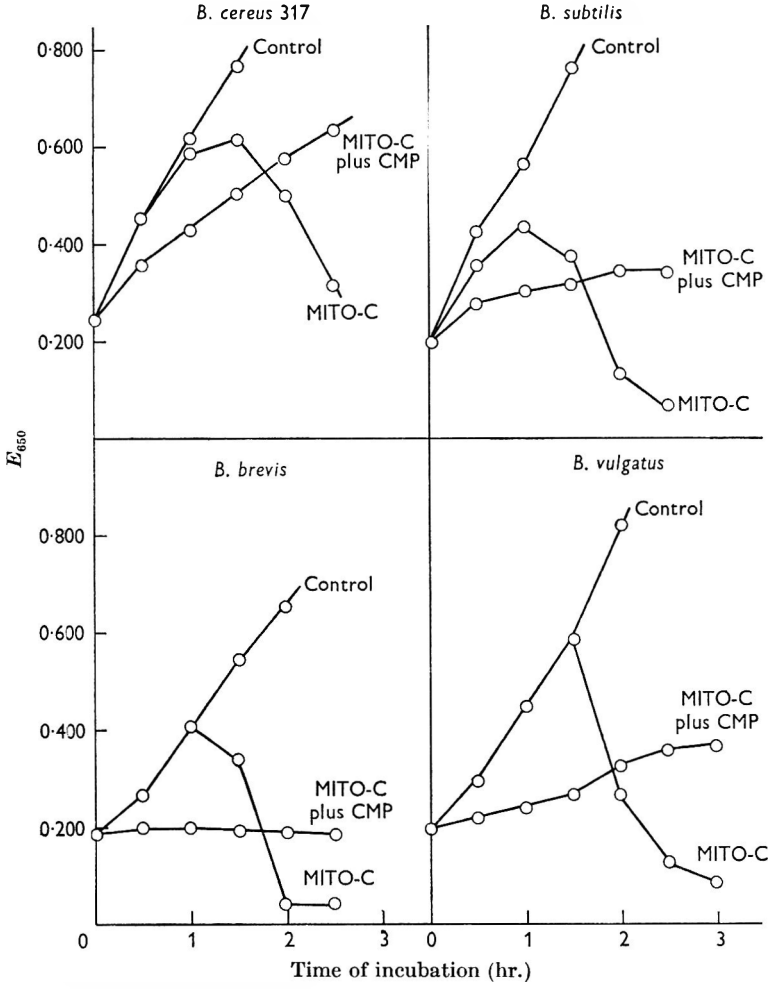

Fig. 4. The effect of chloramphenicol (CMP) on mitomycin C induction of four species of Bacillus. All inductions performed with mitomycin C 0·2 μg./ml. Chloramphenicol added to 10 μg./ml. final concentration at time of adding mitomycin C.

alter the bacilli so that lysis ensued. The rate at which lysis proceeds seems to be independent of the time required for lysis to begin, suggesting that the lytic event *per se* is non-enzymic.

Further work showed that 5-fluorouracil deoxyriboside (5-FUDR) and actinomycin D were potent inhibitors of mitomycin C-induced lysis in *Bacillus cereus* strains 6464 and 317; the data are presented in Fig. 6. Further study of the effect of the time of addition of 5-FUDR or actinomycin D on inhibition of mitomycin C-induced lysis was made. As with chloramphenicol, lysis was prevented by the

addition of 5-FUDR or actinomycin D at any time up to 40 min. after adding mitomycin C (Fig. 6). There was no evidence that the induced lytic system lost sensitivity to an inhibitor of DNA synthesis early, or that inhibition of lysis by chloramphenicol could be effected for a significantly longer time than the inhibition by the two other inhibitors.

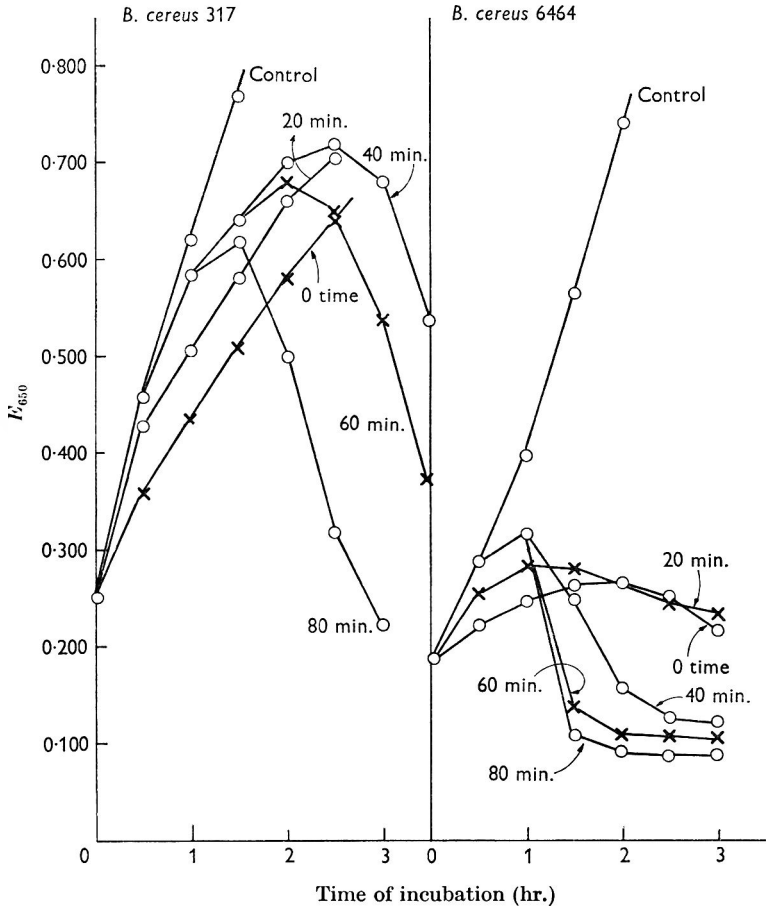

Fig. 5. The effect on the lysis induced by mitomycin C of adding chloramphenicol. All inductions performed with mitomycin C 0·2 μg./ml. Chloramphenicol added to 10 μg./ml. final concentration at the times indicated. Organisms were grown in Casamino acids medium.

Two other substituted deoxyuridines, 5-bromouracil deoxyriboside and 5-iodouracil deoxyriboside, did not prevent mitomycin C-induced lysis of *Bacillus cereus* strains 6464 and 317 even when these compounds were added to the cultures 20 min. before adding mitomycin C. 5-Fluorouracil deoxyriboside prevented mitomycin C-induced lysis in *B. globigii* grown in Casamino acids medium, but 5-FUDR did not inhibit the induced lysis of *B. brevis*, *B. agri* or *B. vulgatus*, all when growing in the nutrient broth + yeast extract medium. The possible effect here of the composition of the medium on the action of this inhibitor was not further investigated.

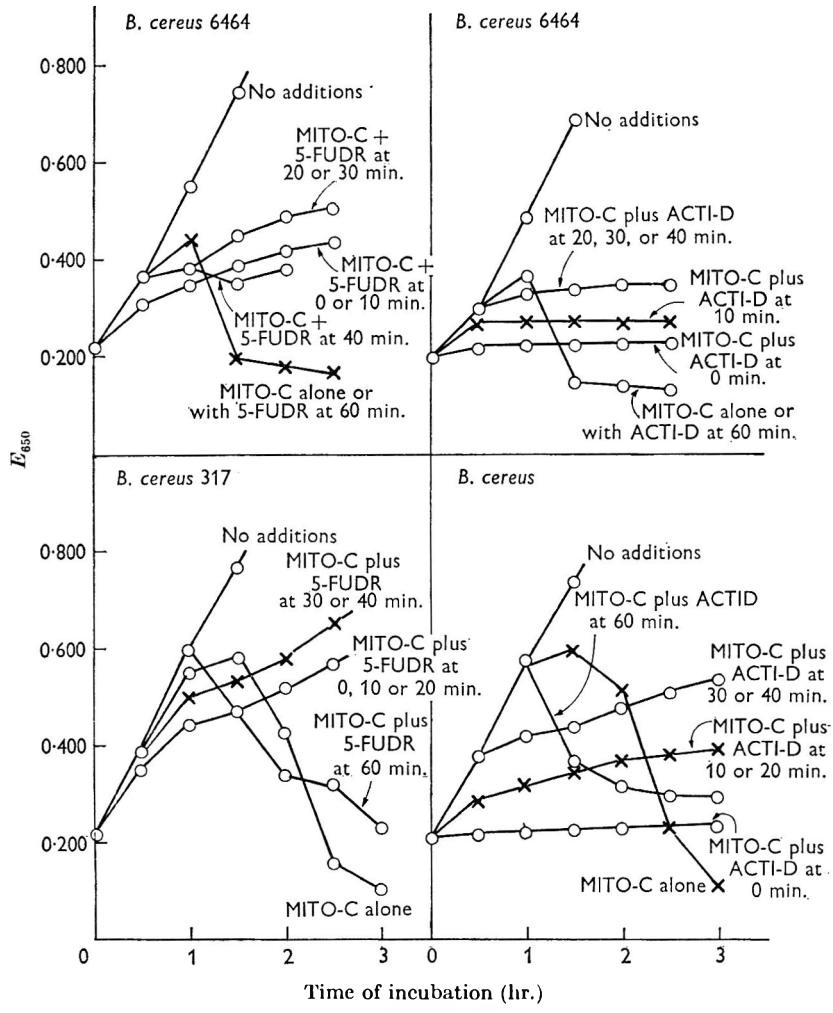

Fig. 6. The effect of addition of 5-fluorouracil deoxyriboside (5-FUDR) or actinomycin D (acti-D) on the lysis induced by mitomycin C. All induction with mitomycin C 0·2 μg./ml. 5-FUDR (10 μg./ml.) or actinomycin D (0·5 μg./ml.) added after induction as indicated. Organisms were grown in Casamino acids medium.

DISCUSSION

Induction of lysogenic bacteria with ultraviolet radiation or with mitomycin C is well known. Induction of lysogenic *Escherichia coli* by mitomycin C was reported by Otsugi, Sekiguchi, Iijima & Takagi (1959) who showed that a small concentration (0·1–1·0 μg./ml.) effected pronounced lysis, whereas larger concentrations (5·0 μg./ml.) caused growth inhibition without inducing lysis. All the strains of the various Bacillus species examined in the present work exhibited such a response, although only one strain (6464) of *Bacillus cereus* possessed a complete prophage which replicated after induction and produced plaque-forming units. Induction of lysis by a radiomimetic agent such as mitomycin C may offer presumptive evidence of

lysogeny even in the absence of a suitable indicator strain to detect free phage. In addition, some types of defective lysogeny might be detected by this technique, whereas plating on selected indicator strains would invariably yield negative results. Electron microscopy of sedimentable material from lysates of these organisms was not used but might reveal defective but morphologically distinct phage particles. In our work no effort was made to detect mature bacteriophage particles in lysates of these cultures by plating filtrates on potential indicator strains. Csuzi & Kramer (1962) showed that there is an u.v.-radiation-inducible lytic system in *B. cereus* strain B-569, and showed by determination of soluble protein that measurement of the extinction of cultures was a valid assessment of the extent and rate of lysis.

The results obtained with inhibitors give indications of the mechanism of mitomycin C-induced lysis. It was assumed that mitomycin C induced a prophage which then directed, through RNA, the synthesis of an enzyme which would lyse the cell. It followed that an inhibitor of DNA synthesis should block replication of the detached (induced) prophage and thus prevent post-induction lysis. Similarly, an inhibitor of RNA synthesis would prevent the formation of new messenger RNA and should thus block synthesis of the lytic enzyme. Finally, chloramphenicol should prevent post-induction lysis by blocking protein (enzyme) synthesis. However, it seemed that there should be a demonstrable sequence of inhibition wherein susceptibility to inhibitors of DNA synthesis should disappear within 10–15 min. after induction, while inhibition by inhibitors of RNA synthesis would be effected 20–25 min. after induction and inhibitors of protein synthesis should prevent lysis when added very late after induction. In contrast to expectation, however, no such sequence of inhibitions was observed and the susceptibility of induced lysis to any of the inhibitors tested seemed to disappear after 40 min. of post-induction. However, DNA, RNA and protein all appear to be involved in the lysis which follows addition of mitomycin C.

Seaman, Tarmy & Marmur (1964) showed that *Bacillus subtilis* 168 possessed an inducible prophage but found no replication of the phage DNA and thus the inducible lysis was not prevented by 5-FUDR, an inhibitor of DNA synthesis. We found that mitomycin C-induced lysis of some Bacillus species was prevented by 5-FUDR, although other strains lysed following exposure to mitomycin C even in the presence of 5-FUDR. It is possible that those cultures in which 5-FUDR had no effect on mitomycin C-induced lysis possessed an inducible but non-replicating prophage as in the case cited above, whereas cultures whose induced lysis was prevented by 5-FUDR possessed an inducible and replicating prophage. Why replication of a prophage is necessary to produce lysis in some cases but not in others is a matter of conjecture. Although our speculation about the nature of the inducible lysis reported here has emphasized the possible involvement of lysogeny, it is recognized that equally plausible interpretations not involving lysogeny may be made.

The authors wish to thank Hoffman-LaRoche, Inc. for the gift of a supply of 5-fluorouracil deoxyriboside and Merck, Sharp & Dohme, Inc. for the generous gift of a sample of actinomycin D.

In the work reported here, the investigators adhered to 'Principles of Laboratory Animal Care' as established by the National Society for Medical Research (U.S.A.).

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Some Carbon-Dioxide Requiring Mutants of *Neurospora crassa*

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SUMMARY

This paper describes some Neurospora mutants which grow on minimal medium when the gas-phase contains 30 % CO_2 (v/v); they are referred to as 'CO_2 mutants'. One is an arginine mutant (*arg*-2; 33442) having a mutation at a different locus from the other arginine CO_2 mutant (*arg*-3; 30300); a second (*arg*-11; 30820) requires arginine + a purine + a pyrimidine for growth in the absence of CO_2; a third (44601) requires arginine + a purine + a pyrimidine + a carboxylic acid for growth in the absence of CO_2. The mutations in mutants 30820 and 44601 are allelic and the carboxylic acid requirement of mutant 44601 is attributable to its genetic background. The remaining CO_2 mutants described here were isolated directly from wild-type Neurospora, and their nutritional requirements in the absence of CO_2 subsequently determined. Two of these mutants, which are allelic, are adenine-requiring mutants sited at the adenine-3 locus, although the adenine-3A and -3B mutants 38709 and Y 112-M2 are not CO_2 mutants. Another two of these new CO_2 mutants, which may be allelic, require carboxylic acids in the absence of CO_2; these mutants are similar in their nutritional requirements to the succinate mutants described by Lewis (1948) and Strauss (1956).

INTRODUCTION

Charles (1962) showed that two mutants of *Neurospora crassa* grew vigorously on minimal medium when the gas phase was air + 30 % (v/v) CO_2. These mutants were the pyrimidine-requiring mutant 1298, and the arginine-requiring mutant 30300, both isolated by Beadle & Tatum (1945). Auxotrophic Neurospora mutants which are able to grow on minimal medium when the gas-phase contains CO_2, but which are unable to grow on minimal medium when the gas-phase does not contain CO_2, will be referred to as 'CO_2 mutants'. After the discovery of the above CO_2 mutants experiments were made to discover other CO_2 mutants of Neurospora. As a result, at least twelve different kinds of CO_2 mutants are now known. Some are well-known mutants which were isolated by Beadle & Tatum (1945), others are CO_2 mutants which have been isolated as such from wild-type Neurospora and their alternative nutritional requirements in the absence of CO_2 subsequently determined. The present paper describes several CO_2 mutants which may be profitably considered together. In the absence of CO_2, these respond, respectively, to: adenine; an aliphatic carboxylic acid; arginine + adenine + uridine; arginine + adenine + uridine + an aliphatic carboxylic acid; arginine. From the number and type of CO_2 mutants now known it is concluded that CO_2 mutants may occur at many or all points in metabolism where a CO_2-incorporation reaction occurs. Possible explana-

tions of how CO_2 in the gas-phase causes the mutants to grow on minimal medium will be considered in the Discussion. The arginine-requiring CO_2 mutant is the second such CO_2 mutant to be described, and has a mutation at the arginine-2 locus, whereas the arginine-requiring CO_2 mutant described by Charles (1962) has its mutation at the arginine-3 locus. Study of the mutants described here, and of those already described (Charles, 1962) leads us to suggest that CO_2 for the synthesis of arginine, purines, pyrimidines and aliphatic acids may either pass along a common pathway, or be under the control of a single gene. Some of the results described here were briefly reported elsewhere (Charles & Broadbent, 1964).

METHODS

Neurospora crassa wild-type strains Emerson 5297a and Emerson 5256A and mutants 30820, 46005, 37807, 66702, 35402 and 55901 were kindly provided by Professor D. G. Catcheside, F.R.S. All other mutants, except those isolated in this department, were kindly provided by Mr W. Ogata of the Fungal Genetics Stock Center, Hanover, New Hampshire, U.S.A.

The medium used was the minimal medium of Ryan, Beadle & Tatum (1943) containing 0·5% sucrose and 0·5% sorbose, and solidified with 1% Difco Bacto agar. A sorbose solution was autoclaved (120°, 10 min.) separately and added to the molten medium to give the required concentration immediately before the medium was distributed into Petri dishes. Unless otherwise stated, supplements were added to final concentration 30 mg./l., except that acetate was added to final concentration 500 mg./l.

Crosses of Neurospora were incubated on slopes of the medium of Westergaard & Mitchell (1947). The slopes were inoculated with one mating type and incubated for 10 days at 25°, during which time protoperithecia formed. These were then fertilized with a suspension of conidia of the opposite mating type. Ascospores were germinated by heating them at 60° in sterile distilled water for 45 min. Newmeyer's (1954) rapid plating method for genetical analysis was used to determine the frequency of wild-type recombinants arising from crosses.

Conidia were obtained from slope cultures grown on the medium of Horowitz (1947) for 3 days at 30°. The conidia were quickly rinsed from the surface of the culture with sterile distilled water, and the resulting suspension was passed through a pad of sterile glass wool to remove mycelial fragments and clumps of conidia.

The auxanographic method (Pontecorvo, 1949) was used to determine the nutritional requirements of mutant strains. Usually a suspension of conidia from the mutant to be tested was added to 500 ml. molten agar medium held at 45°. The medium was then immediately distributed in 10 ml. volumes into Petri dishes. The final conidial concentration was usually about 2000/ml. When it was desired to incubate conidia in CO_2-free air they were sometimes spread on the surface of the solidified medium, rather than being dispersed throughout the medium, because other experiments with yeasts indicated that this method allowed more efficient removal of CO_2 by adjacent potassium hydroxide solutions. In preliminary work unsterilized crystals of various compounds to be tested were used in the auxanographic experiments. Important conclusions were confirmed by testing sterile solutions (pH 5·4) of appropriate substances.

The gas-phase surrounding the Petri dish cultures was controlled by incubating the cultures in 5 l. Pyrex vacuum desiccators, as follows. A shelf of $\frac{1}{2}$ in. mesh wire netting was placed over the well of the desiccator. The Petri dishes, with lids removed and with the exposed surface of the medium facing downwards, were placed in layers of three in the desiccator, with the bottom layer of three Petri dishes resting on the wire gauze. Each Petri dish in the succeeding layers was placed to rest equally on two of the Petri dishes in the layer below it, so that diffusion of gases should not be obstructed. When necessary, a gas-phase free from CO_2 was obtained by placing 100 ml. of 25 % (w/v) potassium hydroxide in a beaker in the well of the desiccator, with a wad of glass wool partly immersed in the potassium hydroxide solution to increase the area of the CO_2-absorbing surface. CO_2 pressures greater than that in air were obtained by removing air from the desiccator until an appropriate decrease in pressure was recorded on a mercury manometer, and then allowing CO_2 to enter the desiccator until atmospheric pressure was restored. Restoration of atmospheric pressure was seen by the swelling of an inflatable polythene bag placed in the gas line between the CO_2 generator and the desiccator. CO_2 was generated by the action of dilute hydrochloric acid on marble chips in a Kipp's apparatus, and was passed through a saturated solution of sodium bicarbonate before it was passed into the desiccator. The gas-phase in the desiccators was renewed every 24 hr.

Growth stimulations observed in the auxanographic experiments are described as 'good' or 'weak'. Stimulations which resulted in growth as vigorous as that of wild-type Neurospora are described as 'good'. Stimulations which were obvious but which gave growth definitely weaker than that of wild-type Neurospora grown under the same conditions are described as 'weak'. All auxanographic experiments were scored by eye; our conclusions were not checked by more objective methods because of the difficulty of controlling the composition of the gas-phase in growth tubes and conical flasks.

RESULTS

CO_2 mutants of Neurospora were sought by two methods, both of which were successful. First, auxotrophs were obtained from other workers and tested to see whether they grew on unsupplemented medium when the gas-phase contained 30 % (v/v) CO_2 and did not grow on unsupplemented medium in CO_2-free air. Secondly, experiments were done to see whether CO_2 mutants could be isolated as such from wild-type Neurospora by using a modification of the filtration-enrichment method (Woodward, de Zeeuw & Srb, 1954; Catcheside, 1954). This second method depended on the fact that wild-type conidia grew vigorously in minimal liquid medium swept through with a current of CO_2-free air, whereas conidia from CO_2 mutants did not, even when in mixed suspension with wild-type conidia. Thus the wild-type conidia, once they had produced hyphae, could be removed from the suspension by filtration through a glass-wool pad. This resulted in a considerable increase in the proportion of CO_2 mutants remaining in the suspension. This method was tested and found effective with mixed ultraviolet (u.v.)-irradiated suspensions of conidia from wild-type Neurospora and from CO_2 mutant 33442. The most effective procedure was then applied to suspensions of conidia from wild-type Neurospora. Conidia were obtained from the Emerson wild-type strains; in some experiments strain 5297a was used, and in others strain 5256A. The u.v.-irradiation

was such as to kill about 97 % of the conidia. The method for isolating CO_2 mutants, and the mutants obtained by it, will be described in a later paper.

CO_2 mutants at the arginine-11 locus

Growth requirements of mutant 30820 (arg-11). The first CO_2 mutant discovered in the present series of experiments was mutant 30820. When the gas-phase contained more than 2 % (v/v) CO_2 this mutant grew almost as vigorously on minimal medium as did wild-type Neurospora. In the absence of CO_2 no growth was observed even after incubation for 9 days, whereas wild-type Neurospora showed vigorous growth within 1 day. Mutant 30820 was obtained by Beadle & Tatum (1945) in their early experiments with Neurospora; it was studied in detail by Srb (1950), who reported that it required arginine (or citrulline) + *either* a purine *or* a pyrimidine.

Table 1. *Growth of the arginine-11 mutant 30820 of Neurospora crassa on various supplemented media in different gas-phases with respect to CO_2*

	Gas-phase			
	CO_2-free	Ordinary air	Air containing 1–2 % (v/v) CO_2	Air containing 30 % (v/v) CO_2
Supplement to minimal medium	Relative amounts of growth			
None	0	±	+ to + +	+ + +
Adenine* *and/or* uridine†	0	±	+ to + +	+ + +
Arginine‡	0	+	+ + +	+ + +
Arginine + uridine *or* arginine + adenine	0	+	+ + +	+ + +
Arginine + adenine + uridine	+ + +	+ + +	+ + +	+ + +

* Or adenosine, adenosine-5′-phosphate; † or cytidine, uracil; ‡ or citrulline.

The only other reference to the nutritional requirements of mutant 30820 in the literature is by Perkins (1959), who reported a personal communication by Newmeyer that 'for optimal growth arginine-11 (30820) requires arginine (or citrulline) plus *both* a purine *and* a pyrimidine'. Since we suspected that even the small amount of CO_2 present in ordinary air (0·02–0·03 %, v/v) affected the nutritional requirements of mutant 30820, experiments were done to see whether this mutant had different nutritional requirements depending on whether the gas-phase was CO_2-free air or air containing CO_2. In these experiments, the conidia of the mutant were spread on the surface of solid minimal medium, rather than being dispersed throughout the medium.

The results of these experiments are given in Table 1. When the CO_2 content of the gas-phase was increased the nutritional requirements of the mutant became simpler. In CO_2-free air the mutant grew only when arginine + a purine + a pyrimidine were present in the medium; growth was then as vigorous as that of wild-type Neurospora. In ordinary air (0·02–0·03 %, v/v, CO_2) arginine and citrulline separately were stimulatory, as established by Srb (1950), but the stimulations were very weak and growth only became visible after 9 days. For vigorous growth in ordinary air the mutant still required arginine + a purine + a pyrimidine. In air containing 1–2 % (v/v) CO_2 arginine (or citrulline) gave fair growth and arginine +

either a purine *or* a pyrimidine gave vigorous growth. When the gas-phase contained more than 2% (v/v) CO_2 the conidia grew vigorously on minimal medium and stimulations were difficult to detect by the auxanographic method. In the absence of arginine, purines and pyrimidines separately or together were not stimulatory under any of the gas-phases. Thus the nutritional requirements of mutant 30820 are not fully expressed when the gas-phase is ordinary air because the small quantity of CO_2 in the air partially satisfies the requirements of the mutant.

Table 2. *Effect of various substances on the growth of Neurospora crassa mutant 44601*

Stimulatory		Not stimulatory
Sodium succinate	2-Oxoglutarate	Sodium citrate
Sodium lactate	Tween 80	Glycollic acid
Sodium acetate	Oleic acid	Sodium pyruvate
Sodium hydrogen malate	Caprylic acid	DL-Isocitrate lactone
Sodium fumarate	Glycerol	Glyoxylate
Oxaloacetate	DL-Aspartate	
Stearic acid	Sodium hydrogen glutamate	
Lauric acid	Sodium propionate	
	DL-α-Aminobutyrate	

Growth requirements of mutant 44601. One of the most interesting mutants which we found to be a CO_2 mutant was mutant 44601, from the original experiments of Beadle & Tatum (1945). This mutant has not been described in the literature, except in a recent stock culture list of the Fungal Genetics Stock Center where it was described as requiring arginine + adenine (Barratt & Ogata, 1963). When the gas-phase was CO_2-free air, the nutritional requirements of mutant 44601 were as follows. It did not grow on unsupplemented medium. On medium supplemented with arginine + adenine it grew slowly, growth becoming visible after 3 days. When the medium was supplemented with arginine + a purine + a pyrimidine growth was more vigorous but not nearly as vigorous as that of mutant 30820 or of wild-type strains under these conditions, or as that of mutant 44601 on unsupplemented medium when the gas-phase contained 2% (v/v) CO_2. For most vigorous growth in CO_2-free air mutant 44601 required acetate in addition to arginine + a purine + a pyrimidine. Vigorous growth did not occur when any one of these four substances was omitted from the medium. Acetate could be replaced by many other substances, most of which were carboxylic acids, although glycerol was also stimulatory. A list of active and inactive substances is given in Table 2. It was concluded from the auxanographic experiments that mutant 44601 had the same nutritional requirements in CO_2-free air as mutant 30820, except that it also required a carboxylic acid. Experiments such as the one shown in Fig. 1 confirmed this conclusion.

Evidence for allelism of mutants 30820 and 44601. The next question to be investigated was whether the mutations in mutants 30820 and 44601 were allelic. The mutants were of different mating type, and a cross between them was fertile. In one experiment 102 ascospores were isolated separately into tubes of complete medium and heat-activated; 70 of these ascospores gave rise to cultures, of which 31 had the same nutritional requirements as mutant 30820 and 39 had the same nutritional requirements as mutant 44601. None of the cultures was wild type, and this suggested that the mutations were allelic. In a subsequent experiment 2500 ascospores were heat-activated and plated on unsupplemented medium. More than

2000 of these ascospores germinated, but not one gave a wild-type colony. The two mutations are therefore almost certainly allelic, unless chromosome abnormalities distorted the segregation. It thus became of interest to know whether the additional requirement for a carboxylic acid shown by mutant 44601 was attributable to its having a different allele at the *arg-11* locus or a second mutation not present in

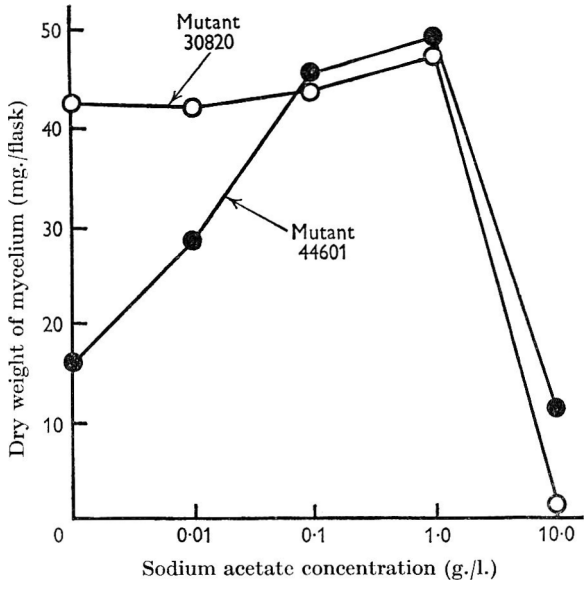

Fig. 1. The effect of sodium acetate on the growth of Neurospora mutants 30820 and 44601. Incubation at 25° for 7 days in 20 ml. liquid medium containing arginine + adenine + uridine (all at 30 mg./l.) + various amounts of sodium acetate. Medium held in 150 ml. conical flasks. Incubation without shaking in a gas-phase of ordinary air (0·03%, v/v, CO_2).

mutant 30820. A cross between mutant 30820 and the wild-type strain Emerson 5297a yielded 50% of auxotrophic segregants, all of which had the same nutritional requirements as mutant 30820. From this cross 97% of the ascospores germinated. A cross between mutant 44601a and wild-type strain Emerson 5256A also yielded about 50% auxotrophic segregants. However, these segregants differed amongst themselves, some requiring a carboxylic acid, others not, although all required arginine + adenine + uridine.

The data from this second cross were as follows: of 228 ascospores transferred individually to culture tubes and heat-activated, 212 germinated (93%); 183 of the resulting cultures were chosen at random and tested further, of these 94 grew on minimal medium in CO_2-free air and were assumed to be wild type; the remaining 89 were auxotrophs which grew on minimal medium only when the gas-phase contained 30% (v/v) CO_2. The nutritional requirements of the 89 auxotrophs were determined auxanographically in CO_2-free air: 41 had the same nutritional requirements as mutant 30820, and did not require acetate; 29 had the same nutritional requirements as mutant 44601, requiring acetate + arginine + adenine + uridine; 19 were intermediate types in that they grew to varying extents when supplied with arginine + adenine + uridine and were further stimulated by acetate. This cross did not yield any segregants which required acetate alone.

It is concluded that the mutations in mutants 30820 and 44601 are allelic and that the carboxylic acid requirement of mutant 44601 is caused by its genetic 'background', which apparently causes a requirement for carboxylic acid only in the presence of the 44601 (and presumably the 30820) mutation. It is quite possible that these mutant strains have different genetic backgrounds, because Beadle & Tatum (1945) obtained mutant 30820 from a cross of the wild type strain 1 A with X-ray-treated conidia from the wild type strain 19a, and they obtained mutant 44601 from a cross of the wild type strain Abbott 4 with ultraviolet-irradiated conidia from the wild type strain 25a.

Table 3. *Adenine mutants of Neurospora crassa which are not CO_2 mutants*

Locus	Mutant no.
ad-1	3254
ad-3*A*	38709
ad-3*B*	Y 112-M2
ad-4	44206
ad-5	71104
ad-6	28610
ad-7	44411
ad-8	Y 193-M22

CO_2 mutants responding to adenine

We suspected that some adenine-requiring mutants might also be CO_2 mutants. Accordingly, mutants representative of seven of the nine known adenine loci were obtained and tested; they are listed in Table 3. None was a CO_2 mutant. However, from our own mutant-isolation experiments we obtained two CO_2 mutants (RE 25, RE 53) which responded to adenine in the absence of CO_2. Both these mutants had the same nutritional requirements and were probably allelic since they did not complement in heterokaryons. Without CO_2 they grew with: adenine, adenosine-5'-phosphate; adenosine; hypoxanthine + histidine; inosine + histidine; they did not grow with hypoxanthine, inosine or histidine separately. A cross between the CO_2 mutant RE 25 and the adenine-3 mutant Y 112-M2 gave no wild-type recombinants from 22,000 ascospores of which more than 90 % germinated. This indicated that the RE 25 mutation was at the adenine-3 locus, and agreed with evidence that the RE 25 mutation was further from the mating type locus than was the arginine-3 locus. However, as shown above, two authentic adenine-3 mutants were not CO_2 mutants. All known adenine-3 mutants produce a purple pigment when the concentration of adenine is insufficient for rapid growth, and high temperatures enhance production of this pigment (Mitchell & Houlahan, 1946; De Serres, 1956, 1958). Conidia of the adenine mutants RE 25 and Y 112-M2 were therefore incubated at 37°, without shaking, in 20 ml. volumes of liquid medium supplemented with a suboptimal concentration of adenine (10 mg./l.). Mutant Y 112-M2 produced large amounts of purple pigment but mutant RE 25 did not produce pigment.

CO_2 mutants responding to succinic acid

The carboxylic acid requirement of mutant 44601 was especially interesting because two CO_2 mutants (RE 50, RE 56) from our mutant-isolation experiments were 'succinate mutants' and required any one of the carboxylic acids and similar

substances which stimulated mutant 44601 (see Table 2) except aspartate, glutamate, 2-aminobutyrate and glycerol. These 'succinate' mutants did not require arginine or a purine or a pyrimidine, nor were they inhibited by these substances. In their nutritional requirements they resembled the 'succinate' mutants studied by Lewis (1948) and Strauss (1956, 1957). They responded to several long-chain fatty acids; there is no record in the literature of the effect of these fatty acids on other succinate mutants. Only one succinate locus is recorded on the linkage maps of Barratt, Newmeyer, Perkins & Garnjobst (1954), although Lewis (1948) reported that one succinate mutation (39311) was on the other side of the centromere from the locus of three other succinate mutants, so there may be more than one succinate locus in Neurospora. So far we have not obtained a culture of mutant 39311, but we have obtained cultures of the allelic succinate mutants 35402, 37807, 46005, 55901 and 66702. When tested in one experiment these mutants did not respond to 30 % (v/v) CO_2. Mutant 66702 was crossed with one of our succinate CO_2 mutants (RE 50) to see whether the mutations were allelic. Sixty-six ascospores were isolated at random; all of the resulting 66 cultures were succinate mutants and, surprisingly, all were CO_2 mutants, although only about half should have responded to CO_2. This problem was resolved when further experiments showed that the succinate mutant 66702 was a CO_2 mutant, as were the other succinate mutants 35402, 55901, 37807, and 46005. We conclude that our succinate CO_2 mutant RE 50 is allelic with, and probably identical with, the succinate mutants 35402, 46005, 37807, 55901 and 66702. The failure of these five mutants to respond to CO_2 in the first experiment is unexplained. In several subsequent experiments these five mutants have all grown vigorously on minimal medium when the gas-phase contained 30 % (v/v) CO_2. On minimal medium in CO_2-free air mutants 35402, 55901 and 46005 showed no growth, and mutants RE 50, RE 56, 37807 and 66702 showed slow ('leaky') growth.

CO_2 mutants responding to arginine

It has been shown that another arginine mutant (33442) is a CO_2 mutant (Reissig & Nazario, 1962; Charles & Broadbent, 1964). This mutant is not allelic with the first arginine CO_2 mutant (30300), being sited at the arginine-2 locus on chromosome IV (Barratt *et al.* 1954). The characters of mutant 30300 have already been considered in detail (Charles, 1964); mutant 33442 is phenotypically indistinguishable from mutant 30300. Both mutants grew vigorously when the gas-phase was air + 30 % (v/v) CO_2, or when the medium contained arginine or citrulline. Pyrimidines inhibited the response to CO_2 but were not inhibitory when the mutants were growing on arginine or citrulline; that is, arginine and citrulline non-competitively overcame the inhibitory effect of pyrimidines. Our mutant-isolation experiments have yielded eleven arginine-requiring CO_2 mutants. Nine of these have been tested in complementation and crossing experiments. Three of them have mutations at the arginine-2 locus, and 6 have mutations at the arginine-3 locus. This suggests that CO_2 mutants may not occur at other arginine loci, except of course at arginine-11 (30820), which is not strictly an arginine locus. Our isolation experiments have not yielded any arginine-11 mutants.

DISCUSSION

The first question to be considered is whether the new CO_2 mutants reveal anything about the mechanism whereby 30 % (v/v) CO_2 in the gas-phase causes the mutants to grow. The only biosynthetic processes known to require carbamoyl phosphate are the syntheses of arginine and of pyrimidines. When only arginine-requiring and pyrimidine-requiring mutants were known to respond to CO_2 it seemed probable that CO_2 mutants might only be associated with biosynthetic pathways which incorporated CO_2 into carbamoyl phosphate. The discovery of many different CO_2 mutants (Charles & Broadbent, 1964), including the succinate-requiring mutants and the adenine-requiring mutants described here, shows that CO_2 mutants are not restricted to pathways which incorporate carbamoyl phosphate, unless carbamoyl phosphate takes part in more reactions than is at present realized.

One possible explanation of CO_2 mutants is that they may have modifications in enzymes which catalyse CO_2-incorporation reactions. Another possible explanation is that they may have enzymes which are altered in such a way that their activity is restored by CO_2 through some physicochemical effect. For example, CO_2 might alter the configuration of the protein produced by the mutant gene, either by combining with it or by altering the intracellular pH value. According to this kind of explanation CO_2 mutants may occur in any metabolic pathway, regardless of whether the pathway has a CO_2-incorporation step. Since several of our CO_2 mutants, especially the arginine mutants at two loci, have nutritional requirements which agree with their having blocks in a CO_2-incorporation process, it is unnecessary to invoke such explanations at present. The adenine-requiring CO_2 mutants are particularly instructive since they are at the adenine-3 locus, which is concerned with two consecutive reactions of purine biosynthesis, the first of these being the reaction of CO_2 or bicarbonate with 5-aminoimidazole ribotide to form 5-amino-4-carboxy-imidazole ribotide (Buchanan, 1960).

Assuming that CO_2 mutants are restricted to CO_2 incorporation reactions, the next question to be considered is how it is that high pressures of CO_2 cause the mutants to grow. One possibility is that the enzymes retain some activity which is expressed in the presence of high concentrations of their substrate, in this case CO_2. Another possibility is that high pressures of CO_2 permit alternative pathways to function. The large number of CO_2 mutants that we have found raises doubts about the general validity of hypotheses which invoke alternative pathways, since it is necessary to invoke so many alternative pathways, unless one pathway may serve as an alternative pathway for several different CO_2 mutants. Since three of the new kinds of mutants described here, and several described elsewhere (Charles & Broadbent, 1964), do not show specific inhibitions by any well-known metabolites, it is in any case unnecessary to invoke alternative pathways to explain these properties. At present, it is not possible to come to a definite conclusion about the nature of CO_2 mutants. We adopt the working hypothesis that CO_2 mutants have damaged mechanisms for incorporating CO_2 and that high CO_2 pressures force CO_2 down alternative pathways which overflow and provide CO_2 or a derivative of CO_2 to the damaged pathway. If the alternative pathway supplies unchanged CO_2, it may either be a pathway having a CO_2-evolving reaction, or be some kind of CO_2-transporting system.

Whatever hypotheses are adopted to explain CO_2 mutants, it is difficult to understand how CO_2 mutants may occur at all if CO_2, which is presumably a product of metabolic processes, has free access to all parts of the protoplasm, because this waste CO_2 should have the same effect as exogenous CO_2 supplied in the gas-phase. One possibility is that CO_2 formed as a product of metabolism may be eliminated from the cell without having direct access to CO_2-fixing reactions, and the CO_2 required for the CO_2-fixation reactions may be brought into the cell by one or more special transport systems. Alternatively, particular CO_2-incorporating reactions may have access to the CO_2 from particular CO_2-evolving reactions only.

Considering other problems raised by the CO_2 mutants, the first question of interest is why CO_2 mutants occur at two different arginine loci. Both mutants have the same nutritional requirements, in that they respond to arginine or citrulline but not to ornithine, which suggests that both are unable to form carbamoyl phosphate from CO_2. The occurrence of two genes which control the synthesis of carbamoyl phosphate for arginine synthesis may be explained in two ways. First, there may be two steps in the proposed arginine-specific pathway for synthesis of carbamoyl phosphate. Secondly, both genes may affect the same enzyme, in which case one gene might determine the structure of the enzyme, and the other gene might be a 'regulator' gene which controls the 'structural' gene.

The significance of CO_2 mutants in the adenine-3 region has already been mentioned. De Serres (1956, 1964) gave evidence that there are two separate loci (*ad-3A*, *ad-3B*) in the adenine-3 region, separated by an unknown region of the chromosome. It is not known which adenine-3 locus is concerned with the CO_2-incorporation reaction. Whichever locus the CO_2 mutations are at, it is clear that that locus must also give mutants which are not CO_2 mutants, because neither of the *ad-3A* and *ad-3B* mutants in Table 3 were CO_2 mutants. This recalls the situation at the pyr-3 locus, where some mutants are CO_2 mutants and others not (unpublished experiments with Professor V. W. Woodward). De Serres (1963) showed that at the ad-3B locus there are two complementation groups, one of which is composed of leaky mutants. Since our mutants are leaky in air (but not in CO_2-free air) it is possible that the CO_2 mutants occur in the 'leaky' ad-3B complementation group.

The mutants responding to carboxylic acids pose an interesting problem when one considers the possible nature of the lesions in them, because of the numerous reactions in which carboxylic acids are involved. Since the mutants grow vigorously with sucrose as carbon source when the gas-phase contains 30% (v/v) CO_2 we assume that the tricarboxylic acid cycle still operates in them. It is known that the operation of the tricarboxylic acid cycle does not generate a supply of 4-carbon substances for syntheses and that for this the cell is dependent upon CO_2-fixation reactions (Wood & Stjernholm, 1962). Perhaps one of these CO_2-fixation reactions is damaged in the 'carboxylic acid' mutants. High CO_2 pressures may therefore bring about effective synthesis of a 4-carbon substance by the damaged pathway, or by an alternative CO_2-incorporation pathway, several of which are known. The response to acetate is not difficult to understand because in many organisms acetate is known to be metabolized via the glyoxylate cycle, the net effect of which is to generate succinate from acetate. These 'carboxylic acid' mutants may be identical with the succinate mutants studied by Lewis (1948) and Strauss (1956,

1957). Strauss (1957) presented evidence that the succinate mutants were deficient in a mechanism for fixation of CO_2 into oxaloacetic acid but he did not investigate the effect of increased CO_2 pressures.

Perhaps the most interesting mutants described here are mutants 30820 and 44601. A possible explanation of why mutant 30820 has three requirements is that its mutation is a deletion covering separate arginine, purine and pyrimidine loci; but this seems unlikely because neither purine or pyrimidine loci are known from the 30820 region. An arginine locus (*arg*-10) is very close to the 30820 locus, but mutation at this locus leads to a requirement for arginine, not citrulline (Newmeyer, 1957). We conclude that a single gene at the 30820 locus is concerned with the biosynthesis of arginine, purines and pyrimidines. An obvious suggestion is that the gene controls the supply of CO_2 to all three pathways; the mutant may require a high CO_2 pressure either because it has an enzyme or permease with a decreased affinity for CO_2, or because the high CO_2 pressure brings an alternative pathway into operation. Although the gene at the 30820 locus may be concerned with three biosynthetic pathways, this does not necessarily mean that the three pathways share a common mechanism for providing CO_2. The gene may be a regulator gene which controls the action of separate systems supplying CO_2 to each pathway. If so, the mutations in the separate arginine, pyrimidine and purine CO_2 mutants might be mutations in 'structural' genes concerned with the individual systems. If an explanation of this type is rejected it becomes difficult to explain how distinct arginine, pyrimidine and purine mutants, as well as mutants which require all three substances, are all able to respond to CO_2.

Our experiments show that the 44601 mutation is allelic with the 30820 mutation and that the carboxylic acid requirement of mutant 44601 is attributable to the genetic background. Barratt & Ogata (1964) also stated that the 44601 and 30820 mutations are allelic, but they gave no further details. The significant facts about the carboxylic acid requirement are as follows: it is only expressed when the 44601 and presumably the 30820 mutation is present; it is satisfied by CO_2; in the absence of CO_2 almost the same array of substances satisfy this requirement as satisfy the requirements of the carboxylic acid mutants described above. It therefore appears that in certain genetic backgrounds not only the supply of CO_2 to the arginine, pyrimidine and purine pathways, but also the supply of CO_2 for one mechanism of carboxylic acid synthesis is under the control of the 30820/44601 locus.

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Growth of *Staphylococcus aureus* in Media of Restricted and Unrestricted Inorganic Iron Availability

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SUMMARY

A new procedure for the removal of iron from complex media is given. With this method, the iron requirements for the growth of *Staphylococcus aureus* in casein hydrolysate medium were investigated. Trypticase medium (0·6–0·8 μg. Fe/ml.) was depleted of iron by treatment with the specific iron-chelator bathophenanthroline; on analysis it was shown to contain 0·01–0·02 μg. Fe/ml. Preliminary studies showed that *S. aureus* grew well in the iron-depleted medium. Further iron restriction of the already depleted medium was accomplished by the addition of conalbumin, a specific iron-chelating protein present in egg white. The initiation of growth, rate of growth, and total crop were all dependent on the concentration of free iron in the medium. The availability of iron was a function of the percentage iron saturation of the conalbumin.

INTRODUCTION

Of the several roles that inorganic compounds in nutrient media may play in the growth of micro-organisms Knight (1936) gave prominent place to the participation of 'elements required as catalysts, or in the manufacture of enzymes for essential growth processes'. Among the metals, iron is an essential element for this purpose in the vast array of micro-organisms whose metabolism involves the use of the cytochrome system for terminal oxidation, catalase, peroxidase, and other iron-containing catalysts. *Bacillus pycnoticus* (Ruhland, 1924), *Azotobacter vinelandii* (Horner & Burk, 1934), *Aerobacter aerogenes*, *Pseudomonas aeruginosa*, *Serratia marcescens*, etc. (Waring & Werkman, 1943), *Escherichia coli* (Ratledge & Winder, 1964) and *Nocardia opaca* (Webley, 1960) are among those organisms whose growth response to the concentration of available inorganic iron in the culture media have been studied in detail. For these studies, well defined simple salts media with sugars or glycerol as carbon and energy sources have generally served as culture media and have lent themselves to procedures for depleting the available iron (Donald, Passey & Swaby, 1952) to low values so that growth responses to measured iron additions could be examined.

Staphylococcus aureus is inhibited in growth in nutrient broth containing a measured amount of hen egg white (Schade & Caroline, 1944). The addition of inorganic iron to such a medium in an amount sufficient to saturate the iron-chelating egg-white protein conalbumin (Alderton, Ward & Fevold, 1946) over-

comes this inhibition completely. The growth-inhibitory properties of conalbumin through its specific iron chelation are paralleled by those of siderophilin (transferrin), the iron-binding β_1 globulin of human and other mammalian sera (Schade & Caroline, 1946; Laurell, 1947). Because of this capacity for specific iron chelation in the serum, siderophilin can function not only as a transporter of iron in the blood but also as a non-specific immunity factor (Schade, 1960). In the latter connexion it has been shown (Schade, 1963) that the rate of aerobic growth of *S. aureus* in human serum as a culture medium is a function of the percentage iron saturation of the siderophilin present.

To aid an examination of the growth and metabolism of *Staphylococcus aureus* grown in a readily prepared complex medium under conditions approximating those found in blood serum, we have used an iron-depleted casein digest medium supplemented with egg white as a serum substitute. To this medium we have added known amounts of inorganic iron to realize several degrees of iron restriction, for comparison with conditions of unrestricted availability of iron. The present paper gives a simple method for the removal of iron from a complex medium and reports the results of growth studies on the iron requirements of *S. aureus* grown in this medium.

METHODS

Organism. A penicillin-resistant, coagulase-positive strain of *Staphylococcus aureus*, phage type 80/81, was used throughout. Stock cultures on nutrient agar (Difco) slopes were refrigerated and subcultured every 2 months.

Media. The basal medium contained: pancreatic digest of casein (Trypticase, Baltimore Biological Laboratories,), 20 g.; biotin, 110 μg.; nicotinic acid, 50 μg.; thiamine hydrochloride, 50 μg.; double glass-distilled water to 1 l., adjusted to pH 7·6 with NaOH, autoclaved (20 min, 121°). On final analysis (Schade, Oyama, Reinhart & Miller, 1954), this medium was found to contain 0·6–0·8 μg. iron/ml. Iron-depleted medium was prepared with a trypticase solution (treated as described below) followed by the vitamin supplementation and adjustment of pH value to conform to the basal medium formula; the final iron concentration of this medium was 0·01–0·02 μg./ml. A 30 % (w/v) glucose solution sterilized by filtration was added as desired to media, to final concentration 2 g./l.

Egg white was removed aseptically from fresh eggs and added as required to media at 1 % (v/v) final concentration. One ml. egg white, through its content of conalbumin, bound approximately 20 μg. iron (Schade *et al.* 1954). A solution of ferrous ammonium sulphate ($Fe(NH_4)_2(SO_4)_2.6H_2O$, Fisher: C. P.), 0·1 % (w/v) with respect to iron, was prepared in 0·06 N-HCl containing 0·006 % (w/v) ascorbic acid, sterilized by filtration, and added to the iron-depleted medium in measured amounts as required. Media were dispensed in 50 ml. volumes in 300 ml. EDTA-rinsed, iron-free, sterile DeLong culture flasks (Bellco Glass, Inc., Vineland, N. J.).

Conditions of growth. Inocula were prepared from *Staphylococcus aureus* grown at 37° in 2 % iron-depleted trypticase medium without glucose or egg white for 12 hr. The resultant suspension had an extinction (600 mμ; E_{600}) of 0·3; this was roughly equivalent to 7×10^8 cocci/ml. This suspension was diluted 1/1000 in sterile saline and 0·3 ml. of dilution used as the inoculum/50 ml. medium. The culture flasks were incubated at 37° and shaken in a gyratory-incubator shaker (Model

G 25, New Brunswick Scientific Co., New Brunswick, N.J.) at a shaking rate of 270 rev./min. with an amplitude of 1 in. Growth was followed by periodic measurement of the extinction E_{600} of the culture with a model DU Beckman spectrophotometer. Cultures containing egg white were passed through a coarse sintered-glass filter before making the extinction measurements.

Procedure for iron removal. Methods of preparation of de-ironized media for microbiological purposes (Donald *et al.* 1952) have in general used inorganic precipitates, e.g. $CaCO_3$ and $Ca_3(PO_4)_2$, as trace element absorbers; specific iron-chelating chromogenic agents followed by separation of stable metal complexes by solvent extractions; biological agents, e.g. *Aspergillus niger*, as iron scavengers (Foster, 1949). The methods which use iron-chelating compounds followed by solvent extraction are the most desirable because of the specificity of the metal removed. However, when the media to be treated are complex (e.g. beef or yeast extract, peptones, brain heart infusion) the usual solvent extraction procedure with 8-hydroxyquinoline and chloroform (Waring & Werkman, 1942) becomes laborious and time-consuming (Rubbo, Albert, & Gibson, 1950). We recommend for reliability and simplicity the following procedure which we used to prepare an iron-depleted pancreatic digest of casein medium for these studies.

For the preparation of 4 l. of 2% (w/v) iron-depleted pancreatic digest of casein (Trypticase) solution the following steps were used.

(1) Trypticase (82 g.) was dissolved in double glass-distilled water to 820 ml. and adjusted to pH 3·3 with 22 ml. concentrated HCl.

(2) A stock solution of bathophenanthroline reagent was prepared by adding 500 mg. 4,7-diphenyl-1,10-phenanthroline to 25 ml. ethanol made 0·1 N with respect to HCl; to this was added 500 mg. ascorbic acid and the solution diluted to 100 ml. with water.

(3) To the acidified Trypticase solution (1) was added, while stirring and heating to 100°, the bathophenanthroline reagent solution in an amount about 120% of that required to bind all the ionic iron present (18 mg. bathophenanthroline will bind 1 mg. iron). Preliminary iron analysis of the concentrated Trypticase solution by any suitable method (e.g. Schade *et al.* 1954) indicated the number of ml. reagent solution needed.

(4) The treated mixture was cooled to room temperature and filtered with suction through 3 layers of Whatman no. 42 paper.

(5) The filtrate was twice extracted with 150 ml. of a mixture of isoamyl alcohol + benzene (50 + 50, by vol.) followed by two extractions with 150 ml. volumes of benzene.

(6) The extracted medium was filtered once through three layers of Whatman no. 42 filter paper and adjusted to desired pH value with saturated NaOH.

(7) The adjusted medium was diluted with double glass-distilled water to 4 l. This was used as the supply of 2% (w/v) Trypticase iron-depleted for further preparation of the media detailed above.

RESULTS

Growth of Staphylococcus aureus in iron-rich and iron-depleted Trypticase media

The growth characteristics of *Staphylococcus aureus* in Trypticase medium before and after iron depletion was first examined, growth in nutrient broth being used as a standard for comparison. Representative results are shown in Fig. 1. *S. aureus* grew well in the iron-rich Trypticase medium (0·67 μg. Fe/ml.) after a lag period of

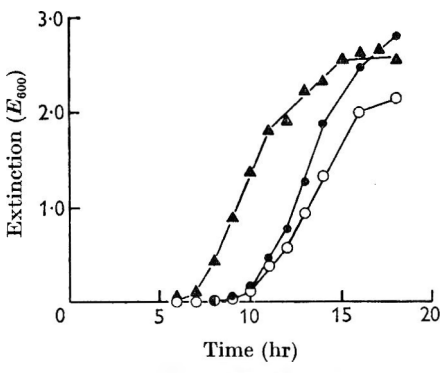

Fig. 1

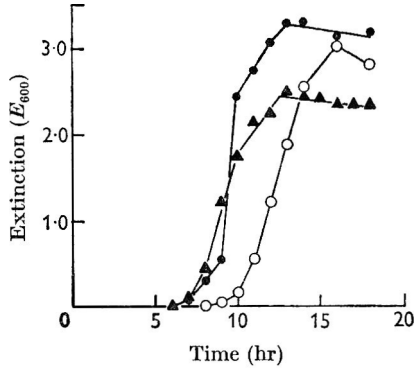

Fig. 2

Fig. 1. Growth of *Staphylococcus aureus* in nutrient broth (0·274 μg./ Fe/ml.), ▲; 2 % iron-rich Trypticase medium (0·67 μg. Fe/ml.), ●; and 2 % iron-depleted Trypticase medium (0·016 μg./ml.), ○.

Fig. 2. Same as Fig. 1, except 0·2 % glucose added to the media.

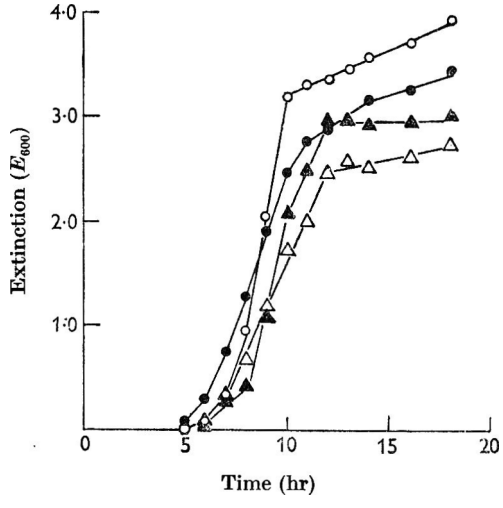

Fig. 3. Growth of *Staphylococcus aureus* in iron-rich and iron-depleted 2 % Trypticase media + 1 % (v/v) egg white, with and without added glucose (0·2 %). Iron-rich Trypticase + egg white (0·46 μg. Fe/ml. excess), ●; iron-rich Trypticase + egg white + glucose (0·46 μg. Fe/ml. excess), ○; iron-depleted Trypticase + egg white (0·037 μg. Fe/ml. excess), △; iron-depleted Trypticase + egg white + glucose (0·037 μg. Fe/ml. excess), ▲.

about 10 hr. In iron-depleted medium (0·016 μg. Fe/ml.) the lag period of growth was similar, but the rate of logarithmic growth and total crop were smaller. Cultures grown in nutrient broth showed a rate of logarithmic growth comparable to that found in iron-rich Trypticase medium, but there was a lag period of only 7 hr. This shortened lag period probably reflected the presence of some readily available carbon and energy sources in the nutrient broth. To test this the above experiments were repeated with 0·2 % (w/v) glucose added to the several media. The results (Fig. 2) show that glucose addition did decrease the lag period in the iron-rich Trypticase medium to that found with the nutrient broth, but did not affect the lag in the iron-depleted medium. The logarithmic growth rates were increased in all three of the glucose-supplemented media. Thus, the lag period and logarithmic growth rate of *S. aureus* in the Trypticase media were dependent on the available iron concentration and the presence of a readily utilizable carbon and energy source such as glucose. It appeared probable that under conditions of limited iron the initial synthesis or activation of iron-requiring enzymes was depressed, with consequent delay in growth response. Further decrease of available iron through the addition to Trypticase medium of an iron chelator should result in increase of the lag period and depression of the logarithmic growth rate.

Growth on iron-depleted Trypticase medium + egg white

To restrict further the amount of nutritionally available iron, the iron-depleted Trypticase medium with 1 % (v/v) egg white was added as a source of the specific iron-binding conalbumin. To this supplemented medium, measured amounts of iron were added to give known degrees (%) of saturation of the conalbumin present. The resulting conditions were similar to those used by Schade (1963) to study the growth of *Staphylococcus aureus* in human serum at different degrees of iron saturation of siderophilin.

Experiments to test the effect of added egg white itself on the growth of *Staphylococcus aureus* in Trypticase medium before and after treatment for iron removal, with and without added glucose, are summarized in Fig. 3. The iron in the untreated medium was sufficient to saturate the conalbumin of the 1 % egg white and to leave an excess of 0·46 μg. iron/ml.; to treated medium, iron was added (as ferrous ammonium sulphate) to provide an excess of 0·037 μg. Fe/ml. Comparison of the appropriate growth curves in Figs. 1 and 2 indicates that no inhibition of growth was occasioned by egg white in any of the four media when iron was present in excess. On the contrary, in the media without added glucose, some enhancement of the logarithmic growth rates resulted and the lag period of about 10 hr in Trypticase medium alone (Fig. 1) was decreased to 5 hr. This decrease in lag period might have been due in part to the glucose introduced into the medium by the egg white; on analysis (Glucostat, Worthington Biochemical Corp., Freehold, N.J.), this was found to contain 10 mg. glucose/ml. egg white. The logarithmic growth rates of the cultures in treated and untreated media with egg white were comparable.

Having established the suitability of egg white-supplemented iron-depleted Trypticase as a growth medium for *Staphylococcus aureus* when iron was added to excess, the growth was examined when the absolute iron concentration was less than that required to exceed the iron-binding capacity of the conalbumin content.

Table 1 shows the concentration of inorganic iron in the media prepared and the various resultant percentage iron saturations of conalbumin. With the exception of the medium used as the control in which the conalbumin was saturated to excess (118 %), the only iron available to the organism was that which was present as free iron in equilibrium with the iron + conalbumin complex,

$$(2\mathrm{Fe} + \text{conalbumin} \rightleftarrows \text{conalbumin}.\mathrm{Fe}_2).$$

Conalbumin has an extremely high affinity for iron and forms a very stable complex; the dissociation constant of the complex is not precisely known but has been estimated to be in the range of 10^{-28} to 10^{-30} M at pH 7·6 (Warner & Weber, 1951). The relative amounts of free ionic iron available in the several media at different percentage iron saturations can be calculated by use of an equation based on conalbumin having two dependent iron-binding sites (Schade, 1963). Table 1, last column, gives the values when the 8 % saturation value is used as a baseline for comparison with the other percentage saturation values.

Table 1. *Egg white-supplemented, iron-depleted 2 % Trypticase media at different percentages iron saturation of conalbumin*

Absolute Fe concentration (μg. Fe/100 ml. medium)	% Fe saturation of conalbumin (20·7 μg. Fe/100 ml. medium = 100 %)	Relative amounts of available Fe (8 % saturation as base)
1·6	8	1
3·7	18	1·59*
5·74	28	2·12
11·95	58	4
18·16	88	9·2
20·23	98	23·8
24·37	118	∞

* As $[\mathrm{Fe}] = \sqrt{\left(\mathrm{K}\frac{[\text{conalbumin}.\mathrm{Fe}_2]}{[\text{conalbumin}]}\right)}$, then by substitution for 18 % saturation,

$[\mathrm{Fe}] = \sqrt{\left(\mathrm{K}\frac{18\,\%}{82\,\%} \text{ or } 0{\cdot}468\sqrt{\mathrm{K}}\right)}$.

For 8 % saturation, $[\mathrm{Fe}] = \sqrt{\left(\mathrm{K}\frac{8\,\%}{92\,\%} \text{ or } 0{\cdot}295\sqrt{\mathrm{K}}.\frac{0{\cdot}468\sqrt{\mathrm{K}}}{0{\cdot}295\sqrt{\mathrm{K}}} = 1{\cdot}59\right)}$.

The results of growth studies with the above media are shown in Fig. 4. The character of the growth patterns observed was markedly affected by increasingly restricted amounts of iron available to *Staphylococcus aureus* through the dissociation of the iron + conalbumin complex at decreasing percentages of its iron saturation. In the media where the conalbumin was 98, 88, and 58 % iron-saturated, almost normal growth curves were obtained, with increasingly longer lag periods and lower logarithmic growth rates associated with the lower saturation values. When the percentage saturation values were 28, 18, and 8, the cultures did not show a normal growth pattern, but grew at constant, increasingly smaller rates as the percentage iron-saturation of conalbumin decreased. Considering that the growth of *S. aureus* in the medium whose conalbumin was 88 % saturated approached that in medium containing an excess of iron (118 % saturation), and that the concentration of available iron in the former case is probably of the order of only 10^{-28} M, it is

clear that very low iron concentrations satisfy the normal nutritional needs of this organism. It is remarkable that, under the specific growth conditions used, the 88% iron-saturated conalbumin medium offered so critical a concentration of available iron that an additional fourfold decrease in available iron concentration (28% iron-saturation) effectively altered the normal growth pattern and the growth rate. Additional evidence that the availability of iron from the iron + conalbumin complex is a function of the percentage iron-saturation of conalbumin was given by Schade (1963), who showed that *S. aureus* grown in given media in which the conalbumin was 10, 50, and 95% iron-saturated contained 0·97, 2·24 and $12{\cdot}7 \times 10^4$ iron atoms, respectively, per coccus.

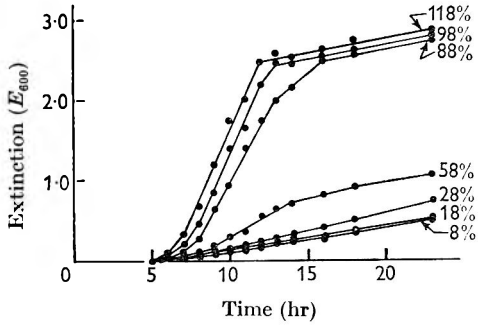

Fig. 4

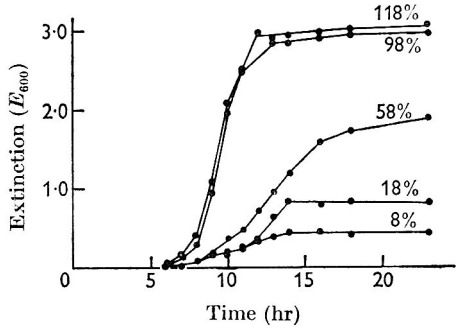

Fig. 5

Fig. 4. Growth of *Staphylococcus aureus* in 1% (v/v) egg white-supplemented, 2% Trypticase iron depleted media at different percentages iron-saturation of conalbumin.

Fig. 5. Same as Fig. 4, except 0·2% glucose added to the media.

Table 2. *Measurements of pH values of cultures of Staphylococcus aureus grown in 1% egg white-supplemented, iron-depleted 2% Trypticase media, with and without glucose, at different percentages iron-saturation of conalbumin*

		Time (hr)							
	% Fe-saturation	8	9	10	11	12	13	14	15
		pH value							
With glucose	8	7·45	7·62	7·39	7·53	7·22	6·74	5·89	5·46
	18	7·45	7·38	7·58	7·57	7·36	7·02	6·16	5·5
	58	7·4	7·21	7·03	6·62	6·07	5·77	5·85	5·97
	98	7·17	6·55	5·87	5·57	5·61	5·67	5·73	5·82
	118	7·03	6·23	5·67	5·57	5·63	5·69	5·76	5·83
Without glucose	8	7·7	—	7·6	—	—	—	7·61	—
	118	7·55	—	7·67	—	—	—	8·1	—

When 0·2% glucose was added to several of the media given in Table 1 (8, 18, 58, 98, 118% iron-saturated) inoculated with *Staphylococcus aureus*, and incubated at the same time as those without added glucose, the results shown in Fig. 5 were obtained. Glucose addition increased the growth rates in all the cultures but most markedly in those in which the conalbumin was 58, 98, and 118% iron-saturated; of particular note is the delayed increase in growth (12 hr) in the 18% iron-saturated

medium. The explanation for this delay is probably related to the change in pH value which accompanied growth. Since the Trypticase medium was poorly buffered, acid production from added glucose can effect a decrease in pH value and so increase the amount of free iron in the medium available for growth. Schade & Caroline (1944) showed that below pH 7·0 iron was increasingly available as a result of the shift in equilibrium of the iron + conalbumin complex. Measurements of pH value of the cultures were made during the incubation period (Table 2). A rapid decrease in pH value occurred in the 8 and 18 % iron-saturated conalbumin cultures between 12 and 15 hr. During this period the cocci in the 18 % iron-saturated culture were capable of responding with increased growth to the greater concentration of available iron until a pH value was reached which halted further development. The 8 % iron-saturated culture lacked this capability. No decrease of pH value occurred in the cultures without glucose.

DISCUSSION

Although there have been reported many studies on the iron requirements of bacteria, few have been done with Gram-positive organisms grown in a complex medium depleted of iron. Schade (1963), in an approach to *in vivo* conditions, used as a growth medium for *Staphylococcus aureus* human serum whose siderophilin acts as a naturally-occurring specific iron chelator. In this iron-restricted medium, the growth of the pathogen was responsive to the additions of known amounts of iron and to the percentage saturation of the chelator. To have a readily available iron-chelating complex medium approximating to human serum, we have used an iron-depleted Trypticase medium supplemented with conalbumin (as from egg white). The results of growth studies with this medium were similar to those obtained with serum and illustrated the profound growth regulatory effects of changes in the availability of iron at extremely low concentrations of it. The use of this medium made possible the harvesting of sufficiently large amounts of *S. aureus* grown in conditions of iron restriction for use in studies of their metabolic activities, to be reported later.

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An Electron Microscope and Biochemical Study of *Neurospora crassa* during Development

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SUMMARY

Mitochondria isolated from protoplasts of conidia, young germinated conidia, and branched mycelia of *Neurospora crassa*, have an active oxidative phosphorylation system which showed no variation in the three stages of development studied; succinate dehydrogenase also did not alter. Cytochrome oxidase activity showed a marked increase at the time of conidial germination; this was not maintained during further growth. An electron microscope study of the conidia showed them to have complex mitochondria analogous to those seen in older mycelial hyphae. A large accumulation of lipid material and extensive vacuolization was observed in these conidia. A rudimentary reticular system with numerous ribosomes lying free in this membrane system were observed. Macroconidia were usually multinucleate; up to 10 nuclei were observed. The outer conidial wall was completely digested by snail extract (*Helix pomatia* digestive juice) and was structurally identical with that of the hyphae as seen with the fixation and embedding conditions used.

INTRODUCTION

Neurospora crassa undergoes different morphological changes depending on the conditions under which it is cultivated. Studies which attempted to correlate progressive alterations in enzyme activities with these morphological transformations in wild-type Neurospora have been made by several workers. Zalokar (1959) examined the effects of hyphal morphology and growth stage on four different enzymes (succinate dehydrogenase, aldolase, β-galactosidase, tryptophan synthetase). Turian (1962) studied the process of conidiation and suggested that an alteration in the concentration of succinate dehydrogenase and isocitrate lyase might occur during conidiogenesis. It seems clear from these studies with Neurospora, and from the work of Yotsuyanagi (1962) with yeast, that significant changes in enzyme activities associated with oxidative metabolism might accompany the processes of biological differentiation.

In the present work an attempt was made to examine several oxidative enzymes unequivocally bound to mitochondrial membranes at the critical stages of conidiation and conidial germination in wild-type *Neurospora crassa*; the phosphorylating function of the mitochondria was also examined. An electron microscope study was made to correlate where possible the biochemical results with the morphological

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changes. Attention was focused on the structure of the conidia, since several studies on the structure of the hyphae of wild-type Neurospora were available for comparison.

METHODS

Growth conditions. Wild-type *Neurospora crassa*, strain Chilton *a*, was grown at room temperature (23°) on 500 ml. solid medium in 2600 ml. Fernbach flasks. The medium contained (g./l.): 10, sucrose; 5, yeast extract; 25, agar; 5, ammonium tartrate; 1·0, NH_4NO_3; 1·0, KH_2PO_4; 0·5, $MgSO_4$; 0·1, NaCl; 0·1, $CaCl_2$. The salts were at the concentration of Fries no. 3 solution (Ryan, Beadle & Tatum, 1943). To ensure adequate aeration, the flasks were covered with thin layers of cottonwool between several layers of cheese cloth. Cultures were harvested 7–10 days after inoculation by washing the hyphae and conidia from the agar surface with cold sterile distilled water. Conidia were separated from hyphae by filtration through glass wool which removed all vegetative filaments.

To obtain a fairly uniform population of young germinated organisms, conidia isolated in the above manner were added to 6 l. Erlenmeyer flasks containing 3·5 l. liquid medium. The absolute number of conidia added to this volume was usually about $1{\cdot}8 \times 10^{10}$. Cultures were vigorously aerated at room temperature by bubbling filtered (cottonwool) air into the medium. To prevent clumping and the formation of mats along the walls of the flask, the medium was stirred with a magnetic stirrer thermally insulated from the flask by a layer of 'rock wool'. To prevent foaming, a silicon anti-foaming agent (Dow Corning antifoam A spray) was sprayed into each flask before autoclaving.

Under these conditions, germination began within 2 hr; the total number of germinated conidia increased at a linear rate for about 8 hr, at which time maximum germination (90 %) was reached. After 10 hr, the majority of organisms had well-formed unbranched germination tubes. When the growth period was extended to 15–16 hr, the hyphae developed into very long and extensively branched mycelial systems.

Preparation of protoplasts from conidia and young germinated conidia. The procedure described by Kinsky (1962) was modified to obtain protoplasts from conidia and germinated conidia in yields large enough for biochemical and ultrastructural analyses on the mitochondria isolated from them. Conidia washed from the surface of one Fernbach flask were separated from hyphal fragments by filtration through glass wool and washed several times with 0·05 M-sodium phosphate (pH 6·8). Germinated conidia were collected on a Buchner funnel and washed similarly. The entire yield (about 2–3 g. wet wt. of organism) was suspended in a 50 ml. Erlenmeyer flask with cottonwool plug in the following incubation mixture: 18 ml. 0·05 M-sodium phosphate (pH 6·8) containing glutathione (0·05–0·1 M) and sucrose (0·44 M); 2 ml. *Helix pomatia* digestive juice preparation. (Suc digestif du *H. pomatia* bought from L'Industrie biologique française, Gennevilliers, Seine, France; supernatant fluid from 2 ml. centrifuged for 30 min. and 8200 ***g*** in 856 head of International centrifuge). The flask was placed in a water bath at 30° and shaken gently for 10–15 hr. Conidia and protoplasts were removed from the incubation mixture by low-speed centrifugation (5 min. at 900 ***g***) and washed 2–3 times with tris + HCl (pH 7·4) containing sucrose (0·44 M).

Disruption procedures and isolation of mitochondria

Mitochondria from protoplasts. Washed protoplasts were broken gently in a glass homogenizer. Whole organisms and debris were removed by centrifugation at about 900*g* for 5 min. periods. Mitochondria were sedimented from the resulting cell-free homogenate by centrifugation at 18,600*g* for 20 min.

Mitochondria from conidia. Conidia and germinated conidia were collected on a Buchner funnel with two to three layers of Whatman no. 1 filter paper. The resulting mat of organisms was washed, weighed and placed in a chilled porcelain mortar with washed and ignited sea-sand equal to 2–3 times the wet weight of the mat. The mixture was ground for about 10 min. in the cold with the addition of a small amount of tris/HCl buffer (0·05 M, pH 7·4) containing 0·44 M-sucrose. The sand was partially removed by allowing it to settle in the cold for a few minutes. The remaining whole organisms, debris, and sand were removed by centrifugation at 900*g* for two 5 min. periods. Mitochondria were separated from the homogenate by sedimentation at 18,000*g* for 20 min.

Mitochondrial suspensions were either used at once or frozen rapidly in liquid nitrogen and stored for short times (1–2 days) before use.

Enzyme assay methods. (1) Succinate dehydrogenase was assayed by the phenazine methosulphate (PMS) procedure of Singer & Kearney (1957) at 37° in micro-Warburg vessels (capacity about 7 ml.). Oxygen uptake was measured at a series of dye concentrations.

(2) *Cytochrome oxidase assay.* Oxygen uptake in the presence of non-enzymically reduced cytochrome *c* was measured by the method of Slater (1949); the reducing agent was ascorbic acid, and manometry was at 37° in double side-arm Warburg vessels (capacity about 15 ml.).

(3) *Oxidative phosphorylation.* A modification of the method of Hunter (1955) was used. To the main compartment of micro-Warburg vessels were added the following reagents: 20 μmole, tris+HCl (pH 7·4); 10 μmole KH_2PO_4 (pH 7·6); 3 μmole ADP; 5 μmole $MgCl_2$; 3 mg. bovine serum albumin; 20 μmole sodium succinate (pH 7·4); enzyme in buffered sucrose; distilled water to a total volume of 1·6 ml. To the side arm was added 90 μmole glucose and 0·5 mg. hexokinase. The centre well contained 0·2 ml. 10% KOH and a filter-paper wick. The reaction mixture was equilibrated at 30° for 7 min., after which the hexokinase+glucose mixture was tipped in. Oxygen uptake was measured every 5 min. for 30 min. The reaction was stopped by adding 0·2 ml. 30% (w/v) trichloroacetic acid solution. In each assay at least two controls were deproteinized immediately after equilibration to provide zero-time phosphate concentrations. Phosphorylation was measured as the disappearance of inorganic phosphate from the reaction mixture by the method of Fiske & SubbaRow (Lindberg & Ernster, 1956).

Protein was measured by the method of Lowry, Rosebrough, Farr & Randall (1951), with a standard curve obtained for human serum albumin.

Electron microscope techniques. Four fixatives were used: (1) potassium permanganate, 4% (w/v) in distilled water; (2) osmium tetroxide, 1% (w/v) in Palade's veronal+acetate buffer adjusted to pH 7·3–7·5 with 0·1 M-HCl; (3) glutaraldehyde, 5% (w/v) in 0·05 M-sodium phosphate (pH 7·0–7·2); (4) formalin, 10% (v/v) in 0·05 M-sodium phosphate (pH 7·0–7·2). For most experiments, particularly those

with isolated particles or protoplasts, 0·44 M-sucrose was added to all fixative solutions.

Dehydration was always done through a graded series of ethanol + water mixtures.

For embedding a 9/1 (by volume) mixture of butyl-and methyl-methacrylate was used. Uranyl acetate (0·035 %, w/v) was routinely added to the monomer mixture, as suggested by Shatkin & Tatum (1959).

The following fixation, dehydration and embedding schedule was used with protoplasts and mitochondrial pellets: (1) Fix in glutaraldehyde or formalin for 2 hr at 4°. (2) Wash twice with 0·05 M-phosphate buffer (pH 7·4) containing 0·44 M sucrose. (3) Let stand overnight at 4° in buffered sucrose. (4) Post-fix in osmium tetroxide for 2 hr at 4°. (5) Wash in buffered sucrose. (6) Dehydrate through a graded series of ethanol + water mixtures. (7) Infiltrate with methacrylate. (8) Polymerize by transferring samples to gelatin capsules containing partially polymerized methacrylate that is quite viscous. Allow further polymerization to proceed very slowly at room temperature or at 37° for 1–3 days.

Blocks were sectioned with a Porter–Blum manual ultramicrotome. Dried sections were stained with uranyl nitrate (saturated solution in distilled water) for 2–5 hr. All preparations were examined and photographed with a Siemens Elmiskop I electron microscope at magnification ×8000 or ×40,000 and a beam potential of 80 kV.

RESULTS

The succinate dehydrogenase system and oxidative phosphorylation

To obtain phosphorylation of ADP coupled to oxidation of succinate it was essential that the mitochondria were relatively intact. The method finally adopted involved the production of protoplasts from hyphae or conidia and their subsequent lysis either by homogenization or osmotic shock. With this method, yields of mitochondrial protein/flask-yield of conidia were low (5–15 mg./flask) but sufficient for enzymic and ultrastructural studies. Table 1 gives P/O ratios for mitochondria from organisms of different stages prepared by the protoplast method and by grinding with sand. Preparations by the latter method gave erratic results with unfractionated suspensions of mitochondria; the P/O ratios were usually less than one and often not measurable at all. With mitochondria isolated from protoplasts, the P/O ratios were consistently greater than one.

Succinate dehydrogenase activity

Succinate dehydrogenase activity was determined in isolated mitochondria from the three stages of growth; pH 7·6 was optimal in these assays. All assays were done with a series of different phenazine methosulphate concentrations, and maximum velocities were calculated by the Lineweaver–Burk double reciprocal method. Mitochondria which had been frozen in liquid nitrogen and stored for 1–3 days at −20° retained full dehydrogenase activity. For a given stage, the mitochondria prepared by grinding with sand or by protoplast lysis showed no significant differences in enzyme activity. The data in Table 2 indicate that the specific activity of succinate dehydrogenase did not differ appreciably in the three stages of growth examined.

Table 1. *Phosphorylative ability of mitochondria prepared from cells at different stages of development and by different methods of isolation*

Stage of growth	Method of cell breakage	P/O ratios	μmoles P_i per 30 min.	μatoms O_2 per 30 min.
Conidia	Protoplast lysis	1·3	5·0	3·8
	Sea sand grinding	0·3	3·2	10·4
		0·6	3·5	5·5
10 hr culture	Protoplast lysis	1·1	0·88	0·79
		1·4	2·0	1·39
		1·9	2·0	1·09
	Sea sand grinding	0·7	3·0	4·5
15 hr culture	Protoplast lysis	—	—	—
	Sea sand grinding	0·6	6·0	10·6
		0·9	6·9	7·4

The reaction mixture consisted of 20 μmole tris+HCl, pH 7·4, 10 μmole KH_2PO_4, pH 7·6, 3 μmole ADP, 5 μmole $MgCl_2$, 3 mg. bovine serum albumin, 20 μmole sodium succinate, pH 7·4, enzyme in buffered sucrose and distilled water to give a total fluid volume of 1·6 ml. To the side arm of each Warburg vessel was added 90 μmole glucose and 0·5 mg. hexokinase. The centre well was filled with 10 % KOH. The reaction was run at 30° and was rapidly terminated after 30 min. by the addition of 30 % trichloroacetic acid.

Table 2. *Succinic dehydrogenase activity as a function of growth*

The main compartment of each micro-Warburg flask contained in a total fluid volume of 2·2 ml.: 60 μmole sodium succinate, pH 7·0, 150 μmole sodium phosphate buffer, pH 7·6, 3 μmole KCN adjusted to pH 8·0 with 0·085 N-HCl, 1·65 μmole $CaCl_2$, enzyme (sufficient to give 30–60 $mm.^3$ oxygen uptake in 5 min.). The KCN was added just before the vessels were attached to the manometers. Phenazine methosulphate (1–4 mg.) in distilled water was added last to each side arm to minimize exposure to light and air. After a 7 min. equilibration, the dye was tipped in, and readings taken 2 and 7 min. later. All assays were performed at 37°.

Stage of growth	Specific activity (μl. O_2/mg. protein/hr)
Conidia	940–1068
10 hr culture	801–1068
15 hr culture	940–1090

Cytochrome oxidase activity

In this assay, maximum velocities were determined by extrapolation to infinite substrate concentration. Assays were made with mitochondria isolated from the same stages of growth as used for succinate dehydrogenase measurements; usually dehydrogenase and oxidase assays were done on each mitochondrial preparation. The data given in Table 3 indicate that a marked increase in cytochrome oxidase activity occurred at the time of conidial germination. A five- to tenfold increase in activity was observed in newly-germinated organisms as compared with ungerminated conidia. This high activity was not maintained; in 14–15 hr cultures, the activity was again at values only slightly higher than those for the conidia.

The ultrastructure of conidia of Neurospora crassa

Initial attempts to study the fine structure of conidia after osmium fixation and methacrylate embedding met with limited success. There were two main difficulties: first, although the ribosomes were well fixed and stained with osmium, the membranous structures, mitochondria and endoplasmic reticulum particularly, were obscured by the enormous quantity of ribosomes present; secondly, polymerization in methacrylate resulted in extensive cell damage. Yotsuyanagi (1959), with yeast cells, had similar difficulties. Organisms fixed in unbuffered 4% (w/v) $KMnO_4$ (which had been shown by him to dissociate ribonucleoproteins in *in vitro* experiments; Yotsuyanagi, 1962) effectively removed the ribosomes and permitted

Table 3. *Cytochrome oxidase activity at different stages of growth*

Each vessel contained in a total volume of 3·2 ml.: 26·4 μmole ascorbate (adjusted to pH 7·0 with NaOH), 0·5–1·5 $\times 10^{-5}$ M cytochrome *c*, 65 μmole sodium phosphate buffer, pH 7·3, enzyme and distilled water to volume. In each experiment, the maximum rate of oxygen uptake was corrected for autoxidation of ascorbate at each concentration of cytochrome c assayed. The corrected rates for oxygen uptake were plotted as $1/V_{max}$ versus 1/cytochrome *c* concentration and extrapolated to infinite substrate concentration to give the values presented in the table.

Stage of growth	Specific activity (μl. O_2/mg. protein/hr.)
Conidia	274
10 hr culture	1890
15 hr culture	461

better visualization of the other cell components. When this method was used with Neurospora conidia, complete removal of the ribosomes was obtained; in fact, the only structures preserved with this strong oxidizing agent were membranous organelles (mitochondria, Golgi apparatus, endoplasmic reticulum; Luft, 1956). The organisms were almost devoid of lipid inclusions. The mitochondria of these organisms were in structure completely analogous to the mitochondria of higher plants and animals; they contained numerous cristae in long parallel arrays, and were often of extremely irregular amoeboid shape. The hypotonicity of the fixative solution undoubtedly was responsible for the swollen appearance of these particles.

All the organisms were surrounded by a thick outer wall. The centre of the organism was occupied by a large vacuole, either clear or filled with granular material; surrounding this vacuole was finely granular cytoplasm. The endoplasmic reticulum was rather sparse and rudimentary, and the presence of a Golgi apparatus is uncertain. Most organisms contained more than one nucleus, and forms with 4 to 6 nuclei were quite common. The nucleoplasm appeared made up of granular light and dense regions. However, because of the nature of the fixative, it is unlikely that the nuclear material was well preserved in these organisms. Pl. 1, figs. 1, 2, and Pl. 2, fig. 3, illustrate the features of $KMnO_4$-fixed conidia described above.

Because of the probable removal of many constituents of the organisms by $KMnO_4$ fixation, they were examined after fixation in other ways. Sabatini, Miller & Barnett (1964) found that several mono- and di-aldehydes provided good morphological preservation of cellular structures in mammalian tissues, and did not impair enzyme activity. Fixation procedures were therefore adopted which used

glutaraldehyde and formaldehyde as primary fixatives of conidia. Post-fixation in osmium was done to increase contrast; it was expected that the aldehydes would penetrate more rapidly than would osmium. To facilitate rapid fixation, conidia treated with the *Helix pomatia* enzyme preparation were used for this work.

The most striking feature of organisms fixed with formaldehyde or glutaraldehyde (as compared with $KMnO_4$ fixation) was the increased complexity seen. Aldehyde-fixed protoplasts contained components not seen in $KMnO_4$-fixed organisms, and those elements common to both showed greater structural variability (see Pl. 2, fig. 4; Pl. 3, fig. 5). These protoplasts contained numerous uniformly dense bodies, ranging from 0·12 to 1·0 μ, and probably lipid in composition. These bodies were not visible in $KMnO_4$-fixed protoplasts. Another unique component was some very electron-dense material found only within vacuolar spaces (Pl. 2, fig. 4). This material was found as a diffuse amorphous mass or densely packed into round bodies which varied in size from 0·15 to 2·5 μ (mean value of 0·44 μ; 79 particles measured). Another prominent structure not seen in $KMnO_4$-fixed organisms was a vesicular body of varying size and internal content, and limited by a single membrane. These structures often contained membranous material or small lipid-like droplets, and varied in size from 0·25 to 1·2 μ (mean value 0·60 μ; 80 particles measured). These particles were usually closely associated with lipid droplets and mitochondria (see Pl. 3, fig. 5). The mitochondria of these protoplasts showed greater variability in shape. Many dumbbell-shaped, hemispherical, and ring-shaped mitochondria were encountered (Pl. 2, fig. 4). The central space enclosed by the ring-shaped particles was usually less granular than the surrounding cytoplasm. The endoplasmic reticulum was almost undetectable in most of these organisms and the coarser granularity of the cytoplasm was probably due to the presence of many free ribosomes. The nucleus did not show the distinct heterogeneity of $KMnO_4$-fixed nuclei, and had a granularity much like the cytoplasm outside it. Most of the nuclei showed a restricted dense aggregation which seemed to be polarized toward the nuclear membrane (Pl. 2, fig. 4). Most of the conidia examined lacked cell wall material or were covered with small remnants of cell wall. This probably means that the *Helix pomatia* enzyme preparation completely digested the cell wall of the conidium and did not merely weaken it at a few select points.

DISCUSSION

The succinate dehydrogenase system at three stages of development of conidia of Neurospora crassa.

In all experiments with the phenazine methosulphate assay system, the specific activity of succinate dehydrogenase was high in conidial mitochondria and did not change significantly in the three stages of growth from which the mitochondria were isolated. These results are at variance with those reported by Zalokar (1956) and Turian (1962). Zalokar reported a tenfold increase in succinate dehydrogenase during the first 8 hr of growth, beginning with ungerminated conidia; Turian reported that the activity of this enzyme was significantly altered depending on growth conditions, and suggested a reciprocal correlation between succinate dehydrogenase activity and the conidiogenic capabilities of wild-type hyphae. It is believed that the succinate dehydrogenase assay used here is a specific one for this

dehydrogenase alone and provides a more accurate measure of it than the methods used by Zalokar and Turian. Singer & Kearney (1957) pointed out that the method used by Zalokar with methylene blue as electron acceptor depended on the presence of enzymes to catalyse the transfer of electrons from the reduced dehydrogenase to methylene blue. The method used by Turian measures succino-cytochrome *c* reductase activity, and not the dehydrogenase alone. In the present work the electron acceptor used was phenazine methosulphate, which has been shown to react specifically with the primary dehydrogenase in both particulate and soluble preparations, regardless of their source. The discrepancy between the results reported here and those of Zalokar and Turian might be explained if the concentration of some other enzyme playing a role in the reduction of methylene blue and the transfer of electrons from succinate to cytochrome *c* underwent change during morphogenesis, rather than the dehydrogenase itself. The growth medium also can profoundly affect the enzyme composition of the organism. The difference in the carbon and nitrogen source used in this work as compared with that used by Zalokar and Turian may also account for the different activities observed.

Cytochrome oxidase, which is localized within the same membranous framework as succinate dehydrogenase, varied independently of it during development of the conidia. The data indicate a five- to tenfold increase in activity, which seems to be maintained only for the short period of time covering conidial germination. Because germination could not be synchronized, 10 hr cultures were at different stages in the formation of their germination tubes. Thus, the activities measured were for a heterogeneous population that ranged from newly germinated conidia to conidia whose initial germination had occurred 6–8 hr earlier. This would suggest that the increase in cytochrome oxidase activity observed in 10 hr cultures would have been greater if germination had occurred simultaneously in all conidia.

With regard to the phosphorylating capacities of Neurospora mitochondria, consistent results were difficult to obtain and, therefore, comparisons between stages are somewhat hazardous. The data suggest that no difference in phosphorylating ability exists between conidia and germinated conidia. Thus, although there is evidence for alterations in the level of specific enzymes associated with the TCA cycle and electron transport, the overall activity of the succinic-oxidase system and the concomitant production of ATP remains high throughout growth.

The ultrastructure of conidia and hyphae

The ultrastructure of wild-type *Neurospora crassa* mycelia has been studied by Zalokar (1961) and Shatkin & Tatum (1959). Comparison between the ultrastructure of hyphae and conidia indicates many similarities. The cell wall of conidia appears to be identical with that of the hyphae. Although the membranes of the endoplasmic reticulum may be associated with ribosomes in the conidia, the large number of these granules free in the cytoplasm suggests that the association is non-specific. Zalokar has shown that in centrifuged Neurospora hyphae the vesicles of the endoplasmic reticulum can be completely separated from the ribosomes, suggesting a weak association. The mitochondria of hyphae and conidia show about the same degree of internal complexity and are approximately the same size. In conidia about 10 % of these particles are in ring- or dumbbell-shaped forms. The variability in the number of nuclei per conidium is in agreement with light-microscope obser-

vations (Huebschman, 1952). Glutaraldehyde- and formaldehyde-fixed conidia have a uniform granular nucleoplasm with a single dense polarized region, the nucleolus. Nuclei of potassium permanganate-fixed conidia do not show this single dense region. The nucleoplasm is divided into several finely granular areas of low density containing fibrillar material. Both methods of fixation probably remove or alter substances within the nucleus. The two fixation procedures may be demonstrating different components or different phases of the nucleoplasm.

Both conidia and hyphae contained large vacuoles. Often these vacuoles were seen to contain large dense bodies of unknown composition. These highly vacuolated conidia may represent an advanced stage in the life of the conidium. Conidia began to form on aerial hyphae within 3 days after inoculation and cultures were harvested 7–10 days after the initiation of growth. Thus, the age of conidia in a given population at the time they were collected varied considerably and might account for the variation in structure seen in the electron microscope. The observation that cultures which were deep orange at the height of conidial formation began to become bleached after 10–15 days suggests that degenerative changes occurred as the spore population ages.

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. Section through a $KMnO_4$-fixed, methacrylate-embedded conidium. Note the thick outer cell wall, sparse reticulum, and abundant mitochondria with typical inner cristae in long parallel arrays. A single nucleus with breaks in the nuclear membrane is evident. The overall fine granularity of the cytoplasm and intra-mitochondrial matrix is typical of these cells. × 22,000.

Fig. 2. A $KMnO_4$-fixed conidium showing the large central vacuole filled with granular material. Note the irregular shape of the mitochondria and the small array of densely stained membranes which may be comparable to the Golgi apparatus of higher plants. × 18,000.

PLATE 2

Fig. 3. Thin section through a $KMnO_4$-fixed conidium illustrating the multinucleate nature of these cells. The typical biphasic composition of the nucleoplasm observed only in $KMnO_4$-fixed cells is evident here. × 19,700.

Fig. 4. A section through a glutaraldehyde-fixed conidial protoplast. Note the complete absence of a cell wall. The cytoplasm is more coarsely granular than in $KMnO_4$-fixed cells. The nuclei also have the same coarse granularity. Four of them in this section show a polarized dense region, which is presumed to be the nucleolus. The ring, hemispheric and dumbbell-shaped mitochondria frequently encountered in wild-type conidia are clearly demonstrated, as are the lipid bodies and dense inclusions within vacuolar spaces. × 20,300.

PLATE 3

Fig. 5. A section of a glutaraldehyde-fixed protoplast of a wild-type conidium. Of particular interest is the large number of vesicles associated with lipid droplets and mitochondria. Two very large lipid bodies are prominent. Particles of this size are atypical. The dense cristae folds and irregularly shaped mitochondria contrast with the thin parallel cristae and regular elliptical shape of the mitochondria of $KMnO_4$-fixed cells. Some remnants of the outer cell wall can be seen in this section. × 27,000.

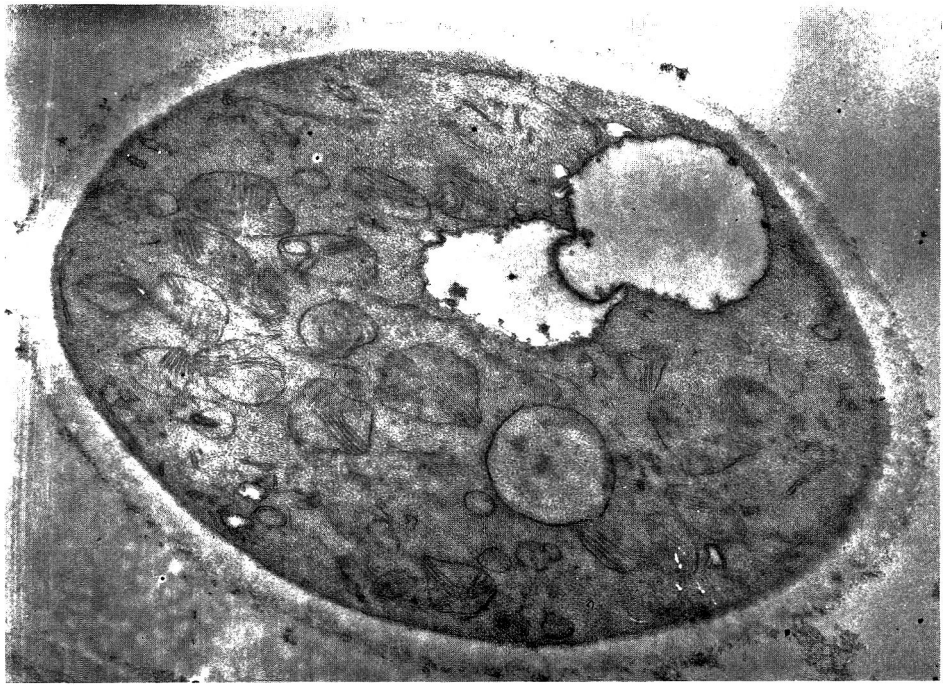

Fig. 1

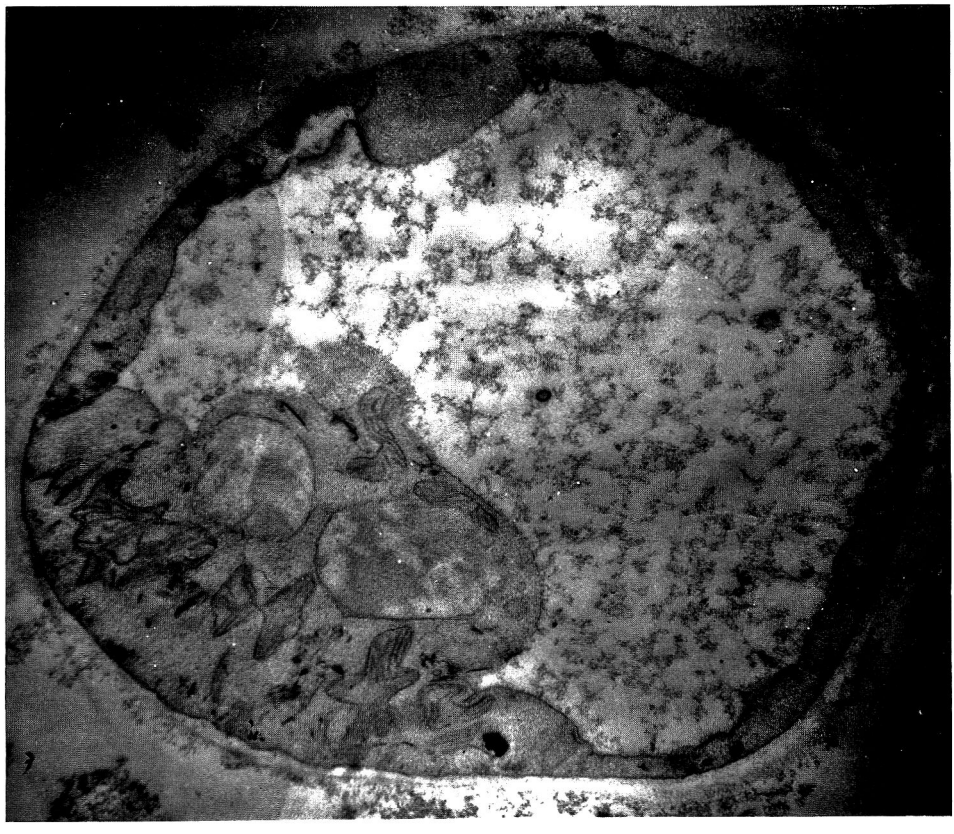

Fig. 2

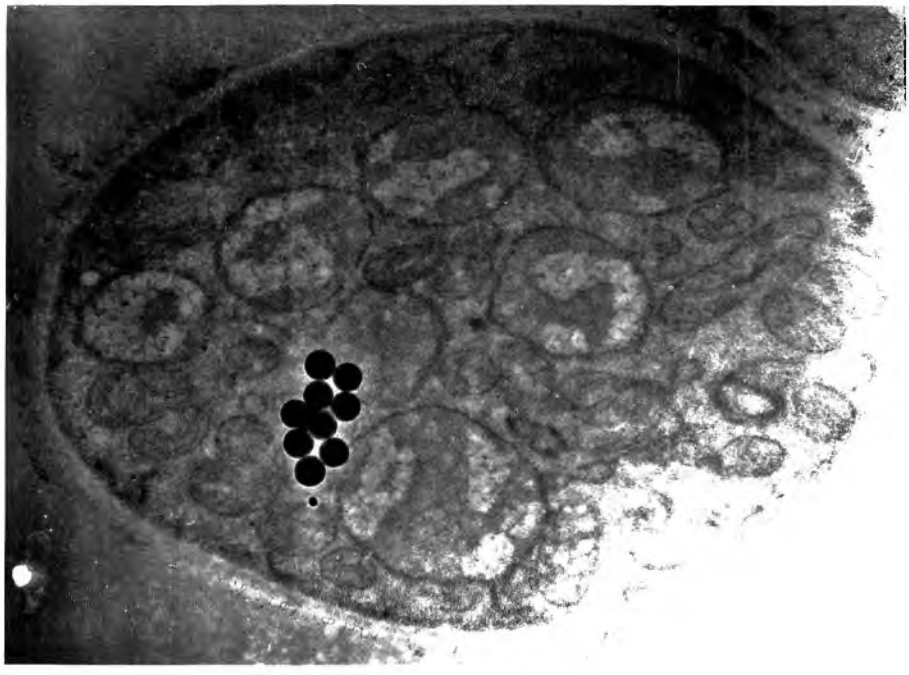

Fig. 3

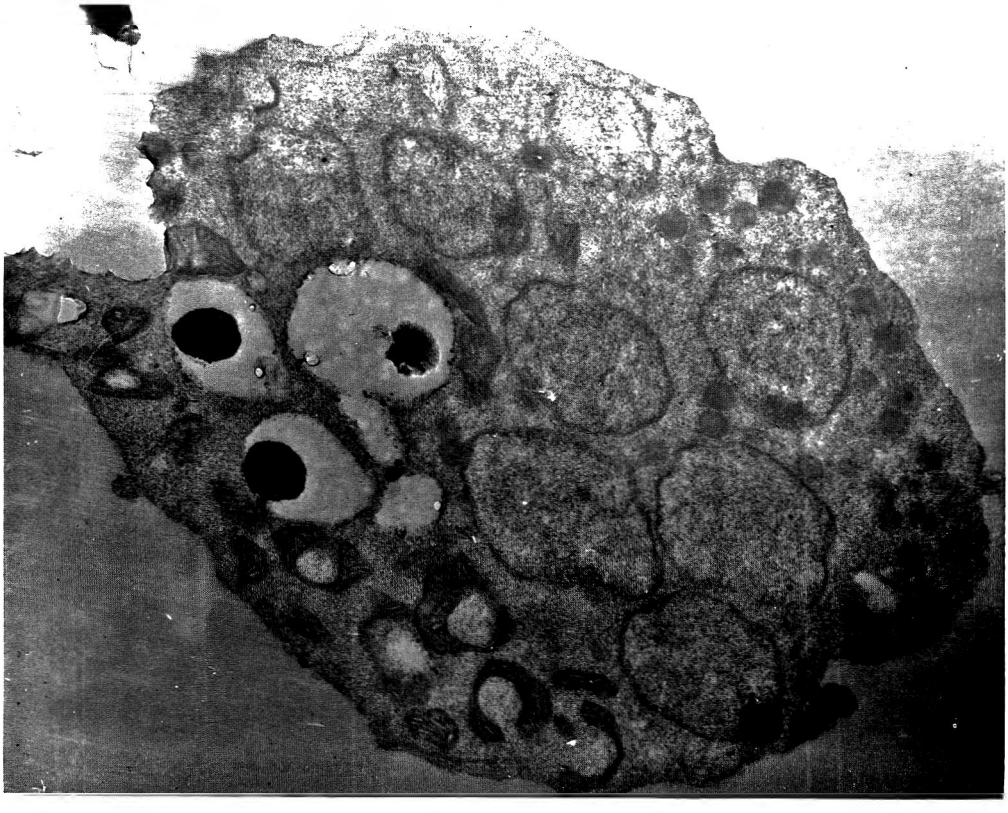

Fig. 4

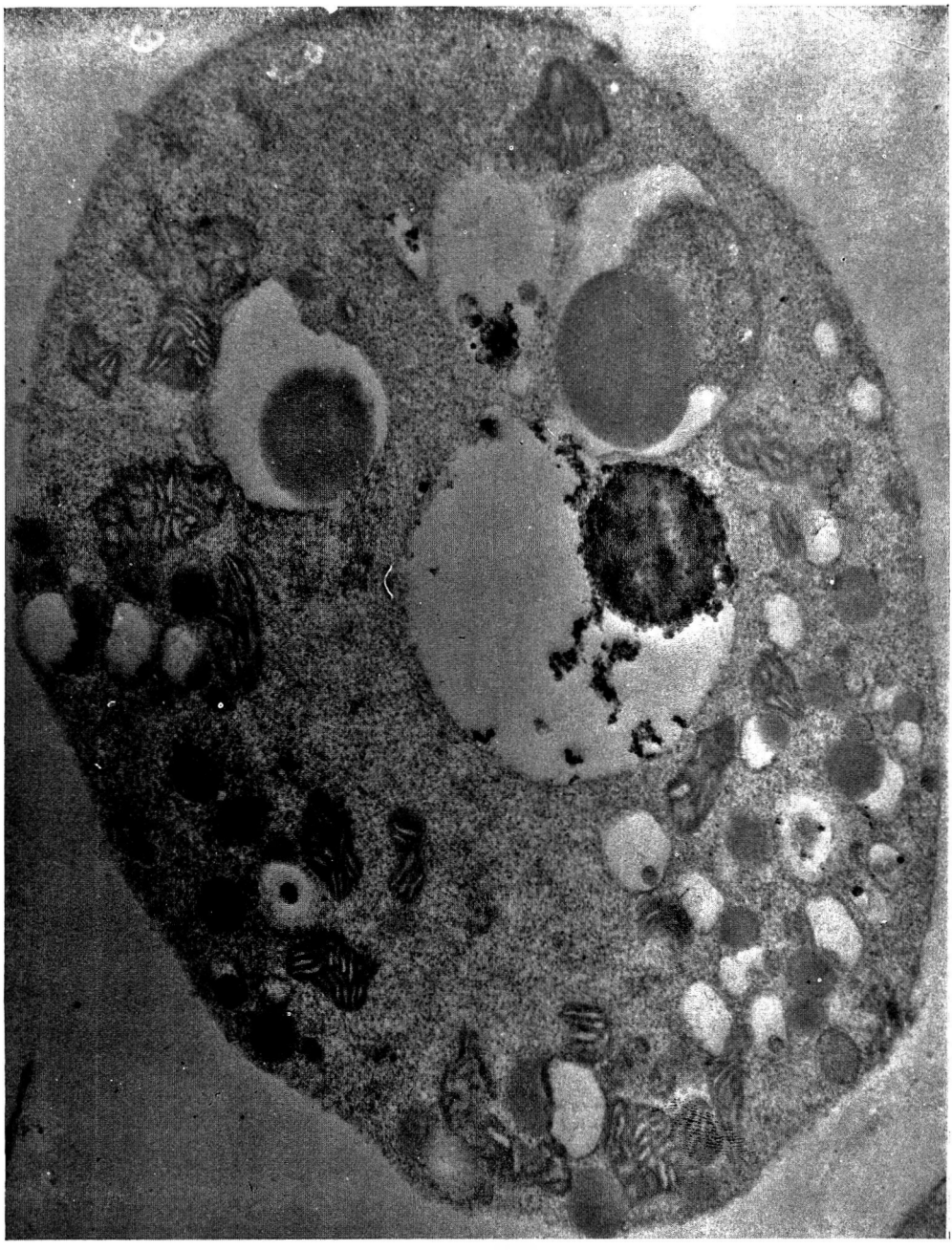

Fig. 5

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Isolation and Characterization of Bacteriophages Active against Stalked Bacteria

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SUMMARY

The isolation and properties of 23 phages lytic for stalked bacteria of the genera *Caulobacter* and *Asticcacaulis* are described. They fall into seven groups on the basis of host range and serological properties. Phages of groups I–VI attack only Caulobacter species; those of group VII, only Asticcacaulis. Groups I–III comprise DNA viruses with long flexible tails and of unusually large size, the heads having dimensions of 50×170 mμ. Groups IV–VI comprise RNA viruses of simple structure, 21–23 mμ in diameter. The specific Asticcacaulis phages (group VII) are tailed DNA viruses, similar in form and size to coliphage lambda. The implications of this study for the taxonomy of the caulobacters are discussed.

INTRODUCTION

Stalked bacteria of the genera *Caulobacter* and *Asticcacaulis* have recently been the subject of a monograph by Poindexter (1964). The consequent availability of an extensive series of strains led us to look for bacteriophages active against these distinctive bacteria. The present paper reports the isolation and general properties of a series of phages which cause lysis of Caulobacter or Asticcacaulis. After this investigation had been undertaken, a phage lytic for *C. vibrioides*, distinctly different from any described here, was reported by Khavina & Rautenstein (1963).

METHODS

Media. The standard complex medium (PYE) of Poindexter (1964), consisting of 0·2% peptone, 0·1% yeast extract, and 0·00045 M-$MgSO_4.7H_2O$ in tap water was used for routine cultivation of Caulobacter and Asticcacaulis strains. This medium was satisfactory for the isolation and propagation of phages. The plating medium contained 1% agar. Phage adsorptions were conducted in PYE broth prepared with de-ionized water (in place of tap water) and containing 0·002 or 0·004 M-$MgSO_4$, instead of the lower concentration used in the standard growth medium.

Bacterial strains. The Caulobacter and Asticcacaulis strains used in this investigation were obtained from the collection of Dr Jeanne S. Poindexter, and will be referred to by her designations (Poindexter, 1964). The Hfr and F^- strains of *Escherichia coli* tested in host range specificity experiments were supplied by Susanne DeWitt.

Isolation of bacteriophages. An enrichment technique was used for isolation of

phages from sewage, soil and pond water, since initial attempts to obtain such phages by direct plating were uniformly unsuccessful. Before setting up the enrichment cultures, sewage samples were usually exposed to 3% (v/v) chloroform to decrease the bacterial content. Equal volumes of sewage or pond water and broth cultures of the prospective host strains were incubated without shaking for periods ranging from 16 to 72 hr. Samples were then centrifuged to sediment bacteria, and the supernatant liquid filtered through a Millipore Type HA filter (0·45 μ). Dilutions of each filtrate were mixed with 5×10^8 colony-forming units of the presumptive host strain used in the enrichment, and overlayer plates were prepared. Plates were examined for plaques after 18- and 48-hr incubation periods at 30°. Pure phage clones were obtained by stabbing single plaques with a platinum needle, suspending the material in PYE broth, and replating a series of dilutions. The procedure was replated several times and single plaques were finally selected for the preparation of stock lysates.

Table 1. *Isolation of Caulobacter and Asticcacaulis phages*

Phage	Original host (enrichment)	Source
ØCb 1	*C. bacteroides* 11a	Chlorinated sewage
ØCb 2	*C. vibrioides* H	Chlorinated sewage
ØCb 3	*C. bacteroides* 11a	Raw sewage no. 1
ØCb 4, 5	*C. vibrioides* H	Raw sewage no. 1
ØCb 6, 7, 8, 9, 10, 11, 19	*C. bacteroides* 11a	Raw sewage no. 2
ØCb 12	*C. vibrioides* H	Contaminating *A. excentricus* 12
ØCb 13, 14, 16, 17, 18	*C. crescentus* 15	Raw sewage no. 2
ØCb 15	*C. crescentus* 15	Pond water
ØCb 23	*C. fusiformis* 27	Raw sewage no. 3
ØAc 20, 21, 22	*A. excentricus* 48	Raw sewage no. 3

A total of 23 phage strains were isolated at the expense of five different Caulobacter and Asticcacaulis species (Table 1). Each phage strain was derived from a single enrichment culture.

Phage assays were carried out by the agar layer method of Adams (1959), with an overlayer that contained 0·4 ml. of a late exponential phase Caulobacter or Asticcacaulis culture, 0·1 ml. phage dilution, and 2 ml. PYE agar. Plates were incubated at 30°.

Preparation of lysates. Attempts to obtain lysates with high phage titres from liquid cultures were not successful. Accordingly, all lysates were prepared from areas of confluent lysis in agar overlayers. The phages were eluted with 4 ml. PYE broth/plate, and filtered. Titres of 5×10^9 to 5×10^{10} plaque-forming units (p.f.u.) per ml. were obtained by this method.

Antiserum preparation and neutralization tests. Seven phages were chosen as immunizing antigens. Each appeared to be representative of a unique group of isolates or unique in itself, on the basis of source, plaque morphology and host-range pattern. These representative phages were: ØCb 3, 6, 8r, 12r, 13, 23r, ØAc 20. Phage stocks containing at least 10^{10} p.f.u./ml. were used to immunize young male albino rabbits by a series of intravenous or subcutaneous injections. Adjuvant consisting of light paraffin oil and Arlacel A (Atlas Powder Co.) was used with the subcutaneous injections. When satisfactory titres of neutralizing antibody had

developed, the rabbits were bled by cardiac puncture and the sera stored at −25°. Neutralization tests were small according to the methods described by Adams (1959), except that mixtures were incubated at 30°.

Partial purification of phages. For electron microscopic examination and nucleic acid determinations, high-titre preparations of phage, free from bacterial debris, were required. Precipitation with ammonium sulphate was sometimes used to concentrate the phages, but differential centrifugation was the main method used. Crude lysates obtained by elution from confluently lysed agar overlayers were centrifuged at 6000 to 12,000*g* for 20 min. to eliminate bacterial debris in part. The supernatant liquid was treated with 4 μg. ribonuclease (California Biochemical Corp.) for 30 min. at room temperature. Most lysates were also treated with deoxyribonuclease (California Biochemical Corp.) at 4 μg./ml. in the presence of 0·0002 M-$MgSO_4$. Ammonium sulphate, when used, was added to 3 M, in the presence of 2 mM-ethylenediaminetetra-acetic (EDTA) acid, and allowed to stand overnight at 4°. The precipitate was sedimented at 27,000*g*, resuspended in 0·01 M-ammonium acetate (pH 7·4) and centrifuged at 6000*g* to remove debris. The supernatant fluid was then centrifuged for 1–2 hr at 70,000*g* in a Spinco Model L ultracentrifuge. The pellets obtained were resuspended in 0·01 M-ammonium acetate, and centrifuged at 6000 or 12,000*g*. The concentrated phage suspensions were sedimented a second time at 70,000*g*, resuspended, and again centrifuged at low speed. When ammonium sulphate precipitation was omitted, nuclease-treated lysates were concentrated and purified by three cycles of differential centrifugation. Approximately 300 ml. of crude lysate provided ample amounts of purified phage suspensions for electron-microscope preparations. In purifications for colorimetric nucleic acid determinations, 2 l. of crude lysate were used as the starting volume, and ammonium sulphate precipitation was not used.

Electron microscopy. Concentrated preparations of phages of ØCb 3, 6, 8r, 12r, 13, 23r and ØAc 20 were examined by the negative staining method of Brenner & Horne (1959) as modified by Bradley (1962). Dilutions of the phage suspensions were mixed with equal volumes of neutral 2% (w/v) potassium phosphotungstate containing 0·4% (w/v) sucrose, or with 1% (w/v) uranyl acetate (pH 4·5), and placed on carbon-stabilized Formvar-covered electron microscope grids. Specimens were observed with a Siemens Elmiskop I operated at 80 kv.

Nucleic acid determinations. The type of nucleic acid present in ØCb 13 and ØCb 23r was determined colorimetrically after extraction with 5% trichloroacetic acid. Deoxyribonucleic acid (DNA) was measured by the modified diphenylamine reaction (Burton, 1956) with deoxyadenosine (California Biochemical Corp.) as a standard. Ribonucleic acid (RNA) was estimated by the orcinol reaction (Mejbaum 1939; Schneider, 1957); standards were deoxyadenosine and purified yeast RNA (Worthington). For purposes of calculation, it was assumed that the ratio of purines to pyrimidines was 1:1, and that only purine-bound deoxyribose would be detected.

Another method of identifying the type of viral nucleic acid, recently described by Bradley (1965), was used with one of each of the seven serological types of caulobacter phages, and with controls of coliphages T2 and T5 obtained from Dr J. W. Banister and MS2 obtained from Dr A. J. Clark. This method differentiates between single-stranded DNA, double-stranded DNA, and RNA. Purified phage preparations stained with acridine orange in the presence of Na_2HPO_4 and

citrate and subsequently examined under ultraviolet irradiation give a yellow-green colour for double-stranded DNA, and orange-red with single-stranded DNA and RNA; single-stranded DNA is subsequently decolorized by treatment with citrate, while RNA retains the orange-red colour.

Sensitivity to ribonuclease. Knolle & Kaudewitz (1963) reported that ribonuclease inhibits the lytic activity of RNA-containing phages. This phenomenon proved useful in preliminary attempts to characterize the caulobacter phages. A mixture of 10^7 phage particles, 10^7 host bacteria and 50 μg. ribonuclease was incubated at 30° for 15 min., after which drops were placed on overlayers containing an inoculum of the host strain. Double controls, omitting phage and RNase, respectively, were included with each test. The inability of RNase to destroy free phage was independently established by incubating 10^9 phage particles with 50 μg. RNase in the absence of host organisms, and determining the plaque count on appropriate dilutions.

Inactivation by ultrasonic treatment. The sensitivity of phages to ultrasonic treatment was determined by treating suspensions for 1 and 2 min. with an ice-cooled $\frac{3}{8}$ in. probe ultrasonicator (MSE Ultrasonic Disintegrator, 60 W. output), and determining survival by plaque count.

Phage adsorption. Broth cultures of bacteria in the exponential phase of growth were sedimented at 8000*g* and resuspended in a small volume of adsorption medium at a concentration of about 5×10^9 colony-forming units/ml. (Among caulobacters the viable count does not accurately describe the total number of viable organisms in the population, since a large but variable proportion of the organisms occur in many-celled rosettes, each of which gives rise to a single colony on plating.) Addition of phages at a multiplicity of infection of 0·01 preceded incubation on a slow rotary shaker at 30°. Adsorption was followed by determining residual free phage in filtrates from samples of the incubation mixture, after 1/100 dilution and passage through a Millipore HA (0·45 μ) filter, held in a Swinney adapter. Phage lysates containing no added host organisms were filtered to determine the phage concentration at zero time and the proportion of phage passed through the filters.

Single-step growth and burst-size experiments. The procedures used were modelled after those described by Adams (1959). The temperature of incubation was 30°, the time allowed for phage adsorption was 15 min., and platings were made from the growth tube at intervals of 30 min. during 4–6 hr. Platings were made after 5 hr in the single-burst experiments.

RESULTS

The 23 Caulobacter phages isolated fall into seven distinct groups in terms of host range and serology (Tables 2, 3). We shall refer to these groups by Roman numerals. Additional properties have in most cases been determined for only a single phage strain belonging to each group. The first six groups comprise phages isolated by enrichment with various Caulobacter species and active only against species belonging to this genus. Phages of groups I–III are DNA-containing phages which share a common gross structure and are distinctive by virtue of their unusually large particle size. They share certain similarities in their relatively wide host ranges (Table 2), and some degree of serological inter-relationship (Table 3). Phages of groups IV, V and VI are small polyhedral RNA-containing phages, closely alike in morphology but completely distinct in serology and host range. Each group of

RNA phages has a very limited host range. The phages of group VII were isolated by enrichment with an Asticcacaulis strain, and can attack only Caulobacter strains which belong to this genus. They contain DNA, and have a morphology similar to that of many other DNA-containing bacterial viruses, such as coliphage lambda.

Table 2. *Host ranges of Caulobacter and Asticcacaulis phages*

	Phage groups							
	I	II	III	IV	V	Va	VI	VII
Host strains	ØCb 1, ØCb 3	ØCb 6, 7, 10, 11, 19	ØCb 13, 14, 16, 17, 18	ØCb 8r, ØCb 9	ØCb 2, 5, 12r, 15	ØCb 4	ØCb 23r	ØAc 20, 21, 22
C. vibrioides H, CB 5, CB 18	−	−	+	−	+	+	−	−
C. vibrioides G	−	+	−	−	+	−	−	−
C. vibrioides limonus CB 16, 21	−	−	+	−	−	−	−	−
C. crescentus CB 1	−	−	−	−	+	+	−	−
C. crescentus CB 2	−	−	+	−	+	−	−	−
C. crescentus CB 15	−	+	+	−	+	+	−	−
C. crescentus KA 2, KA 3	+	+	+	−	+	−	−	−
C. henricii KA 6, CB 4, 25, 36	−	−	+	−	−	−	−	−
C. henricii CB 23, KA 1	−	−	−	−	−	−	−	−
C. henricii aurantacus R	−	−	−	−	−	−	−	−
C. subvibrioides albus CB 88, CB 89	−	+	−	−	−	−	−	−
C. fusiformis CB 27, 29	−	−	−	−	−	−	+	−
C. leidyi CB 37	−	−	−	−	−	−	−	−
Type II CB 24, 26	+	+	−	−	−	−	−	−
C. bacteroides CB 6, 7	−	+	−	−	−	−	−	−
C. bacteroides CB 9, 10	−	−	−	−	−	−	−	−
C. bacteroides CB 8, 11	+	+	−	+	−	−	−	−
Type III KA 5	+	+	−	−	−	−	−	−
Type IV CB 35	−	−	−	−	−	−	−	−
Type V CB 28	−	−	−	−	−	−	−	−
Bacteroid strain CB 3	+	+	−	−	−	−	−	−
Vibroid strains CB 52, 54, 61	−	−	+	−	+	+	−	−
Vibroid strain CB 87	−	−	−	+	+	−	−	−
Henricii strain CB 34	−	−	−	−	−	−	−	−
A. excentricus AC 48	−	−	−	−	−	−	−	+
A. excentricus AC 12, KA 4	−	−	−	−	−	−	−	−
Asticcacaulis S-1; C-19	−	−	−	−	−	−	−	+

Host ranges

The host range of each phage was determined by placing 0·05 ml. of lysate containing 10^7 to 10^8 p.f.u. on an agar overlayer inoculated with the bacterial strain to be tested. Plates were examined after 18 and 48 hr. Results of tests with 23 Caulobacter phages and 46 strains of stalked bacteria are shown in Table 2. The generic specificity of Caulobacter and Asticcacaulis phages is absolute. The Caulobacter phages of groups I, II, III and V are capable of lysing more than one species in this genus.

The host ranges of 11 phages, representing all the Caulobacter phage groups isolated, were tested by Dr M. P. Starr against Flavobacterium (6 species), Erwinia (2 species), Xanthomonas (6 species), phytopathogenic Pseudomonas (13 species) and fluorescent soil Pseudomonas (26 species). No lytic action by the Caulobacter phages was found against any of these bacteria. Six *Escherichia coli* F^- and Hfr strains were also tested as possible hosts and gave negative results.

Table 3. *Antiserum neutralizaton of Caulobacter phages; first-order reaction constants*

Homologous systems are indicated by bold figures.

Group	Phages	Antiserum: As to Ø 3	As to Ø 6	As to Ø 13	As to Ø 8	As to Ø12	As to Ø 12	As to Ø20
I	ØCb 1	**258**	**540**	**23**	< 1	< 1	< 1	< 1
	3	**276**	**720**	**15**	< 1	< 1	< 1	< 1
II	ØCb 6	46	**4000**	17	All < 1	All < 1	All < 1	All < 1
	7	52	**3500**	24				
	10	42	**3140**	18				
	11	44	**3760**	13				
	19	40	**4500**	12				
III	ØCb 13	All < 1	200	**154**	All < 1	All < 1	All < 1	All < 1
	14		170	**154**				
	16		123	**130**				
	17		205	**133**				
	18		220	**180**				
IV	ØCb 8r	< 1	< 1	< 1	**690**	< 1	< 1	< 1
	9	< 1	< 1	< 1	**746**	< 1	< 1	< 1
V	ØCb 2	All < 1	All < 1	All < 1	All < 1	**346**	All < 1	All < 1
	4					**102**		
	5					**386**		
	12r					**460**		
	15					**330**		
VI	ØCb 23r	< 1	< 1	< 1	< 1	< 1	**1450**	< 1
VII	ØAc 20	All < 1	All < 1	All < 1	All < 1	All < 1	All < 1	**2860**
	21							**2880**
	22							**2580**

Serology

The results of the antiserum neutralization tests are shown in Table 3, expressed as first order reaction constants. It was possible to place the 23 phage isolate into seven serological groups. The pre-immune sera, included as controls, gave constants of less than 1 with homologous immunizing antigens, indicating that no 'normal' neutralizing antibodies were present before immunization had begun. The constant for phage ØCb 4 with antiserum 12 is somewhat lower than those obtained with the other phages of group V; the host range pattern of phage ØCb 4 also differs slightly from that of phages ØCb 2r, 5r, 12r and 15r. The phages of groups I, II and III show distant serological cross-relationships to each other. In an exceptional case, antiserum to phage ØCb 3 (group I) did not neutralize plaque-forming ability of phages of group III to any measurable extent, but in the reciprocal tests, antiserum to

phage ØCb 13 (group III) neutralized phages of group I. The remainder of the phages, which make up groups IV, V, VI and VII, were not neutralized by antisera to any other groups.

Electron microscopy of phages

Caulobacter phage ØCb 13 is shown in Pl. 1, fig. 1. It possesses an unusually large and elongated head. The dimensions of an intact phage head are 170 × 50 mμ; tails are 200–250 mμ in length. Plate 1, fig. 2, shows structures that are interpreted to be cross-sectional fragments of the phage ØCb 13 tail. The hollow core is observable. The outer diameter of the cross-section is about 10 mμ, in agreement with the width of the intact tail. Apices of the head structure can be observed in the empty heads of preparations precipitated with ammonium sulphate (Pl. 1, fig. 3). Most of the ØCb 13 particles in such preparations were inactivated, having been subjected to osmotic shock, and appear as tailless empty heads. Phages ØCb 3 and 6 are similar in morphology to ØCb 13.

Caulobacter phage ØCb 23r is shown in Pl. 1, fig. 4. It has a diameter of 21–23 mμ and no tail has been observed. Morphologically it resembles the coliphage R-17 (Crawford & Gesteland, 1964). In Pl. 1, fig. 4, the narrow filaments to which the phage particles adhere may be DNA fibres, since they could be removed from the preparations by treatment with deoxyribonuclease. Phages ØCb 8r (Pl. 1, fig. 5) and ØCb 12r are similar in size (22–23 mμ diameter) to phage ØCb 23r and also do not appear to have tails. Phosphotungstate stains of these two phages caused most phage particles to lose their structural integrity, and very few intact phage particles could be identified in such preparations. However, uranyl acetate stains gave satisfactory results.

Plate 2, fig. 6, shows Asticcacaulis phage ØAc 20; the hexagonal shape of the ØAc 20 head is more evident in empty particles. A circular electron-transparent area can be seen just inside the hexagonal boundary of many of the empty heads, and is probably due to the way in which the protein coat of the head collapsed after becoming emptied of its nucleic acid. Most of the empty heads lack a tail. The dimensions of the intact phage head are 65 × 70 mμ, and the tail is 150 mμ in length.

Phage-plaque morphology

Four distinct plaque morphologies, shown in Pl. 2, figs. 7–10, were seen among the seven phage groups. Phages ØCb 3 and 6 with their host *Caulobacter bacteroides* strain 11, and phage ØCb 13 with its host *C. crescentus* strain 15 gave clear plaques with a diameter of 0·5 to 1 mm. (Pl. 2, fig. 7). Phage ØAc 20 also produced clear plaques but these were extremely minute, with an average diameter of less than 0·5 mm. (Pl. 2, fig. 8). Phages ØCb 8r, 12r and 23r produced cloudy diffuse plaques on their respective host strains. The plaque sizes of phages ØCb 8r and ØCb 23r (Pl. 2, fig. 9) varied from 0·5 to 2 mm. in diameter, but despite their cloudy character were not difficult to detect. Phage ØCb 12r and phages related to it (ØCb 2r, 4r, 5r, 15r) formed extremely cloudy plaques (Pl. 2, fig. 10).

Nucleic acid determinations

The results of colorimetric analysis of purified preparations of phage ØCb 13 showed that all the orcinol-reacting material present could be ascribed to the presence of DNA as determined by the diphenylamine reaction. The other phage

tested by these procedures, ØCb 23r, contained only RNA; no DNA was detectible in purified preparations of it.

The results of the acridine orange-staining technique are shown in Table 4.

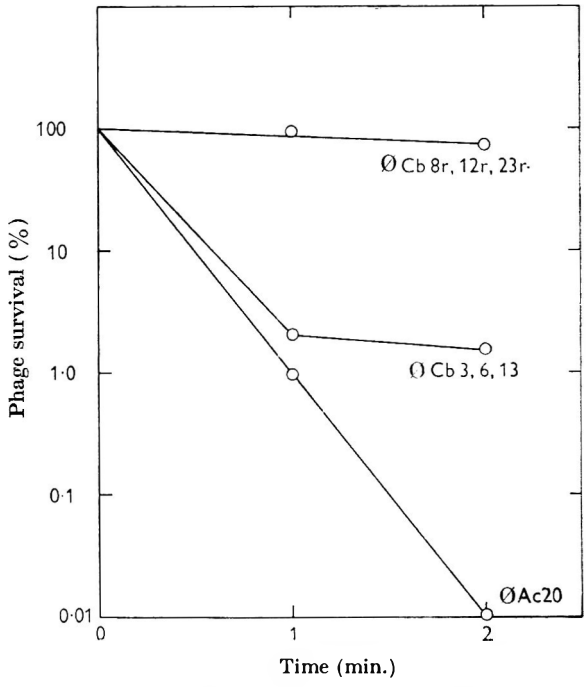

Fig. 1. Inactivation of Caulobactor phages by ultrasonic treatment.

Table 4. *Results of acridine orange colour test (Bradley, 1965) for nucleic acid identification in Caulobacter and other phages*

Phage	Group*	Primary acridine orange reaction	Decolorization with 0·1 M-citrate	Nucleic acid
ØAc 20	VII	Yellow-green		DNA
ØCb 3	I	Yellow-green		DNA
ØCb 6	II	Yellow-green		DNA
ØCb 13	III	Yellow-green		DNA†
T 2 (control)	III	Yellow-green		DNA
T 5 (control)	III	Yellow-green		DNA
ØCb 8r	IV	Orange-red	–	RNA
ØCb 12r	V	Orange-red	–	RNA
ØCb 23r	VI	Orange-red	–	RNA†
MS 2 (control)		Orange-red	–	RNA

* See Tables 2, 3.

† Nucleic acid also identified by other colorimetric methods.

Phage inactivation

The plaque-forming ability of phages ØCb 8r, 12r and 23r was inhibited by ribonuclease in the presence of host bacteria. However, when the phages were exposed to RNase in the absence of the host, followed by dilution before plating, phage inactivation did not result. The other phages tested were resistant to RNase.

The small RNA phages (ØCb 8r, 12r, 23r) which seem to lack tails, were more resistant to inactivation by ultrasonic treatment than were tailed phages (Fig. 1). The three large phages (ØCb 3, 6, 13) which resemble each other morphologically also have similar ultrasonic inactivation curves. These three phages, which have flexible tails, were less sensitive than was phage ØAc 20, which has a relatively rigid tail. Marvin & Hoffman-Berling (1963) studied the sensitivity of phages to ultrasonic treatment in relation to their structure; filamentous phages and phages with tails were much more sensitive to ultrasonic treatment than were the polyhedral phages lacking tails, such as fr. Caulobacter phages ØCb 8r, 12r and 23r, examined in the present work, gave results similar to the polyhedral phages studied by Marvin & Hoffman-Berling (1963).

None of the Caulobacter phages was sensitive to chloroform. Only phages ØCb 8r and 23r withstood osmotic shock following exposure to 10% NaCl. In heat-inactivation experiments phage ØCb 12r was inactivated by exposure to 40° for 1 hr, while phages ØCb 3 and 8r were stable at that temperature. Higher temperatures caused inactivation of all three phages. Phages ØCb 3, 8r, 12r and 13 were inactivated by 0·02 M-phosphate buffer (pH 7) and by 0·02 M-tris-(hydroxymethyl)-aminomethane buffer (Sigma 121; pH 7).

Adsorption of Caulobacter phages

Results of phage adsorptions done in the presence of 0·002 or 0·004 M-$MgSO_4$ are shown in Table 5. Because the concentrations of caulobacters in the adsorption mixtures could not be accurately determined because of rosette formation, first-order reaction constants were not calculated. The use of filtration to obtain unadsorbed phage particles was preferable to the removal of adsorbed phages by sedimentation, since stalked caulobacters and rosettes were difficult to sediment

Table 5. *Phage adsorption to Caulobacter and Asticcacaulis strains*

Phage	Group*	Host	$MgSO_4$ concentration (M)	Free phage at time (min.) 2	5	10
				% free phage		
ØCb 3	I	*C. bacteroides* 11	0·002	51	30	19
ØCb 3	I	*C. bacteroides* 11	0·004	22	14	10
ØCb 6	II	*C. bacteroides* 11	0·002	75	47	35
ØCb 6	II	*C. bacteroides* 11	0·004	32	22	14
ØCb 13	III	*C. crescentus* 15	0·004	35	19	10
ØCb 8r	IV	*C. bacteroides* 11	0·002	18	6	4
ØCb 12r	V	*C. crescentus* 15	0·002	55	31	25
ØCb 23r	VI	*C. fusiformis* 27	0·002	40	28	21
ØAc 20	VII	*A. excentricus*	0·002	50	46	43

* See Tables 2 and 3.

quantitatively. The presence of rosettes also prevented determination of numbers of infected caulobacters as an efficient measure of adsorption, since more than one caulobacter per large rosette might be infected, but the rosette would plate as only one infective centre. Since most of the Caulobacter phages were adequately absorbed

within 15 min. after their addition, single-step growth experiments could be done satisfactorily, and the optimal adsorption conditions for each phage were not determined.

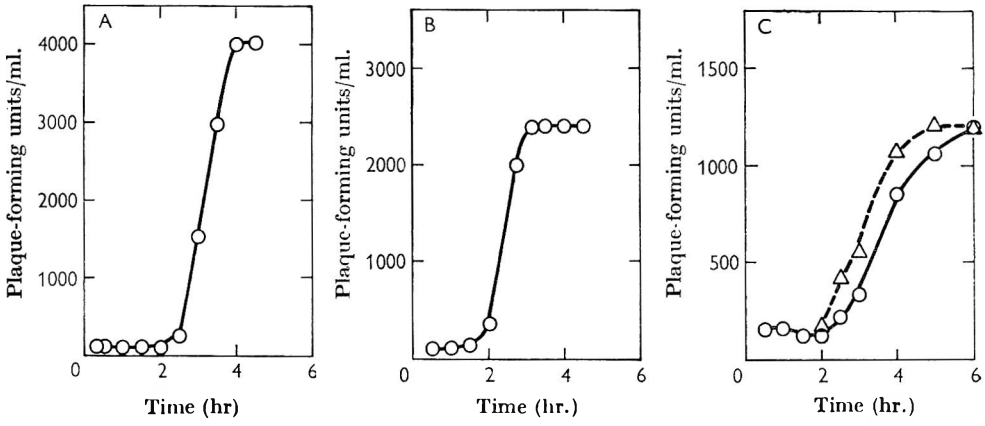

Fig. 2. Single-step growth curves of phages on Caulobacter hosts. A. phage ØCb 8r, on *C. bacteroides* 11; B. phage ØCb 13 on *C. vibrioides* 15; C. phage ØAc 20 on *A. excentricus* 48. ○——○, infective centres; △---△, chloroform treatment.

Table 6. *Latent period and burst sizes of selected Caulobacter phages*

Phage	Group*	Host	Phage latent period (min.)	Host generation time (min.)	Burst size
ØCb 3	I	*C. bacteroides* 11	150	215	10
ØCb 6	II	*C. bacteroides* 11	150	215	8
ØCb 13	III	*C. crescentus* 15	105	108	.
ØAc 20	VII	*A. excentricus* 48	150	120	.
ØCb 8r	IV	*C. bacteroides* 11	150	215	33
ØCb 12r	V	*C. crescentus* 15	150	108	22
ØCb 23r	VI	*C. fusiformis* 27	180	300	14

* See Tables 2 and 3.

Single-step growth and single-burst experiments

A summary of data obtained from single-step growth and single-burst experiments is shown in Table 6. The latent period of all the Caulobacter phages tested was relatively long (2 hr or more) and the burst sizes were very small. Single-step growth curves are shown for three representative Caulobacter phages in Fig. 2. In the phage ØAc 20 single-step growth experiment, the curve in which samples were exposed to 5 % chloroform represents mature intracellular phage particles in addition to phage particles already released in normal bursts.

DISCUSSION

It is notable that the first systematic search for phages active against caulobacters has yielded, out of a total of 23 strains, no less than eight phages which contain RNA. Furthermore, these eight phages fall into three entirely distinct

serological groups, and are also readily distinguishable from one another in terms of specific host ranges. Hitherto, only two species of bacteria have been reported to serve as hosts for RNA-containing viruses: *Escherichia coli* (Loeb & Zinder, 1961; Dettori, Maccacaro & Piccinin, 1961; Davis & Sinsheimer, 1963; Marvin & Hoffman-Berling, 1963; Crawford & Gesteland, 1964; Bradley, 1964), and *Pseudomonas aeruginosa* (Feary, Fisher & Fisher, 1963). These facts suggest that the natural phage populations active against Caulobacter species may contain an unusually large proportion of RNA viruses.

The discovery of three new types of RNA-containing phage provides a somewhat broader basis for drawing conclusions about the properties of this interesting category of bacterial viruses. Like the types which have been earlier described, the RNA phages active against caulobacters are small (21–23 mμ in diameter) and apparently simple in structure. All of them share the property of susceptibility to the action of RNase in the presence of host bacteria, a character which Knolle & Kaudewitz (1963) claimed to be useful in determining the type of nucleic acid present in newly-isolated phages.

One of the initial goals of our work was to obtain a viral system that might be used to mediate gene transfer in the Caulobacter group. To this end, several attempts were made to detect lysogeny in Caulobacter and Asticcacaulis strains, particular attention being given to those strains which were resistant to the virulent phages which had been isolated. Such strains were exposed to ultraviolet irradiation and subsequently incubated in growth medium for several hours, after which filtrates were plated on randomly selected potential indicator strains. No evidence of lysogeny was detected. Attempts to use Caulobacter phage ØAc 20 to lysogenize its host *Asticcacaulis excentricus* strain 48, also failed. Following exposure of this host to phage ØAc 20, many phage-resistant clones were isolated, but none of the purified phage-resistant strains yielded detectable phage ØAc 20 particles after ultraviolet irradiation.

Taxonomic implications

The caulobacters are a group of Gram-negative polarly-flagellate bacteria, until recently little known, and distinguishable from other bacteria that conform to this general description only by their ability to form cellular stalks and to secrete holdfasts. The study of the group by Poindexter (1964), based on a detailed comparative analysis of many pure cultures, led for the first time to an understanding of their biological properties. Poindexter's work showed that in many physiological respects the caulobacters resemble the aerobic chemoheterotrophic pseudomonads. At the same time, studies on their development (Poindexter, 1964; Stove & Stanier, 1962) showed that stalk formation is not a facultative phenomenon, but a morphogenetic event intimately geared with the process of cell division: in effect, no caulobacter can divide until it has formed a stalk. There is, accordingly, a deep difference in the reproductive cycle between the caulobacters and all other eubacteria of the pseudomonas line. On these and other grounds, Poindexter (1964) recommended the retention of a special family, the Caulobacteraceae, for these organisms. In this context, phage specificity is relevant. Dr M. P. Starr & Mrs G. R. Cosens (personal communication) have tested our Caulobacter phages against many strains of Pseudomonas, Xanthomonas and Erwinia, and we have tested them against strains

of *Escherichia coli*. In no case has lytic activity been observed. Conversely, Dr Starr & Mrs Cosens have tested 65 phages active against members of the Pseudomonas Xanthomonas and Erwinia groups on 60 representative isolates of Caulobacter, with completely negative results. Thus far, therefore, the caulobacters emerge as a completely isolated group with respect to host/phage interactions.

In the family Caulobacteraceae, Poindexter recognized two genera: the original genus *Caulobacter* Henrici and Johnson, and a new genus, *Asticcacaulis*. In Asticcacaulis strains, there is a topological separation between stalk and holdfast. While the holdfast occupies the same position as in the caulobacters, at one pole of the cell, the stalk develops as a lateral rather than as a polar extrusion, and consequently does not have holdfast material at its base. Base ratio analyses of DNA from Caulobacter and Asticcacaulis strains, performed by Dr M. Mandel (Poindexter, 1964) also revealed significant differences. The moles per cent of guanine+cytosine in eight Caulobacter strains ranged from 62 to 67, substantially coinciding with the range characteristic of aerobic pseudomonads; the value obtained for one Asticcacaulis strain was 55, significantly lower than the values either for Caulobacter species or for the aerobic pseudomonads. As we have shown, there appears to be an absolute separation between these two genera of stalked bacteria with respect to host specificity for bacteriophages: none of our numerous phages active against members of the Caulobacter group lysed any strain of Asticcacaulis, and the group of phages active against Asticcacaulis was unable to lyse any of the Caulobacter strains. Accordingly, Poindexter's general taxonomic conclusions on the stalked bacteria are very well supported by the results of the present study.

We wish to thank Dr J. H. McAlear, Director of the Electron Microscope Laboratory, for his helpful advice and Mr Philip Spencer for his preparation of the phage photographs. One of us (J.M.S.) was supported by a National Institutes of Health Traineeship.

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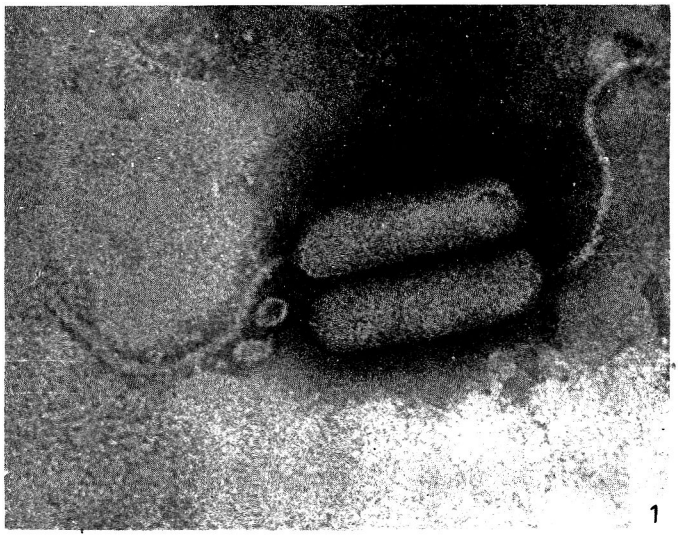

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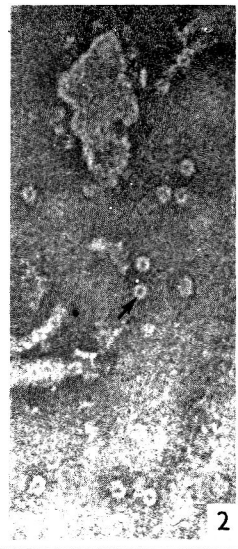

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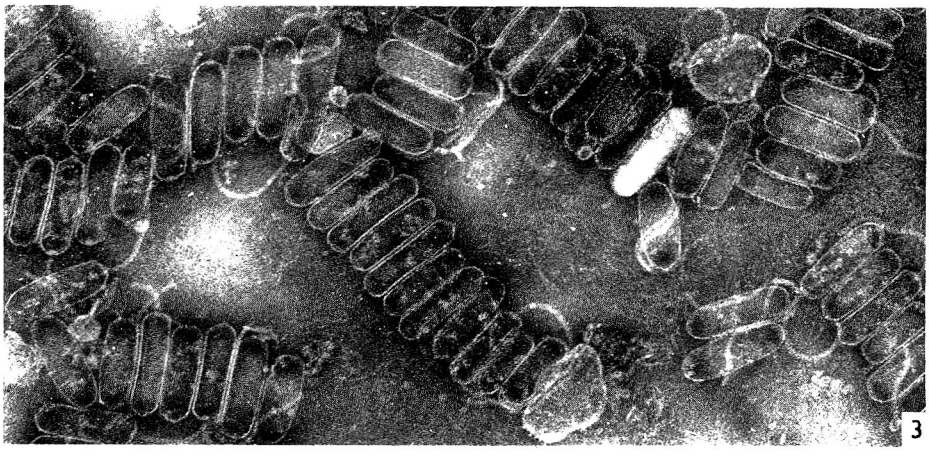

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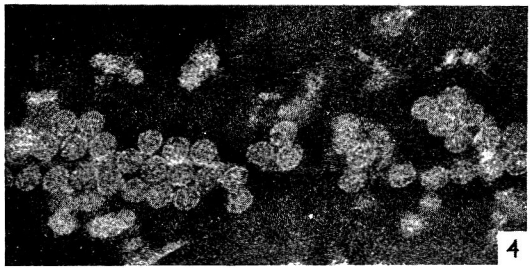

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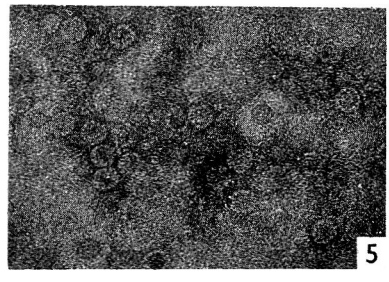

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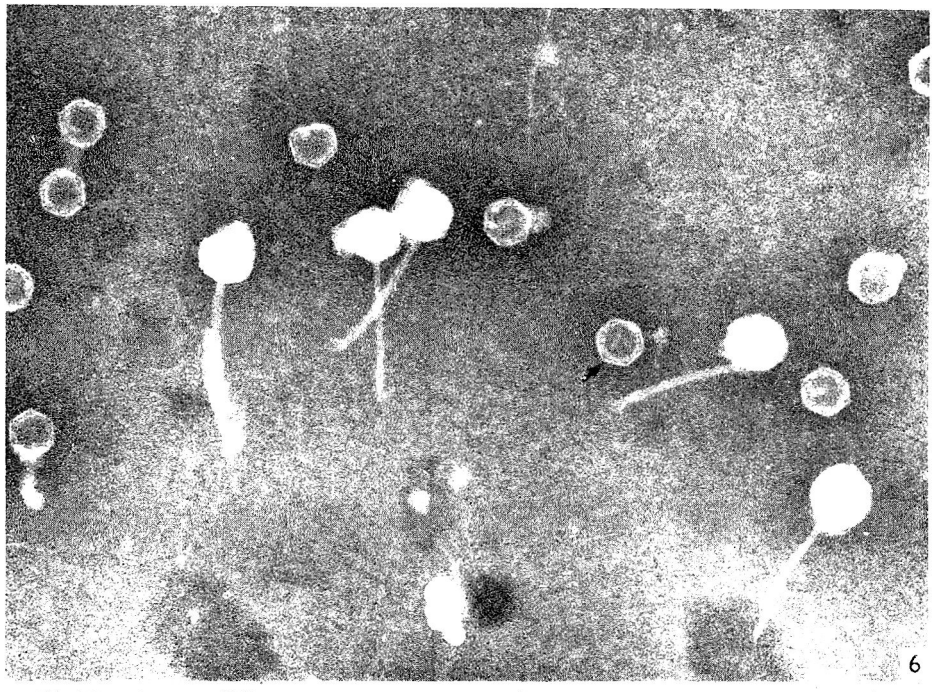
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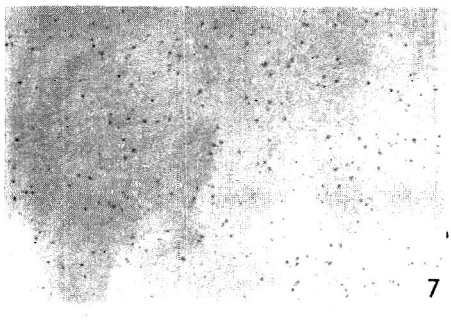
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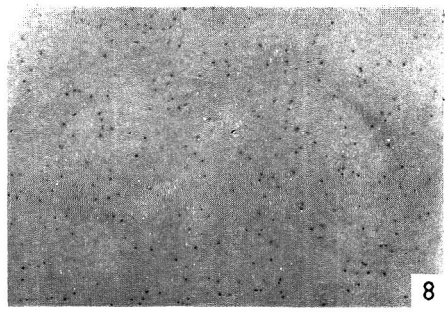
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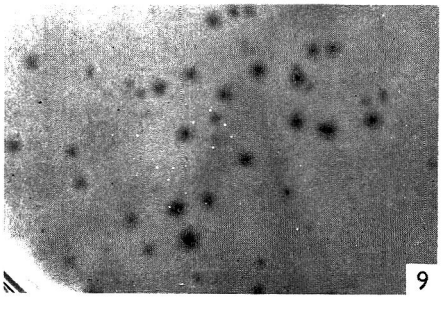
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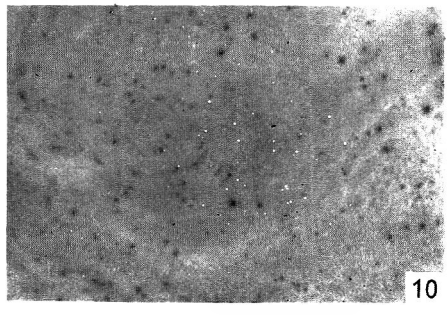
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EXPLANATION OF PLATES

PLATE 1

Fig. 1. Caulobacter phage ØCb 13. Intact phage particles. Phosphotungstate stain. × 200,000.

Fig. 2. Caulobacter phage ØCb 13. Cross-sectional fragments of phage tails. Phosphotungstate stain. × 200,000.

Fig. 3. Caulobacter phage ØCb 13. Phage preparation inactivated with ammonium sulphate precipitation. × 96,000.

Fig. 4. Caulobacter phage ØCb 23r. Phosphotungstate stain. × 160,000.

Fig. 5. Caulobacter phage ØCb 8r. Uranyl acetate stain. × 160,000.

PLATE 2

Fig. 6. Asticcacaulis phage ØAc 20. Phosphotungstate stain. Arrow: empty head with interior electron-transparent ring. × 120,000.

Figs. 7–10. Caulobacter phage plaque morphology. × 1.

Fig. 7. ØCb 13 plated on *C. crescentus* strain 15.

Fig. 8. ØAc 20 plated on *A. excentricus* 48.

Fig. 9. ØCb 8r plated on *C. bacteroides* 11.

Fig. 10. ØCb 12r plated on *C. crescentus* strain 15.

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Growth Characteristics of Some Gram-negative Bacteria

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SUMMARY

The growth of individual organisms of four Gram-negative species has been measured. The growth rate of the individual was not in general constant, and its mean value differed from one organism to another in the same culture. There was positive correlation between the size of an organism at termination and the size of its mother at termination. These findings are contrary to the major postulates of the Koch & Schaechter hypothesis, but are so far no more than qualitatively established. Fission is unsymmetrical in respect of growth rate and size; the growth rate and size of sisters differ systematically according to their relative positions in the family tree.

INTRODUCTION

In this paper we record some measurements of the growth of individual organisms, indicate a number of seeming regularities in the pattern of growth, and compare our findings with the postulates of the Koch & Schaechter (1962) hypothesis. Koch & Schaechter compared some of the derivative consequences of the hypothesis with experimental results obtained by Schaechter, Williamson, Hood & Koch (1962) but neither group of authors seems to have made a direct test of the postulates themselves. In pointing out deficiencies in the hypothesis we must not be thought captious; it is not to be expected at present that any 'model' of bacterial growth will agree more than very roughly with the known phenomena. In our opinion the Koch & Schaechter hypothesis is likely to prove at least as fruitful and suggestive as any older view, and it certainly deserves as much consideration.

We have worked entirely with rod-like organisms, and in common with many authors we have accepted the approximation of taking the length of an organism as a measure of its volume. Collins & Richmond (1962) discussed the difficulty of measuring volume accurately, and Powell & Errington (1963*b*) showed that there can be detectable differences between the diameters of organisms drawn from the same steadily growing culture. Our curves for the lengthwise growth are thus not strictly paralleled by the true curves of growth in volume.

EXPERIMENTAL METHOD

Our numerical results consist of repeated measurements of length made at known times on single organisms in steadily growing cultures. The measurements were made with a Dyson image-splitting eyepiece (Dyson, 1959, 1960) in exactly the same way and under the same conditions as those of Powell & Errington (1963*b*). Six series of experiments were carried out (Table 1) with four species and two media: tryptic

Table 1. *Organisms and growth conditions*

Series reference no.	Organism	Medium	Temperature
Sm 1	*Serratia marcescens*	TMB	35°
Pm 1	*Proteus morganii*	TMB	30°
Ec 1	*Escherichia coli*	TMB	30°
Ec 2	*E. coli*	TMB	30°
Pa 1	*Pseudomonas aeruginosa*	TMB	35°
Pa 2	*P. aeruginosa*	H 12P	30°

meat broth (TMB) and a chemically defined medium (H 12P) described by Powell & Errington (1963*a*). The *Escherichia coli* in series Ec 1 had at the time been recently isolated from human faeces. In series Ec 2, done a year later with nominally the same strain, the organisms grew more slowly and were larger and less uniform in size. It was discovered independently that the antigenic structure of the strain altered greatly on serial subculture; we therefore feel justified in treating the two series separately. The long generation times (mean 52 min.) in series Pa 2 enabled us to make 50–100 length measurements during the life-span of an organism; no special details of the law of growth were thereby brought to light. Young cultures of *Proteus morganii* contained many filamentous organisms which broke down or were diluted out as the culture aged. Few remained in the inocula transferred to the culture chamber; organisms of moderate size were selected for examination, and their progeny, as far as we traced them, behaved regularly (Table 2).

The measurements in a typical experiment were conducted as indicated in Fig. 1; they were begun on a conveniently situated organism which was followed to its termination, then continued on one of its daughters, and so on, until crowding made the measurements difficult and inaccurate. The process was repeated on other

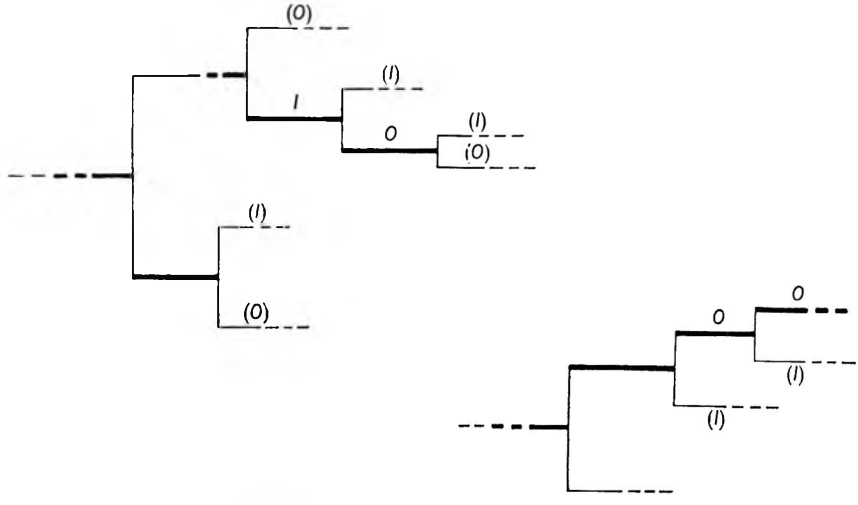

Fig. 1. Scheme of observations n a typical experiment. Measurements were made on the organisms represented by heavy horizontal lines. The organisms are marked *I* (inner) or *O* (outer) according to their relative position in the family tree.

parent organisms. Each experiment provided data on 1–3 such sequences of organisms. Powell & Errington (1963*a*) showed that rod-like organisms might be classified as 'inner' (*I*) or 'outer' (*O*) according to their position in the family tree. We therefore recorded, where it was known, the *position symbol* (*I* or *O*) of each of the organisms measured.

A few experiments were also made to provide additional information on the relative sizes of sisters at inception. The measurements, made alternately on the members of each pair for a short time only, gave estimates of initial size probably less accurate than the rest, and these are not included in column 3 of Table 2.

RESULTS

Growth of individual organisms

There are two systematic errors of which we shall have to take account in treating length measurements as equivalent to volume measurements. First, some erratic changes in length occur at fission, and these can be ascribed in part to the rounding of the ends of the newly-formed organisms, the sum of whose lengths is always (in our experience) greater than the length of the mother (see also Powell & Errington, 1963*b*). Second, the length (x) is not directly proportional to the volume (v). The shape of the organism is roughly that of a cylinder terminated by hemispherical caps, and

$$v = \pi r^2(x-2r/3),$$

where r is the transverse radius. If the increase in volume is strictly exponential with growth rate ν,

$$\frac{d\log v}{dt} = \nu$$

and we find

$$\frac{d\log x}{dt} = \nu(1-2r/3x).$$

That is, the rate of increase of $\log x$ increases with x. At worst, when the length (x_i) at inception is $2r$ and the organism is then a sphere, the acceleration is very small (Fig. 2); it is probably of no account in our work, in which no x_i was less than $4r$. We noted, however, that positive values of $d^2\log x/dt^2$ were more frequent than negative values.

We found that in most instances the logarithm of the length of an organism was a nearly linear function of time (t), but in some the plot was certainly curved—the curvature could take either sense (Fig. 3). Viable organisms having a strongly curved characteristic were not obviously remarkable in other ways, and there was therefore no point in establishing limits of statistical significance for the various degrees of curvature.

With some exceptions we fitted an equation of the form

$$\log x = \alpha+\beta t+\gamma t^2 \qquad (1)$$

to the set of measurements on each organism. The fitting was carried out by the method of weighted least squares, based on the assumption that the random error in measuring x was independent of the absolute value of x. The coefficients α, β, γ were thus determined by the three normal equations

$$\Sigma t_r^n x_r^2 \log x_r = \alpha\Sigma t_r^n x_r^2+\beta\Sigma t_r^{n+1} x_r^2+\gamma\Sigma t_r^{n+2} x_r^2 \quad (n = 0, 1, 2)$$

in which the x_r are the measured lengths at corresponding times t_r. The normal equations were solved for each organism by means of an electronic digital computer. The computer was so programmed as to yield, besides the three coefficients α, β, γ the residual

$$x_r - \exp(\alpha + \beta t_r + \gamma t_r^2)$$

at each measured value, the root-mean-square (r.m.s.) residual, and the estimated length of the organism at inception (x_i) and termination (x_t'), the times t_i, t_t of these events being known:

$$x_i,\ x_t' = \exp(\alpha + \beta t_{i,t} + \gamma t_{i,t}^2). \qquad (2)$$

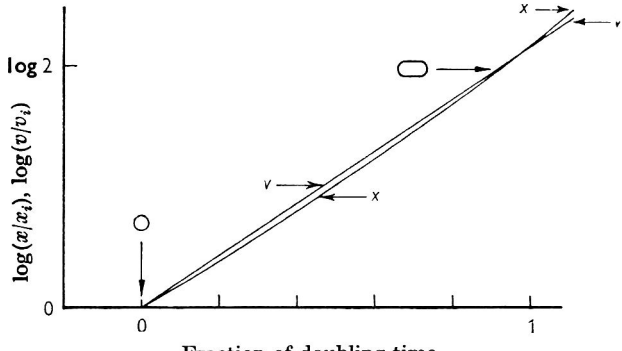

Fig. 2

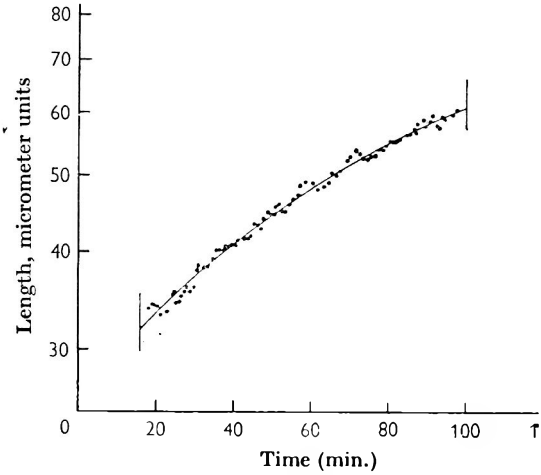

Fig. 3

Fig. 2. Growth of an organism initially spherical. If the law of growth in volume (v) is strictly exponential, the rate of growth in length (x) is slightly accelerated.

Fig. 3. An organism of *Pseudomonas aeruginosa* showing a diminishing growth rate (series Pa2). The vertical bars indicate the times of inception and termination, and the continuous line was obtained by fitting equation (1) to the observations. The deviation from linearity, Δ, is $-0{\cdot}113$. Logarithmic vertical scale.

Our results included some sequences of measurements covering only part of the life-span of an organism: its approach to termination or its growth for a short time after inception. Such sequences were used only to obtain estimates of x_i or x_t; those which included less than sixteen t_r, x_r pairs of co-ordinates were fitted to a simpler equation

$$\log x = \alpha + \beta t$$

in order not to embarrass a χ^2 test, subsequently applied, for the overall goodness of fit of equation (1).

The grand r.m.s. residual from equation (1) for the total of 6000 length measurements was $0{\cdot}0762\ \mu$, i.e. about $2\frac{1}{2}$ times the standard deviation to be expected from the Dyson eyepiece under the best conditions (Powell & Errington, 1963*b*). The circumstances of the experiments were such that the full sensitivity of the eyepiece might well not be attained, but the large residual suggested that the goodness of fit of (1) should be investigated in a more detailed way. We therefore made an examination of the signs of the residuals (Jeffreys, 1961).

Suppose we have a series of n measurements to which a polynomial equation containing m parameters has been fitted, and suppose that the probability of a change of sign between successive residuals is z. There are $n-1$ possible changes of sign, and m are ensured by the fitting of an m-parameter curve. Thus the effectively variable number of changes of sign will be $n-m-1$, and the probability of finding actually s changes will be given by the binomial term

$$\binom{n-m-1}{s-m} z^{s-m}(1-z)^{n-s-1}$$

—provided that the assumed equation is a true representation of the law underlying

Table 2. *Mean, standard deviation* (S.D.) *and range of generation time* (τ), *length at inception* (x_i), *length at termination* (x_t) *and mean growth rate* (κ); *deviation from linearity* (Δ) *and corrected ratios* (p_I, p_O) *of* x_i (*daughter*) *to* x_t (*mother*). *Unit of time* : 1 *min.; unit of length:* 1 μ; *number of observations: n*

Series		τ	x_i	x_t	$10^2\,\kappa$	$10^2\,\Delta$	p_I	p_O
Sm 1	Mean	22·4	2·86	5·59	3·02	—	0·491	0·509
	S.D.	6·51	0·47	0·85	0·36	—	—	—
	Range	13	2·04	4·29	1·92	−13·1	0·431	0·491
		42	4·42	8·77	3·44	+14·4	0·545	0·543
	n	20	23	27	19	19	12	8
Pm 1	Mean	27·1	4·53	8·79	2·35	—	0·499	0·501
	S.D.	5·72	0·67	1·15	0·20	—	—	—
	Range	20	3·36	6·44	1·93	− 4·2	0·470	0·451
		41	6·30	11·87	2·69	+11·7	0·532	0·549
	n	27	38	38	26	26	35	30
Ec 1	Mean	25·8	3·36	6·67	2·64	—	0·494	0·506
	S.D.	3·27	0·33	0·61	0·17	—	—	—
	Range	21	2·94	5·54	2·37	− 5·0	0·456	0·461
		33	4·45	7·92	2·90	+13·2	0·531	0·575
	n	20	23	28	18	18	11	9
Ec 2	Mean	31·1	4·54	8·99	2·19	—	0·499	0·504
	S.D.	5·91	1·08	2·28	0·29	—	—	—
	Range	22	3·17	5·89	1·37	− 6·4	0·413	0·434
		45	7·51	16·35	2·68	+13·2	0·629	0·594
	n	25	40	42	25	25	30	26
Pa 1	Mean	32·7	3·26	6·32	2·04	—	0·493	0·508
	S.D.	4·64	0·40	0·76	0·19	—	—	—
	Range	24	2·48	5·11	1·74	−11·3	0·453	0·474
		40	4·04	7·89	2·47	+13·0	0·535	0·552
	n	30	31	33	30	30	5	16
Pa 2	Mean	52·4	2·82	5·53	1·18	—	0·494	0·507
	S.D.	11·23	0·47	0·73	0·21	—	—	—
	Range	39	1·86	3·76	0·75	−11·3	0·410	0·450
		83	3·45	6·94	1·56	+11·1	0·550	0·611
	n	20	38	33	20	20	21	16
Sm 1 omitting 1 fission	Range	—	2·04	4·29	—	—	—	—
		—	3·51	6·82	—	—	—	—
Ec 2 omitting 4 fissions	Range	—	3·39	6·69	—	—	—	—
		—	6·43	13·37	—	—	—	—

the measurements. The expected value of s is then $m+(n-m-1)z$. We formed a χ^2 for each fitting of (1):

$$\chi^2 = \frac{\{(s-m)-(n-m-1)z\}^2}{(n-m-1)z} + \frac{\{(n-s-1)-(n-m-1)(1-z)\}^2}{(n-m-1)(1-z)}$$

(1 degree of freedom). As Powell & Errington (1963*b*) explained, the method of measurement we adopted resulted in association between successive residuals, and the appropriate value of z was consequently about 1/3; m was 3, whence

$$\chi^2 = (3s-n-5)^2/2(n-4).$$

Of 163 curves thus tested, 29 gave a χ^2 exceeding the 0·05 probability limit; in only 2 of the 29 did s exceed its expectation. The sum of χ^2 for the 163 was 326. We conclude that (1) is usually a satisfactory representation of the law of growth, but that

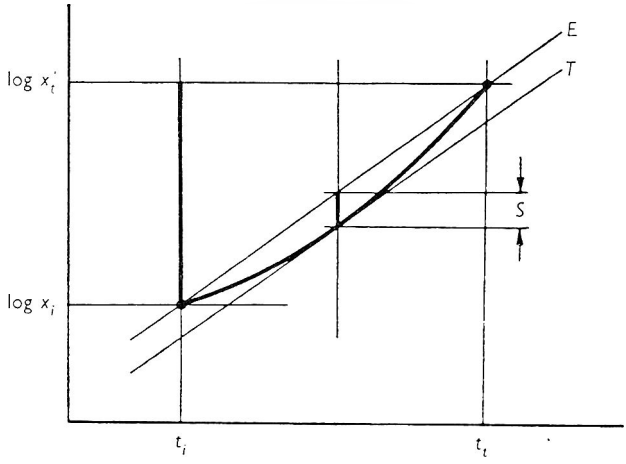

Fig. 4

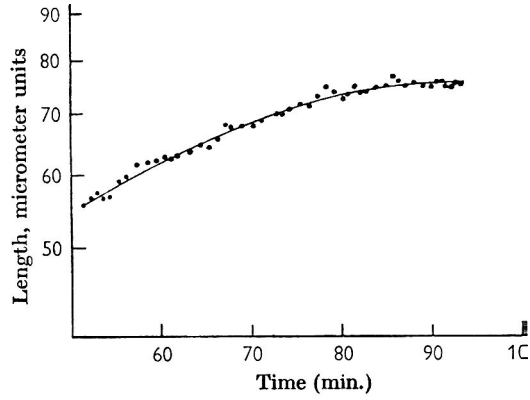

Fig. 5

Fig. 4. The definition of Δ. The heavy curved line represents a typical growth curve. Through its end points $(t_i, \log x_i)$, $(t_t, \log x_t')$ draw a straight line E. Draw T parallel to E and tangent to the curve. The vertical distance between T and E, measured upwards from T, is S. Then

$$\Delta = \frac{S}{\log(x_t'/x_i)}.$$

Fig. 5. Early growth of a non-viable organism of *Pseudomonas aeruginosa* (series Pa 1).

runs of residuals of the same sign are improbably frequent. The fluctuations in growth rate differ from one organism to another, and cannot be assimilated to a quite simple formula. This may not be so for the law of growth in volume, to which length measurements give only an approximation.

The crude numerical results are summarized in Table 2. Instead of recording the coefficients of equation (1) (of which α and β are not independent of the time origin) we have characterized the individual growth curves by the two quantities κ and Δ. Of these, κ is the mean growth rate over the life-span of the organism:

$$\kappa = \frac{\log(x_t'/x_i)}{\tau}. \qquad (3)$$

Here the x_i and x_t' belong to the same organism, not the same fission, and they were

estimated from the fitted curves (equation (2)). The definition of Δ is given in Fig. 4; it appears unnecessarily complicated, but Δ gives a more immediate and appealing impression than does γ of the degree of curvature over the life-span of the organism. For equation (1)

$$\Delta = \gamma\tau/4\kappa \tag{4}$$

and so it has the same sign as γ. In Fig. 2, the curve of accelerated growth in length has $\Delta = +0{\cdot}028$.

In the course of the experiments we happened upon two organisms which turned out to be non-viable. The growth of one of them is illustrated in Fig. 5. Although its size did increase during the earlier stages of observation, the initial growth rate was only about 0·014 min.$^{-1}$—smaller than the mean rate for any other organism in the series. The other was the daughter of a mother notable for having much the smallest x_t in the series (Ec 2).

Some general features of the family tree

To permit synoptic examination we made lists of the following quantities (where they were known) belonging to each organism:

(i) The position symbol, *I* or *O*.

(ii) The generation time, τ.

(iii) The length at inception and termination, estimated from the fitted growth curves.

(iv) The mean growth rate of the individual, κ.

(v) The sign of the coefficient γ in equation (1).

(vi) A quantity q relating the length of the organism at inception to the length of its mother at termination:

$$q = 2x_i - x_t(\text{mother}). \tag{5}$$

(vii) The ratio, p, of length of daughter to length of mother. Actually a small correction (discussed below, p. 116) was deducted from the estimated lengths before forming the p. The correction did not alter the ranking of the p in order of magnitude.

(viii) The sign of $(x'_t/x_i - 2)$, x_i and x_t belonging to the same organism. This quantity is positive or negative according as the organism increases in size by more or less than a factor of 2 over its life-span.

Table 3. *Serratia marcescens, series Sm* 1

Association between position symbol and mean growth rate. $V = +0{\cdot}387$.

	$\kappa >$ sample median	$\kappa <$ sample median	Row total
I	6½	3½	10
O	1½	4½	6
Column total	8	8	16

From pairs of some of these quantities we computed, for each series of experiments, Yule's coefficient of absolute association, V (Kendall, 1947). In order to form the classes of a 2×2 contingency table, the range of metrical quantities was dichotomized at the sample median (Table 3 gives an example). Our sample sizes were too small to justify the computation of a large number of product-moment

correlations, and we expected to detect only the cruder and more regular features of the growth pattern. Many of the individual V were statistically non-significant, but the combined significance of the V of any one kind was tested by forming the χ^2 from the class totals in the usual way. In the first place we accepted as suggestive only those V which had the same sign in every series of experiments.

We found the following to be true of every series:

(*a*) Position symbol I is associated with $p < \frac{1}{2}$. In pairs of sisters, the inner organism is usually the smaller at inception. $\chi^2 = 0{\cdot}9$. Also, in 26 pairs of sisters of which both members were measured, the inner was the smaller in 19 instances. $\chi^2 = 5{\cdot}5$.

(*b*) Position symbol I is associated with large κ. In pairs of sisters, the inner usually grows the faster. $\chi^2 = 6{\cdot}4$.

(*c*) Large κ is associated with $p < \frac{1}{2}$. In pairs of sisters, the smaller usually grows the faster. $\chi^2 = 7{\cdot}1$.

Although the χ^2 is small, the first part of (*a*) is significant at the 0·05 probability level by virtue of the uniform sign of the V alone, and it is supported by the second part. The other two statements have much greater weight because of the large χ^2. The following two generalizations are less striking because only five of the six V were of the same sign; the χ^2 are both significant at the 0·05 level:

(*d*) Position symbol I is associated with a positive value of $(x_t'/x_i - 2)$. In pairs of sisters, the inner usually increases in size by more, the outer by less, than a factor of 2. $\chi^2 = 4{\cdot}4$.

(*e*) A positive value of $(x_t'/x_i - 2)$ is associated with $p < \frac{1}{2}$. In pairs of sisters, the smaller usually increases in size by more, the larger by less, than a factor of 2. $\chi^2 = 4{\cdot}1$.

If two of $V(x, y)$, $V(y, z)$, $V(z, x)$ are of like sign, it does not follow that the third is positive; the triads $\{(a)\,(b)\,(c)\}$, $\{(a)(d)(e)\}$ do not include a redundant statement. We thus have evidence for two interesting properties of the growth pattern. First, fission is unsymmetrical in respect of size at inception and mean growth rate; the lack of symmetry is not random but is systematically related to the position symbol. Secondly, there appears to be some regulation of the individual growth rate which partially compensates for inequality in the size of sisters at inception. The regulation is imperfect; within each series of experiments we found the generation time still to be negatively associated with absolute size at inception. On the other hand, the excess or deficit in κ is more than enough to offset the tendency of inner organisms to have shorter generation times than outer organisms (Powell & Errington, 1963*a*).

Fission into organisms of unequal size

The use of the quantity q (equation 5) as a measure of the relative sizes of mother and daughter at fission was suggested by Powell & Errington's (1963*b*) experience that the estimated x_i of sisters were together greater than the x_t of the mother. In our experiments, the number of inner and outer organisms measured were not usually equal, but we also found that in every series, the mean q associated with inner ($\bar{q}_I$) and outer ($\bar{q}_O$) organisms were together greater than 0. The excess can be ascribed to two causes: an unknown systematic error of the micrometer and a systematic increase due to the rounding of the ends of the organisms at fission,

making them for a given volume longer than cylinders of the same diameter bounded by plane ends. We had no means of estimating these errors separately, and we therefore computed for each series a length correction δ given by

$$2\delta = \bar{q}_I + \bar{q}_O$$

which, since it was small (0·05–0·15 μ), we assumed to apply throughout the series.

In the Koch & Schaechter hypothesis it is more convenient to use the ratio p instead of q:

$$p = \frac{x_i(\text{daughter})}{x_t(\text{mother})}. \tag{6}$$

The frequency function (say $k(p)$) of p and its parameters play a large part in quantitative inferences from the hypothesis. The dispersion of p is in practice quite small, and we considered it desirable to attempt a more accurate estimate of the volume ratio than that given by (6). We therefore worked with p based on corrected lengths, i.e. with

$$p = \frac{x_i - \delta}{x_t - \delta}.$$

Table 2 shows that the mean values $\bar{p}_I$, $\bar{p}_O$ differed systematically, but their sum was very nearly unity in each series. The separate distributions of p_I and p_O must be unsymmetrical, each a mirror image of the other in the line $p_I = p_O = \frac{1}{2}$. But in a growing culture equal numbers of inner and outer organisms are formed and the distribution $k(p)$ of p generally is symmetrical.

Table 4. *Sample statistics of the distribution of the corrected ratios p, the correlation* $\rho(x_t, \theta)$ *(equation (7)), and the correlation between the logarithms of p and terminal size at succeeding fission. Number of observations in parentheses*

Series	$-\mu_1'(\log p)$	$10^3\mu_2(\log p)$	$\rho(x_t, \theta)$	$\rho(\log p, \log x_t')$
Sm 1	0·69471	3·210	+0·465 (22)	−0·029 (17)
Pm 1	0·69419	2·098	−0·212 (38)	+0·354 (26)
Ec 1	0·69450	2·771	−0·170 (23)	+0·062 (18)
Ec 2	0·69623	6·216	+0·023 (40)	−0·195 (25)
Pa 1	0·69439	2·482	+0·113 (22)	−0·067 (20)
Pa 2	0·69626	6·290	+0·199 (38)	+0·158 (20)

For purposes of record, the first and second sample moments of $\log p$ are listed in Table 4.

In its simplified form, the Koch & Schaechter hypothesis requires the distribution of p to be the same for all x_t (Powell, 1964). We might expect to detect a trend in the dispersion of p by forming the correlation coefficient between x_t and $|p-\frac{1}{2}|$. The distribution of $|p-\frac{1}{2}|$, however, is J-shaped and highly skew, and the customary significance test cannot be legitimately applied to $\rho(x_t, |p-\frac{1}{2}|)$. Graphical experiment with our data showed that the variate

$$\theta_t = \log\left\{\frac{1}{2|p-\frac{1}{2}|} - 1\right\}, \tag{7}$$

(whose range is $-\infty$ to $+\infty$) was much more nearly normal and not significantly skew. Therefore the six series of p were converted to θ and the correlation coefficients $\rho(x_t, \theta)$ determined. Of the six ρ (Table 4) only the first differs from zero

at the 0·05 probability level—not an unlikely event among six examples—and not all are of the same sign. So far, we can conclude that the distribution of p is roughly the same for all x_t.

Another requirement of the hypothesis is that the size of an organism at termination (x_t') should be independent of the p associated with its inception; in particular, that $\mathrm{cov}(\log p, \log x') = 0$ (Powell, 1964). We therefore computed also the $\rho(\log p, \log x_t')$ and found none to be significant at the 0·05 probability level; three were positive and three negative (Table 4).

The dispersion of individual mean growth rates

The dispersions of generation time and individual mean growth rate are compared in Table 5. It will be seen that the coefficient of variation $c(\kappa)$ of κ is always less, but not always much less, than that of τ. The fact that the experimental κ are systematically associated with p and with position symbol makes it almost certain that the dispersion of κ is real, but we may ask how much of the sample variances

Table 5. *Comparison of the variances* (σ^2) *and squared coefficients of variation* (c^2) *of generation time* (τ) *and mean growth rate* (κ)

Series	$\sigma^2(\tau)$	$10^2\, c^2(\tau)$	$10^6\sigma^2(\kappa)$	$10^2 c^2(\kappa)$
Sm 1	42·34	8·477	12·89	1·417
Pm 1	32·75	4·443	4·182	0·7547
Ec 1	10·69	1·607	2·944	0·4226
Ec 2	34·94	3·608	8·802	1·836
Pa 1	21·54	2·019	3·435	0·8256
Pa 2	126·04	4·590	4·247	3·029

may have been contributed by simple errors of measurement. From the definition of κ (equation (3)) it can be seen that estimates of it can be affected both by errors in measuring length and by errors in estimating times of fission. Temporarily, we shall use subscripts x, t to distinguish components of variance due to these errors.

First, κ is hardly affected by errors in estimating times of fission. From (2) and (3)

$$\begin{aligned}\kappa &= \{(\alpha+\beta t_t+\gamma t_t^2)-(\alpha+\beta t_i+\gamma t_i^2)\}/(t_t-t_i)\\ &= \beta+\gamma(t_t+t_i).\end{aligned}$$

The uncertainties, say δt, in t_t and t_i are alike and independent, hence the uncertainty in κ is

$$\delta\kappa = \sqrt{2}\gamma\,\delta t$$

and the expectation of $(\delta\kappa)^2$ namely $\sigma_t^2(\kappa)$, is

$$2\overline{\gamma^2}(\delta t)^2.$$

In series Ec 1, for which $\sigma^2(\kappa)$ was smallest, $\overline{\gamma^2}$ was $3{\cdot}2\times10^{-8}$, giving

$$\sigma_t^2(\kappa) = (6{\cdot}4\times10^{-8})(\delta t)^2.$$

It is difficult to obtain an estimate of δt except by comparing the judgement of several observers about the occurrence of particular fissions. Experience suggests $\delta t \doteq 1$ min. for fast growing cultures, and then

$$\sigma_t^2(\kappa) = 6{\cdot}4\times10^{-8}.$$

This is quite negligible in comparison with $\sigma^2(\kappa)$.

Secondly, we can obtain a rough but simple estimate of $\sigma_x^2(\kappa)$ by neglecting the small curvature and treating the length measurements as uniformly spaced in time (they were nearly so). If the uncertainty $\delta \log x$ in $\log x$ were constant, we should then have (see e.g. Jeffreys, 1961)

$$(\delta\kappa)^2 = \frac{12}{n\tau^2}(\delta \log x)^2, \tag{8}$$

where n is the number of co-ordinate pairs t_r, x_r. Now $\delta \log x \doteq \delta x/x$, and for any one organism x varies from x_i to x_t'. An upper limit to $\sigma_x^2(\kappa)$ is therefore given by putting minimum values for the variable terms in the denominator of (8). In series Ec 1 these were $n = 23$, $\tau = 21$, $x_i = 2{\cdot}9\ \mu$, and

$$\sigma_x^2(\kappa) < (1{\cdot}4 \times 10^{-4})(\delta x)^2.$$

Under the best conditions, δx is about $0{\cdot}03\ \mu$, but since the κ were determined through the application of equation (3), it is proper to take as δx the r.m.s. residual from that equation, namely $0{\cdot}076\ \mu$. Then

$$\sigma_x^2(\kappa) < 8{\cdot}1 \times 10^{-7}.$$

In series Ec 1, therefore, not more than a quarter of the observed variance of κ can be ascribed to errors of measurement, and in the other series the fraction is still smaller.

There remains the possibility that $\sigma^2(\kappa)$ is inflated by imperfect replication of the experimental conditions. We therefore carried out two-way analyses of variance for within- and between-experiment components of variance. Because the sample sizes were small, and because we might expect τ and κ to be affected to a similar extent (though in opposite senses) by imperfect replication, the analyses were carried out on the τ as well as the κ. Only one of the 12 analyses (for κ in Ec 2) yielded an F-ratio above the 0·05 probability limit, and six of the ratios were less than unity. Previous, less happy, experience suggests that this result was no more than fortunate; but we are left with the conclusion that $\sigma^2(\kappa)$ cannot for the most part be attributed to recognizable experimental error.

Statistical independence of size of organism at termination

According to the Koch & Schaechter hypothesis, if an organism has attained a size x, its chance of dividing at the greater size x_t is independent of its history before attaining x. Powell (1964) showed that the x_t will not then be statistically independent of one another in general, but that they will be independent if the distributions of x_i and x_t do not overlap. It will be seen from Table 2 that Powell's condition is satisfied in Series Pm 1, Ec 1, Pa 1 and Pa 2: the extremes of x_i stand in a ratio less than 2:1; the same is true of x_t; the largest x_t is less than four times the smallest x_i; the largest x_i is less than the smallest x_t; no τ is greater than twice the mean.

But Powell did not notice that if by chance or selection we obtain a group of organisms in which the sample x_i and x_t obey his condition, the x_t will be statistically independent of one another, even if in the whole culture the condition is not met—provided the Koch & Schaechter hypothesis is true. We can make use of our data in series Sm 1 and Ec 2 by omitting the x_i and x_t associated with 5 fissions, and so reducing the range sufficiently (Table 2). In this section, therefore, the outlying

x_i and x_t have been omitted from the calculations for these two series. Sample values of two correlation coefficients

$$\rho(X_{tt}) \equiv \rho(\log x_t, \log x'_t)$$

and

$$\rho(X_{it}) \equiv \rho(\log x_i, \log x'_t)$$

are given in Table 6; here x_t, x'_t distinguish sizes associated with successive fissions (and therefore with mother and daughter), while x_i, x'_t distinguish sizes associated with the same organism. All the ρ are positive and all but one significantly different from zero at the 0·05 probability level.

Table 6. *Sample values of the crude* (σ^2) *and inter-experiment* (σ^2_E) *variances of* $\log x_i$ *and* $\log x_t$*; the ratios* $R_{tt} = \sigma^2_E(\log x_t)/\sigma^2(\log x_t)$ *and* $R_{it} = \sigma_E(\log x_i)\sigma_E(\log x_t)/\sigma(\log x_i)\sigma(\log x_t)$ *and the correlation coefficients,* $\rho(X_{tt})$, $\rho(X_{it})$ *between* $\log x_t$ *and* $\log x'_t$, $\log x_i$ *and* $\log x'_t$

Series	$10^2\sigma^2(\log x_i)$	$10^2\sigma^2_E(\log x_i)$	$10^2\sigma^2(\log x_t)$	$10^2\sigma^2_E(\log x_t)$	R_{tt}	$\rho(X_{tt})$	R_{it}	$\rho(X_{it})$
Sm 1	2·284	0·378	1·870	0·123	0·066	0·546 (18)	0·104	0·431 (20)
Pm 1	2·161	0	1·798	0	0	0·568 (26)	0	0·596 (26)
Ec 1	0·873	0·016	0·815	0·119	0·146	0·537 (19)	0·051	0·682 (18)
Ec 2	3·546	2·053	3·572	2·380	0·666	0·756 (20)	0·621	0·794 (20)
Pa 1	1·562	0·614	1·445	0·775	0·536	0·652 (21)	0·459	0·620 (30)
Pa 2	2·115	0·722	1·798	0·936	0·521	0·672 (19)	0·422	0·750 (20)

As in all investigations of this kind, the comparison of hypothesis with observation is bedevilled by the possibility that imperfect replication of experimental conditions has inflated the variances and biased the correlations; there is the further complication that family likenesses may produce a genuine lack of homogeneity which will be interpreted as imperfect replication. The analyses of variance carried out on the κ and τ (see previous section) seemed to show that we had been successful in reproducing the experimental conditions. But it by no means follows that the sizes of organisms are also unchanged in a replicate experiment which leaves the κ and τ unchanged. It is logically possible, for example, to have two cultures in which the κ and τ have identical distributions and an identical structure of inter-relations, but in one of which the sizes are a constant multiple of those in the other. In fact, two-way analyses of variance on $\log x_i$ and $\log x_t$ showed that in three series there were significant (and very large) components $\sigma^2_E(\log x_i)$, $\sigma^2_E(\log x_t)$ of apparent inter-experiment variance (Table 6).

It follows from Powell's (1958) formulae that if $\rho(X_{tt})$ is really zero, the value to be expected as a result of inter-experiment variance is $\sigma^2_E(\log x_t)/\sigma^2(\log x_t)$, $= R_{tt}$, say; and, by simple extension of his reasoning, if $\rho(X_{it})$ is really zero, the value to be expected is $\sigma_E(\log x_i)\sigma_E(\log x_t)/\sigma(\log x_i)\sigma(\log x_t)$, $= R_{it}$, say. All the ρ in Table 6 exceed the corresponding variance ratios, and the excess is nowhere marginal, as is shown by an inverse hyperbolic tangent (z) transformation. The σ, σ_E and ρ were not based on exactly the same samples of x_i and x_t, however, so that the statistical significance of the excess is uncertain; but we can argue from the uniformity in the sign of $\rho(X_{tt}) - R_{tt}$ and $\rho(X_{it}) - R_{it}$. Since the ρ were in large part calculated from x_i and x_t belonging to the same fissions, and since x_i/x_t never differs much from $\frac{1}{2}$, the two sets of ρ are not independent; they have the weight not of twelve but only

of rather more than six independent estimates. The probability to be attached to the uniformity in sign of the $\rho - R$ is thus something less than $2^{-5}(= 1/32)$, but not as little as $2^{-11}(= 1/2048)$.

We can also show by a non-parametric test, independent of errors of replication, that the x_t at successive fissions are associated. Though it can tell us less about the intensity of the association, it gives a result of higher statistical significance than the foregoing discussion of the ρ. We can say that if x_m is the median terminal size in an individual experiment, then if x_t, x_t' are successive terminal sizes also belonging to the experiment, the product $(x_t - x_m)(x_t' - x_m)$ will be positive or negative according as x_t and x_t' lie on the same or opposite sides of x_m, and the probabilities of these events will be equal if there is no association between x_t and x_t'. The frequencies of positive and negative products are collected together in Table 7. In every series

Table 7. *The sign of the product* $(x_t - x_m)(x_t' - x_m)$, *where* x_t, x_t' *are the terminal sizes at successive fissions, and* x_m *is the experiment sample median*

	Frequency with which $(x_t - x_m)(x_t' - x_m)$ was		
Series	+ve	0	−ve
Sm 1	9	1	7
Pm 1	16	2	8
Ec 1	10	4	5
Ec 2	12	5	4
Pa 1	13	3	5
Pa 2	7	8	6
Sum	67	—	35
	102		

positive signs are in excess, and the total frequencies differ by 32, more than six times the standard deviation $\sqrt{(102)}/2$ on the hypothesis of independence: probability less than 0·003. This estimate of significance is really conservative; the use of sample medians instead of the unknown true medians in the test biases the sign frequencies towards equality.

We thus have fairly strong evidence that the size of an organism at termination is not independent of the circumstances of its inception.

CONCLUSION

It now seems that both of the major postulates of the Koch & Schaechter hypothesis are wrong. But we have not yet shown that they are quantitatively so far wrong as to make the hypothesis valueless; our experiments are exploratory only. We expect the hypothesis to remain as a framework into which refinements can be introduced, and to be more adaptable in this respect than older hypotheses about the pattern of generation times.

According to the original hypothesis we have (Powell, 1964)

$$\nu^2\sigma^2(\tau) = 2\sigma^2(\log x_t) + \sigma^2(\log p)$$

and approximately

$$c^2(\tau) = 4c^2(x_t). \tag{9}$$

But if we admit that κ may be dispersed and $\rho(X_{tt})$ not zero, we must now write (from (3))

$$\begin{aligned}\sigma^2(\kappa\tau) &= 2\sigma^2(\log x_t) - 2\,\mathrm{cov}(\log x_t, \log x'_t) + \sigma^2(\log p) \\ &= 2\{1-\rho(X_{tt})\}\,\sigma^2(\log x_t) + \sigma^2(\log p).\end{aligned}$$

Neglecting $\sigma^2(\log p)$ as small compared with $\sigma^2(\log x_t)$, and $c^2(\kappa)$ as small compared with $c^2(\tau)$, we now have instead of the approximation (9)

$$c^2(\tau) = 4\{1-\rho(X_{tt})\}\,c^2(x_t): \tag{10}$$

if $\rho(X_{tt})$ is positive, $c(\tau)$ will be less than twice $c(x_t)$. But if in (10) we substitute sample values such that the τ on the left belong to the same organisms as the x_t on the right, we necessarily obtain approximate agreement, and the comparison adds nothing to a discussion of the significance of sample ρ computed directly from the data. (We can obtain a useful comparison, however, by taking the $c^2(\tau)$ from one sample of organisms and the $c^2(x_t)$ and $\rho(X_{tt})$ from an independent sample; our own data were not numerous enough to sustain this subdivision.) On the other hand, Schaechter *et al.* (1962) do not record sample values of $\rho(X_{tt})$, and we can apply (10) to their published data in order to obtain at least a rough impression of the magnitude of $\rho(X_{tt})$. In six of seven experiments for which Schaechter *et al.* record both $c(\tau)$ and $c(x_t)$, $c(\tau)$ is less than $2c(x_t)$ (in two of them, much less); thus $\rho(X_{tt})$ seems to be on the whole positive (Table 8). If it were zero, we might expect a tendency for $c(\tau)$ to exceed $2c(x_t)$ because of the neglected variances $\sigma^2(\log p)$ and $\sigma^2(\kappa)$.

Table 8. *Coefficients of variation of x_t and τ in seven experiments from Schaechter et al.* (1962). *Estimates of $\rho(X_{tt})$ obtained from $c(x_t)$ and $c(\tau)$ by means of equation* (10)

Expt. no.	$c(x_t)$	$c(\tau)$	$\rho(X_{tt})$
A–1	0·085	0·176	−0·07
A–3	0·089	0·137	+0·41
B–4	0·099	0·169	+0·27
B–5	0·107	0·152	+0·50
F–1	0·118	0·228	+0·07
F–2	0·130	0·144	+0·69
F–3	0·090	0·179	+0·01

We cannot be surprised if $\rho(X_{tt})$ should turn out to be always appreciably positive; we know already that the pattern of growth exhibits hereditary elements extending beyond those implied by the Koch & Schaechter hypothesis, as witness the correlations between the generation times of cousins and second cousins.

The more striking outcome of our experiments is the indication that fission is systematically unsymmetrical in several respects. Powell & Errington (1963*a*) were able to detect an ordered difference in the generation times of sisters only by working with thousands of observations. The ordered differences in κ and p are detectable at about the same level of significance in less than 200 observations; they are too large to be at once dismissed as trivial consequences of the cells' mechanical structure (though they may yet be so). The manner in which the Gram-negative cell-wall is laid down is not yet settled, and may differ from one species to another; it is almost certainly more complex than Powell & Errington supposed (Chung, Hawirko & Isaac, 1964). While we are dubious about Chung, Hawirko & Isaac's interpretation in terms of cross-walls and multicellular organisms, it is clear

that the cell-wall structure of *Escherichia coli* at least is unsymmetrical at inception, and hence that at termination the daughters may exhibit an ordered difference in properties. The banded wall structure which Chung, Hawirko & Isaac demonstrate may well correspond to such fluctuations in individual growth rate as would account for the failure of our equation (1) to represent the growth accurately enough.

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Occurrence and Population Densities of Yeast Species in the Digestive Tracts of Gulls and Terns

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SUMMARY

From the intestinal tracts of 37 out of 69 birds belonging to four species of gulls (*Larus fuscus*, *L. genei*, *L. argentatus*, *L. ridibundus*) and three species of terns (*Sterna sandvicensis*, *S. hirundo*, *S. minuta*) 62 yeast isolates belonging to 16 species were obtained. The occurrences of individual yeast species were (% of positive birds in brackets): *Saccharomyces cerevisiae* (20%), *Torulopsis glabrata* (12%), *S. oleaginosus* (10%), *Candida tropicalis* (9%), *C. albicans* (9%) and *T. pintolopesii* (7%), other yeast species (1–4%). Average numbers of colony-forming units/g. wet intestinal contents in the seven host species were: *Larus fuscus* 5210; *Larus genei* 22,200; *L. argentatus* 5620; *L. ridibundus* 3180; *Sterna sandvicensis* 50; *S. hirunda* 211,000; *S. minuta* 9200.

INTRODUCTION

In recent years attention has been given to the intestinal yeast flora of several mammal and bird species (van Uden, 1963), but little to marine birds. High numbers of viable yeasts were found in the digestive tract of *Larus occidentalis* (Western Gull) by van Uden & Castelo-Branco (1963). It was thought of interest to examine whether gulls and terns are, in general, suitable hosts for intestinal yeasts and to obtain information on the population densities and specific composition of their intestinal yeast flora. The possible role of marine birds in the dispersal of marine-occurring yeasts (Taysi & van Uden, 1964; van Uden, 1964) gave additional stimulus to seeking such information. To be reported here are qualitative and quantitative findings on intestinal yeasts in 69 birds belonging to 7 species of gull and tern.

METHODS

Isolation medium (%, w/v): glucose, 2; peptone (Difco), 1; yeast extract (Difco), 0·5; agar, 2; distilled water. The medium was adjusted to pH 4·5 to discourage bacterial growth.

Isolation. Free-living gulls and terns were caught along the coast of Portugal and samples were taken from rectal contents. Weighed portions of the samples were suspended in sterile water and serial dilutions plated in 1 ml. volumes in isolation medium. After incubation at 37° for 48–72 hr, the numbers of yeast colonies were recorded according to macroscopic and microscopic morphology. Representatives of each type were subcultured for later identification.

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Identification of the yeast isolates. The methods described by Lodder & Kreger-van Rij (1952), Wickerham (1951), van Uden & Farinha (1958), and Buckley & van Uden (1963), were used.

RESULTS

Of 69 birds sampled, 37 harboured yeasts in detectable numbers. The total number of isolated strains was 62, which corresponds to a total-yeast index (van Uden, 1960) of 0·9. These figures indicate that gulls and terns are hosts of medium suitability for intestinal yeasts, similar to other omnivorous host species (e.g. humans, swine; van Uden, 1960, 1963).

Table 1. *Yeast species from the intestinal tracts of 69 gulls and terns*

Yeasts isolated	No. of birds positive	Percentage incidence of yeast species
Saccharomyces cerevisiae	14	20
Torulopsis glabrata	8	12
Saccharomyces oleaginosus	7	10
Candida tropicalis	6	9
C. albicans	6	9
T. pintolopesii	5	7
C. krusei	3	4
C. norvegensis	3	4
C. tenuis	3	4
C. lusitaniae	1	2
C. parapsilosis	1	2
C. utilis	1	2
T. famata	1	2
Saccharomyces sp.	1	2
Prototheca sp.	1	2

Table 2. *Yeast population densities in the digestive tracts of 7 gull and tern species*

No. of birds tested	Species of bird	No. of birds with yeasts	Average numbers of colony-forming units/g. wet intestinal contents: (*a*) Positive birds	(*b*) All tested birds
26	Lesser Black-Backed Gull (*Larus fuscus*)	12	11,280	5.210
4	Slender-Billed Gull (*L. genei*)	3	29,600	22,200
4	Herring Gull (*L. argentatus*)	1	22,500	5,600
11	Black-Headed Gull (*L. ridibundus*)	5	7,000	3,180
3	Sandwich Tern (*Sterna sandvicensis*)	2	70	50
6	Common Tern (*S. hirunda*)	5	253,100	211,000
15	Hooded Tern (*S. minuta*)	9	11,500	9,200
Total 69		37	44,600	23,900

The species of yeast isolated and their respective incidences are listed in Table 1. The six most frequently isolated species included *Torulopsis glabrata* and *Candida tropicalis*. These two species were also predominant in a group of Western Gulls

studied earlier (van Uden & Castelo-Branco, 1963). In the present survey *Saccharomyces cerevisiae* was the most frequent species. Van Uden & Carmo-Sousa (1962) found that this yeast was capable of multiplication in the digestive tract of swine and must be regarded as a facultative intestinal saprophyte. *T. glabrata* and

Table 3. *Occurrence and population densities of yeast species in the digestive tracts of 7 gull and tern species*

Host species	No. of birds tested	No. of birds with yeasts	Yeast species	No. of birds with species	Average population density of each yeast species in the digestive tracts of the birds harbouring this species (colony-forming units/g. wet intestinal contents)
Lesser Black-Backed Gull (*Larus fuscus*)	26	12	*Candida albicans*	5	26,716
			Saccharomyces cerevisiae	4	35
			Torulopsis glabrata	3	135
			C. tropicalis	2	49
			C. krusei	1	48
			C. norvegensis	1	1,110
			T. pintolopesii	1	25
			S. oleaginosus	1	13
Slender-Billed Gull (*L. genei*)	4	3	*C. norvegensis*	1	33,916
			C. parapsilosis	1	2,796
			C. tropicalis	1	1,004
			T. pintolopesii	1	11,000
			T. glabrata	1	24,667
			S. oleaginosus	1	15,417
Herring Gull (*L. argentatus*)	4	1	*T. glabrata*	1	2,800
			T. pintolopesii	1	200
			C. krusei	1	200
			S. cerevisiae	1	19,000
			Saccharomyces sp.	1	300
Black-Headed Gull (*L. ridibundus*)	11	5	*T. glabrata*	2	890
			T. pintolopesii	2	6,850
			C. albicans	1	1,770
			C. lusitaniae	1	620
			C. tenuis	1	22,500
			S. cerevisiae	1	2,060
			Trichosporon capitatum	1	295
Sandwich Tern (*Sterna sandvicensis*)	3	2	*C. tropicalis*	1	40
			S. cerevisiae	2	60
Common Tern (*S. hirundo*)	6	5	*T. glabrata*	1	134,880
			C. tropicalis	1	78,680
			C. krusei	1	880,000
			C. norvegensis	1	10
			S. oleaginosus	3	57,373
Hooded Tern (*S. minuta*)	15	9	*C. tropicalis*	1	240
			C. utilis	1	640
			C. tenuis	2	3,000
			T. candida	1	6,700
			S. oleaginosus	2	31,850
			S. cerevisiae	6	10,053
			Prototheca sp.	1	300

C. albicans are polyspecific obligatory intestinal saprophytes with potential pathogenicity; they occur in a wide range of hosts (van Uden, 1963). The occurrence of the oligospecific *T. pintolopesii* in gulls and terns is surprising since this yeast is known as an obligatory saprophyte of small rodents; however, it has also been obtained from pigeons (van Uden, 1960). *Saccharomyces oleaginosus* (*S. italicus* var. *melibiosi*) is probably a facultative intestinal saprophyte; it was isolated previously from humans and horses (van Uden & Assis-Lopes, 1957) and from swine (van Uden & Carmo-Sousa, 1962). Outside the animal body this yeast is frequent in the watery discharge from fermenting stacked olives (Santa Maria, 1963).

The average number of colony-forming units/g. wet intestinal contents was 44,600 for the 37 birds which harboured yeasts at all, and 23,900 for the total of 69 birds studied (Table 2). Among the seven host species the yeast population densities varied between 50 and 211,000 colony-forming units/g. wet intestinal contents. Whether this wide range of population densities is indicative of differences in host suitability for yeasts between the bird species cannot be ascertained from the relatively small numbers of specimens tested within each host species. Nevertheless, the yeast species distribution among the birds and their respective population densities (Table 3) suggest that host suitability for intestinal yeasts is a property of gulls and terns as a group rather than of certain species of these birds.

Gulls and terns must inoculate yeasts with their faeces into natural water bodies the world over. *Torulopsis glabrata*, *Candida tropicalis*, *C. krusei*, and other yeasts which occur in these birds, have been encountered in tropical and subtropical marine water bodies as predominant yeasts (Fell & van Uden, 1963; van Uden & Castelo-Branco, 1963; van Uden, 1964). In temperate and cold-water bodies such intestinal yeasts have so far not been detected, regardless of the presence of gulls and terns or other sources of intestinal yeasts (sewage). There is some evidence that intestinal yeasts are outgrown in temperate and cold-water bodies by yeasts which have lower cardinal temperatures (Taysi & van Uden, 1964).

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Cellular Physiology during Logarithmic Growth of a Streptococcal L-Form

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SUMMARY

Certain synthetic processes of a stable L-form and its parent streptococcus were compared to examine whether conversion was accompanied by significant alterations in the growth pattern. DNA and RNA were isolated from the streptococcus and its derived L-form, degraded, and molar base ratios measured and compared. Conversion to the L-form apparently did not result in a disturbance of synthetic processes related to DNA and RNA rates of syntheses and growth measured by extinction of cultures, colony count and dry weight increases. The stable L-form, although almost twice as slow growing, as compared with the streptococcus, retained the ability to function in an orderly manner and was capable of balanced growth. Conversion from streptococcus to L-form did not result in an addition to the DNA base complement (5-methylcytosine, 5-hydroxymethylcytosine) nor in a quantitative alteration in the molar base ratio of either nucleic acid in the resulting L-form. The disorganization in L-form division characteristic of L-form growth was not directly related to an obvious disturbance in any of the parameters examined.

INTRODUCTION

It has always been recognized that L-forms grow slower, display a smaller maximal growth response and result in yields of organisms (dry weight) appreciably lower than those of the parent bacterial form from which they are derived. Microscopically, the extreme diversity of sizes of the large bodies which comprise the structural components of the L-form, and the complete lack of morphological similarity to the parent bacterial form, suggest that a permanent disorganization in cellular division has also occurred after conversion to the L-form. A stable L-form was found to possess altered physical, structural and metabolic properties following its derivation from a group A streptococcus (Panos, 1964*a*). However, biochemical information about the growth pattern of this L-form is lacking. It has been shown by Schaechter, Maaløe & Kjeldgaard (1958) that *Salmonella typhimurium* can exist in one of a number of possible stable physiological states under conditions of balanced growth; similar information for the L-form considered here was lacking. The present work was undertaken to examine whether alterations in the synthetic processes (i.e. balanced growth) underlying the growth of the L-form had occurred after its conversion from the bacterial form. Part of these results were reported in preliminary form (Panos, 1964*b*).

METHODS

The group A streptococcus and its derived stable L-form were the same as that used previously (Panos, 1962). This non-pathogenic group A, type 12 β-haemolytic streptococcus, lacking M protein, has been successfully typed by its T antigen (W. Hijmans, personal communication). The medium and growth conditions for each of these organisms were described by Panos & Barkulis (1959). Identical results were obtained with the stable (i.e. not reverting to the bacterial form) L-form when it was grown in the presence and in the absence of penicillin. Five l. medium were inoculated with 5 or 10% (v/v) overnight inoculum of the streptococcus or its L-form, respectively, and 0·3 l. of culture removed at intervals for analyses. L colony counts were made as previously described (Panos, Barkulis & Hayashi, 1960). Growth was followed by measuring the increase in extinction at 660 mμ (E_{660}) in a Beckman DU spectrophotometer. Organisms were harvested and washed as described by Panos, Barkulis & Hayashi (1959). For dry weight determinations, portions of washed suspensions of organisms were pipetted into tared beakers, dried (90°), placed over P_2O_5 and NaOH and weighed. Since the L-form organism is osmotically fragile, the L-form content of L-form + NaCl preparations was determined by weight difference and/or by determinations of nitrogen (Kjeldahl).

Before nucleic acid analyses, the organisms were extracted with 5% (w/v) trichloroacetic acid at 4° until all material absorbing at 260 mμ was removed. Extraction and colorimetric methods for these nucleic acid analyses were as given by Panos *et al.* (1959). Amounts of nucleic acids are expressed as % of dry weight of total cellular material.

L-form organisms and streptococci were obtained from 18-hr cultures for ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) base composition studies. The nucleic acids were extracted by several methods (Lee, Wahl & Barbu, 1956; Sherratt & Thomas, 1953; Sevag, Lackman & Smolens, 1938), hydrolysed, and chromatographed and measured in the usual way (Lynn & Smith, 1957). DNA was also isolated by the method of Marmur (1961). The streptococcus was disintegrated, either by ultrasonic treatment, grinding with alumina or shaking with glass beads in an International Centrifuge shaker head (Shockman, Kolb & Toennies, 1957), for complete extraction of total nucleic acid content. The data presented are averages of at least two different experiments performed in duplicate.

RESULTS

Figure 1 illustrates the % nucleic acid of the L-form organism and its parent streptococcus. The arrows indicate the prevailing concentrations during the respective logarithmic growth periods. The DNA content remained relatively constant for both organisms over the period examined. The RNA content reached a maximum of 20·6% for the streptococcus and only 13% for the L-form. The cellular dry-weight yields increased proportionally during logarithmic growth for both organisms. From extinction E_{660} of 0·050–0·300, the yields were 1·8–11·5 mg. and 2·6–15·4 mg./100 ml. medium respectively for the L-form and the streptococcus. The RNA/DNA ratio of the L-form changed as the cellular mass increased throughout most of logarithmic growth, as can be calculated

from the data in Fig. 1 and the dry-weight yields cited. The maximal RNA/DNA ratio reached within the L-form was about one-half that of the parent streptococcus. Since DNA/unit mass remained relatively constant in both organisms, the changes in these ratios were mostly the result of alterations in the RNA content/unit mass. In earlier work (Panos *et al.* 1959) done with cultures considerably beyond their logarithmic growth periods, RNA/DNA ratios were found to be lower, 4·1:1 for the streptococcus and 4·6:1 for the L-form. Although the

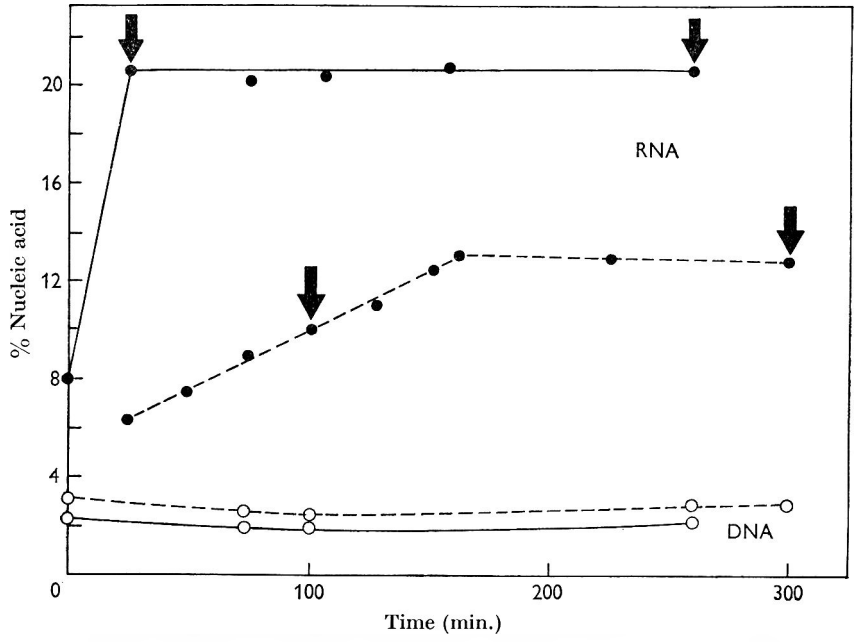

Fig. 1. RNA and DNA content of the derived L-forms (– – –) and the parent streptococcus (——). Arrows indicate concentrations during logarithmic growth phases. Cellular growth rate (doubling time): 50 and 80 min. for the streptococcus and L-form, respectively.

DNA content of these older organisms agrees closely with those of logarithmically growing L-forms and streptococci, the RNA/DNA ratio was double (10:1) in the logarithmically growing streptococci. The logarithmically growing L-forms showed only a slight increase in their RNA/DNA ratio over that of older organisms.

Figure 2 shows that increases in the rates of nucleic acid syntheses, extinction and colony counts paralleled each other during logarithmic growth of the L-form and of the parent streptococcus. The colony count data show that an increase in numbers of L-form bodies had occurred. A definition of 'growth' in terms of an increase in mass in place of an increase in numbers has already been used in protoplast investigations (Shockman & Lampen, 1962). The rates of nucleic acid syntheses observed were much lower in the L-form. Over a comparable increase in extinction during the logarithmic growth period, the maximum amount of DNA synthesized by the L-form was about 66% of that of the streptococcus. Similarly, the maximum amount of RNA synthesized by the L-form was one-half that of the streptococcus. Although not illustrated, the stationary phase of growth was usually attained at an

extinction (E_{660}) between 0·8 and 0·9 for the streptococcus and 0·4 and 0·5 for its L-form.

RNA and DNA preparations from the streptococcus and L-form were isolated and degraded by various methods and the molar base ratios determined. Tables 1 and 2 show the analytical data for the nucleic acid bases obtained from both

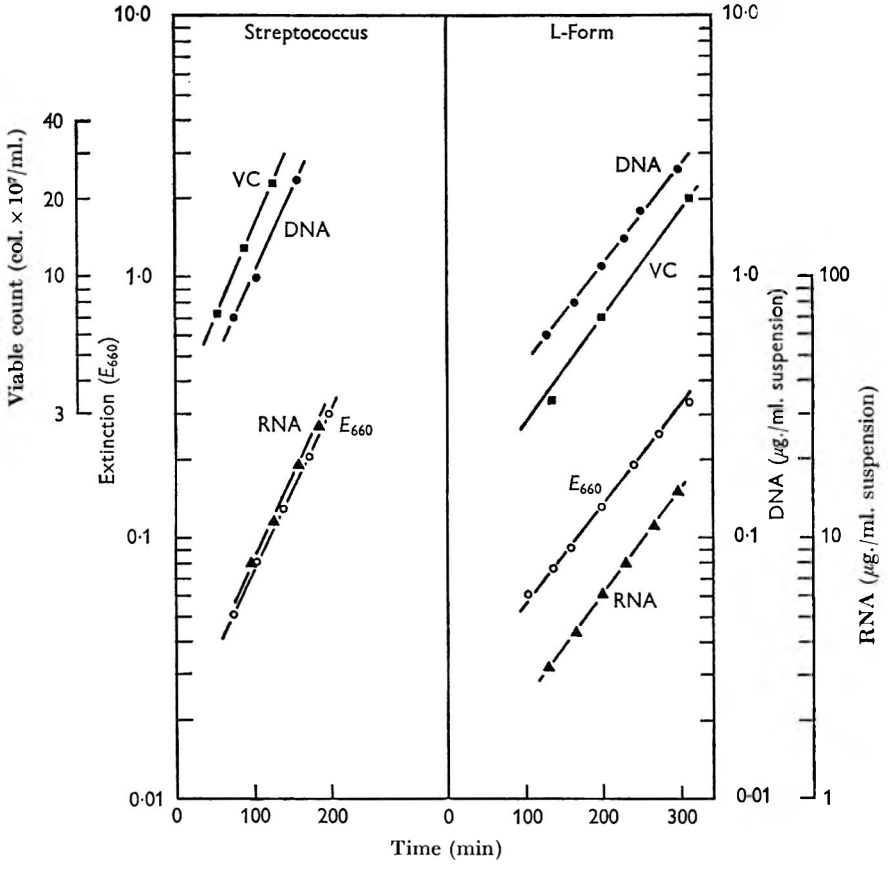

Fig. 2. Rates of nucleic acid syntheses, viable count and extinction increases during logarithmic growth of both organisms. VC = viable count.

Table 1. *Analytical data (moles %) of the deoxyribonucleic acid isolated from the streptococcus and its derived L-form*

Organism	Guanine (G)	Adenine (A)	Cytosine (C)	Thymine (T)	$\frac{A+T}{G+C}$	Pu/Py
Streptococcus	17·1	32·5	17·6	32·8	1·88	0·98
L-form	16·7	32·9	17·0	33·4	1·96	0·98

Pu = purines; Py = pyrimidines.

organisms. No 5-methylcytosine or 5-hydroxymethylcytosine was observed in the DNA base complements from either organism. Failure to disrupt the streptococcus before these determinations by either of the methods mentioned (see Methods)

prevented complete extraction of the nucleic acids and led to inconsistent results. The DNA base composition of the parent streptococcus agrees closely with values previously published for *Streptococcus pyogenes*.

The doubling times for each of the parameters studied were obtained from semi-logarithmic plots of the respective data; these are tabulated in Table 3. These doubling times were found to be greater for the L-form, and revealed a marked decrease in the rates of the cellular processes examined as compared with those of the streptococcus.

Table 2. *Analytical data (moles %) of the ribonucleic acid isolated from the streptococcus and its derived L-form*

Organism	Guanine (G)	Adenine (A)	Cytosine (C)	Uracil (U)	$\frac{G+U}{A+C}$	$\frac{G+C}{A+U}$	Pu/Py
Streptococcus	29·6	25·6	25·0	19·9	0·98	1·20	1·23
L-form	29·7	26·1	24·0	20·2	0·99	1·16	1·15

Pu = purines; Py = pyrimidines.

Table 3. *Doubling time (min.) of the parameters of the two forms studied*

	Streptococcus	L-form
E_{660}	50	80
RNA	50	76
DNA	51	80
Viable count	45	75
Dry weight	50	80

DISCUSSION

Much information has accumulated about the biochemistry of bacterial L-forms. With regard to the stable streptococcal L-form used in the present work, it has been found that changes have occurred at the subcellular level (Panos, 1962; Edwards & Panos, 1962) but that, for example, the vitamin requirements have remained unaffected following derivation from the parent streptococcus (Panos & Hynes, 1964). There is little in the literature, however, about the growth physiology of coccal L-forms which lack the rigid bacterial cell wall. As is apparent from Fig. 1, the RNA content of this osmotically protected and structurally intact L-form does not approach that of the parent streptococcus. Since protein synthesis is related to RNA content, the proportional decrease in mass (i.e. protein synthesis) RNA content, as well as the decreased growth rate (Fig. 2), show that this relation has not been obviously disturbed by conversion of the streptococcus to an L-form. For *Escherichia coli* it has been shown that the soluble RNA/DNA ratio is a constant irrespective of the growth rate; the ribosomal RNA/DNA ratio, however, increases proportionally with the growth rate (Kjeldgaard & Kurland, 1963). It is not known whether the decreased growth rate of the fragile streptococcal L-form represents an alteration in the ribosomal RNA content or is a manifestation of a less efficient ribosomal protein-synthesizing system. The RNA content during the stationary growth phase of each organism was not examined.

It has been shown that cellular DNA content has a tendency to remain nearly constant under different experimental conditions which affect bacterial

growth rate. The derived L-form examined here displayed a decreased rate of DNA synthesis; the total cellular DNA content, as compared with the parent streptococcus, was nearly the same and was not affected by the inability of the L-form to synthesize the rigid bacterial cell wall.

The present results show some differences from those of Weibull & Beckman (1960) for *Proteus vulgaris* and an L-form derived from it. The RNA content of both the Proteus and its L-form increased during early growth, followed by an abrupt and continuous decrease over the remainder of their growth cycles; also, there was no major difference between the growth rate and RNA content of the parent Proteus and its L-form. In contrast to their results, the RNA content of the streptococcus and its L-form studied here, while also increasing sharply, did not decrease but remained relatively constant once it had attained its maximum. Similarly, an appreciably slower growth rate (Table 3) and lower RNA content (Fig. 1) were observed for the L-form as compared with its parent streptococcus. The lower RNA content found in the streptococcal L-form is in agreement with a similar finding for a Proteus L-form by Kandler, Zehender & Müller (1956) and Vendrely & Tulasne (1953). Weibull & Beckman (1960) suggested that the difference in the RNA content of their L-form from that found by Kandler *et al.* (1956) and Vendrely & Tulasne (1953) might have been due to the latter authors having used L-form material '...consisting to a large degree of vesicles apparently more or less empty'. In the present work very few such 'empty' vesicles were observed, yet the RNA content of the L-form was lower than that of the parent streptococcus (Fig. 1).

The data presented here illustrate that in a nutritional medium similar to that of the parent streptococcus, conversion to the L-form apparently did not result in a disturbance of any of the synthetic processes underlying cellular perpetuation which were studied. The resulting L-form, although almost twice as slow in its rate of growth, continued to retain the ability to function in an orderly manner and was capable of balanced growth. These findings mimic the balanced growth results of Schaechter *et al.* (1958) with *Salmonella typhimurium* grown in a variety of media, from nutritionally complex to chemically defined. With the L-form used here, however, this decrease is associated with the permanent loss of ability to synthesize cell wall. Although our previous results suggest that conversion to the stable L-form results in a permanent disorganization of the cellular division process, these results could not be directly related to an obvious disturbance in any of the parameters examined; this suggests that the division process is probably distinct from that of balanced growth.

The DNA base findings given here represent the first such comparisons between a streptococcus and a derived stable L-form. No alterations were observed in an addition to the DNA base complement (5-methylcytosine, 5-hydroxymethylcytosine), nor in a quantitative alteration in the molar base ratios of DNA or RNA, on conversion to the L-form. The DNA from each source is of the 'AT-type', containing more adenine and thymine than guanine and cytosine; guanine was found to equal cytosine and adenine to equal thymine. In a similar study of a pleuropneumonia-like organism (PPLO), Lynn & Smith (1957) observed that although the DNA base complement was of the 'AT-type', guanine was not equivalent to cytosine nor was adenine equal to thymine. The DNA purine/pyrimidine (Pu/Py)

ratio was 0·98 for the streptococcal L-form examined in the present work as compared with 0·86 for the PPLO.

The present RNA results are in accord with those of Mandel & Sensenbrenner (1958) for *Proteus vulgaris* and an L-form derived from it. In both instances, no apparent differences were noted in the RNA base composition of these L-forms as compared with their parent bacteria. The pentose nucleic acid of the present streptococcal L-form is of the 'GC-type', containing more guanine + cytosine than adenine + uracil. The RNA Pu/Py ratio from the PPLO (also a 'GC-type') studied by Lynn & Smith (1957) was 0·98 as compared with 1·15 for the present streptococcal L-form.

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Cellulolytic Bacteria in some Ruminants and Herbivores as Shown by Fluorescent Antibody

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SUMMARY

A method is described for the demonstration and enumeration *in situ* of antigenically related cellulolytic bacteria in the intestinal contents of some herbivores and ruminants, by means of a fluorescent antibody staining technique.

INTRODUCTION

A comprehensive study of cellulolytic bacteria from the ruminant has been made by many workers in recent years (Hungate, 1950); much less attention has been paid to the same group of organisms in simple stomach herbivores and omnivores. Because of the technical difficulties involved in the isolation and identification of cellulolytic bacteria, an attempt has been made to demonstrate their presence in colon contents of pig, rabbit, guinea pig, sheep and bovine by the use of fluorescent antibody and to determine whether similar or antigenically related types were present in both ruminants and herbivores.

METHODS

Antisera. These were prepared in rabbits by intravenous injection of living cultures of four different types of cellulolytic bacteria isolated from the horse. Three of these organisms were Gram-negative bacilli belonging to the genus *Bacteroides*, and the fourth was a Gram-negative coccus (Davies, 1964). The bacilli were designated I, II and III and the coccus IV.

Washed bacteria suspended in normal saline to give about 1700 million organisms/ml. were used for a series of ten injections of 0·25 ml. each, given over a period of 4 weeks. Agglutination tests with homologous organisms showed that the titres of the antisera were low, ranging from 1/120 to 1/960.

Fluorescent staining. This was done by the indirect method, by using the prepared rabbit antiserum and Bacto (Difco) fluorescent goat anti-rabbit globulin. After rehydration according to the manufacturers' instructions, it was found advantageous to dialyse the fluorescent globulin against 200 ml. Coons buffered saline (Coons & Kaplan, 1950) kept in the refrigerator for 6 days. This removed any free fluorescein released by dissociation. The dialysed antibody was absorbed with Bacto (Difco) mouse liver powder (Hobson & Mann, 1957) immediately before use.

Rhodamine B 200, conjugated with bovine serum albumin (Nairn, 1962) was used as an intermediate stain to provide a background contrast.

Preparation of test slides. Samples of caecum, colon and rumen contents taken

from freshly killed animals, were diluted one part by weight in ten parts of Coons saline. Smears were made on thin (0·8 mm.) glass slides, allowed to dry in air, and fixed by gentle heat. Each of the antisera was diluted 1/100 in Coons saline to minimize background fluorescence, and layered over the prepared smears, which were then incubated at 39° for 30 min. in closed Coplin staining jars. They were stained first with rhodamine B and then with fluorescein conjugates, for 30 min. periods, being washed for 10 min. in two changes of Coons buffered saline between each stage of staining. The smears were blotted, mounted in Bacto (Difco) FA mounting fluid, with ordinary coverslips, and examined for fluorescent bacteria by dark-field illumination, with a 250-watt high-pressure mercury arc light. Controls used to establish the validity of the technique consisted of smears of the samples treated with normal instead of immune serum, and stained with fluorescent conjugates, and of other smears stained with the fluorescent conjugates without previous treatment with serum. Smears of the organisms I–IV, treated with their homologous antiserum and stained with the fluorescent conjugates, were included in every set of samples examined.

Counts of organisms I, II, III and IV were made as follows. Suspensions of gut contents were prepared by diluting one part by weight in ten parts Coons saline, and volumes of 0·001 ml. were dropped on to slides from a micrometer syringe (Agla; Burroughs Wellcome). These drops were stained and mounted as above, and the fluorescent bacteria present in the whole drop were then counted.

RESULTS

Each of the four organisms used for preparing antisera was examined for fluorescence with antisera to the other three, and it was found that organisms I and II possessed a common antigen. Organisms II, III and IV also shared some antigens, but differed from each other and from organism I.

Table 1. *Occurrence of four types of cellulolytic bacteria in five animal species*

	Animal species							
	Rabbit (10)		Guinea pig (10)		Pig (12)		Cattle (10)	Sheep (10)
Organism	Caecum	Colon	Caecum	Colon	Caecum	Colon	Rumen	Rumen
I	5	6	3	4	9	7	7	5
II	5	7	5	7	8	10	6	4
III	4	5	7	2	9	6	9	4
IV	5	4	6	4	8	9	9	5

Figures in parentheses indicate the number of animals examined.

The number of organisms/g. sample varied; the average value for the six animals being 3·5 million for organism I; 4·7 million for organism II; 15·0 million for organism III and 17·0 million for organism IV. In all cases therefore the numbers exceeded the minimum of 1 million/g. postulated by Gall & Huhtanen (1951) as a criterion of significance for organisms in the rumen.

Samples from the caecum and colon of ten rabbits, ten guinea pigs and twelve

pigs and from the rumen of ten cattle and ten sheep were examined for fluorescent bacteria. The number of times each organism was found in samples from the different animal species is shown in Table 1.

These results showed that fluorescent antibody technique could be used satisfactorily to study the distribution of cellulolytic bacteria in the intestinal contents of different animal species. They also indicated that the four antigenic types isolated from the horse, or others very closely related antigenically to them, were present in the large intestine of the other animal species investigated.

The technique could also be applied to the enumeration of individual bacterial species in colon samples, although as with other counting methods, considerable variation was found in the numbers of each species in the colon contents of different animals. This method would appear to have the advantage of accounting for the 'fixed' as well as the 'free' bacteria present in any sample. In his monograph Oxford (1964), stated that most counting methods so far used have not taken into account the fixed organisms (those closely attached to plant fibres) which may be the most important. The method would also allow counts to be made of any one species without the difficulties of culture.

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Principles and Practice of Bacterial Taxonomy—a Forward Look

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SUMMARY

Taxonomy is divisible into three parts: (1) classification, (2) nomenclature, (3) identification. There are rules of nomenclature but none for classification or identification. Six principles are postulated for classification of bacteria and three ways of making identifications are discussed. Both classification and identification depend on characterization of the bacterium, but each makes different use of the individual feature. In classification equal weight is given to each independent character; in identification characters are weighted, some as important (distinguishing), others less so. Exception is taken to the retroactive application of the rules of nomenclature, and the unrealistic starting date (1753) of bacterial nomenclature is criticized. Names act merely as labels and it is suggested that a sequential code should be used, not only as a substitute for a name, but as a means of conveying information about the characters of the organism.

INTRODUCTION

Systematics is the study of multiple items, units or individuals with the aim of finding common factors and differences; lines of cleavage are made so that the like fall on the same side of the dividing line, and the unlike on the other. Biological systematics bears the special name taxonomy, and the subject can be subdivided into three sections.

(i) *Classification*, the orderly arrangement of units into groups of larger units. A simple analogy can be found in a pack of cards; the individual cards can first be sorted by colour, then into suits. Within each suit the cards can be arranged in a numerical sequence, and the face cards placed in some order of seniority.

(2) *Nomenclature*, the naming of the units defined and delineated by the classification. In the example of cards, the face cards are given names and more than one name, for example, jack or knave, may be given to the same card.

(3) *Identification* of unknown units with known units of the classification developed in (1) and bearing names given in (2).

These three facets, or the trinity that is taxonomy, are to some extent interdependent, but in an orthodox scheme they are considered in the order given above. It is arguable whether the hen or the egg came first, but since the end of the nineteenth century bacteriological ethics have demanded that we should not name a bacterium before we have allotted it to a unit in an orderly classificatory system. This is not only ethical but common sense, for we cannot identify an organism until

the preparatory work has been done; this means that identification depends on adequate characterization, description and comparison with published work.

The end result of a classification is often presented in a deceptive form, deceptive because we read downwards and it appears that the scheme starts by taking one large group, say Bacteria, and breaking it down progressively into small and smaller groups; this is a hierarchical system (Fig. 1). In fact the converse has occurred; the units have been built up from individual isolates, similar isolates have been united as species, similar species as genera, and so on. Represented diagrammatically ,this build-up looks like a tree (Fig. 2), but when we describe it we always invert the tree so that it looks like a genealogical chart (Fig. 1).

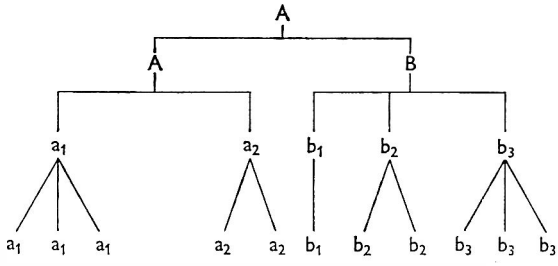

Fig. 1. A hierarchical system shown in the form of a genealogical table. Division of a large unit A into smaller subunits A and B, with further subdivision into sub-subunits (a_1, a_2, b_1, b_2 and b_3) made up of individuals.

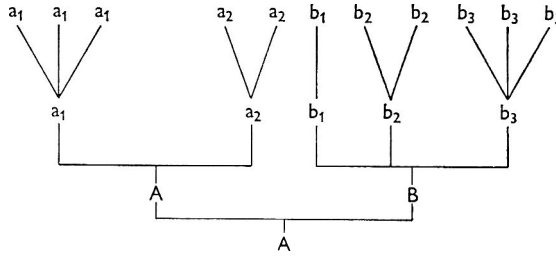

Fig. 2. A 'tree' which shows how groups of similar individuals are united as larger groups, which themselves are combined to form still larger groups.

PRINCIPLES OF BACTERIAL CLASSIFICATION

I. *Purpose*

The first principle is that there must be a purpose or reason for making a classification; it follows that there can be several classifications of the same objects, each scheme being created for a particular (and usually different) reason.

II. *Subdivisions*

The subdivision of a large unit does not follow any law; for example, the subdivisions do not need to be of equal size, or to be equally spaced. The philosophy of taxonomists themselves is reflected in the subdivisions they make. They can be subdivided (or classified) into clumpers and splitters; again, the division is not an equal one, neither is it clear-cut; on analysis it appears that when bacterial taxo-

nomists deal with their own group they are splitters, but when surveying bacteria as a whole they are clumpers of those organisms they know least about.

The first two principles of taxonomy give the taxonomist complete freedom to make his subdivisions as he will. It follows that it is unlikely that two workers will have identical reasons for making a classification, or will agree on all the lines of cleavage.

III. *Uniformity of units*

The third principle is that there should be some parallelism in the subdivisions of the hierarchy, but because of the subjectivity of taxonomy this principle is seen as much in the breach as in the observance. It is observed in the subdivision of the pneumococci into serological types dependent on the chemical nature of the capsular polysaccharides, and of *Streptococcus pyogenes* by the M and T surface proteins. It is breached when we compare the serotypes of streptococci with those of *Salmonella* which some regard as species and others (Kauffmann, 1963*a*) as units above species.

IV. *Circumscription*

Precision of definition is the fourth principle of classification. The boundaries of each unit must be defined; so that they are clearly recognizable by other workers. Thus, in the second half of the twentieth century the definition of *Escherichia coli* must be more than a Gram-negative rod that produces acid and clot when grown in milk; on the other hand, all the antigenic details do not need to be defined; indeed, a definition based solely on antigenic structure is as inadequate and unacceptable as any made by Escherich and his contemporaries.

V. *Characterization*

A unit cannot be defined until it has been characterized, which means until we have studied the morphology (including cytology), physiology, chemical make-up, enzymic constituents, genetic and other factors. The detail yielded by these studies will vary with different organisms, but the aim should be to produce as much information as possible; from this we look for pairs of correlated characters. There has been, and still is, much argument about the value of different characters and this point will be discussed later (see Principle VI). We often speak of 'adequate characterization' but seldom say what we mean by that term; one imagines that to a cytologist a characterization without details of cell walls, membranes, septa and nucleus would be inadequate, and that a serologist would regard a description that did not include antigenic structure as quite unacceptable. A characterization cannot be adequate unless it is fairly comprehensive; thus the descriptions of *Mycoplasma* species (PPLO) are inadequate because we know so little about their characters; in the same way, but to a lesser degree, the characterizations of *Neisseria* species are inadequate because these organisms may not grow in media developed for the characterization tests used for most organisms. Among the viruses characterization of the organism (if viruses are organisms) is limited, although cytopathic effects can be determined and may become part of the virus characterization.

The adequacy of characterization of a bacterium is a reflexion of time; it should be as full as modern techniques make possible. Unfortunatcly, one now regarded as adequate is likely, in ten years time, to be hopelessly inadequate!

VI. *Weighting of characters*

The weight to be attached to different characters is the sixth principle of classification; this is so debatable a subject that it requires more detailed consideration than we have given to the other principles. The early bacteriologists used the minimum of features in describing their organisms and, as the bacteria most studied were thought to cause disease in man or other animal, the organism was named after the disease. There was little attempt to create a systematic classification or nomenclature; the greatest stress was placed on morphology and, because only few characterization tests were then available, on a few simple cultural characters such as growth in various media, liquefaction of nutrient gelatin, and the changes induced in milk. By virtue of the length of time for which they have been used, these characters have assumed an importance out of all proportion to their usefulness. It was also found that certain characters easily shown by simple tests (for example the ability or inability to produce acid from lactose) seemed to be associated with certain bacteria; some of these bacteria (lactose-fermenters) were found in healthy people, others (non-lactose-fermenters) mainly in the sick. The tests then became important and the characters they revealed were regarded as important. Thus there developed a climate in which tests (and characters) were regarded as important or not important; in general the important ones were those that we should now call 'distinguishing', and they still retain all their importance in identification. But as classificatory criteria they are now under a cloud.

Adansonism. Sneath (1957*a*, *b*) drew attention to the theories and ideas of the French biologist Adanson (1727–1806), and applied his principle that all features had equal merit in making up the characterization of the whole to the characters of chromobacteria, in which Sneath showed that there were two major subgroups. The analysis was made easier by using a computer, and an extension of the 'electrotaxonomy' to a wider range of bacteria showed that the principles enunciated by Adanson were applicable to the classification of bacteria (Sneath & Cowan, 1958). It was a tribute to the work of the older taxonomists that a classification made by giving equal weight to all characters produced results which, by and large, confirmed the empirical classifications that have been used for so long, such as those proposed by Winslow *et al.* (1917, 1920), *Bergey's Manual* (1923–57) and *Topley & Wilson's Principles* (1929–64), to mention but a few.

Few taxonomists would now deny that Sneath made a most valuable contribution to bacteriology when he drew our attention to the principles of Adanson, for he was not only able to develop numerical taxonomy (Sneath & Sokal, 1962), but he made bacteriologists pause and question ideas that, by constant repetition, were becoming accepted as facts.

Anti-Adansonism or Kauffmannism. The most forceful and repetitious champion of unequal weighting of characters is Kauffmann, who in 1937 wrote: 'Salmonellabakterien sind gramnegative Bakterien, die auf Grund ihrer Antigenstruktur in das Kauffmann-White-Schema eingefügt werden können.' On this basis any Gram-negative organism sharing an antigen with a known salmonella would also be regarded as a salmonella. Since the specificity of antigens is determined by their chemical nature, the same (or closely similar) antigen may be found in more than one group defined on other characters. With this definition of the Salmonella

group, one could include in it many quite different bacteria including pasteurellas, and the group would become something quite different. Emphasis on antigenic structure has been apparent in nearly all Kauffmann's writings. He showed greater appreciation of other characters when, as chairman of the International Enterobacteriaceae Subcommittee, and perhaps influenced by the views of other workers, he wrote: 'Tribes, genera and species should be established by biochemical methods and then sub-divided serologically' (Kauffmann, 1954). Later he retracted and antigenic structure again dominated his thoughts, indicated by his statement that each serotype was to be regarded as a species (Kauffmann, 1959). His complete antipathy to biochemical characters and classifications based on them became clear when he wrote: 'The higher groups are only biochemically defined and therefore badly defined' (Kauffmann, 1963*b*). Among biochemical tests he thought that some (e.g. the organic acids; Brown, Duncan & Henry, 1924, 1926) were more important than others; of fermentation reactions he took a particularly poor view (Kauffmann, 1963*a*). Kauffmann does not limit the stressing of antigenic structure to the Salmonella group; he thinks that it should apply to all the Enterobacteriaceae, to the pneumococci and other organisms that are known to be divisible into subunits on serological grounds. By his persistent advocacy of a classification based on heavily weighted characters, he is clearly the leader of those who stress the importance of one kind of character above all others. This anti-Adansonian attitude seems to deserve the name Kauffmannism.

Kauffmann is a determined advocate and because he is such an authority on salmonellas his views must be considered seriously. He spoils his case by the extremism with which he presents it, as when he says that biochemically defined groups are badly defined. He is not on firm ground even when he quotes Bruce White as an authority for equating serotypes with species. Bruce White was a man of great perception but he regarded the serotype as a stable unit and he did not conceive that what we now call transformations and transductions could occur.

Before we leave the sixth principle it is clear that we must decide for or against the Adansonian concept, but in doing so I want to refer to the subdivision of taxonomy into three sections: classification, nomenclature, and identification. This is necessary because I believe that in classification we should give equal weight to all characters, but in identification we can legitimately put different weightings on different characters. If I am Adansonian in my approach to classification there is no necessity for me to use the same approach to identification, and in fact few diagnosticians would attempt to be so unpractical in trying to identify a bacterium.

Relation of classification to nomenclature

These then, are the general principles that govern classification; they have not been codified and are likely to remain plastic and to serve more as guides than as precepts. It is perhaps unfortunate that the attempt to formulate these principles has never been made, because the development of an official code of nomenclature may suggest that naming has precedence over classification, an almost classic example of the cart being before the horse. To some bacteriologists the most important aspect of taxonomy is nomenclature, or the labelling of units. As the world's

foremost authority on bacterial nomenclature, it is not unnatural that Buchanan holds this view and he would base classification on the nomenclatural type culture; he has written that a bacterial species is 'the type culture together with such other cultures or strains of bacteria as are accepted by bacteriologists as sufficiently closely related' (Buchanan, 1955). This idealistic view (Buchananism) assumes that all bacteriologists are equally competent to pass opinions on all kinds of bacteria, and blurs the distinction between identification and classification.

PRINCIPLES OF IDENTIFICATION

The true taxonomist is a man with a mission; he often leads a cloistered life, protected from the vexations and frustrations of the everyday world, and he may well wear blinkers as opaque as any worn by a horse. He is more likely to have academic interests than to be an applied worker, a follower of Adanson rather than of Kauffmann, and an Englishman rather than an American. Living a life of seclusion, safe in his small laboratory, and surrounded by his books, his microscope (and perhaps his computer tape), he affects an unconcern for the mundane application of his work. But science has a way of making itself useful, and the useful application of classification is identification.

In the utilitarian laboratory there is probably more specialization: the individual worker concentrates on a more limited field, and may be unaware of what is going on in other, often adjacent or parallel, fields.

Identification is dependent on an earlier classification, of which it is the complement. There are three methods by which an identification can be made. (1) The method in which many tests are made before attempting to compare the unknown with known (and named) units; I have named this the *blunderbuss* method, but it would have been equally apt to call it the unthinking method. (2) The method in which the approach is *intuitive* and is often followed when we think we know what organism we shall isolate (e.g. from pus from a boil we might expect to isolate *Staphylococcus aureus*) but occasionally we are shocked into abandoning this lazy approach to identification. (3) The step-by-step or *progressive* method which uses dichotomous keys; the dichotomous keys given in *Bergey's Manual* (1957) are not altogether satisfactory, but the excellent keys drawn up by Skerman (1959) take the identification down to the generic level, those of Manclark & Pickett (1961) to species.

In *Bergey's Manual* dichotomous keys are supplemented by descriptive text, and in Manclark & Pickett's scheme by tables of characters which are much more informative. A newer progressive method uses only diagnostic tables; we first carry out a few screening tests, then consult primary diagnostic tables (Fig. 3) which lead on to secondary tables made up of more specialized tests (Cowan & Steel, 1961, 1965). In using the progressive method we put different stress on different characters; in fact a good differential test becomes an important test. The screening tests of the primary tables are all simple but important because they reveal characters of great diagnostic value. By the selection of a few important tests we can often identify the genus very quickly; for the medical bacteria the primary tables are based on less than ten characters. The more a group has been studied, the more subdivisions will have been made. It follows that the diagnostic tables of the Enterobacteriaceae,

the streptococci and the sporing bacilli are large, but those dealing with staphylococci, micrococci, and neisserias are much smaller.

I have described bacterial identification as the complement of classification; it is also its antithesis because differential weighting of characters forms an essential part, and there is a deliberate selection of features or characters for descriptive purposes. Descriptions of bacteria often leave much to be desired because the author has not been selective enough. It is surprising how few characters are needed

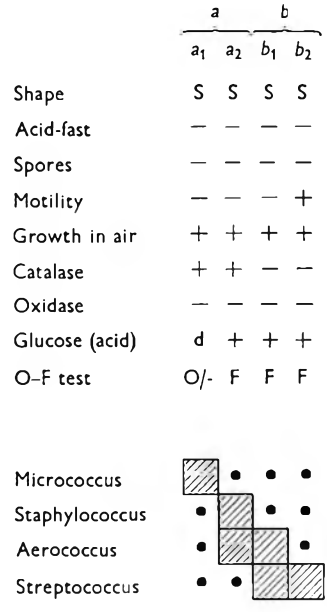

Fig. 3. Part of a primary diagnostic table which, by a few characters, will identify a Gram-positive bacterium to the generic level (from Cowan & Steel, 1965).

for miniature definitions (minidefinitions) which contain the fewest possible characters to define bacterial genera (Cowan & Steel, 1965); those used must be characters that are stable and constant when looked for by different techniques. Drawing up the minidefinitions spot-lighted similarities known for some time, but ignored because their acceptance would offend the innate conservatism of taxonomists. A good example is the close similarity of *Serratia marcescens* to the motile Gram-negative rods now forming the genera *Enterobacter* and *Hafnia*, first noticed by Pederson & Breed as long ago as 1928.

The influences of media, indicators, and technical methods in establishing the characters of bacteria are now well recognized, and every effort should be made to keep the test conditions constant. Workers who devise new characterizing tests should describe their techniques in detail. Cowan & Steel (1965) adapted the nomenclatural type culture concept to cultures for characterizing tests, by designating different strains (biotest type strains) that are positive and negative in the tests used.

RULES OF NOMENCLATURE

Classification and nomenclature form two parts of taxonomy and the result of taxonomic work is conveyed to other workers by the application of labels to the microbial units. As so often happens communication is the weakest link in the taxonomic trinity, and there may be much discussion about the correctness of names given to different taxa. To try to bring order to this problem an International Committee on Bacteriological Nomenclature was formed in 1930, and a Code of Nomenclature approved in 1947 (Buchanan, St John-Brooks & Breed, 1948); this was revised and annotated by Buchanan and published (Buchanan, Cowan, Wikén & Clark, 1958). The nomenclatural Code consists of Rules and Recommendations but its weakness lies in an inability to enforce the rules, which thus become precepts of good behaviour and professional ethics. To many people the Code is unacceptable because it recognizes 1753 as the starting date for bacteriological nomenclature, and the rules are to be applied retroactively; a starting date in the era of pure cultures with automatic invalidation of many old names would be much more acceptable.

Nomenclature is only a means of labelling individual units and need not commit a worker to more than a label which must, however, be unique for each different organism. The advantages of a binomial system are twofold: (1) fewer unique names are needed than in a uninomial system; (2) relations between units can be indicated by the first (or generic) name. A disadvantage of any system of naming is that it is limited to pronounceable words; this limit has apparently been reached in epithets used for some salmonellas and in names such as *Bdellovibrio* recently proposed by Stolp & Starr (1963).

ALTERNATIVE TO NOMENCLATURE

It is possible to devise a method of labelling microbial units in which names are avoided altogether; the unique label can be a code either in the form of letters and numbers as in the telephone system, or a series of figures as in the Zip numbers used by the United States postal service. Empirical codes of this kind are not limited to pronounceable words, and figures, unlike letters in word form, can be arranged in any order as in these examples.

142148 = *Staphylococcus aureus*
142247 = *Streptococcus pyogenes*
241146 = *Escherichia coli*
241245 = *Shigella dysenteriae* 1

Six figures might not suffice to show the delicate nuances of, say, the various serotypes of *Salmonella*, but provision could be made for these finer details to be indicated after a decimal point, colon or dash.

A more elaborate descriptive code can be developed in which a few simple basic characters are given single digit numbers to form a sequential code. Of the characters used by Cowan & Steel (1961), those dealing with morphological and tinctorial features are given numbers 1 to 9 and 0; and five of these appear before the dash. Catalase, oxidase, and attack on glucose are expressed as four numbers 1 to 9 and are shown after the dash; the cipher 0 would be added for those bacteria that are

strict anaerobes. The two groups of figures are sufficient to characterize many bacteria to the generic level of an orthodox classification; more individualistic (? specific) features can be indicated by a code figure or figures after a colon. Alternatively the subgeneric unit can be shown by a purely arbitrary number (Table 1).

Table 1. *A descriptive code and examples*

	Before dash	After dash
1	Gram-positive	Catalase-positive
2	Gram-negative	Catalase-negative
3	Sphere	Oxidase-positive
4	Rod shaped	Oxidase-negative
5	Acid-fast	Glucose not attacked
6	Not acid-fast	Glucose attacked by fermentation
7	Spore forming	Glucose attacked by oxidation
8	Not spore forming	Gas produced from glucose
9	Motile	Gas not produced from glucose
0	Non-motile	Does not grow in air

Number after colon = empirical number allotted to a species

13680–1469:1 = *Staphylococcus aureus*
13680–2469:1 = *Streptococcus pyogenes*
14670–1479:7 = *Bacillus anthracis*
24689–1469:1 = *Salmonella typhi*
14679–24590:1 = *Clostridium tetani*

DISCUSSION

If we are looking for a new approach to systematics in general and the classification of bacteria in particular, we must be prepared to abandon hierarchical systems which imply natural relationships, and seek new ways of building up the bigger units from the smaller subunits. First we should seek similarities of fundamental characters, and here transferable genetic material seems at the present time to be of the utmost importance. Next we may think of similarities in the cellular substance of different units; we can collect evidence from independent sources, such as chemical analysis of cell walls (Cummins & Harris, 1956; Westphal, Kauffmann, Lüderitz & Stierlin, 1960) or of antigenic structure by serological analysis. Other similarities that will help us to form our larger groups will be the metabolic activities of the smaller units; thus it is convenient to group together those bacteria that use gaseous nitrogen; in the same way those that ferment carbohydrates can be separated from those that oxidize or do not attack sugars. A build-up such as this will lead to groupings many of which are in use today, but there will not be any suggestion that the individual units making up a group are related. A classification built up in this way, utilitarian in concept, would not be hidebound by outmoded ideas on relations other than those of basic similarities. Kauffmann (1963*a*) advocated such building-up, but, in my view, his conservatism in using such terms as species, genus and so on decreases the chances of having his views accepted.

The comparison of characters is simple only as long as the known characters of the different units are few; mechanical devices help when a moderate number of characters is known (Cowan & Steel, 1960, 1961), but when many characters are to be compared a computer becomes invaluable, if not essential (Sneath, 1957*b*). The

usefulness of a computer has been shown in classification (Sneath & Cowan, 1958) and in identification (Payne, 1963), but a computer is an expensive luxury and we are now seeking ways by which it can be transferred from the cloisters of systematics to the utilitarianism of the diagnostic laboratory.

The expression of characters can be achieved better by a sequential code than by a name descriptive of some characters. Table 1 is a utilitarian system of codification rather than an elegant form of nomenclature associated for so long with classical taxonomy, but it is an example of a concept that must be given serious thought if we are to attempt to label all the permutations of characters of which bacteria are capable.

This paper is based on one read on 1 May 1964 in Washington, D.C., U.S.A. at a Symposium organized for the dedication of new laboratories for the American Type Culture Collection.

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