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THE JOURNAL OF GENERAL MICROBIOLOGY

THE PREPARATION OF PAPERS

'The more words there are, the more words there are about which doubts may be entertained.'

JEREMY BENTHAM (1748–1832)

'What can be said at all can be said clearly; and whereof one cannot speak thereof one must be silent.'

LUDWIG WITTGENSTEIN: *Tractatus Logico-philosophicus* (tr. C. K. Ogden)

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(1) Papers should be written with the utmost conciseness consistent with clarity. The best English for the purpose of the *Journal* is that which gives the sense in the fewest short words, spelt according to the *Shorter Oxford English Dictionary*.

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

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

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

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

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The following books may be found useful:

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

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

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

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Studies on Cholera Toxin and Antitoxin

By J. UNGAR, M. STANIĆ, N. CHARIATTE AND
S. J. VARALLYAY

Swiss Serum and Vaccine Institute, Berne, Switzerland

(*Accepted for publication 2 September 1970*)

SUMMARY

The preparation, purification and properties of cholera toxin type 2 are described. When the toxin was given to experimental animals it induced a high level of toxin-neutralizing antibodies. Cholera toxoid given to human volunteers induced antibodies consisting of agglutinins, vibriocidal and toxin-neutralizing antibodies. The relevant titres depended on the purity of the antigen administered.

INTRODUCTION

The protective value of whole-cell cholera vaccines has been demonstrated in the last six years in field trials conducted in the Philippines (Azurin *et al.* 1967) and in East Pakistan (Oseasohn, Benenson & Fahimuddin, 1965; Benenson, Mosley, Fahimuddin & Oseasohn, 1968*b*; Mosley *et al.* 1969) when 50 % of the people inoculated were shown to be protected but only for a short time. The duration of the protection can be prolonged up to 18 months either by using vaccines of extremely high mouse potency or by repeated inoculations (Oseasohn *et al.* 1965; Benenson *et al.* 1968*b*). As the protection produced by whole-cell cholera vaccines and endotoxin preparations seems to be only partially effective (Watanabe & Verwey, 1965), it is of particular importance to determine the protective effect of the cholera toxin preparation in man. In a recent review Burrows (1968) summarized the characteristics of three toxins of *Vibrio cholerae*. Dutta & Habbu (1955) introduced the suckling rabbit test which later was developed by Finkelstein, Norris & Dutta (1964) for the studies of the thermolabile, non-dialysable type 2 'choleragenic' toxin which plays an important role in the pathogenesis of cholera. Craig (1965, 1966) found a factor in the filtrate of vibrios, thermolabile and non-dialysable. He later described a method for the titration of this factor, which he referred to as the 'permeability factor' (1966).

We decided to investigate the protective value of antigens prepared from the type 2 toxin. For this purpose we prepared the toxin according to the method described briefly in a previous communication (Ungar, Chariatte, Stanić & Varallyay, 1969) and given below in detail.

METHODS

Strains. All the strains used in our investigation were freeze-dried after isolation and stored at 4°. The toxigenic strain *Vibrio cholerae* 569B serotype INABA came from Dr R. A. Finkelstein. To determine the prevalence of toxin production among classical and EL TOR vibrios isolated from cholera patients, various vibrios (D34580 and D34580GP from East Pakistan) and EL TOR biotypes (no. 133, 282 and 929 from the Middle East) were cultivated and their culture filtrates tested for toxicity.

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Media. In the early experiments syncase medium (Finkelstein, Atthasampunna, Chulasamaya & Charunmethee, 1966) was used. The peptone water medium contained 30 g. Bacto-peptone (Difco)/l. and 5 g. NaCl/l. at pH 8·0. The dialysed peptone water used in later experiments contained the same amount of Bacto-peptone and NaCl but the peptone had been previously dialysed against deionized water. A 30 % (w/v) solution in deionized water was dialysed against 20 vol. of deionized water, with vigorous magnetic stirring for 24 h. at 4°. The dialysate was concentrated and freeze-dried. These small dialysable molecules of peptone were used instead of the untreated product in order to facilitate the separation by dialysis of unaltered medium from the bacterial products in the crude supernatant of the cholera cultures. The dialysis tubing (Kalle AG, 6206 Wiesbaden-Biebrach, West Germany) was sterilized by boiling in distilled water. The stock cultures were rehydrated from the freeze-dried state by the addition of nutrient broth and inoculated on Bacto-peptone agar containing peptone water plus 2 % Bacto agar (Difco).

Cultures. The vibrios were cultivated in 1 l. Erlenmeyer flasks containing 200 ml. medium on a rotating shaker (130 rotations/min. (rev./min.)) at 37° for 7 h. Alternatively, a stainless steel 5 l. laboratory fermenter was used in which the medium was stirred at 1400 rev./min and aerated with air (500 ml./min.). The air was sterilized by a closed system of 2 Berkefeld N-15 filters. To prevent the formation of foam, 1 ml. of a 25 % (w/v) silicone–paraffin oil emulsion (Dow Corning Antifoam A) was added.

Toxin production

Inoculation of flasks and fermenters. The freeze-dried *Vibrio cholerae* strain 569B was recovered, streaked on to 3 % (w/v) peptone agar and cultivated at 37° for 18 h. The surface growth was washed off with physiological saline, and the suspension adjusted to $1{\cdot}4 \times 10^9$ vibrios/ml. using Brown's opacity tubes (Wellcome). Two ml. of the suspension were used as inoculum for the 200 ml. shaken culture, grown at 37° for 7 h. and then used as inoculum for the 5 l. fermenter. The fermenter was incubated at 36° for 6·5 h. When the culture reached the end of the logarithmic phase it was harvested by centrifugation at 6100 *g* using a Sorvall RC-2B superspeed refrigerated centrifuge at 4°.

To achieve rapid filtration through the final GS (0·22 μm.) membranes, prefiltration of the supernatant was necessary through RA (1·2 μm.), DA (0·65 μm.) and HA (0·45 μm.) membranes, with AP 32 Dacron mesh between each pair of membranes. All the membranes were put in a single, 142 cm. filter holder with an AP 25 glass fibre prefilter. The product was our crude syncase choleragen or, in the case of the peptone media, the crude peptone water supernatant (PSUP) toxin. The products were stored at 4° until purification. Table 1 summarizes the yields of the toxin obtained under various growth conditions.

Purification of the toxin. The crude cholera toxin was precipitated by 90 % saturation with ammonium sulphate or concentrated by flash evaporation (Rotavapor R, Büchi AG, Flawil/SG, Switzerland).

Purification was first tried on Sephadex G-200 by eluting with 0·1 M-tris buffer, pH 7·5. But as the flow rate was slow, the gel was replaced by Sepharose 4B and the elutions were performed with 0·1 M-tris buffer pH 8·6. The extinction of the eluates was monitored at 253·7 nm. with an LKB 8300 A Uvicord II (LKB Produkter AB, Stockholm, Sweden). At a later stage, the cholera toxin was purified on DEAE

cellulose by stepwise elution with distilled water, 0·05 and 0·5 M-NaCl. The properties of the purified material were assessed by immunoelectrophoresis and gel diffusions and measurements of loop toxicity. For the characterization of the purified toxin, total nitrogen and protein determinations were of little value.

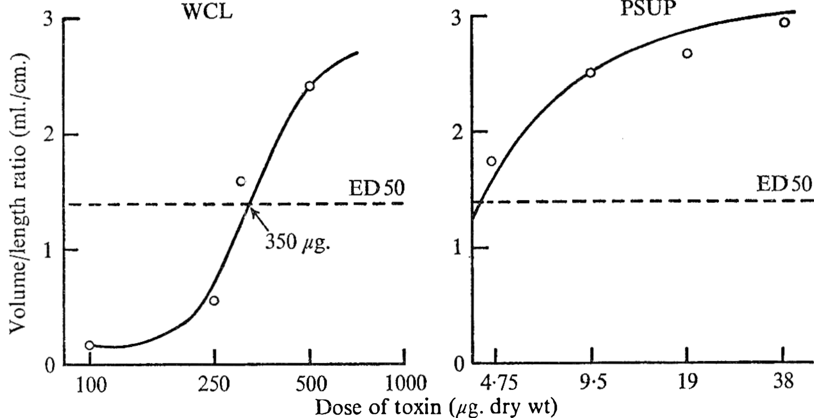

Fig. 1. Typical toxin titration curves of a whole cell lysate (WCL) and a crude peptone water supernatant (PSUP) toxin in the adult rabbit ileal loop. 1 unit of WCL toxin = approximately 350 μg. dry wt of toxin *and* 1 unit of PSUP toxin = 2 μg. dry wt of toxin.

Table 1. *Yield of the toxin from Vibrio cholerae under different growth conditions*

Cultures were grown as described in Methods (p. 2) for 7 h. (shaken cultures) or 6·5 h. (fermenter cultures). The bacteria were removed by centrifugation and the supernatant passed through membrane filters. The toxin content of the filtrate was assayed using the ileal loop technique.

	Yield of toxin (loop units/ml.)	
Medium	Shaken flasks	Five-litre fermenter
Syncase	50 to 400	50 to 600
Peptone	400 to 1000	200 to 1000
Dialysed peptone	400 to 1200	200 to 1200

Titration of the toxin and antitoxin. All toxin and antitoxin titrations were performed according to the method of Kasai & Burrows (1966) and are designated in the text as toxin loop units and AT (antitoxic) units respectively. We used non-inbred albino rabbits of both sexes, aged 8 to 12 weeks, from the Tierzuchtinstitut, Zürich, Switzerland. Other strains of rabbits of the same age were also tried but their resistance to the toxin was irregular. The animals were starved for 48 h. before the operation but were always allowed free access to water. The animals were killed by intravenous injection of sodium pentothal, and autopsy was performed between 18 and 20 h. after injection of the toxin. The volume of fluid (V) in each loop (expressed in ml.) and the length (L) of the loops (expressed in cm.) was measured and the ratio (V/L) calculated. Values higher than 2·75 were not considered in the calculation of the averages. The ED 50 reaction was therefore a V/L value of 1·375, and this was taken as one unit of type 2 cholera toxin, as described by Kasai & Burrows (1966).

Fig. 1 illustrates two typical toxin titrations. The titration of toxin-neutralizing

antibodies was also performed in the ligatured ileal loop of adult rabbits, but in this case a neutralization coefficient (*NC*) was calculated from the *V/L* ratios ($NC = 1-[2\cdot75/V/L]$). The *NC* was plotted on a log scale against the dilutions of the antiserum. In these neutralization tests a sonic lysate of cholera toxin, kindly supplied by Dr W. Burrows, was used as a standard.

Preparation of the toxoid. The crude and purified cholera toxins from syncase, peptone water and dialysed peptone water medium were treated with 0·25 % formaldehyde (E. Merck, 35 % (w/v) analytical grade) at room temperature for 1 week. After detoxification, the residual formaldehyde was removed by dialysis at 4° against distilled water for 3 days; the toxoid was tested in the ileal loop for its innocuity.

Preparation of antisera. Antisera were prepared by immunizing rabbits. A single injection of 1 or 2 ml. toxin (0·5, or 2 mg.) without adjuvant was given to each rabbit intramuscularly or intraperitoneally and blood specimens taken 2 or 3 weeks later. The presence of specific antibodies against the crude PSUP toxin could be shown using the agar immunodiffusion technique.

As large amounts of high titre antiserum were needed to study the role of the antibodies in the prevention and treatment of cholera, a horse was hyperimmunized intramuscularly with purified cholera toxin, using aluminium phosphate as an adjuvant. When the titre of the antitoxin in the serum was found to be satisfactory (2000 to 2500 AT units/ml.) the horse was bled and the plasma purified.

Precipitation of the low molecular weight proteins from the horse plasma was performed by adding 0·5 % rivanol at pH 8·0, followed by fractionation of the non-precipitated immunoglobulins with either (*a*) 2 moles sulphate/l. at neutral pH, or (*b*) ethanol at −7° and neutral pH. In both cases filtration and lyophilization of the precipitate was performed and the protein content adjusted to 10 %; the yield of gamma-globulin was 65 to 70 % by method (*a*) and 83 to 85 % by method (*b*). The solution was made isotonic by the addition of sodium chloride and then sterilized by filtration through Millipore GS membrane filters. Thiomersal (0·01 %) was added as a preservative.

Serological methods

Agglutinins were determined using a suspension of live vibrios according to the method of Goodner, Smith & Stempen (1960).

Vibriocidal antibodies were determined either by the method of Finkelstein (1962) or by the microtechnique of Benenson, Anisa Saad & Mosley (1968*a*) using the Microtiter System (Cooke Engineering Co., Alexandria, Va, U.S.A.) as recommended by Sever (1962).

Antitoxins were titrated by the ileal loop technique described by Kasai & Burrows (1966).

RESULTS

Strains. Fig. 2 shows the results of comparative titrations carried out in duplicate. The abscissa give the dilution of the PSUP toxin from cultures of different 'classical' (D34580 and D34580GP) and EL TOR (133, 282, 929) vibrios. The variations in the quantities of toxin produced by the strains agreed with the findings of other workers (De, Ghose & Sen, 1960; Finkelstein *et al.* 1964; Burrows, 1968).

Media. A culture supernatant containing crude toxin could be obtained with the strain 569B by incubating in syncase medium for 18 to 20 h. at 37° on a rotating shaker.

The amount of the toxin varied between 100 to 400 loop units/ml. We noted that for toxin production the ratio of the surface of the medium to the volume in the Erlenmeyer flask was of considerable importance (200 ml. medium in 1 l. Erlenmeyer flask). Static cultures did not yield supernatants with measurable loop toxicity. Cultures (either shaken or fermenter) grown on Bacto-peptone and the dialysed Bacto-peptone media always produced toxin in the logarithmic phase of the growth and the culture filtrates contained 100 to 1000 loop units/ml. On the other hand, in the syncase medium toxin was only occasionally produced in the logarithmic phase, but a toxicity comparable to the peptone media was reached after 18 to 20 h. incubation when the culture was entering the stationary phase.

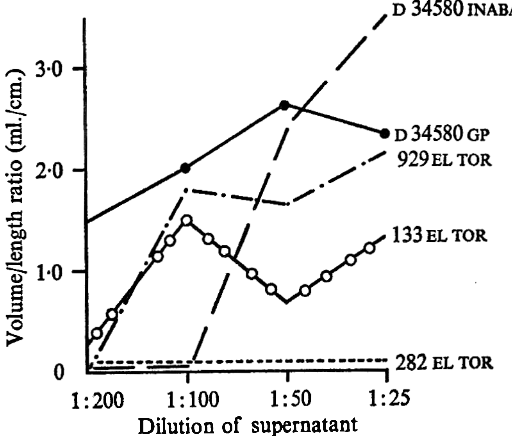

Fig. 2. Comparison of the toxigenicity of various classic and EL TOR biotypes of *Vibrio cholerae*. Crude peptone water supernatant toxin was isolated from shaken cultures and assayed in the ileal loop of adult rabbits.

Purification of the crude toxin. When the crude syncase or PSUP toxin preparation was chromatographed on Sephadex G-200 two peaks of material were eluted. The first contained the toxin and the second was unchanged ingredients of the culture medium and contained no toxin. The material in the first peak gave two precipitin lines on gel diffusion. When the antigenic properties of these peak materials were determined it was found that the material in the first peak stimulated the formation of toxin-neutralizing antibodies and the material in the second peak gave rise to vibriocidal antibodies. Fractionations on Sepharose 4B with 0·1 M-tris buffer, pH 8·6, gave similar results. On gel diffusions no interference reaction (Ouchterlony) was found with the two precipitin lines produced by material derived from the Sephadex G-200 fractionation.

Considerably better resolution was obtained when DEAE cellulose was used. The material eluted in deionized water contained the major portion of toxicity and gave a single precipitin line on gel diffusion. Further elution with 0·05 and 0·5 M-NaCl gave two more fractions. The latter gave a broad band of precipitin and two distinct precipitin lines on gel diffusion but no biological (loop) activity was detected. The precipitin line of the water subfraction gave an interference reaction (Ouchterlony) with the precipitin lines of the NaCl fractions. Immunogenic studies in rabbits showed that the water fraction produced antitoxin, whereas the 0·5 M-NaCl fraction produced vibriocidal antibodies.

Characteristics of the toxin

Stability. The crude PSUP toxin or syncase choleragen was stored at +4° and its loop toxicity tested at intervals of 4, 8 and 12 weeks. This activity diminished by between 80 and 90 % after 8 to 12 weeks. After freezing the toxin at −70° for 4 weeks, the toxin showed a similar loss of activity. Lyophilization studies showed that when the final drying was carried out at 23° up to 60 % of the activity was lost, and if done at 30° as much as 70 % was lost.

Toxicity. When 0·1 ml. (5 units) of the toxin was injected intradermally into a guinea pig, a reddening and induration was produced after 12 h. which reached a diameter of 25 mm. after 48 h. No necrosis was noted.

Table 2. *Types and titres of antibodies present in purified globulins prepared from horse antiserum*

Antiserum was prepared by hyperimmunization of a horse, using purified cholera toxin (from *Vibrio cholerae* 569B). The immunoglobulins were precipitated (Methods, p. 2) and lyophilized. A solution containing 10 % protein was prepared, made isotonic with NaCl and sterilized. The titre of agglutinating antibodies was determined using suspensions of *V. cholerae* INABA strain 569B and OGAWA strain C 38383.

	Reciprocal titres			
			Agglutinating antibodies	
Batch no. and method of preparation	Antitoxic antibodies	Vibriocidal antibodies	INABA	OGAWA
A-2/67 Rivanol–ammonium sulphate fractionation	24,000	10^7* 5×10^4 to $1\cdot5\times10^5$†	1280	1280
A-1/68 Rivanol–ammonium sulphate fractionation	22,000	10^7	5120	5120
B-1/68 Rivanol–ethanol fractionation	30,000 to 40,000	10^5	2560	2560

* By the method of Finkelstein (1962).
† By the method of Sack *et al.* (1966).

Pyrogenicity. When injected intravenously into rabbits, 0·1 ml. (5 units)/kg. body weight produced a rise in temperature of 0·6 to 0·8°, i.e. the toxin was slightly pyrogenic.

Detoxification. Formaldehyde concentrations greater than 0·25 % destroyed the ileal loop activity after 6 h. at temperatures from 4 to 22°.

Tests in animals. The antigenicity of the toxin and of the toxoid was tested in rabbits immunized intramuscularly. The serological response of rabbits to a single dose of 50 loop-units of the toxin was as follows: agglutinin titres varied between 1:1280 and 1:10,240, while vibriocidal antibody titres varied between $1:10^4$ and $1:5\times10^6$. Antitoxin titres 3 weeks after immunization were between 1600 and 2400 antitoxin (AT) units. The agglutinin and vibriocidal antibody titres in rabbits immunized with the same amount of toxin (previously treated with 0·1 % formaldehyde for 24 h.) showed titres of 1:1280 to 1:2560 and $1:10^4$ to $1:10^5$ respectively. Immunization with the partially inactivated toxin, which was obtained by treatment with 0·05 % formaldehyde for 1 h., also produced agglutinins, vibriocidal antibodies and titres of

antitoxin (200 to 400 units/ml.) similar to those following injection of toxin treated with 0·1 % formaldehyde.

During the hyperimmunization of the horse, doses of the purified toxin (50 to 100 ml. of the eluate derived from Sephadex G-200 and containing 5 to 10 mg. protein/ml.) produced general symptoms, such as rise in temperature and increased perspiration, which disappeared in 4 to 6 h. After six repeated doses of this toxin, the antitoxin level was usually between 1500 and 2500 AT units/ml. which decreased after 3 to 4 weeks to 500 to 600 units/ml. Repeated immunization was needed to maintain the high antitoxin titres. As in the rabbit sera, the agglutinin and vibriocidal antibody titres of the horse serum increased to $1:10^4$ and $1:10^5$ respectively.

Biological control tests of the horse antitoxin conformed to the requirements laid down by the U.S. PHS Regulations Title 42 regarding the General Safety Test (part 73.72) in mice and stability conformed to the N.I.H. Minimum Requirements for Gammaglobulins.

Pyrogen tests (according to part 73.74, 'purity' of the above U.S. PHS Regulations) with the rivanol–ammonium sulphate purified serum showed a slight pyrogenicity (1·0 to 1·5° rise in temperature). The rivanol–ethanol purified material was pyrogen-free (rise in temperature less than 0·5°). The latter method was therefore used subsequently for purification.

The antitoxin titres of the horse globulins purified by the rivanol–ethanol method were considerably higher, namely 30,000 to 40,000 AT units/ml. compared with 20,000 to 24,000 AT units/ml. using the rivanol–ammonium sulphate method (Table 2). All of these partially purified and non-adsorbed antitoxin sera contained, in addition to antitoxins, agglutinins and vibriocidal antibodies (respective titres 1:1280 to 1:5420 and $1:10^5$ to $1:1 \times 10^5$). In order to obtain a pure antitoxic product, absorption was performed with living suspensions of *Vibrio cholerae* strain 569 B. This reduced the agglutinin and vibriocidal antibody titres to 1:80 and 1:1000 respectively without loss of the antitoxic titre.

Tests in human volunteers

Innocuity and immunogenicity. Purified toxin (fraction eluted from Sephadex G-200) was injected subcutaneously into three humans (25 loop units/person) and the results are shown in Table 3. High levels of the toxin-neutralizing antibodies were found in one person and lower levels in the other two. A simultaneous production of the agglutinins and vibriocidal antibodies also occurred, which indicated that additional bacterial fractions were present in this preparation. Apart from a slight mild local reaction at the site of inoculation, no untoward reactions were noted in the three volunteers. The investigation was then extended to 33 volunteers (Table 4). Reactions to the first injection of the toxoid were observed in only 10 persons (group 1) inoculated subcutaneously in the upper arm with the purified toxoid without adjuvant. Of these, six had increased temperatures up to 38°, three suffered from chills, headache and lassitude, and six had local reactions such as erythema, swelling and tenderness of the skin at the site of the injection which subsided after 48 h. No local reaction occurred in volunteers injected intramuscularly with either adsorbed or inadsorbed toxoid.

The antibody response to two injections of the crude and partially purified toxoid is shown in Table 4. As can be seen, the increase in titre of agglutinins and vibriocidal antibodies was greater in the two groups immunized with the crude toxoid than in

Table 3. *Antibody response of three volunteers to purified toxin from Vibrio cholerae 569B*

A single dose of 25 loop units of purified toxin of *Vibrio cholerae* 569B (preparation 113/67, the fraction eluted from Sephadex G-200) was given subcutaneously without adjuvant. AGGL = titre of agglutinating antibodies for INABA strain 569B and for OGAWA strain C 38383. VA = titre of vibriocidal antibodies, and AT = titre of antitoxic antibodies (as units/ml.). n.t. = Not tested.

Serum titres	Volunteers 1 INABA	Volunteers 1 OGAWA	Volunteers 2 INABA	Volunteers 2 OGAWA	Volunteers 3 INABA	Volunteers 3 OGAWA
Before immunization						
AGGL	1:20	< 1:10	< 1:10	< 1:10	< 1:10	< 1:10
VA	< 1:10		< 1:10		< 1:10	
AT	< 25		< 25		< 25	
Two weeks after immunization						
AGGL	1:20	1:40	1:20	1:160	1:40	1:80
VA	10^{-3}	10^{-3}	10^{-5}	10^{-6}	10^{-5}	10^{-5}
AT	*c.* 1000		260		400	
Five weeks after immunization						
AGGL	1:80	n.t.	1:320	n.t.	1:640	n.t.
VA	10^{-4}	10^{-3}	10^{-4}	10^{-5}	10^{-4}	10^{-4}
AT	*c.* 280		100		*c.* 300	

Table 4. *Antibody response of volunteers to cholera toxoid*

The volunteers (33) were divided into four groups: only group 1 received the toxoid subcutaneously; the other groups received toxoid intramuscularly. Purified toxoid (15 loop units in 0·5 ml. physiological saline, preparation 113/67 from *Vibrio cholerae* strain 569B) was given to groups 1 and 2, and crude toxoid (30 loop units in 0·5 ml. physiological saline, preparation 05F/68) to groups 3 and 4. The adjuvant was aluminium phosphate (2 mg./dose). All volunteers received a booster dose of crude toxoid (05F/68) with adjuvant 3 weeks after the first injection. AGGA = agglutinating antibodies; VA = vibriocidal antibodies determined by the microtechnique of Benenson *et al.* (1968); AT = antitoxic antibodies (as units/ml.).

Serum titres	Purified toxoid: Group 1 (toxoid alone)	Purified toxoid: Group 2 (toxoid with adjuvant)	Crude toxoid: Group 3 (toxoid alone)	Crude toxoid: Group 4 (toxoid with adjuvant)†
Before immunization				
AGGL	< 1:10	< 1:10	< 1:10	< 1:10
VA	< 1:10	< 1:10	< 1:10	< 1:10
AT	< 25	< 25	< 25	< 25
Three weeks after immunization (before booster)				
AGGL	1:120	1:80	1:480	1:640
VA	1:160	1:160	1:320	1:1280
AT	*c.* 100	*c.* 50	*c.* 50	< 25
Six weeks after immunization				
AGGL	1:480	1:80	1:240	1:640
VA	1:320	1:160	1:320	1:1280
AT	*c.* 200	*c.* 100	*c.* 50	*c.* 150

those immunized with the partially purified toxoid. The antitoxin response was less satisfactory, the maximum titre reached being about 200 units/ml. The addition of the aluminium phosphate seems to have increased only the titres of the agglutinins and vibriocidal antibodies.

DISCUSSION

The present procedure for immunization against cholera is unsatisfactory as the bacterial vaccine currently in use gives an immunity of short duration (3 to 6 months) and in only 50 to 60 % of those vaccinated. As it is now known that cholera toxin plays a role in the pathogenesis of cholera, it seemed reasonable to investigate the value of antigens prepared from cholera toxin as immunizing agents. In our study we have prepared cholera toxin in various media according to the methods of Finkelstein *et al.* (1964) and Burrows (1968). In the first instance we have ascertained that the toxin is produced to a different degree by all the strains which we have studied. We have chosen the strain 569B as the one producing maximal quantities of the toxin and Bacto-peptone water as the medium for the toxin production. Our attempts to purify the toxin from the crude metabolic fluid have resulted in a relatively pure toxin; but in human volunteers and in immunized animals low titres of agglutinins and vibriocidal antibodies were found, indicating that small amounts of antigens other than toxin were present in the inoculum. In this context it is interesting to note that the addition of an aluminium phosphate adjuvant increased the titres of agglutinins and vibriocidal antibodies but not of the antitoxin. The toxin injected parenterally to human volunteers was well tolerated and can therefore be used as such for immunization. It seemed to us, however, desirable to have it stabilized by formaldehyde and to use the toxoid for our immunological investigations. It is desirable to have further studies in experimental animals and in man to assess the immunizing capacity of cholera whole-cell vaccines compared with the action of toxoids alone and toxoids combined with other fractions present in the culture supernatants.

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Characteristics of Ruminococcus and Cellulolytic Butyrivibrio Species from the Rumens of Sheep Fed Differently Supplemented Teff (*Eragrostis tef*) Hay Diets

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SUMMARY

Twenty-four representative isolates of cellulolytic cocci and ten of cellulolytic rods from the rumens of sheep fed differently supplemented teff hay diets were characterized. Nine of the coccal isolates could be classified as *Ruminococcus albus* and twelve as *R. flavefaciens*. Three were classified as a new variety of *R. flavefaciens*, *R. flavefaciens* var. *lacticus*, on the basis of the preponderance of lactic acid among the products of cellulose fermentation. Two of the isolates did not fit into any of these groups. The rods were identified as *Butyrivibrio fibrisolvens*. Tests for the presumptive identification of cellulolytic bacteria from the rumen are discussed.

INTRODUCTION

The effect of variously supplementing low-protein teff hay on the numbers and types of predominant cellulolytic bacteria in the rumen was described in a previous paper (van Gylswyk, 1970), but only a presumptive identification of the bacteria was made. One group, *Cillobacterium cellulosolvens*, has since been described in detail (van Gylswyk & Hoffman, 1970). In the present communication the characteristics of bacteria presumptively identified as butyrivibrios, *Ruminococcus albus* and *R. flavefaciens* are reported in greater detail and compared with those of bacteria isolated by other workers from sheep and cattle fed on other diets (Hungate, 1950; Bryant & Small, 1956; Bryant, Small, Bouma & Robinson, 1958; Dehority, 1963; Kistner & Gouws, 1964; Jarvis & Annison, 1967; Shane, Gouws & Kistner, 1969).

METHODS

The culture techniques, media and identification tests used in this study were as described by van Gylswyk & Hoffman (1970) except that succinate was determined according to the method of Kmetec (1966). All determinations were done at least in duplicate, and data in Tables 1 and 2 represent mean values. Aggregation and cell dimensions were noted on at least nine occasions, using Gram-stained preparations.

RESULTS

Isolates 19, 20, 21, 22, 23, 24 and 41 were obtained from sheep fed on unsupplemented low-protein teff hay; 15, 17, 18, 28 and 46 from sheep fed on hay supplemented with urea; 12, 27, 38, 39 and 40 from sheep fed on hay supplemented with protein and the

remainder from sheep fed on hay supplemented with urea plus branched-chain volatile fatty acids. The diets were described in detail by van Gylswyk (1970).

All the isolates fermented cellobiose and cellulose (Whatman no. 1 filter paper). None grew at 22°, all grew at 30 and 38° and some growth of all was observed at 45° in at least one run. None produced catalase, indole or H_2S, and nitrite was never detected after growth in nitrate-containing medium. All the isolates gave a weakly positive Voges–Proskauer reaction in at least one run.

Cocci. The cocci were obligate anaerobes. Cell dimensions varied from 0·5 to 1·2 μm. (typically 0·6 to 1·0 μm.). All were Gram-variable except for 33 and 34 which were always Gram-negative. None was motile. On the basis of the types and proportions of end-products formed during the fermentation of cellulose the isolates were placed into four different groups (Table 1). Groups 1 and 2 were distinguished principally on the basis of their production of ethanol, succinate, lactate and hydrogen. Isolates 35 and 40 were anomalous in this grouping. In the case of strain 35, acetate, ethanol and hydrogen production were somewhat low while formate was relatively high for group 1. Isolate 40 produced more hydrogen and less acetate than was characteristic of its group. Group 3 comprised organisms that produced predominantly lactic acid. Group 4 consisted of only two strains that were very similar in fermentation products. They were isolated from the same animal on the same diet on two successive sampling dates. Fairly high figures for ethanol, no succinate for isolate 34, and very little for isolate 52 could perhaps have been criteria for inclusion in group 1. However, unusually high figures for lactate and low production of hydrogen made this less appropriate. Furthermore, the isolates showed higher production of formate and much lower production of acetate than group 1 except for isolate 35 which was similar in some respects to group 4.

All group 1 isolates, as well as isolates 25, 34, 40 and 52, grew well in medium without carbon dioxide (final pH 5·3 to 5·8). The remaining isolates grew poorly (final pH 6·0 or higher). All grew poorly in medium containing Trypticase and yeast extract instead of rumen fluid. That some growth occurred may have been due to transfer, with the inoculum of unwashed organisms, of growth factors from the cellobiose/rumen fluid agar slants on which the cells were grown. All the isolates fermented pectin and all except 39, 40, 41 and 52 fermented xylan. D-Galactose was fermented only by isolates 28, 34 and 40 and only isolates 39 and 43 fermented esculin. None of the isolates fermented L-rhamnose, maltose, trehalose, raffinose, dextrin, starch, inulin, salicin, glycerol, mannitol, inositol or DL-sodium lactate. Final pH of cultures showing a positive test was never below 5·4.

Other characteristics, in which both interstrain and interspecies differences were observed, are listed in Table 2.

Butyrivibrio-like rods. All the isolates (12, 15, 17, 18, 19, 20, 21, 22, 23 and 24) grew anaerobically but they often also showed considerable growth in the upper part of deep agar stab cultures, the surface of which was exposed to air containing 10 % CO_2 (van Gylswyk & Hoffman, 1970). In the case of isolates 12, 15, 20, 22, 23 and 24, growth was noticed up to the surface of the medium in at least one of the runs. The bacteria were slightly curved to curved, motile rods with a single polar or subpolar flagellum. They were 0·7 to 2·5 μm. long and 0·2 to 0·4 μm. wide (typically 0·8 to 2·0 μm. long and 0·3 to 0·4 μm. wide), occurring singly, in pairs or in short chains. The diameter of clearings in cellulose agar films after incubation for 2 to 4 weeks was

Table 1. *End-products of cellulose fermentation by isolates of cellulolytic cocci*

		Compounds produced (+) or utilized (−)										
Group	Isolate no.	Formate	Acetate	Propionate	Isobutyrate	Butyrate	Isovalerate	Valerate	Lactate	Succinate	Ethanol	Hydrogen
1	27	+1·14	+1·99	−0·09	0	−0·03	0	−0·01	+0·11	+0·10	+3·66	+11·9
	28	+1·05	+2·23	−0·07	0	0	0	0	+0·09	+0·09	+3·32	+10·9
	29	+1·31	+2·68	−0·06	0	0	0	0	+0·09	+0·05	+3·63	+9·8
	30	+1·22	+2·65	−0·05	0	0	0	0	+0·10	0	+3·12	+13·6
	31	+1·22	+2·36	−0·10	−0·01	−0·01	0	0	+0·10	0	+3·54	+11·4
	32	+0·99	+2·50	−0·08	−0·01	−0·01	0	0	+0·11	+0·05	+3·65	+12·1
	33	+0·73	+2·84	−0·03	0	0	+0·01	0	+0·06	+0·05	+3·38	+10·7
	35	+1·71	+0·37	−0·04	−0·01	0	0	0	+0·16	0	+1·84	+3·3
	36	+0·99	+2·48	+0·01	0	0	+0·01	0	+0·05	+0·06	+3·37	+11·6
2	38	+1·41	+0·53	−0·12	0	−0·07	−0·04	−0·02	+0·10	+1·44	0	+2·0
	39	+0·86	+0·65	0	−0·03	0	−0·04	0	+1·09	+1·35	0	+1·7
	40	+0·37	+0·01	+0·01	−0·05	0	−0·05	0	+0·08	+1·97	0	+5·1
	41	+0·62	+1·08	+0·12	−0·02	0	−0·04	0	+0·05	+1·51	0	+2·2
	43	+0·21	+0·73	0	−0·03	0	−0·03	0	+1·98	+1·33	0	+2·2
	44	+0·70	+1·21	+0·01	−0·05	−0·02	−0·04	0	+1·25	+1·52	0	+1·6
	45	+0·73	+1·24	+0·01	−0·05	−0·02	−0·05	0	+0·23	+1·73	0	+1·9
	46	+0·57	+0·90	−0·03	−0·05	−0·06	−0·05	0	+0·55	+1·49	0	+1·7
	47	+0·40	+1·12	+0·10	−0·03	0	−0·03	0	+0·36	+1·85	0	+1·6
	51	+0·57	+0·58	−0·10	−0·04	−0·07	−0·05	−0·02	+0·26	+1·48	0	+1·7
3	25	0	−0·13	−0·07	−0·02	0	−0·03	−0·02	+2·81	+0·10	+0·47	+11·9
	37	+0·07	0	−0·07	−0·05	−0·04	−0·05	−0·01	+4·78	+0·05	+0·30	+1·3
	49	+0·14	+0·74	+0·06	−0·01	0	−0·02	0	+4·44	+0·03	0	+1·3
4	34	+1·72	+0·34	−0·14	−0·02	−0·02	0	0	+3·17	0	+1·59	+1·9
	52	+1·35	+0·16	−0·16	−0·01	−0·05	−0·01	−0·01	+2·88	+0·11	+1·33	+1·6

Values expressed as mmole/100 ml. medium except in the case of hydrogen concentrations which are given as percentage by volume of the gas phase in the culture bottles. Mean values for the uninoculated medium were as follows (mmole/100 ml.): formate, 0·29; acetate, 4·24; propionate, 1·03; isobutyrate, 0·05; butyrate, 0·51; isovalerate, 0·05; valerate, 0·10; lactate, 0·07; succinate, 0; ethanol, 0; hydrogen (v/v % of gas phase), 0·03.

Table 2. *Some variable characteristics of isolates of cellulolytic cocci*

Group	Isolate no.	Approximate size of clearings on cellulose agar films (mm.)	Pigment production	Occurrence in chains (9 observations)	Gelatin liquefaction	Acid from L-Arabinose	Acid from D-Xylose	Acid from D-Glucose	Acid from D-Mannose	Acid from D-Fructose	Acid from Lactose	Acid from Sucrose
1	27	1·0	−	−	+	+	+	±	+	+	+	+
	28	3·5	−	−*	+	+	+	+	+	+	+	+
	29	4·5	−	−	+	+	+	+	+	−	−	+
	30	3·0	−	−	−	+	+	−	+	+	+	−
	31	5·0	−	−	+	+	+	+	+	−	+	+
	32	4·0	−	−	+	+	+	+	+	−	+	+
	33	3·0	−	−	+	+	+	+	+	−	+	+
	35	1·0	−	−	+	−	−	−	−	−	+	+
	36	3·0	−	−	+	+	+	−	+	−	−	+
2	38	2·0	+	+	+	−	−	−	−	−	−	−
	39	1·0	−	+	−	+	−	+	−	−	−	+
	40	1·0	+	+	−	−	−	−	−	−	+	−
	41	2·0	+	+	−	−	−	−	−	−	−	−
	43	1·0	−	+	−	+	−	−	−	−	−	−
	44	1·5	+	+	−	+	−	+	−	−	−	−
	45	2·0	+	+	−	+	−	±	−	−	−	−
	46	1·0	+	+	−	+	−	+	−	−	−	−
	47	1·5	+	+	+	+	−	+	−	−	−	−
	51	2·0	+	+	−	+	−	±	−	−	−	−
3	25	2·0	−	+	−	−	−	−	−	−	−	−
	37	1·0	−	+	−	−	−	−	−	−	+	−
	49	2·0	−	+	−	−	−	−	−	−	+	−
4	34	2·0	−	+	+	+	+	+	−	−	+	+
	52	2·5	±	+	+	−	−	−	−	−	+	+

* Short chains noted in two cases. ± Variable.

usually less than 1 mm. but clearings were well defined. No pigment was found. All the isolates liquefied gelatin, grew in the absence of carbon dioxide (final pH 5·2 to 5·5) and in medium containing Trypticase and yeast extract instead of rumen fluid. All fermented the following sugars, the figures in parentheses indicating the mean final pH values; L-arabinose (5·3), D-xylose (5·3), D-glucose (5·2), D-galactose (5·5), D-mannose (5·7), D-fructose (5·2), maltose (5·2), lactose (5·3), sucrose (5·1), raffinose (5·2), dextrin (5·8), starch (5·2), esculin (5·9), salicin (5·5) and pectin (5·9). All gave weakly positive reactions in the case of inulin and xylan (final pH 6·2 or higher). In the case of glycerol isolate 22 gave negative tests in both runs while the others were weakly positive, often negative in one run and weakly positive to positive in the other (pH 6·5 to 5·7). None of the isolates except 15 (pH 5·5) fermented trehalose nor did any ferment L-rhamnose, mannitol, inositol or DL-sodium lactate.

Types and quantities of products formed (+) or utilized (−) during growth in medium containing filter-paper cellulose were as follows (mean and range in mmoles/100 ml. medium): formate, +0·44 (+0·25 to +1·09); acetate, −2·13 (−1·73 to −2·82); propionate, −0·17 (−0·13 to −0·27); isobutyrate, +0·01 (−0·01 to +0·03); butyrate, +3·70 (+2·23 to +5·28); isovalerate, +0·03 (+0·01 to +0·04); valerate, +0·01 (0 to +0·03); lactate, +1·14 (+0·85 to +1·63); hydrogen (percentage by volume of the gas phase in culture bottles), +6·9 (+5·9 to +8·0). None of the strains produced succinate or ethanol. The mean values for the uninoculated medium are the same as those given in Table 1. The quantities of the acid products of cellobiose fermentation in a medium containing 1·2 % (w/v) of this substrate were similar to those found when cellulose was used as substrate except that lactate production was almost three times as high.

DISCUSSION

All the coccal isolates were strict anaerobes, fermented cellulose and cellobiose, occurred singly, in pairs or in chains, were Gram-negative to Gram-variable and non-motile. No endospores were observed. According to these characteristics they belong to the genus *Ruminococcus* (Sijpesteijn, 1951), the definition of which was amended by Hungate (1957) and further amended by Bryant *et al.* (1958). The group 1 isolates of the current study clearly belong to the species *R. albus* (Hungate, 1957) in that they produced no pigment, did not occur in chains and produced hydrogen, ethanol, formate, acetate and lactate. Some of the isolates produced a little succinate but Bryant *et al.* (1958) and Jarvis & Annison (1967) also classified such isolates, which otherwise conformed to the characteristics of *R. albus*, as belonging to this species. The present isolates differed from previously described strains of *R. albus* principally in that most of them fermented L-arabinose, D-xylose, D-glucose, D-mannose, lactose and sucrose. Such organisms have been found infrequently by other workers. The group 2 isolates can be regarded as belonging to the species *R. flavefaciens* (Sijpesteijn, 1951) as redefined by Hungate (1957) and Bryant *et al.* (1958), on the basis of chain formation, production of succinate and lack of production of ethanol.

Five of the isolates could not, on the basis of fermentation products, be assigned to either of the two known species of the genus *Ruminococcus*. The three group 3 isolates produced mainly lactate. Bryant *et al.* (1958) also found a strain that produced mainly lactate, and said that this strain, B_133, could not be placed in either species. Jarvis & Annison (1967) found two strains, LY8 and LY13, that produced mainly lactate and

both were classified as 'intermediates similar to *Ruminococcus flavefaciens*'. On the basis of chain formation all six strains could be classified as *R. flavefaciens*, especially in the case of LY8 and LY13, which produced yellow pigment. The strains have been isolated from both sheep (present work and that of Jarvis & Annison, 1967) and cattle (Bryant *et al.* 1958) in three widely separated parts of the world, Australia, United States of America and South Africa. On these grounds it is proposed that the group 3 isolates as well as strains $B_1 33$, LY8 and LY13 be named as a new variety of *R. flavefaciens*, *R. flavefaciens* var. *lacticus*. This variety differs from *R. flavefaciens* in that the principal end-product of cellulose or cellobiose fermentation is lactic acid. Small quantities of formate, succinate and ethanol may or not be produced and acetate may be formed in varying amounts.

The group 4 isolates also produced unusually large quantities of lactate. They formed considerable quantities of formate and ethanol and this together with low succinate excluded them from group 2. High production of lactate, low production of hydrogen and chain formation excluded them from group 1. Up to the present no strains with similar properties have been reported and an attempt to name these strains is considered premature.

The cellulolytic curved rods can be placed in the species *Butyrivibrio fibrisolvens* (Bryant & Small, 1956). However, they showed a greater resemblance to the 'less actively cellulolytic rumen rod' described by Hungate (1950) and to the group 1 curved rods of Shane *et al.* (1969) than to the type strain, especially in the case of cell dimensions. The dimensions of the type strain were given as 0·4 to 0·6 μm. wide and 2 to 5 μm. long. Other characteristics of our strains were rather similar to those of the type strain. It is noteworthy that Butyrivibrio strains similar to the group 2 strains of Shane *et al.* (1969) were never found. In the current study only isolates that had retained ability to degrade cellulose after repeated storing and culturing on cellobiose-containing agar slopes were used. In this way strains, similar to those of group 2 of Shane *et al.* (1969), that lost the ability to degrade cellulose could have been excluded.

The Butyrivibrio isolates described here all produced small amounts of isobutyrate and isovalerate. In the rumen the production of these acids is important for the growth of ruminococci (Bryant & Robinson, 1961) and isobutyrate and isovalerate were utilized by the majority of our Ruminococcus isolates. Whether these acids produced by our Butyrivibrio strains were synthesized from cellulose, or whether they were formed from proteinaceous material (in the rumen fluid of the medium) after hydrolysis and subsequent decarboxylation and deamination of the branched-chain amino acids (El-Shazly, 1952), remains to be determined.

The tests employed by van Gylswyk (1970) for the presumptive identification of a large number of cellulolytic isolates were found to be fairly reliable. Representative strains of the group described as 'Butyrivibrio-like rods' were subsequently all found to belong to the species *Butyrivibrio fibrisolvens* and those described as 'coccoid rods' were identified as *Cillobacterium cellulosolvens* (van Gylswyk & Hoffman, 1970). The ruminococci were easily recognized, but perfect agreement was not obtained in separating *Ruminococcus albus* from *R. flavefaciens*. The results could nevertheless be regarded as satisfactory. Of the 24 isolates the presumptive and final identifications agreed in 17 cases. Isolates 34 and 52, now placed in group 4, were presumptively identified as *R. albus* and atypical *R. flavefaciens* respectively. Isolates 25, 37 and 49, now assigned to the variety '*lacticus*', were presumptively identified as *R. albus*,

atypical *R. flavefaciens* and *R. flavefaciens*. Isolates 46 and 47, presumptively identified as atypical *R. albus*, were shown by the more detailed testing to be *R. flavefaciens*.

Presumptive tests that proved valuable in differentiating between the different species included examination for cell morphology and arrangement, motility and the Gram-reaction. In the case of the ruminococci the quantitative test for ethanol and semi-quantititative test for succinate production from cellulose gave fairly reliable information on whether an isolate could be classed as *Ruminococcus albus* or as *R. flavefaciens*, and the production of yellow pigment provided additional information in confirming the identity of the latter species. Testing for ability to ferment glucose, xylose or xylan was found to be of little value. Further knowledge of the identity of ruminococci may be obtained by determining lactate in addition to succinate and ethanol. This would facilitate detection of *R. flavefaciens* var. *lacticus* and group 4-like isolates. A tendency to form chains is in itself a fairly good criterion for separating *R. albus* from *R. flavefaciens* as shown by the current work and that of Bryant *et al.* (1958) and Jarvis & Annison (1967).

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Variant Forms of Saprophytic Mycelium Grown from Uredospores of *Puccinia graminis* f. sp. *tritici*

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SUMMARY

Saprophytic cultures obtained from uredospores of *Puccinia graminis* f. sp. *tritici* race 126-ANZ-6,7 varied. Cultures were placed in two general categories: those which formed macroscopic colonies within 2 weeks of inoculation, and those which formed colonies 4 or more weeks after inoculation. The fast-appearing colonies contained binucleate cells and were the more common; growth ceased by staling or formation of spore-bearing stromata. Slow-appearing colonies were formed at an erratic and low incidence; they were uninucleate, showed much less tendency to stale or form stromata than did fast-appearing colonies, and could be propagated by serial subcultures. Variation of uninucleate colonies during maintenance in axenic culture is described.

INTRODUCTION

An understanding of the nutrition, metabolism, and physiology of plant rusts has been restricted by the types of mycelium previously available. Vegetative mycelium has been isolated from flax cotyledons infected with *Melampsora lini* (Turel & Ledingham, 1957) and from bean leaves infected with *Uromyces phaseoli* (Dekhuijzen, Singh & Staples, 1967; Dekhuijzen & Staples, 1968). Since such mycelium is dependent on host tissue for continued growth, this approach cannot provide the experimental scope possible with free-living forms of the fungus, particularly in the study of nutrition. Another approach is to use mycelium grown on an artificial medium. Hotson & Cutter (1951) and Cutter (1951, 1952, 1959, 1960*a*, *b*) were successful in obtaining axenic cultures of *Gymnosporangium juniperi-virginianae*, *U. ari-triphylli*, and *Puccinia malvacearum* from infected plant callus tissue. Other investigators have not been able to repeat this work or obtain axenic cultures of a rust from other two-membered callus systems (reviewed by Scott & Maclean, 1969). However, Cutter's isolates could be maintained by serial subculture, and results are available on studies of the nutrition (Cutter, 1951; and unpublished experiments cited by Scott & Maclean, 1969) and metabolism (Wolf, 1956) of one axenic isolate of *G. juniperi-virginianae*.

More recently, a number of rusts have been cultured from uredospores on artificial media: *Puccinia graminis tritici* (Williams, Scott & Kuhl, 1966; Williams, Scott, Kuhl & Maclean, 1967), *P. recondita* (Singleton & Young, 1968), *P. graminis avenae* (J. L. Kuhl, D. J. Maclean, K. J. Scott & P. G. Williams, unpublished observations, *Melampsora lini* (Turel, 1969).

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Work in this laboratory to devise methods for the routine production of vegetative mycelium of *Puccinia graminis tritici* race 126-ANZ-6,7 has been hampered by the inconsistent growth pattern of cultures. The formation of saprophytic mycelium from uredospores is unpredictable (J. L. Kuhl, D. J. Maclean, K. J. Scott & P. G. Williams, unpublished observations), and even when such mycelium is formed its subsequent development is variable. Variant forms of saprophytic mycelium have been described in previous work. Williams *et al.* (1967) found that mycelium formed a dense stroma which bore uredospores and teliospores in a manner similar to uredial infections of wheat plants. In repeating this work, Bushnell (1968) obtained a dense stroma with an outer sporophore-like layer, but very few spores formed. Coffey, Bose & Shaw (1969) obtained mycelium which was less dense than the stroma obtained by the other workers, and clusters of non-pigmented spores were found within it.

In the present communication we distinguish the isolation and morphology of two kinds of mycelium, the growth characteristics of which are described.

METHODS

Media. The media used were modified from that of Williams *et al.* (1967). Medium I: 0·1 % yeast extract (Difco-Bacto, Detroit, U.S.A.), 0·2 % peptone (Evans Medical, Liverpool), 2 % (w/v) glucose, Czapek's mineral solution (Ainsworth & Bisby, 1945) to 100 %. Medium II: medium I plus 0·3 % sodium citrate ($Na_3C_6H_5O_7 . 5\frac{1}{2}H_2O$). Medium III: medium I with yeast extract and peptone each at 0·15 %. Medium IV: medium II but with glucose at 4 % (w/v) and peptone at 1·0 %. The pH was adjusted to 6·4. When solid media were required, 1·5 % agar (Difco-Bacto) was added before autoclaving. All media were sterilized by autoclaving for 10 min. at 121°.

Unless otherwise stated, plastic Petri dishes (8·5 cm. diameter) contained 30 ml. of solidified medium. Slants comprised 8 ml. of medium in 150 mm. × 17 mm. tubes plugged with cotton wool. Erlenmeyer flasks (150 ml.) containing 25 to 35 ml. of medium were used in liquid culture experiments.

Preparation and inoculation of uredospores. Uredospores of *Puccinia graminis* (Pers.) f. sp. *tritici* (Eriks. and E. Henn.) race 126-ANZ-6,7 were obtained by methods of aseptic leaf culture previously described (Williams, *et al.* 1966). The leaves were dried under reduced pressure and uredospores removed by vibration. Each liquid culture was inoculated with approximately 10^6 uredospores (from 2 to 3 leaf cultures) and the spores were dispersed by gentle agitation. Agar surfaces were inoculated with uredospores with the aid of a camel-hair brush.

Subculture. Pieces of mycelium were transferred to new medium in slants at 1 to 2 month intervals. Alternatively, uniform pieces of mycelium from the margins of colonies were transferred to Cellophane on agar in plastic Petri dishes. Pl. 1, fig. 2 shows such a culture 15 days after subculture. Thin commercially available Cellophane was boiled in distilled water for 10 min. and rinsed; this process was repeated three times. The Cellophane was immersed in distilled water and sterilized by autoclaving for 10 min. at 121°.

Dry weight was used as a parameter of growth, samples being dried for 15 h. at 90°. Results are expressed as mean dry wt/colony.

Conditions of growth. Cultures were incubated in the dark at high humidity. Uredospore-inoculated media were maintained at 17°. Initially subcultures were maintained at 17°, but later studies of growth were carried out at 23°.

RESULTS

Saprophytic mycelium derived from uredospores either staled, entered a reproductive phase, or formed a persistently vegetative mycelium. *Staled cultures* had a dull brown necrotic appearance and excreted brown pigments into the medium. This contrasted with *reproductive cultures* where the fungal thallus formed a dense brown–black stroma which usually bore uredospores or teliospores or both. *Persistently vegetative cultures* could be propagated by serial subculture, and had little tendency to stale or form stromata.

Development of staled or reproductive cultures on agar media

The formation of staled or reproductive cultures from a uredospore mass-inoculum seemed to follow a common pattern of early development. Sporelings formed macroscopic colonies within 2 weeks of inoculation, and growth centres expanded to form a combined mycelium within 3 weeks of inoculation. At this stage, cultures ranged from a spongy prosenchyma with profuse aerial hyphae to a dense interlocked mycelium; the former eventually staled (Pl. 1, fig. 5; Pl. 2, fig. 8), while the latter usually formed stromata (Pl. 1, fig. 4; Pl. 2, fig. 9 to 11). Uredospores or teliospores or both were formed in stromata in localized regions either on the outer surface (Pl. 2, fig. 11; cf. Williams, *et al.* 1967) or in clusters within the stromata (Pl. 2, fig. 9, 10). These spores showed the same pigmentation as found in uredial infections of wheat plants. The close arrangement of hyphae in the centre of a typical stroma is shown in Pl. 2, fig. 14; large thin-walled vesicles were often present. Sometimes spores formed in localized regions of a predominantly staled mycelium (e.g. Pl. 2, fig. 8).

Table 1. *Morphology of cultures derived from uredospores on agar medium II*

A sterile filter-paper template with holes was placed on the agar surface and uredospores spread over the exposed agar at a density of 500 to 1000 spores/mm². Each treatment was duplicated. The experiment was repeated with a different batch of uredospores. Results were consistent between replicates.

Volume of agar in Petri dish	Diameter of uredospore mass-inoculum (mm.) — Morphology after 6 weeks incubation at 17°				
	1	3·5	5	8	13
7 ml.	−	−	−	−	−
12 ml.	±	±	±	±	−
20 ml.	+	+	+	±	−

− = Loose, spongy, and mostly staled tissue; + = dense stroma with spore formation; ± = texture of mycelium intermediate between + and −.

As shown in Table 1 and in Pl. 1, fig. 1 stroma formation was favoured by a greater volume of agar and by confining the uredospores inoculum to a small area. This indicates that mycelial development was modified by a fungus–medium interaction in which nutrient depletion or accumulation of fungal excretory products or both would be important factors. However, small colonies staled on rare occasions, even when grown on a relatively deep layer of agar.

Both staling and reproductive colonies stopped growing within 4 to 6 weeks of inoculation. Subcultures ceased growth after one or two transfers.

Previous work has shown that rust mycelium which develops in a way similar to that described above contains binucleate cells at early (Williams *et al.* 1966) and later (Coffey *et al.* 1969) stages of growth.

Occasionally, colonies which appeared to be persistently vegetative formed on agar medium when the uredospore inoculum produced uneven and erratic growth. No subcultures were made from these colonies. This type of colony was more closely studied during a series of liquid culture experiments.

Growth from uredospores on liquid medium

All the types of growth mentioned above have also been observed in liquid cultures. In a typical experiment, uredospores were floated on liquid medium (Table 2), and after 17 days incubation 7 of the original 15 flasks remained uncontaminated. Only three of these flasks produced significant amounts of saprophytic growth (Table 2). An attempt to subculture the mycelium from one flask by fragmentation was unsuccessful. During incubation of the remaining flasks for a further six days, the colonies staled or formed stromata.

Table 2. *Growth of rust mycelium from uredospores on liquid medium*

Each flask (150 ml.) contained 36 ml. of medium III and was inoculated with approximately 10^6 uredospores.

	Number of flasks exhibiting condition specified in left column	
Condition of culture	After 17 days	After 23 days
Contaminated	8	8
Germination only	4	4
Mycelium over part of liquid surface	1	1 (stroma)†
Mycelium over entire liquid surface	2*	1 (staled)†

* Mycelium in one flask was harvested.
† The combined fresh weight of mycelium from these two flasks was 1·2 g.

Extended incubation of liquid cultures occasionally led to the appearance of persistently vegetative colonies, particularly when uredospores had germinated but produced little or no saprophytic growth within the first 3 weeks. Such colonies were first isolated in 1967 as a result of two experiments designed to produce large quantities of mycelium. At the end of 3 weeks incubation growth was erratic in all flasks. Re-examined 2 to 3 months after inoculation, a total of 13 small white mycelial tufts 1 to 2 mm. in diameter were visible in three flasks on the liquid surface amidst the necrotic remains of sporelings and primary mycelium. These tufts were transferred to agar slants where they grew to a diameter of 3 to 8 mm. 68 days after transfer. None had staled or formed stromata. One was propagated by serial subculture and has now been maintained for 3 years; this isolate is termed V1 (i.e. vegetative isolate 1).

The occurrence of persistently vegetative colonies was unexpected and we have experienced difficulty in re-observing this phenomenon. However, an experiment in June 1968 resulted again in the formation of persistently vegetative colonies (Table 3). As shown in this table, the formation of these colonies was favoured in flasks where least growth took place during the first 3 weeks of incubation. Pl. 1, fig. 6 shows flask

no. 8 (Table 3) 60 days after inoculation; the discrete white tufts could be clearly distinguished from the necrotic remains of sporelings. Incubation for a further 20 days resulted in the appearance of more tufts, and some flasks then contained up to 30 colonies.

The persistently vegetative colonies described in Table 3 were similar to those described in the previous experiment as they could be propagated by serial subculture and showed no tendency to stale or form stromata. Three isolates from this experiment are still being maintained and are designated V2, V3 and V4.

Table 3. *Incidence of persistently vegetative rust isolates from uredospores on liquid medium*

Each flask (containing 25 ml. liquid medium II) was inoculated with approximately 10^6 uredospores and incubated for 59 days. The degree of staling was estimated visually by the intensity of brown pigments in the medium. This was roughly correlated with the amount of saprophytic mycelium formed within 2 to 3 weeks of inoculation. Flasks which had produced no visible saprophytic mycelium in the first three weeks gave a colour rate + to ++ on the staling scale.

Flask no.	Degree of staling	No. of macroscopically visible mycelial tufts after 59 days
1	+++++	0
2	++++	0
3	++++	0
4	+++	0
5	+++	2
6	+++	23
7	++	6
8	++	14
9	++	21
10	++	3
11	++	23
12	++	13
13	+	2
14	+	4
15	+	20
16	+	10
17	+	13

Growth characteristics of persistently vegetative colonies

Subcultures form compact white colonies when grown in the dark (Pl. 1, fig. 2). Colonies become orange-yellow on exposure to the light, the pigment being localized in lipid globules in the hyphae (Pl. 2, fig. 13). All isolates are now maintained by placing pieces of mycelium on a Cellophane layer over an agar medium. Hydrolysis of Cellophane by the fungus has never been observed.

Attempts were made to grow subcultures of V1, 15 months after its isolation, in submerged liquid culture (medium I, medium II). These cultures were inoculated with surface-grown colonies, either as large pieces or as a suspension of fragments. This resulted in a few viable cultures, growth being diffuse and extremely slow. After optimal conditions for surface growth had been determined (25°, 4% w/v glucose, 1% peptone—cf. medium IV; D. J. Maclean & K. J. Scott, unpublished observations),

and provided that autoclaving time was restricted to a maximum of 10 min. at 121° (N. K. Howes & K. J. Scott, unpublished observations), submerged growth was greatly improved.

Nuclear content

An examination of V1 2 years after its isolation, and V2, V3 and V4 1½ years after their isolation, showed that constituent cells were uninucleate (Pl. 1, fig. 3). The nuclei appeared to be in the expanded condition described by Savile (1939). The development of persistently vegetative colonies when first isolated differed markedly from that of mycelium which staled or formed stromata. This suggested that the persistently vegetative colonies were uninucleate even when first isolated, but no study was made of the nuclear condition of these isolates at that time.

Sectoring

Sectoring was not often observed. However, when V1 was serially subcultured for a few generations on a medium of high peptone concentration (medium IV) for the first time about 2 years after its isolation, some colonies grew faster than others. Slow-growing (V1A) and fast-growing (V1B) colonies were selectively subcultured for 6 months. Then, after 43 days growth on medium IV, colonies of V1A had a mean dry weight one-third that of V1B (11 mg. as compared with 32 mg.). On medium II, which had a lower peptone concentration, the difference in growth rates was less but still significant.

For more than a year after isolation subcultures of V2 formed small heaped colonies, the mycelium being relatively dense and fine in texture (V2A). Later subcultures of this isolate occasionally formed faster-growing colonies (V2B) which were more diffuse and showed no tendency to revert to the slow-growing type. Sectors of V2B were observed to arise from the edge of colonies of V2A in contrast to the slow separation of sectors in V1.

Isolates V3 and V4 were distinguished from the other isolates. Colonies of V3 grown on medium II became water-logged more easily. Isolate V4 showed some resemblance to V1A and V1B in general appearance, but larger pieces of mycelium were required for its successful subculture. We have not yet observed sectors in cultures of V3 and V4.

The age and size of the mycelium piece used in subculturing was important. Subcultures from aged colonies often failed to grow, whereas pieces of young colonies (2 to 4 weeks after subculture) usually did. A dry weight of 0·15 mg. or more was required for the successful subculture of V2, V3 and V4. However, growth has been obtained with V1B from an inoculum of as little as 0·02 to 0·06 mg. dry weight.

Sporulation of uninucleate isolates

On rare occasions colonies of V1 growing on Cellophane on an agar medium bore uredospores and teliospores dispersed throughout the thallus (Pl. 2, fig. 12). Neither this nor any other uninucleate isolate formed a dense stroma. Although the uredospores were similar to those produced under natural conditions, some teliospores were abnormal in shape (Pl. 2, fig. 12). The nuclear condition of these spores was not studied. Uninucleate mycelium can interact with wheat leaves to form uredospores and teliospores which are uninucleate (D. J. Maclean & K. J. Scott, unpublished observations).

DISCUSSION

The variant forms of saprophytic mycelium that we have observed fall into two general categories based on differences in morphogenesis and nuclear content. Both categories were obtained from wild-type uredospores of *Puccinia graminis*. These spores are characteristically binucleate; however, uninucleate uredospores have been observed in predominantly binucleate uredosori of rust fungi (Little & Manners, 1969). Mycelia in the first category formed macroscopic colonies within 2 weeks of inoculation which contained binucleate cells and terminated growth either by staling or formation of stromata. We were not able to obtain viable subcultures. Mycelia in the second category (persistently vegetative) did not form macroscopic colonies until 4 or more weeks after inoculation. These colonies did not tend to stale or form reproductive tissues, and serial subcultures were made. Although their nuclear condition was not determined immediately that they were isolated, subsequent subcultures were found to be uninucleate.

Cutter (1959, 1960*a*; and unpublished observations cited by Scott & Maclean, 1969) examined the nuclear condition of axenic isolates of *Gymnosporangium juniperi-virginianae* and *Uromyces ari-triphylli*. Telial galls, the material from which the cultures of *G. juniperi-virginianae* were obtained, characteristically contained a binucleate mycelium. Four of seven axenic strains were predominantly binucleate within 30 days of isolation, the others being completely uninucleate. After serial subculture, two of the previously binucleate strains became uninucleate. However, all five strains of *U. ari-triphylli* contained binucleate cells and were remarkably stable in culture. Our results show that *Puccinia graminis* resembles *G. juniperi-virginianae* in tending to lose the binucleate condition.

Bushnell (1968) and Coffey *et al.* (1969) obtained axenic cultures of the same race of rust used in the present study. The 60 day cultures of Coffey *et al.* were seen to contain binucleate cells and resembled the staled mycelium of our first category. The cultures described by Bushnell seem to be intermediate between our two categories both in morphology and in time taken for the mycelium to develop from sporelings. Subcultures of Bushnell's mycelium were viable, and on ageing they formed stromata. We have not observed stroma formation during ageing of our uninucleate cultures. Bushnell's results are difficult to interpret in view of our inability to subculture successfully mycelium of our first category.

It is informative to examine the conditions under which uninucleate mycelium was formed. This mycelium was derived from 'persistently vegetative' colonies. These colonies were formed when sporelings produced little or no primary mycelium within 3 weeks of inoculation. Under these conditions variant cells would have been relatively free of competition. A maximum of 30 small discrete colonies were isolated from any individual inoculum of about 10^6 uredospores.

The mechanisms whereby uredospores gave rise to the various forms of saprophytic mycelium, particularly the uninucleate cultures, are obscure. Uninucleate hyphae could have arisen in ways such as: (1) formation of a diploid; (2) septation of a dikaryon followed by growth of one of the cells; (3) presence of uninucleate uredospores in the inoculum; (4) recombination followed by haploidization and septation. Preliminary results of an investigation by the authors with I. Tommerup indicate that nuclei in our uninucleate strains are haploid.

Variation has also been observed during the maintenance of uninucleate isolates by subculture. Two isolates (v 1, v 2) each produced faster-growing strains. If v 1 and v 2 contained a mixture of 'fast' and 'slow' cells when first isolated, one would expect the fast-growing cells soon to predominate. Since the new strains were observed more than a year after isolation, we conclude that new cell types arose during maintenance. Both the old 'slow' and the new 'fast' strains were uninucleate. Possible causes of this variation include mutation, segregation of cytoplasmic factors and parasexual mechanisms.

Mechanisms of somatic variation which occur in rust fungi maintained by uredial infection have not yet been satisfactorily elucidated (e.g. Watson & Luig, 1962; Bartos, Fleischmann, Samborski & Shipton, 1969; Little & Manners, 1969). Haploid isolates in axenic culture may be of value in clarifying them.

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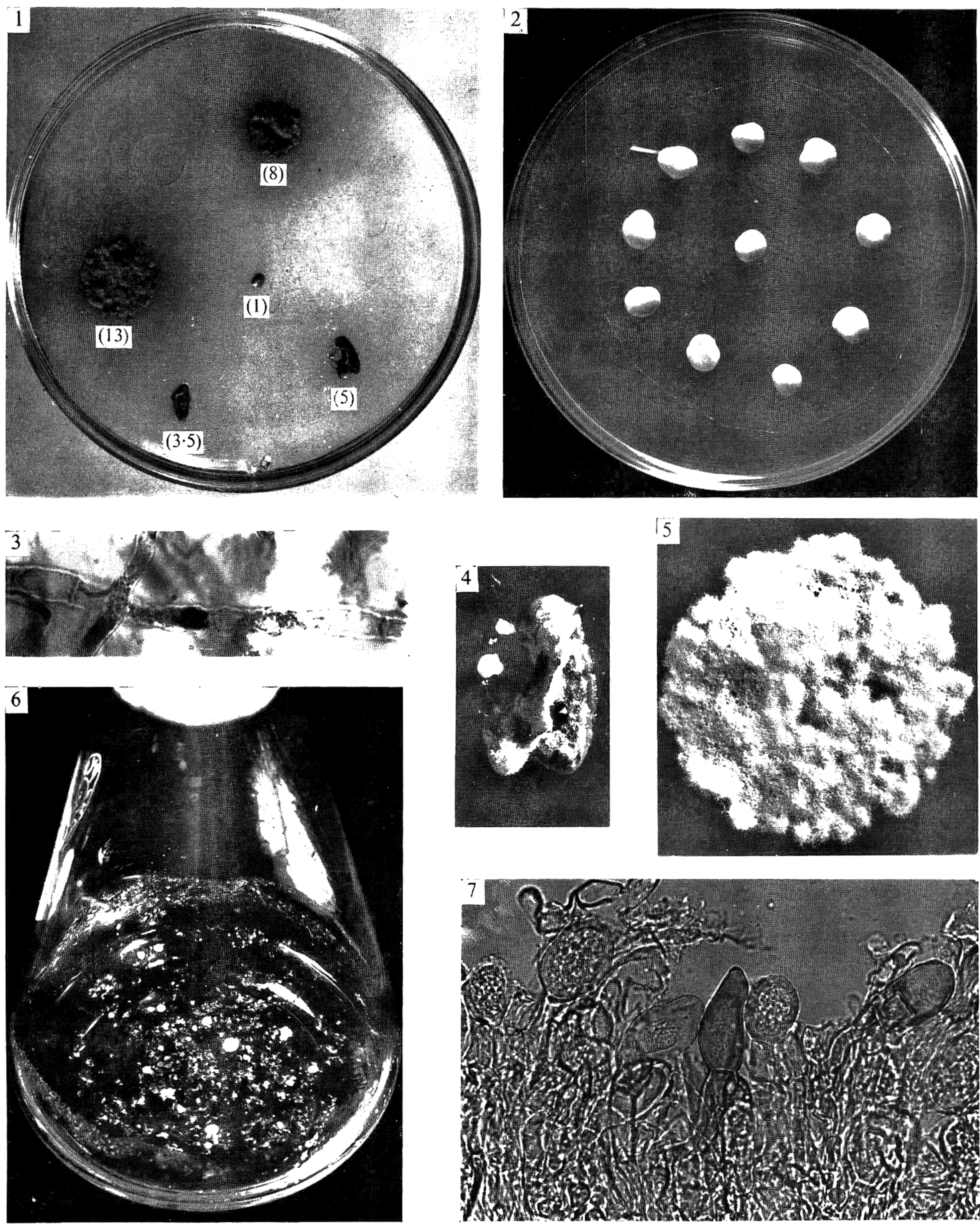

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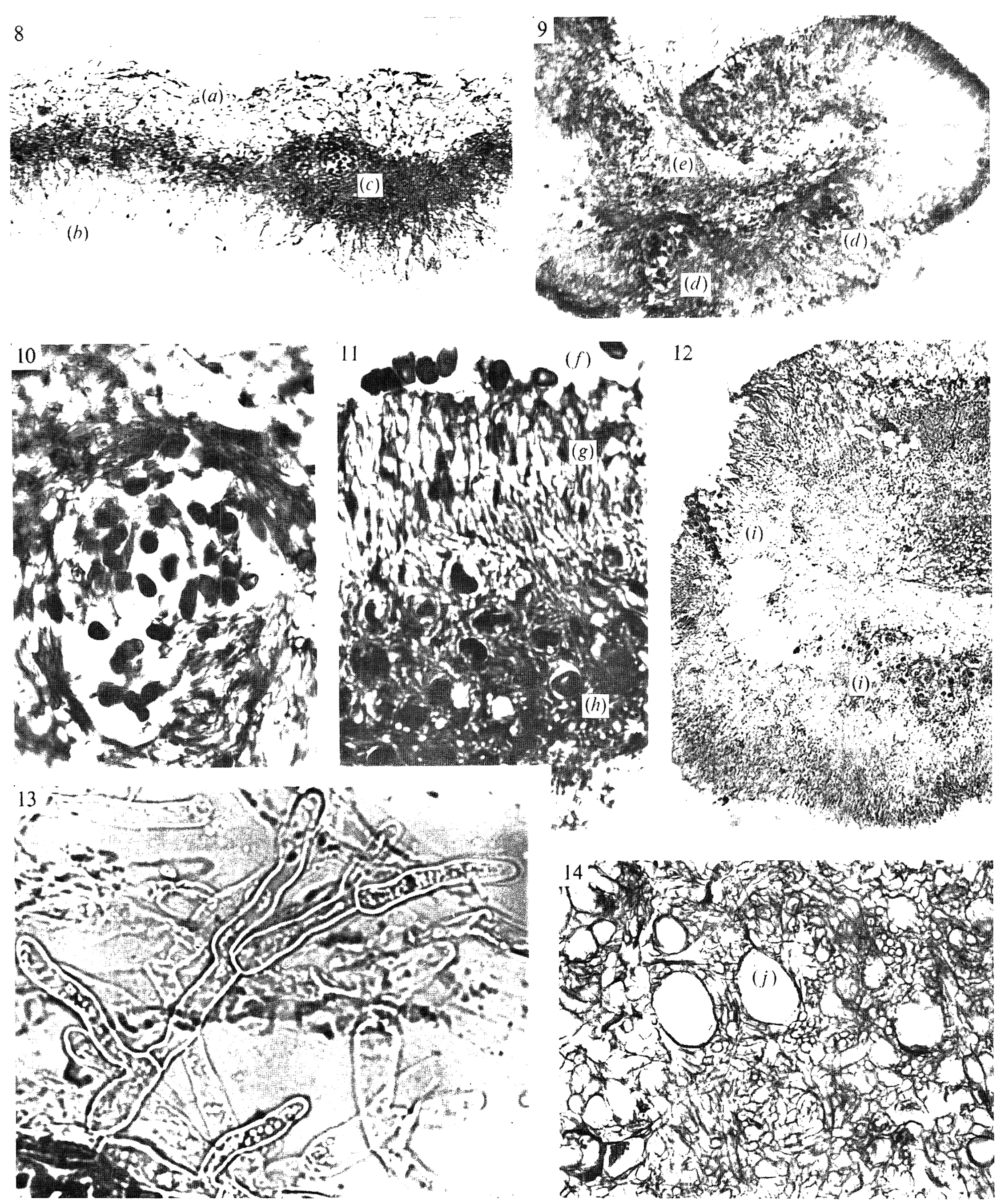

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. Petri dish (20 ml. medium, cf. Table 1) 44 days after inoculation with uredospores. Diameter (mm.) of inoculum is shown in parentheses. Some colonies have excreted brown substance(s) into the medium. × 0·85.

Fig. 2. Subcultures of isolate V1 growing on Cellophane disc on 30 ml. agar medium II. × 0·85.

Fig. 3. Uninucleate cell of isolate V1. Stained by Giemsa technique after 8 min. hydrolysis at 60° in *N*-HCl. × 750.

Fig. 4. Stroma formation (5 mm., fig. 1) showing fungus curling up from the agar. × 3·3.

Fig. 5. Staling mycelium (13 mm., fig. 1). × 3·3.

Fig. 6. Flask containing persistently vegetative colonies 60 days after inoculation with uredospores. The discrete white colonies can be distinguished from masses of necrotic sporelings. × 0·75.

Fig. 7. Uredospore and teliospore formation in a subculture of V1. × 400.

PLATE 2

Fig. 8. Staled culture: section through 13 mm. colony, fig. 1. Note aerial hyphae (*a*), loosely arranged hyphae in the agar (*b*). A cluster of small uredospores was formed (*c*), but such regions are not general in staled cultures. × 50.

Fig. 9. Stroma: section through 3·5 mm. colony, fig. 1. Uredospores and some teliospores were formed in clusters (*d*). This colony has curled up, enclosing aerial hyphae (*e*). × 50.

Fig. 10. One of the spore clusters in fig. 9. × 200.

Fig. 11. Uredospore formation (*f*) on outer surface of sporophore layer (*g*). Original inoculum at (*h*). × 200.

Fig. 12. Spores (*i*) formed in a subculture of isolate V1. Frozen section, unstained. × 33.

Fig. 13. Monopodial branching of V1 showing lipid globules in hyphae. Frozen section, unstained. × 730.

Fig. 14. Tightly interwoven hyphae in centre of a stroma. Hyphae are often swollen, sometimes forming large vesicles (*j*). × 200.

Figures 8, 9, 10, 11 and 14 are from 10 μm. wax sections, hydrated, and stained with aqueous crystal violet.

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The Tricarboxylic Acid Cycle in *Thiobacillus denitrificans* and *Thiobacillus-A2*

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SUMMARY

The enzymes involved in the tricarboxylic acid cycle and some other related reactions were measured quantitatively in cell-free extracts from the obligate autotroph *Thiobacillus denitrificans* grown anaerobically, and in the facultative autotroph *Thiobacillus-A2* grown under various conditions. The presence of an incomplete tricarboxylic acid cycle in *T. denitrificans* and in *Thiobacillus-A2* (when grown autotrophically on thiosulphate) is described. The cell-free extracts from these two organisms lacked α-ketoglutarate dehydrogenase and succinyl CoA synthetase. The data from *Thiobacillus-A2* grown on succinate, aerobically or anaerobically, and on glutamate aerobically suggest that both the enzymes are repressed during autotrophic growth. Results are further discussed in relation to possible reasons for autotrophy.

INTRODUCTION

It is generally assumed that the pathways of intermediary metabolism in autotrophic organisms are essentially the same as those in heterotrophic organisms with the exception of the unique ability of the autotrophs to use inorganic electron donors as the source of energy. However, the lack of one or more enzymes required for the metabolism of organic compounds has been proposed as a hypothesis to explain the biochemical basis of autotrophy.

In order to reach a conclusion about the relationship, if any, between enzyme deficiency and autotrophy, it was necessary to conduct detailed enzymic analyses. The present report presents a comprehensive study of the enzymes of the tricarboxylic acid cycle and the glyoxylate pathway (and two related enzymes, glutamate dehydrogenase and 3-ketoacyl CoA transferase) in *Thiobacillus denitrificans* and *Thiobacillus-A2*. Certain enzymes of the tricarboxylic acid cycle have previously been measured in *T. thioparus* by Cooper (1964), in *T. thioparus* and *T. thiooxidans* by Smith, London & Stanier (1967) and in *T. thioparus* and *T. neapolitanus* by Johnson & Abraham (1969*a*, *b*).

METHODS

Growth of bacteria and preparation of cell-free extracts. Thiobacillus denitrificans was grown on thiosulphate under anaerobic conditions with nitrate as the final electron acceptor in an inorganic medium described by Sargeant, Buck, Ford & Yeo (1966).

Thiobacillus-A2 was grown according to the method described by Taylor & Hoare (1969) using thiosulphate, succinate or glutamate as individual substrates for autotrophic or heterotrophic growth. With succinate as the carbon and energy source, the bacteria were grown aerobically as well as anaerobically and in the latter case nitrate served as the terminal oxidant.

The organisms were harvested during the early log phase using a DeLaval continuous flow centrifuge maintained at 6°. In the case of *Thiobacillus-A2* the bacterial paste was washed three times with 0·05 M-tris-HCl buffer pH 8·0. The wet packed organisms were suspended in the same buffer (30 %, w/v) containing 0·3 M-sucrose, 5 mM-$MgCl_2$, 0·5 mM-Na_4EDTA and 0·5 mM-glutathione (reduced form). Organisms were broken by sonic disruption for 3 min. in a Bronson Sonifier (Heat Systems Ultrasonics, Inc.) operated at full power; the temperature was maintained at 5°. The suspension was centrifuged first at 30,000 *g* for 30 min. and further at 144,000 *g* for 60 min. yielding the appropriate cell-free preparations. The cell-free extracts from *T. denitrificans* were prepared as described previously (Peeters & Aleem, 1970).

Enzyme assays. All determinations, unless otherwise indicated, were made with the 144,000 *g* supernatant and the various enzymes were measured using well-established methods. The optical procedure described by Ochoa (1955) was used for the assay of citrate synthetase (EC 4.1.3.7). Isocitrate dehydrogenase (EC 1.1.1.42), malate dehydrogenase (EC 1.1.1.37) and glutamate dehydrogenase (EC 1.4.1.4) were assayed according to the method described by Sottocasa, Kuylenstierna, Ernster & Bergstrand (1967); succinyl CoA synthetase (EC 6.2.1.5) was determined as described by Bridger, Ramaley & Boyer (1969). For succinate dehydrogenase (1.3.99.1) the spectrophotometric adaptation of the manometric method with phenazine methosulphate was used (Veeger, DerVartanian & Zeylemaker, 1969). The decline in fumarate concentration was followed at 300 nm. as described by Hill & Bradshaw (1969) for the assay of fumarase (EC 4.2.1.2). The α-ketoglutarate dehydrogenase was measured by the method of Kaufman (1955) and also by the method of Amarasingham & Davies (1965) using 3-acetylpyridine-NAD. Other enzymes assayed were: aconitase (EC 4.2.1.3), Afinsen (1955); malate synthetase (EC 4.1.3.2), Dixon & Kornberg (1965); isocitrate lyase (EC 4.1.3.1), Olson (1959) and 3-ketoacyl CoA transferase (EC 2.8.3.5), Pearce, Leach & Carr (1969). As the cell-free preparations from the two organisms showed a powerful NADH oxidase activity, those determinations which involved NADH oxidation or NAD^+ reduction were performed anaerobically or in the presence of 1 mM-potassium cyanide; the latter completely blocked the NADH oxidase activity.

Specific activities are reported as nanomoles substrate converted/min./mg. protein. The following millimolar extinction coefficients were used in the calculations: reduced NADP and NAD (340 nm.), 6·2; *cis*-aconitic acid (240 nm.), 4·88; thio-ester bond in succinyl CoA and acetyl CoA (230 nm.), 4·5; 2,6-dichlorophenol indophenol (600 nm.), 20·6; glyoxylic semicarbazone (252 nm.), 12·4. For fumarate the millimolar extinction coefficient at 240 nm. is 2·11. We have observed that the ratio between the extinction of fumarate at 240 and 200 nm. was 63; and, since fumarate measurements were made at 300 nm., we used a millimolar extinction coefficient of 0·0335 in our calculations. Finally, a value of 11·9 was used for the magnesium complex of acetoacetyl CoA (Stern, Coon, DelCampillo & Scheider, 1956). Protein was determined by the method of Gornall, Bardawill & David (1949).

Chemicals. All chemicals were obtained from commercial sources and were of the highest purity available. The ATP, CoA, acetyl CoA, acetoacetyl CoA, $NADP^+$, NADPH, NAD^+, NADH and 3-acetylpyridin-NAD were purchased from Sigma Chemical Co., St Louis, Missouri, U.S.A.

RESULTS

The cell-free extracts from *Thiobacillus denitrificans* contained most of the tricarboxylic acid cycle enzymes and their activities were comparable with those found by Smith *et al.* (1967) and Johnson & Abraham (1969*a*) in *T. thioparus* (Table 1).

Table 1. *Tricarboxylic acid cycle and some related enzymes in cell-free extracts from Thiobacillus denitrificans as compared to Thiobacillus thioparus*

Activities are expressed as nanomoles/min./mg. protein.

Enzyme	*T. denitrificans*	*T. thioparus*
Citrate synthase	30·1	40·7*
Aconitase (isocitrate as substrate)	23·1	12·0*
Isocitrate dehydrogenase (NADP-specific)	45·0	204·4* 74·0†
α-Ketoglutarate dehydrogenase	0·0	0·0*
Succinyl CoA synthetase	0·0	—
Succinate dehydrogenase	0·5	7·5†
Fumarase (fumarate as substrate)	215·0	79·6*
Malate dehydrogenase (oxaloacetate as substrate)	32·3	223·5* 28·0†
Isocitrate lyase	91·5	—
Malate synthetase	12·0	—
3-Ketoacyl CoA transferase	16·3	—
Glutamate dehydrogenase	2·6	—

* Smith *et al.* (1967). † Johnson & Abraham (1969*a*).

Assays for α-ketoglutarate dehydrogenase were repeated several times using both the methods described by Kaufman (1955) and Amarasingham & Davis (1965), but without success. In addition, we failed to demonstrate the presence of succinyl CoA synthetase in *Thiobacillus denitrificans*. The latter enzyme was also absent in the autotrophically grown blue-green algae *Anabaena variabilis* and *Anacystis nidulans* (Pearce *et al.* 1969). Succinate dehydrogenase activity was quite low, but was increased by a factor of 1·5 when pellet was added back to the cell-free extract. It should be noted that, while the apparently higher succinate dehydrogenase activity in *T. thioparus* was based on the rate of reduction of cytochrome *c*, we have expressed the specific activity of this enzyme on the basis of the rate of reduction of phenazine methosulphate. Among the other enzymes assayed, isocitrate lyase was present at a high level, while the activity of glutamate dehydrogenase was quite low.

The same enzymes were measured in extracts of *Thiobacillus-A2* grown under various conditions (Table 2). Cell-free extracts from the organism grown autotrophically showed some distinct but important similarities with the *T. denitrificans* enzyme pattern: citrate synthase, isocitrate dehydrogenase, fumarase and malate synthetase were present at about the same level of activity while α-ketoglutarate dehydrogenase and succinyl CoA synthetase could not be detected. Succinate dehydro-

genase and glutamate dehydrogenase, however, were present at about the same level in both autotrophically and heterotrophically grown organisms.

Among the aerobic and anaerobic heterotrophic growth conditions examined significant differences in the levels of various enzymes were observed in the case of organisms grown anaerobically on succinate. These organisms contained comparatively greater aconitase, succinate dehydrogenase, fumarase and malate synthetase activities. Under all of the heterotrophic growth conditions both the α-ketoglutarate dehydrogenase and the succinyl CoA synthetase were easily detected.

Table 2. *The tricarboxylic acid cycle and some related enzymes in cell-free extracts from Thiobacillus-A2*

Activities are expressed as nanomoles/min./mg. protein.

	Organism grown			
	Aerobically with			Anaerobically with
Enzyme	thiosulphate	glutamate	succinate	succinate
Citrate synthase	23·2	10·2	6·0	NT
Aconitase	35·4	NT	21·5	513·0
Isocitrate dehydrogenase	34·8	140·8	258·0	270·0
α-Ketoglutarate dehydrogenase	0·0	95·1	10·6	35·3
Succinyl CoA synthetase	0·0	NT	267·0	300·0
Succinate dehydrogenase	7·5	6·4	8·7	42·5
Fumarase	195·0	174·0	211·0	1065·0
Malate dehydrogenase	213·0	85·5	196·3	195·9
Isocitrate lyase	16·2	17·3	9·7	8·7
Malate synthetase	10·3	NT	13·2	144·9
3-Ketoacyl transferase	50·2	31·3	37·6	42·1
Glutamate dehydrogenase	22·0	21·6	20·0	30·0

NT = Not tested.

DISCUSSION

Smith *et al.* (1967) advanced the hypothesis that obligate autotrophs lack both NADH oxidase and α-ketoglutarate dehydrogenase activities and proposed that the absence of these enzymes could provide a biochemical explanation for the inability of strict autotrophs to use organic compounds as sources of energy.

However, NADH oxidase has been demonstrated in many obligate autotrophs. Its presence in substantially high levels has been reported in *Nitrobacter agilis* (Aleem, 1968; Smith & Hoare, 1968), in *Nitrosomonas europaea* (Hooper, 1969), in *Thiobacillus neapolitanus* (Hempfling & Vishniac, 1965; Aleem, 1969), in Thiobacillus strain C (Trudinger & Kelly, 1968), in *T. thioparus* and *T. thiooxidans* (Johnson & Abraham, 1969 *a*), and in *T. denitrificans* (Peeters & Aleem, 1970 *a*). Although the role of NADH oxidase in the obligate autotrophs has not yet been well established, the enzyme has been shown to catalyse the process of oxidative phosphorylation with remarkable efficiency in *Nitrobacter wynogradskii* (Kiesow, 1964) and in *N. agilis* (Aleem, 1968).

Based upon direct enzymic analyses, and the study of the incorporation of labelled acetate, the existence of an incomplete tricarboxylic acid cycle, lacking α-ketoglutarate dehydrogenase, has been fairly well established in several of the thiobacilli (Smith *et al.* 1967; Kelly, 1967; Johnson & Abraham, 1969 *b*), blue-green algae (Hoare & Moore,

1965; Smith *et al.* 1967; Pearce *et al.* 1969), and also in two photosynthetic bacteria (Callely, Rigopoulos & Fuller, 1968; Fuller, Smillie, Sisler & Kornberg, 1961).

Our results further substantiate the hypothesis that in most of the autotrophic organisms the tricarboxylic acid cycle is incomplete. In agreement with Taylor, Hoare & Hoare (1969) we were unable to detect the α-ketoglutarate dehydrogenase in *Thiobacillus denitrificans*. We also failed to demonstrate this enzyme in cell-free extracts from *Thiobacillus-A2* when grown autotrophically. Our results further indicate that the absence of α-ketoglutarate dehydrogenase cannot be considered as a causative feature of obligate autotrophy. Indeed, the fact that this enzyme is present in *Thiobacillus-A2* when grown heterotrophically suggests that it is repressed under autotrophic growth conditions. As already suggested by Smith *et al.* (1967), such a repression might be expected, because in autotrophic organisms the Krebs cycle appears to lose its energetic function and may thus fulfil only biosynthetic needs for which the presence of α-ketoglutarate dehydrogenase is not necessary. The same authors could not find such a repression in the facultative autotroph *Hydrogenomonas eutropha*, but it is interesting to note that Amarisingham & Davis (1965) reported a repression of α-ketoglutarate dehydrogenase in *Escherichia coli* when this organism was grown on carbon sources that do not require terminal respiration. Although the mechanism of repression under autotrophic growth conditions is not clearly understood at present, one might still expect to find a low α-ketoglutarate dehydrogenase activity even in obligate autotrophs. Smith & Hoare (1968) already reported low levels in *Nitrobacter agilis*, although Aleem (1970) failed to observe any activity of α-ketoglutarate dehydrogenase in pure cultures of this organism. Quite puzzling is the finding of Butler & Umbreit (1969), who reported that the α-ketoglutarate dehydrogenase activities in *T. thiooxidans* were twice the highest figures published for *E. coli*; while Smith *et al.* (1967), working with the same organism, could not detect any activity.

One might further expect that, if the Krebs cycle serves only the biosynthetic needs during chemosynthesis (or autotrophy), the levels of all the enzymes involved would decrease; this was indeed the case in *Thiobacillus denitrificans* and for most of the enzymes in *Thiobacillus-A2* grown autotrophically. Surprising is the absence of succinyl CoA synthetase in both organisms, under autotrophic growth conditions especially, in view of the important biosynthetic role of this enzyme. Pearce *et al.* (1969) did not find this enzyme in *Anacystis nidulans* and *Anabaena variabilis*, and suggested that its function was taken over by the enzyme 3-ketoacyl CoA transferase. Although the latter enzyme was present under both autotrophic and heterotrophic growth conditions, it is not clear why this should be so. It is apparent, therefore, that the control exerted by autotrophic conditions on the heterotrophic cellular metabolism warrants further investigations.

The glyoxylate pathway is present in these organisms, but no evidence can be deduced from our results that this pathway is more important in autotrophic organisms.

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Formulation of Cell Growth Inhibition by Chemicals and Environmental Agents

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SUMMARY

Equations are developed in which the inhibition of cell growth by different levels of a given chemical or environmental agent is expressed by a single straight line. The formulation provides a simple, economic, and consistent way of characterizing different growth inhibitors and evaluating their possible mechanisms of action, e.g. structure-activity relationships.

INTRODUCTION

The growth rate of bacteria and cells in general is decreased by a variety of chemicals and environmental agents (Clifton, 1957; Dean & Hinshelwood, 1966). As no satisfactory or consistent procedure exists for comparing the degree of growth inhibition by different agents, investigators have often presented numerous graphs in which they simply plotted some measure of cell growth versus time for each concentration or energy unit employed. Others, in an attempt to obtain a straight line relationship, plotted an arbitrarily chosen index of growth at a given time versus the logarithm of the concentration, or energy units. Monod (1949) employed a growth lag constant, L, defined as the difference in number of divisions between observed and ideal growth during the exponential phase.

The approaches cited above, and others which have been employed to evaluate growth inhibition (see Dean & Hinshelwood, 1966, for details) possess many disadvantages, e.g. (*a*) they are useful only for comparisons under a given set of experimental conditions; (*b*) the concentrations of inhibitors are expressed on a volume basis without regard to the number of cells initially present; (*c*) the choice of growth phase used as an index of base line is often arbitrary.

It seems best to evaluate true growth inhibition at a time as close to the initial conditions as is practically possible, a point stressed by Monod (1949). In this connexion it is important to recognize that the concentration per cell of a chemical agent is constantly changing as cell number increases. Therefore, criteria of growth inhibition which encompass the exponential phase become meaningless when one considers that exponential growth following inhibition may reflect additional factors such as destruction or modification of the primary action.

In investigations on the effects of irradiated media and chemical agents on the growth of *Salmonella typhimurium* LT2 (Schubert, Watson & White, 1967; Schubert & Watson, 1969; Schubert, Watson & Baecker, 1969; Watson & Schubert, 1969) it was necessary

to compare the inhibitory actions of different agents on bacterial growth. The formulation described here has been found to be a simple, economic and consistent formulation of expressing the degree of growth inhibition as a linear relationship over practically the entire range of concentration of inhibitor.

FORMULATION

Consider the bacterial growth curve (Fig. 1) in which the number of bacteria in $\log_2$ units is plotted against time. Curve o represents bacterial growth in absence of added inhibitors, and curve A represents bacterial growth when inhibitor A is present. One $\log_2$ unit corresponds to a doubling of cell number.

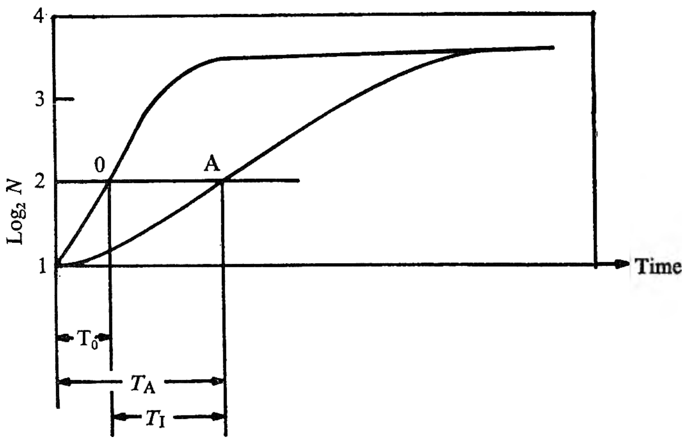

Fig. 1. Schematic growth curve for bacteria following inoculation in which N represents number of bacteria. Curve o is the growth curve when the concentration of inhibitor is zero; T_0 represents the doubling time, T_A is the doubling time in the presence of inhibitor A; and T_I is the net initial doubling time.

In the control samples the concentration of inhibitor is zero, and the doubling time is expressed by T_0, while T_A is the doubling time when a finite concentration of inhibitor A is present. The initial doubling time, T_I, is defined as follows:

$$T_I = T_A - T_0, \tag{1}$$

where

$$T_A > T_0.$$

It is seen, therefore, from equation (1) and from Fig. 1 that T_I represents the net increment of growth inhibition or delay produced by a given concentration of inhibitor. As the concentration of inhibitor A increases, the value of T_I increases. Expressed as a simple differential equation we may assume that

$$\frac{dT_I}{T_I} = \lambda dC, \tag{2}$$

where λ is a constant and C is inhibitor concentration in μmoles/cell.

Integration gives

$$\ln T_I = \lambda[C] + \ln T_{ext} \tag{3a}$$

or expressed in logs to the base ten

$$\log T_I = \frac{\lambda[C]}{2\cdot303} + \log T_{ext} \tag{3b}$$

where $\lambda/2\cdot303$ and $\log T_{ext}$ are the slope and intercept respectively of the straight line obtained when $\log T_I$ is plotted against $[C]$.

Expressing equation (3*b*) in terms of the doubling concentration $[C_2]$, i.e. the incremental concentration of $[C]$ which doubles T_I, we obtain

$$\log T_I = \frac{0\cdot30}{[C_2]}[C] + \log T_{ext}. \tag{3c}$$

Accordingly, a semilog plot of T_I *v.* $[C]$ should yield a straight line with slope proportional to $[C_2]$. Upon extrapolation to $[C] = 0$, the intercept on the T_I ordinate gives the value of T_{ext} (Fig. 2). To simplify, note that $T_I = 2T_{ext}$ when $[C] = [C_2]$.

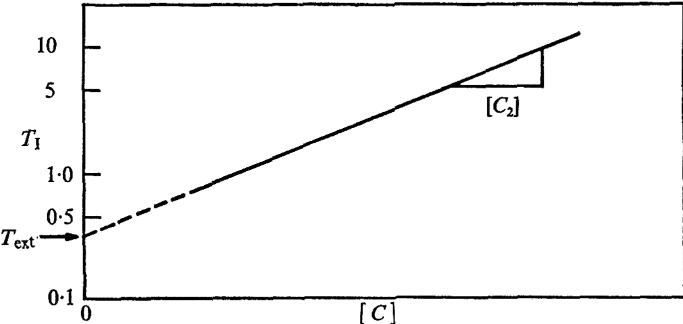

Fig. 2. Schematic diagram of a plot of $\log T_I$ *v.* initial concentration, $[C]$, following equation (3*c*).

Equation (3*c*) applies over practically the entire range of measured inhibition for finite positive values of $[C]$. However, since $T_I = 0$ when $[C] = 0$, deviation from the straight line must occur at very low values of $[C]$, and usually does in the region where T_I is too small to be measured reliably, e.g. where T_I differs by 5% or less from T_0. More precisely, deviation from the straight line will occur at concentrations approaching the threshold concentration $[C_t]$, the value of which can usually be estimated from the experimental data.

The relative inhibitory activity of different substances is conveniently expressed by a single term, namely the concentration of inhibitor for which $T_I \approx 10\,T_0$. Thus, in my system, I find that self-consistent results are obtained by the use of C_{10h}, i.e. the concentration at which $T_I = 10$ hours.

DISCUSSION

The formulation described here has provided a convenient means for evaluating the relative growth inhibition by varying concentrations of chemical agents. An added advantage of the formulation is its expression in terms immediately identified with the experimental system. Further, less time is needed to evaluate relative growth inhibition since it usually suffices to measure growth rates only for the time needed for the cells to undergo one doubling time following inoculation.

I have applied equation (3*c*) to data on the inhibitory action of different classes of chemical agents including carbonyl compounds on the growth of *Salmonella typhimurium* LT2 using methods described previously (Schubert *et al.* 1967). Some representative values of $[C_2]$ (in units of 10^{-8} μmoles/cell) and T_{ext} for carbonyl compounds are formaldehyde, $[C_2] = 0{\cdot}11$, $T_{ext} = 0{\cdot}053$; acetaldehyde, $[C_2] = 6{\cdot}4$, $T_{ext} = 0{\cdot}25$; glycolaldehyde, $[C_2] = 1{\cdot}6$, $T_{ext} = 0{\cdot}031$; and glyceraldehyde, $[C_2] = 4{\cdot}0$, $T_{ext} = 0{\cdot}18$. The experimental data for a variety of chemical agents including H_2O_2 will be published at a later date.

Equation (3*c*) has been found to apply to systems other than those mentioned here, e.g. *Escherichia coli* (Együd, 1967), and to the effect of irradiation on the growth of mammalian cells in culture (Elkind & Whitmore, 1967). In the latter case, we can plot log T_I *v.* energy units absorbed. However, if it is suspected or known that the radiation-induced inhibition is due to the formation of a given compound or class of compounds and the radiation yield is known, then the energy units can, of course, be converted to μmoles. For example, I have obtained linear log T_I *v.* $[C]$ plots for the antibacterial action of irradiated sucrose solutions for radiation doses in the range of 0·05 to 5·0 Mrad. where $[C]$ was expressed in terms of the total concentration of carbonyls produced by radiolysis.

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Structure and Composition of Walls of the Yeast Form of *Verticillium albo-atrum*

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SUMMARY

Walls of the yeast form of *Verticillium albo-atrum* showed a granular appearance on the outer surface. The granular components could be extracted by alkali, revealing a fibrillar wall fabric. A region of circularly oriented microfibrils with a minute central 'orifice' was commonly found at one of the cell poles and probably represented a bud scar. The material dissolved by alkali was a heteropolysaccharide–protein complex containing mannose, galactose, glucose, glucuronic acid, glucosamine and the common range of amino acids. The alkali-insoluble microfibrillar network was made of a β-linked glucan and chitin. The glucan was digested by endo-β-glucanases yielding glucose, β-1,3-linked glucose oligomers and cellobiose, but no evidence for cellulose was found. Most of the glucan was also soluble in hot acid. The acid-insoluble glucan (hydroglucan) contained β-1,6-links. Acid treatment produced coarse microfibrils resembling those in *Saccharomyces cerevisiae* walls treated similarly. The hydroglucan was soluble in alkali leaving an insoluble microfibrillar network composed mainly of chitin. A small amount of lipid (2·7 to 3·4 %), mostly of the bound type, and traces of phosphate were also found.

INTRODUCTION

Little is known about the biochemical basis of differentiation in *Verticillium albo-atrum*, a fungus which causes wilt diseases of a wide range of economically important plants. Brandt and his associates (Brandt, 1962, 1964, 1965; Brandt & Reese, 1964; MacMillan & Brandt, 1966) have clarified some of the events leading to microsclerotial formation but nothing is known about the biochemistry of vegetative development in this organism.

Verticillium albo-atrum is a dimorphic fungus. In stationary culture media, it grows as a typical mycelium; in shake cultures it can develop chiefly as budding yeast-like organisms (Malca, Erwin, Moje & Jones, 1966). Significantly, the yeast form (also referred to as conidial or spore form) also occurs in infected tissues of cotton plants (Garber & Houston, 1966). The dimorphism of *V. albo-atrum* is somewhat analogous to that of animal pathogens where the parasitic phase is usually the yeast phase. The dimorphic capacity of *V. albo-atrum* plays an important role in pathogenesis; colonization of the upper parts of infected plants is by movement of the yeast form (conidia) through vascular tissue and not by mycelium spreading (Presley, Carns, Taylor & Schnathorst, 1966).

This report describes the walls of the yeast form of *Verticillium albo-atrum* grown in shake culture. This is an initial effort to correlate cellular morphogenesis with wall

structure, as done in the study of morphogenesis of other fungi (Nickerson & Bartnicki-Garcia, 1964; Bartnicki-Garcia, 1968). The ultimate objective of this project is to elucidate the biochemical basis of dimorphism of *V. albo-atrum* and thus contribute to a better understanding of host–parasite relationships in Verticillium wilt.

METHODS

Microbiological techniques. A microsclerotial isolate (V_3H) of *Verticillium albo-atrum* which causes defoliation of cotton (Mathre, Erwin, Paulus & Ravenscroft, 1966) was used. Stock cultures were routinely maintained on yeast extract + potato + glucose-agar slopes at 25°. Slopes were inoculated with single cells to maintain morphological stability. The yeast from was grown in shake cultures in glucose nitrate medium (Malca *et al.* 1966) prepared by dissolving the ingredients, excluding phosphates, in 4/5 final vol. water. A solution of KH_2PO_4 and K_2HPO_4 (pH 6·4) was prepared separately, both were autoclaved 20 min. at 121°, then mixed aseptically. Unless otherwise specified, 50 ml. medium in a 250 ml. Erlenmyer flask was inoculated with 1 ml. of a 7 day liquid culture which had been freed of a small proportion of mycelium by passing through a double-layer of sterile cheesecloth. The yeast suspension which emerged was centrifuged at 1000 *g*, rinsed with sterile water and suspended to 10^6 to 10^7 organisms/ml. and used as an inoculum. Cultures were incubated in a rotary shaking incubator at 25° for 5 to 7 days. The yeast form was harvested after filtration through nylon mesh (in place of cheesecloth to avoid contamination with cellulose fibres) to remove hyphae; centrifuged at 10,000 *g* for 10 min. at 1° and rinsed twice with 10 vol. cold 0·05 M-tris-HCl buffer, pH 7·5.

Isolation of walls. The organisms were suspended in 3 vol. cold 0·05 M-tris-HCl buffer, pH 7·5, and the slurry transferred to a 50 ml. Bronwill MSK homogenizer glass container to which an equal vol. of acid-washed glass beads (0·25 to 0·30 mm. in diameter) had been added. After four 15 sec. treatments with 5 sec. intervals for cooling, while liquid CO_2 was flushed continuously around the flask (at the end of a run, the temperature of the container seldom exceeded 15°) the glass beads were allowed to sediment and the broken cell suspension was decanted into a centrifuge tube. The walls were centrifuged at 500 *g* for 5 min. at 0°. The pellet was suspended in 5 vol. buffer and blended at the top speed of a Virtis 45 homogenizer for 2 min. and centrifuged again at 500 *g*.

The entire breakage and washing procedure was repeated four or five times to obtain a wall preparation with less than 0·1 % unbroken cells. Cytoplasmic debris was eliminated after 20 to 30 cycles of centrifugation and homogenization. Walls, stained with Lugol's iodine solution, were examined with a Reichert phase-contrast microscope and glass bead fragments were removed by consecutive 1-min. centrifugation at 50, 100 and 200 *g*. The pellet resulting from each centrifugation was suspended in water and centrifuged again at the same speed, while the supernatant fluid was combined with the residue sedimenting at the next higher speed. Purified walls were lyophilized and stored in a desiccator.

Chemical analyses. Two types of hydrolysate were prepared. *H_2SO_4 hydrolysate*: dry wall samples were soaked with 22·5 N-H_2SO_4 and incubated at 30° for 3 h. The acid was diluted to 0·85 N and heated at 97° for 4 h. *HCl hydrolysate*: dry wall samples were suspended in 6 N-HCl, sealed in ampules under N_2 and hydrolysed at 105° for

different times. The HCl was evaporated to dryness under vacuum in a rotary evaporator, or in a desiccator over NaOH pellets under vacuum at 45° overnight.

H_2SO_4 hydrolysates containing neutral sugars were neutralized with $BaCO_3$ and de-ionized with a double bed of Dowex-1 (acetate form)+Dowex-50 (H^+ form). Sugars were separated on paper chromatograms (Whatman 3 MM) irrigated in descending fashion with either butanol+pyridine+water (BPW) (6+4+3, by vol.) or ethyl acetate+acetic acid+water (EAW) (9+2+2, by vol.) for 36 h. or longer. Sugar spots were revealed with aniline phthalate (Dawson *et al.* 1959). For quantitative measurements, unsprayed portions of the chromatogram containing suspected sugars were cut out, eluted, and the sugar estimated by the anthrone method.

For gas-liquid chromatography (g.l.c.), hexose samples were converted to their corresponding alditol acetates. They were reduced to the corresponding alditols by sodium borohydride at room temperature for 3 h.; the resulting mixture was decationized through a Dowex-50 (H^+ form) column and the borate in the filtrate was evaporated off with methanol+1 % (v/v) acetic acid. The alditols were acetylated with acetic anhydride and pyridine (Hause, Hubicki & Hazen, 1962). The alditol acetates were separated by a Perkin-Elmer gas chromatograph, model 881, using a stainless steel column (10 ft × $\frac{1}{8}$ in. o.d.) packed with Gas Chrom Q 60 to 80 mesh, coated with 3 % ECNSS-M, an organosilicone polyester phase resulting from the combination of ethylene glycol succinate and a silicone of a cyanoethyl type (Kim, Shome, Liao & Pierce, 1967).

Amino sugars were determined in the HCl hydrolysate by the Elson-Morgan method (Tracey, 1955). Hexosamine was identified as glucosamine by oxidation with ninhydrin (Stoffyn & Jeanloz, 1954) and by paper electrophoresis in 1 % borate (Maley & Maley, 1959).

Hexoses were estimated by Dreywood's anthrone method (Bartnicki-Garcia & Nickerson, 1962). Reducing sugars were determined by the method of Somogyi & Nelson (Hodge & Hofreiter, 1962). Glucose was assayed by glucose oxidase reagent (Glucostat 'special'; Worthington Biochemical Corp., New Jersey, U.S.A.).

Uronic acids in the neutralized H_2SO_4 hydrolysate were separated by column chromatography. After neutralization with $BaCO_3$ the cleared supernatant fluid was placed on a Dowex-1 (acetate form) column. Uronides were eluted in 10 ml. fractions by a linear gradient of acetic acid (0 to 2 M; total vol. 1000 ml.). A modified carbazole reagent (Bitter & Muir, 1962) was used to detect uronic acid. Uronic acids and aldobiouronic acids were identified by reduction to the corresponding hexoses and neutral disaccharides, respectively, by way of the methyl ester methyl glucoside which was reduced with sodium borohydride (Lewis, Smith & Stephen, 1963) and acid hydrolysed to liberate the free monomers.

Total amino acid-N in HCl hydrolysates was determined by ninhydrin after elimination of interfering glucosamine (Bartnicki-Garcia & Nickerson, 1962). Individual amino acids and amino sugars were estimated in a Spinco automatic amino acid analyser, model 120 C. Total phosphate was determined spectrophotometrically (Dryer, Tammes & Routh, 1957). Free and bound lipids were extracted as described previously (Bartnicki-Garcia & Nickerson, 1962).

Physical techniques. Infrared spectra of purified walls in KBr pellets, and of alditol hexa-acetates in chloroform, were determined with a Perkin-Elmer spectrophotometer. Optical rotations were measured with a Bendix automatic polarimeter. X-ray diagrams

of purified wall samples spread on glass microslides were obtained with a Norelco Philips recording diffractometer using CuKα radiation. Chemically extracted wall residues were shadowed with Pd and examined in a RCA electron microscope, model EMU-3B.

Enzymic digestion. A glucanase preparation from *Streptomyces* sp. QMB 814 containing chiefly β-1,3- and β-1,4-endoglucanases was obtained through the courtesy of Dr E. T. Reese (U.S. Army Quartermaster Research and Engineering Center, Natik, Massachusetts, U.S.A.). Cell walls were digested in 12-ml. glass-stoppered centrifuge tubes at 50° for 36 h. A typical digestion mixture consisted of 5 ml. 0·05 M-sodium citrate buffer (pH 5·75), 1 mg. Streptomyces endoglucanases and 10 mg. cell walls. Digestion was stopped by heating at 97° for 5 min., and the mixture centrifuged at 1000 *g* for 10 min. The supernatant was de-ionized with Dowex resins as indicated above, and the soluble digestion products then separated by paper chromatography.

RESULTS

At harvest time, our cultures of *Verticillium albo-atrum* had very little mycelium and contained mainly a heterogeneous assortment of single cells in different stages of development. Some of the cells were identical in appearance to conidia or phialospores (Cole & Kendrick, 1969) and may have originated in part from true conidiophores formed on the mycelium prevalent in the first days of incubation. The large majority, however, were 'secondary conidia' produced by a budding process from large single cells, which in turn were derived from the initial conidia. The buds were frequently formed, not on the surface of the mother cell but at the end of a tubular process of variable length, which when well developed, resembled the phialide of a true conidiophore (Pl. 1, fig. 1). Thus the development of these buds has been considered equivalent to true conidation and the daughter cells named 'secondary conidia' (Buckley, Wyllie & DeVay, 1969). In addition to conidia, a large portion of the culture consisted of single vegetative cells in different stages of budding.

Chemical composition

Chemical fractionation. Purified walls were chemically fractionated into alkali-soluble, acid-soluble, acid-insoluble but alkali-soluble and insoluble residue fractions. The composition of these fractions is summarized in Table 1 and a description of the principal components follows.

Heteropolysaccharide complex. This appeared to be the major component of the alkali-soluble fraction. On hydrolysis with N-acid it yielded D-glucuronic acid, D-mannose, D-galactose and D-glucose. In addition, D-glucosamine (Table 2) and the range of amino acids commonly found in fungal walls were detected in HCl hydrolysates of the alkali-soluble fraction (Table 3). The hexosamine was identified as glucosamine by oxidation with ninhydrin which yielded arabinose; the presence of mannosamine which also yields arabinose was ruled out by paper electrophoresis of the amino sugar in 1 % sodium borate. Up to 2 % glucosamine was detected, but its polymeric state is unknown. To exclude the possibility that this glucosamine arose from chitin, the alkali-soluble fraction was put through a Millipore filter (type HA; pore size 0·45 μm) to remove any contaminating wall fragments. The glucosamine content of the filtrate remained essentially the same.

Neutral sugars were separated by paper chromatography and by g.l.c. after conversion to their corresponding alditol acetates. In both instances, mobility was verified by co-chromatography with authentic samples. The properties of the alditol acetates are summarized in Table 4. Glucose was also identified by the glucose oxidase assay. The relative proportions of the neutral sugars were glucose:galactose:mannose = 3·5: 2·6:1·0.

Table 1. *Fractionation of Verticillium albo-atrum walls*

Consecutive fractions	Composition: Monomers	Composition: Main polymers
1. Alkali- soluble (N-KOH at 60°)	D-Glucose D-Galactose D-Mannose D-Glucuronic acid D-Glucosamine 15 Amino acids	Heteropolysaccharide Protein
2. Acid-soluble (N-HCl at 97°)	D-Glucose D-Glucosamine 15 Amino acids	β-Glucan Protein
3. Acid-insoluble, alkali-soluble (0·75 N-NaOH at 60°)	D-Glucose D-Glucosamine (trace) 15 Amino acids (traces)	β-Glucan (hydroglucan)
4. Insoluble residue	D-Glucose (trace) D-Glucosamine Lysine, histidine	Chitin

Table 2. *Quantitative composition of Verticillium albo-atrum walls**

Component	Wall dry weight (%)
Glucuronic acid†	1·2 to 1·3
Glucose†	41·0 to 56·0
Galactose†	8·0 to 8·5
Mannose†	6·0 to 6·4
Glucosamine (non-chitinous)‡	2·0 to 3·3
Chitin	7·6 to 10·0
Protein	11·0 to 14·0
Lipid, readily extracted	0·4 to 0·6
Lipid, bound	2·3 to 2·8
Phosphate (as H_2PO_3)	0·1 to 0·2
Sum	79·6 to 103·1

* A range of values obtained with at least four different wall preparations.
† Expressed as anhydrosugar.
‡ Computed by subtracting the glucosamine content of insoluble residue (chitin) from that of unextracted wall.

The uronides present in the sulphuric acid hydrolysates were separated by column chromatography with Dowex-1, acetate form (Fig. 3). The separation was performed on the alkali-soluble fraction as well as on a large sample of unextracted walls. Peak A contained mannose, galactose and glucose. Peak B contained an aldobiouronide of D-glucuronic acid and D-galactose. After reduction of the aldobiouronic acid to the neutral disaccharide, followed by acid hydrolysis, glucose and galactose were obtained in a 1:1 ratio and about 50% yield. The compound of Peak C was identified as D-glucuronic acid by: (*a*) detection of both glucuronic acid and glucuronolactone on the

the paper chromatogram; (*b*) formation of D-glucose upon reduction of the uronic acid.

Glucan(s). After removing alkali-soluble components, chiefly heteropolysaccharide and protein, wall residues consisted mainly of glucan(s) and chitin. Two glucan fractions were separated (Table 5). By treatment with hot N-HCl, 25 % (wall basis) glucan

Table 3. *Amino acid analysis of Verticillium albo-atrum walls and fractions thereof**

	% μmoles				
Amino acid	Alkali-soluble	Acid-soluble	Acid-insoluble, alkali-soluble	Insoluble	Whole cell walls†
Lysine	3·9	3·6	T	35·4	4·8
Histidine	3·2	3·4	T	64·6	2·2
Arginine	1·5	1·0	T	—	0·9
Aspartic acid	10·5	8·7	T	—	8·2
Threonine	8·7	12·7	T	—	14·0
Serine	6·4	5·3	T	—	8·6
Glutamic acid	11·3	10·6	T	—	8·5
Proline	12·5	15·7	T	—	13·2
Glycine	10·9	7·1	T	—	10·0
Alanine	12·5	10·9	T	—	7·9
Cystine	T	T	T	—	1·9
Valine	8·1	10·5	T	—	8·4
Methionine	—	—	—	—	T
Isoleucine	3·3	3·2	T	—	2·7
Leucine	5·1	3·2	T	—	2·9
Tyrosine	X	2·9	T	—	3·8
Phenylalanine	2·1	1·1	T	—	1·9
Total amino acid mg./g. cell wall	32·02	44·94	Traces	0·56	90·80†
Glucosamine mg./g. cell wall	1·36	16·88	Traces	65·56	—

* Average of two determinations made on a 6N-HCl hydrolysate at 105° for 12 h. Fractions obtained as indicated in Table 1.

† The wall sample was hydrolysed with 6 N-HCl at 105° for 24, 48 and 72 h., respectively. Per cent μmoles of each amino acid were determined from either average, maximum, and/or extrapolated values.

T = Traces. X = Inseparable from glucosamine residue.

Table 4. *Alditol acetates prepared from the H_2SO_4 hydrolysates of Verticillium albo-atrum walls**

	Melting point		$[\alpha]_D^{25}$ in $CHCl_3$		G.l.c. retention
Compound	Observed	Reference	Observed	Reference	time (min.)
Glucitol hexa-acetate	99	98	+9·1 (*c* = 2·05)	+10·0	44
Mannitol hexa-acetate	124	124 to 125	+25·5 (*c* = 2·59)	+25·2	33
Galactitol hexa-acetate	169	168 to 169	—	—	38

* Reference values from Lohmer (1949).

was solubilized. Glucose was the only hexose detected in the acid hydrolysate (0·85 N-H_2SO_4 at 105° for 10 h.) by paper chromatography using the BPW solvent system. This finding was confirmed by glucose oxidase assays. The remaining acid-insoluble glucan was essentially all dissolved by extraction with alkali (0·75 N-NaOH at 60° for 30 min.). Evidently the acid treatment not only dissolved most of the glucan but also rendered

the rest alkali soluble. This alkali-soluble glucan reprecipitated on acidification. Following two cycles of solution and precipitation, the glucan was examined in the X-ray diffractometer (Fig. 2B). The spectrum was similar to that of the 'hydroglucan' of *Saccharomyces cerevisiae*, with a strong reflexion in the 1·3 to 1·4 nm. region (Houwink & Kreger, 1953) which was not seen in the unextracted walls or alkali-extracted walls of *Verticillium albo-atrum*. It appeared when the walls were treated with hot acid

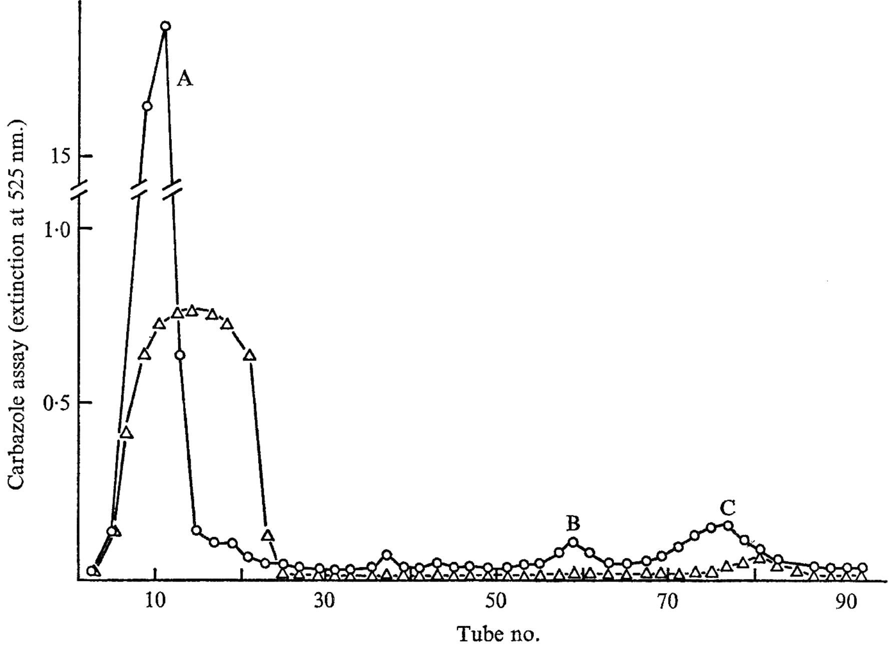

Fig. 1. Separation of uronides on a Dowex-1 (acetate form) column from an unextracted sample (○——○) and a N-KOH soluble fraction (△——△) of *Verticillium albo-atrum* walls. A gradient of 0 → 2 N-acetic acid was applied beginning with tube no. 16 for the unextracted walls and tube no. 24 for the alkali-soluble fraction.

Table 5. *Distribution of hexose polysaccharides in Verticillium albo-atrum walls*

Consecutive fractions	Hexosan* % wall dry weight
1. Alkali-soluble (N-KOH at 60°)	24·2
2. Acid-soluble (N-HCl at 97°)	25·1
3. Acid-insoluble, alkali-soluble (0·75 N-NaOH at 60°)	9·1
4. Insoluble residue	1·8
Sum	60·2
Unfractionated walls	66·0

*Determined with anthrone using glucose as standard; values expressed as % anhydrosugar.

(compare Fig. 2A with 3E), and disappeared upon subsequent alkali extraction (compare Fig. 2A with 3F). Glucose was the only sugar obtained after complete acid hydrolysis of the 'hydroglucan' with 22·5–0·85 N-H_2SO_4. The 'hydroglucan' optical activity was $[\alpha]_D^{25} = -10{\cdot}5^\circ$ ($c = 0{\cdot}36$ in 0·1 N-NaOH).

On partial acid hydrolysis the 'hydroglucan' yielded glucose and gentiobiose,

indicating the presence of β-1,6-linked residues. As with the acid treatment, a substantial (28 % wall dry weight) amount of glucan(s) was also dissolved by treatment of the alkali-extracted cell walls with the β-glucanase mixture from *Streptomyces* sp. The solutions resulting from enzymic hydrolysis were de-ionized and chromatographed on Whatman 3 MM paper irrigated by BPW (Table 6). The major products were β-1,3-linked oligosaccharides in addition to glucose and cellobiose. Cellobiose and laminaritriose, which moved at the same rate (R_{glu} = 0·65), were eluted and separated by chromatography with the EAW system. Glucose was identified by purified glucose

Table 6. *Digestion of the alkali-insoluble fraction of the walls of Verticillium albo-atrum with Streptomyces* QMB *814 glucanases in the presence of* [^{14}*C*]*glucose**

Compound	Relative abundance†	Specific activity (d.p.m./μg.)
Glucose	13·9	2081
Laminaribiose	13·2	17
Cellobiose	25·1	2
Laminaritriose	28·6	6
Laminaritetraose	19·2	0·7

* The alkali-insoluble wall (56·8 mg.) incubated with 1·9 μCi of [^{14}C]glucose (0·63 μCi/0·28 μmoles). The oligosaccharides were separated by paper chromatography with BPW (6+4+3, by vol.) at 24° for 24 h. followed by elution with H_2O and by rechromatography with EAW (9+2+2, by vol.) at 24° for 48 h.

† Determined by anthrone with glucose as standard; values are % of the sum of the five components.

oxidase reagent (Glucostat 'special'). The oligomers were identified by: (i) paper co-chromatography with authentic compounds in at least two solvents; (ii) susceptibility to a purified β-glucosidase (Worthington Biochemical Corp.) with negligible α-glucosidase activity; (iii) lead tetraacetate oxidation (Perlin, 1955). In the latter, oxidized β-1,3-sugars gave glucose and arabinose after acid hydrolysis; erythrose and glucose were the major products from cellobiose. The identity of cellobiose was confirmed through the preparation of cellobiose octa-acetate (m.p. and mixed m.p. = 196°).

To exclude the possibility that cellobiose may have arisen by trans-glucosidation during the enzymic digestion, such as that noted in β-glucosidase (Reese, Maguire & Parrish, 1967), digestion of the alkali-insoluble wall residues was repeated in the presence of [^{14}C]glucose. The low specific activity of each of the resulting oligosaccharides indicated that cellobiose and other β-1,3-linked oligosaccharides were not likely transglucosylated products (Table 6).

Despite the presence of cellobiose, no evidence for cellulose was obtained. Extraction with 17·5 % NaOH (under N_2) (commonly used to separate hemicelluloses from α-cellulose in plant materials; Corbett, 1963), did leave a substantial amount of insoluble glucan (50·5 %) in the walls of *Verticillium albo-atrum*. However, this result did not show that the alkali-insoluble glucan was α-cellulose since other β-glucans from fungal walls are known to be equally insoluble (Northcote & Horne, 1952; Bartnicki-Garcia, 1966). Furthermore, other more specific tests for cellulose were negative: (*a*) Schweizer's reagent dissolved nearly the same amounts of carbohydrate as NaOH (17·5 %) but no cellulose was regenerated upon neutralization; (*b*) absence of X-ray reflexions of crystalline cellulose (I or II) from any of the cell wall fractions (Fig. 2, 3).

Chitin. The reflexions of crystalline chitin were not clearly evident on the diagrams of the unextracted walls (Fig. 3D) but the two broad peaks, about 0·95 nm. and 0·45 nm., probably corresponded to the major reflexions (0·98 nm. and 0·45 nm.) of authentic chitin. The reflexions remained essentially unchanged after removal of the alkali-soluble components from the wall (Fig. 3E), but became more pronounced following consecutive extractions by hot N-acid and again by alkali (Fig. 3F). Seemingly, this last residue consisted almost exclusively of chitin. The chitin content was estimated

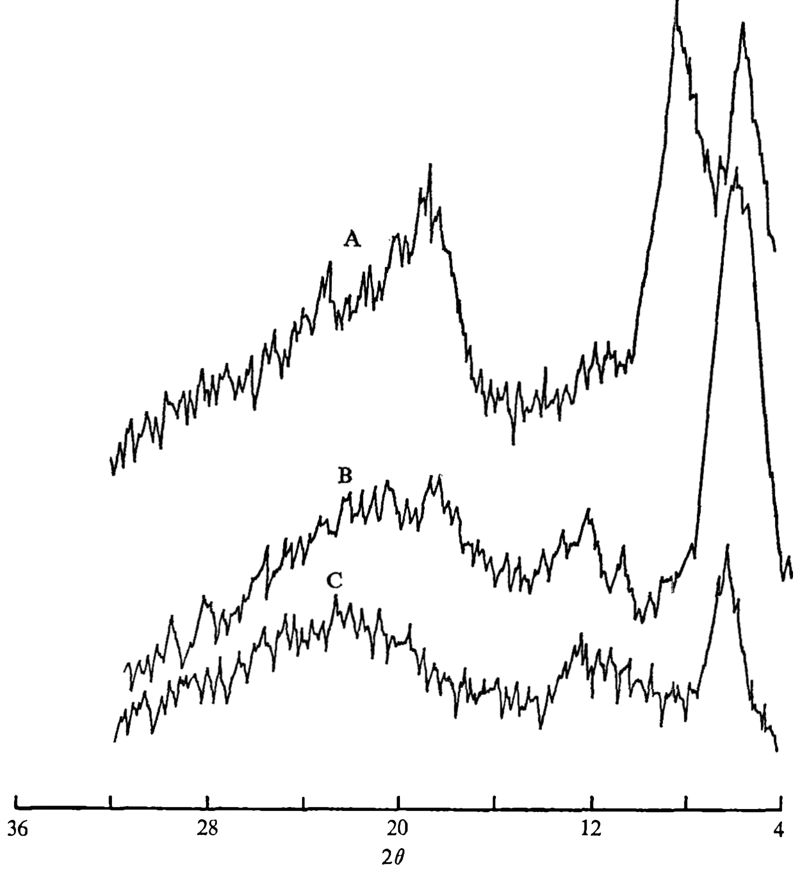

Fig. 2. 'Hydroglucan' in *Verticillium albo-atrum* walls. X-ray diagrams of alkali-extracted walls after treatment with hot N-acid (A); purified 'hydroglucan' from *V. albo-atrum* (B) and from *Saccharomyces cerevisiae* walls (C).

directly from the acid hydrolysate of the acid and alkali-insoluble residue in order to exclude glucosamine residues from non-chitinous constituents; consequently, the figures shown in Table 2 probably represent minimum estimates, since they do not account for losses incurred during both fractionation and hydrolysis.

Protein. Values of overall protein content estimated from total amino acid-N determinations ranged from 11·0 to 14·0 % (Table 2) and were higher than those calculated from the sum of individual amino acids by the automatic analyser (Table 3). The latter analyses showed that about 42 % of the total amino acid content was associated with

the heteropolysaccharide in the cold alkali-soluble fraction. The rest appeared together with the glucan in the acid-soluble fraction. Only traces of amino acids were recovered from the acid-insoluble alkali-soluble fraction (hydroglucan). Only lysine and histidine residues were detected in the hydrolysate of the insoluble fraction comprised almost exclusively of chitin fibrils.

Lipids. Extraction of dry walls with ethanol + ether (1 + 1, v/v) followed by chloroform yielded 0·4 to 0·6 % of 'readily extracted lipid' (Table 2). An additional 2·3 to

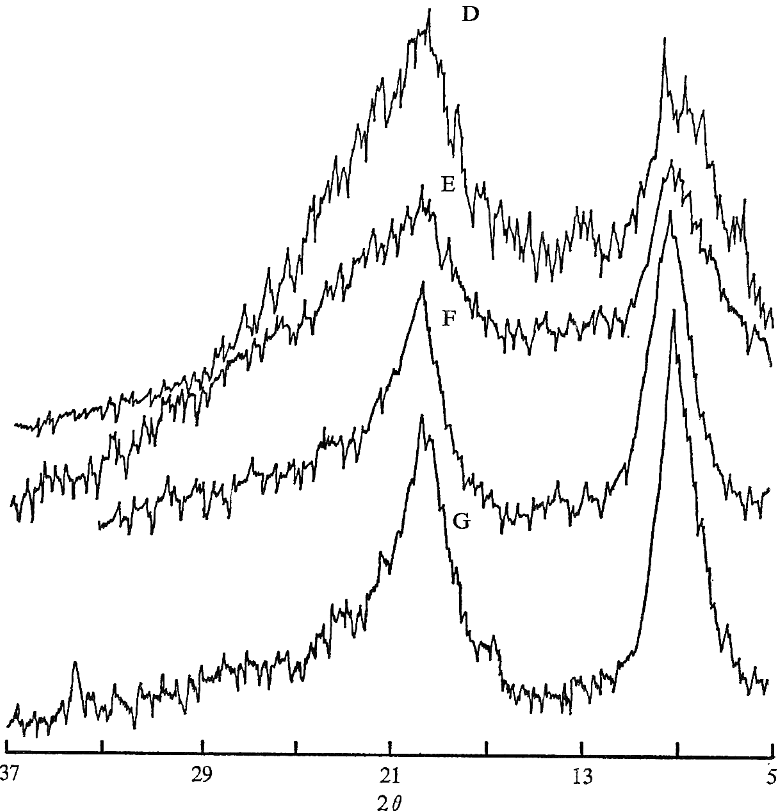

Fig. 3. Chitin in *Verticillium albo-atrum* walls. X-ray diagrams of unextracted walls (D); walls after removal of alkali-soluble components (E); final residue after consecutive alkali, acid and alkali extractions (F); purified lobster chitin (G).

2·8 % of 'bound lipid' was extracted after a mild hydrolysis of the walls in acidic ethanol + ether (1 vol. 12 N-HCl in 100 vol. ethanol + ether, 1 + 1, v/v mixture) at 50° for 5 h., followed by extractions with ethanol + ether and with chloroform.

Phosphate and ash. Wall samples were ignited at 450° in an electric muffle furnace; a slightly grey ash comprising 0·1 to 0·5 % of the initial weight was obtained. The total phosphate content of spore walls accounted for 0·1 to 0·2 % of the wall dry weight.

Physical structure

Walls were extracted consecutively and examined under the electron microscope in shadow-cast specimens; the following observations were recorded:

(1) The outer surface of unextracted yeast walls of *Verticillium albo-atrum* had a coarsely and irregularly granulated appearance (Pl. 2, fig. 6); there was only a vague microfibrillar pattern underlying the surface granulation.

(2) After extraction with N-alkali (at 97° for 30 min.) the surface granules disappeared and the wall texture became fibrillar (Pl. 2, fig. 7). The microfibrils were randomly oriented except at one of the cell poles where there was a region of circularly arranged microfibrils with a raised outer annulus and a minute central orifice or depression. These areas resemble the bud scars of *Saccharomyces cerevisiae* (Houwink & Kreger, 1953). The central 'orifice' might be the septal pore observed by Buckley, Wyllie & DeVay (1969) in thin sections.

(3) Upon subsequent extraction with hot N-acid (at 97° for 30 min.), the interwoven network of microfibrils became more sharply defined as individual microfibrils had become thicker (Pl. 3, fig. 8). The fibrillar structure resembled the 'hydroglucan' network obtained from *Saccharomyces cerevisiae* walls (Houwink & Kreger, 1953) (Pl. 3, fig. 10). The increased thickness of the microfibrils in *S. cerevisiae* and in *Verticillium albo-atrum* probably resulted from aggregation of the original microfibrils during the acid treatment (Houwink & Kreger, 1953). In *V. albo-atrum* the microfibrillar network, at this stage in the extraction sequence, consisted of roughly equal proportions of chitin and glucan. It was not possible, however, to distinguish two types of microfibrils in the electron micrographs.

(4) Upon another extraction with 0·75 N-alkali (at 60° for 30 min.) the glucan ('hydroglucan') was dissolved and the final residue contained almost exclusively randomly oriented chitin microfibrils (Pl. 3, fig. 9). The soluble 'hydroglucan' was reprecipitated twice (see above) and the resulting purified microfibrils are shown in Pl. 3, fig. 11.

DISCUSSION

Although the cultures studied contained some cells that may be regarded as true conidia, we prefer the term yeast to describe the overall appearance and developmental behaviour prevalent in our cultures. Buckley, Wyllie & DeVay (1969) were of the opinion that the process of 'secondary conidium' formation was not budding. However, since the cytological details of budding differ considerably among genuine yeasts (Streiblová & Beran, 1965), we feel justified in using the term budding to describe the mutiplication of single cells in *Verticillium albo-atrum*. By using the term yeast we seek specifically to contrast this development with mycelial growth, and to point out that the duality of vegetative morphogenesis of *V. albo-atrum* falls within the realm of mycelial-yeast dimorphism, a widespread phenomenon among fungi (Romano, 1966).

The walls of *Verticillium albo-atrum*, like the walls of other fungi, have a complex chemical composition consisting of polysaccharides, proteins and lipids. The polysaccharides, which are the principal components, are built from different monosaccharides: hexoses, amino sugar and uronic acid. The present work provides a further example of the existence of uronic acid in fungal walls, a fact which was only recently

recognized (Gancedo, Gancedo & Asensio, 1966; Bartnicki-Garcia & Reyes, 1968). At least three different polysaccharides are present in the walls of *V. albo-atrum*: an alkali-soluble heteropolysaccharide of D-glucuronic acid, D-glucose, D-mannose and D-galactose, β-linked glucan(s) and chitin. Similar heteropolysaccharides have been found in the walls of other fungi. A heteropolymer of D-glucuronic acid, D-glucose, D-mannose and D-galactose was isolated from *Aureobasidium pullulans* (Brown & Lindberg, 1967) and Mahadevan & Tatum (1965) detected a heteropolysaccharide of glucose, galactosamine, and glucuronic acid in *Neurospora crassa*. The heteropolysaccharide and protein components extracted by alkali from the walls of *V. albo-atrum* were probably surface and/or interfibrillar components. Thus upon alkali treatment the external surface of the walls lost its granular appearance and a randomly interwoven network of microfibrils was revealed.

This microfibrillar network consisted of β-glucan(s) and chitin. The glucan appeared to be mainly β-1,3-linked with some β-1,6-linkages as is true of most other fungi (Bartnicki-Garcia, 1968). The glucan could be separated into two fractions by treatment with hot acid. However, there was no conclusive evidence that two distinct glucans were present. Conceivably, the glucan fraction dissolved by the hot acid was that portion susceptible to acid hydrolysis, while the hydroglucan represented the acid-resistant core of the glucan molecule. A similar behaviour was noticed for the alkali-insoluble wall glucans of *Saccharomyces cerevisiae* (Houwink & Kreger, 1953), Endomyces (Kreger, 1954), *Phytophthora cinnamomi* (Bartnicki-Garcia, 1966) and *Schizophyllum commune* (Wessels, 1965), all of which formed 'hydroglucan' upon acid treatment. The coarsely fibrillar appearance of the 'hydroglucan' might be an artefact (Houwink & Kreger, 1953) resulting from chain aggregation after removal of the acid-soluble portion (side branches?) of the glucan molecule.

Although cellobiose was isolated from the enzymic digestion of the alkali-insoluble glucan of *Verticillium albo-atrum*, the polymeric nature of β-1,4-linked glucose units remained uncertain. Protein was found associated with the heteropolysaccharide and with the alkali-insoluble network of chitin-glucan microfibrils. Presumably the walls contained polysaccharide-glucosamine-protein complexes analogous to those described for the walls of yeast (Nickerson, Falcone & Kessler, 1961; Sentandreu & Northcote, 1968). The final insoluble residue made of chitin contained only two amino acids, lysine and histidine. These amino acids might represent the linking bridge between protein and chitin chains.

In view of the presence of alkali-soluble glucosamine-containing components, estimates of chitin content based on the total amount of glucosamine (Isaac & Milton, 1967) may have to be revised.

It was recently noted that the wall composition of any fungus could be correlated with its taxonomic position (Bartnicki-Garcia, 1968). Euascomycetes (and also Homobasidiomycetes and Chytridiomycetes) were characterized as fungi with the chitin-β-glucan type of cell walls. The present findings on *Verticillium albo-atrum* agree with this generalization.

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EXPLANATION OF PLATES

Plate 1

Fig. 1. Yeast cells of *Verticillium albo-atrum* from a 5 day liquid culture. Marker = 10 μm.

Fig. 2. Isolated yeast walls of *V. albo-atrum*. Arrow points at a cell with a phialide-like tube. Magnification same as in fig. 1.

Plate 2

Fig. 6. External surface of unextracted walls of the yeast form of *Verticillium albo-atrum*. × 23,000.

Fig. 7. Microfibrillar texture and 'budding scars' in alkali-extracted walls of *V. albo-atrum*. × 16,000.

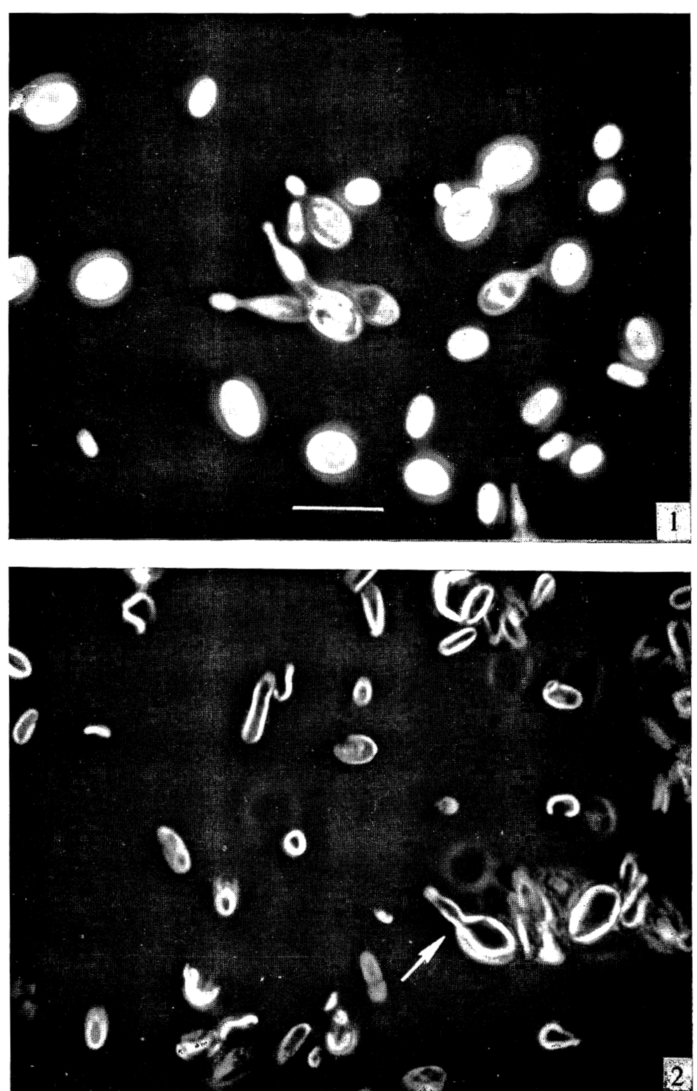
Plate 3

Fig. 8. Microfibrillar network of *Verticillium albo-atrum* walls following consecutive alkali and acid extractions. × 27,000.

Fig. 9. Microfibrillar network of *V. albo-atrum* walls following consecutive alkali-acid-alkali extractions. × 31,000.

Fig. 10. Microfibrillar network of *Saccharomyces cerevisiae* walls following same treatment as that in Fig. 8. × 26,000.

Fig. 11. Purified 'hydroglucan' microfibrils of *V. albo-atrum*. × 34,000.



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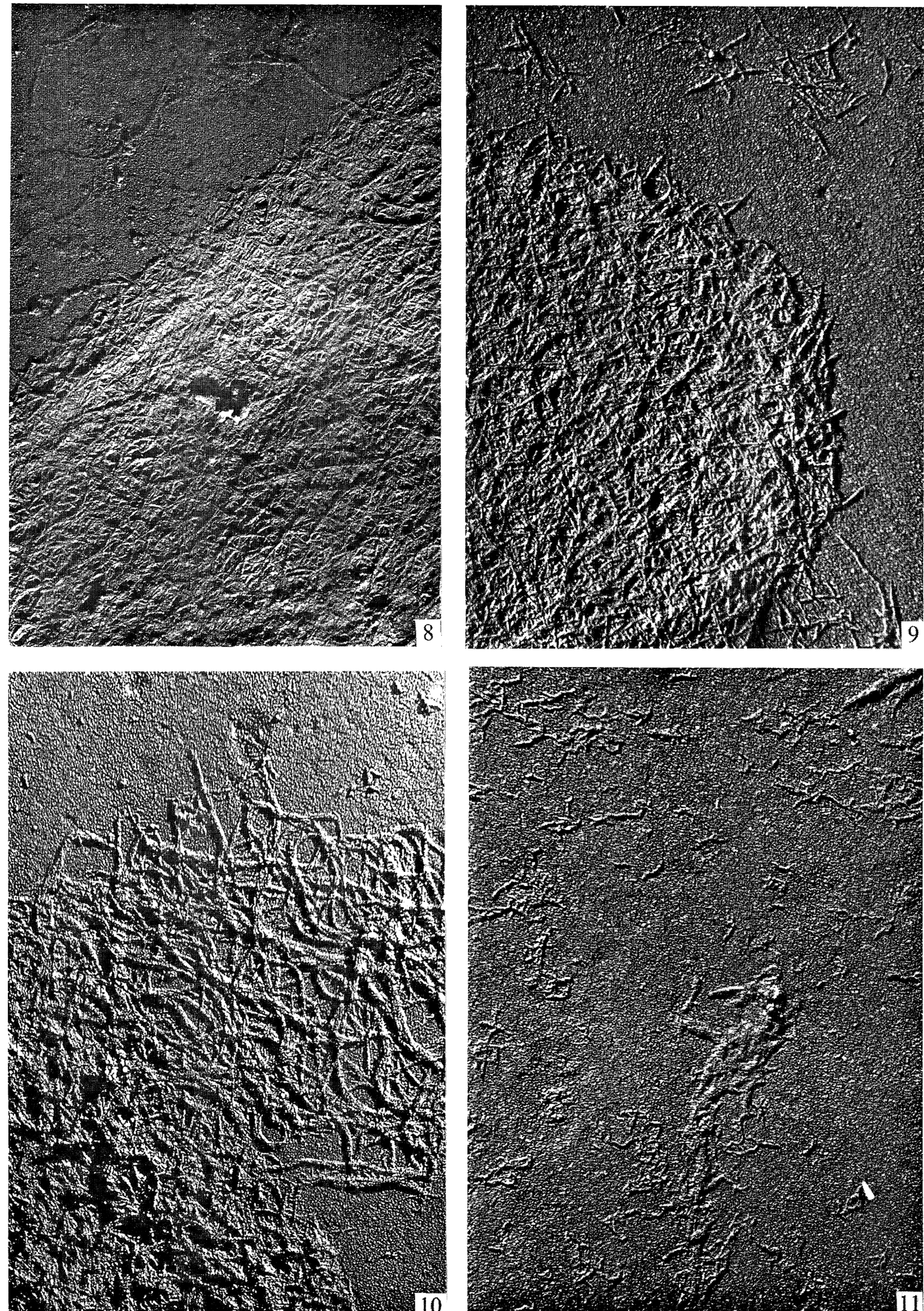

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Electron Transport and Oxidative Phosphorylation in the Blue-green Alga *Anabaena variabilis*

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SUMMARY

The respiratory electron transport system involved in NADPH (2·0 nmoles/min./mg.) and NADH (1·0 nmoles/min./mg.) oxidation in the dark that was operative in extracts of light-grown *Anabaena variabilis* has been examined. NADPH oxidation was inhibited 50% by cyanide (2×10^{-2} M), rotenone (10^{-4} M); antimycin A, amytal and azide were markedly less inhibitory. NADPH:ferrocytochrome *c* oxidoreductase, NADPH:menadione oxidoreductase, ferrocytochrome *c*:oxygen oxidoreductase and succinic dehydrogenase were detected. A phosphorylation (0·4 nmoles/min./mg.), associated with NADPH oxidation, was measured, NADPH could be replaced by NADP and isocitrate. This phosphorylation was absolutely dependent upon oxygen and was inhibited 25% by carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (5 μM).

Blue-green algae, in common with other autotrophic micro-organisms, are capable of growth in an inorganic environment. However, autotrophs will utilize to some extent reduced carbon compounds (Carr & Pearce, 1966; Hoare, Hoare & Moore, 1967; Kelly, 1967; Smith, London & Stanier, 1967; Pearce & Carr, 1967, 1969) and it is their dependence upon a specific energy source (an inorganic oxidation or photosynthesis) that distinguishes them most clearly from heterotrophic bacteria. Typically, growth and respiratory rates do not increase when organic substrates are supplied to obligate autotrophs. However, when the rate of respiration of *Anabaena variabilis* is reduced by prior starvation of CO_2, oxygen uptake is partially restored by addition of glucose (Kratz & Myers, 1955; Pearce & Carr, 1967). Although respiratory rates in blue-green algae are low, Q_{O_2} values of 5 to 10 being common, some respiration does occur; its inhibition by cyanide, azide or carbon monoxide suggests an electron transport chain at least comparable to that found in aerobic bacteria (Webster & Frankel, 1953).

The observation by Smith *et al.* (1967) that obligate chemoautotrophs and blue-green algae lack an NADH oxidase led these workers to suggest that such a metabolic lesion is the characteristic and general feature of obligately autotrophic micro-organisms. However, oxidation of reduced pyridine nucleotides by extracts of autotrophic thiobacilli (Hempfling & Vishniac, 1965; Trudinger & Kelly, 1968) and blue-green algae (Horton, 1968; Leach & Carr, 1968) suggests that these organisms can oxidize organic substrates, and is consistent with their low but reproducible respiratory oxygen uptake. Furthermore, in *Anabaena variabilis* the coupling in the dark of NADPH oxidation to ATP synthesis has been shown in cell-free extracts (Leach & Carr, 1969)

and a proton translocation, outwards through the membrane, in response to oxygenation of intact cells has been found (Scholes, Mitchell & Moyle, 1969). In view of the lack of information on the respiratory activity of blue-green algae and because of the relevance of such data to the understanding of autotrophic physiology, a study of respiratory enzymes and oxidative phosphorylation in *A. variabilis* is here presented. Preliminary accounts of some of these results have been reported elsewhere (Leach & Carr, 1968, 1969).

METHODS

Growth of Anabaena variabilis. Organisms were grown autotrophically in light as previously described (Pearce & Carr, 1967) and were harvested by centrifugation in the late log phase of growth.

Preparation of cell-free extracts. Three procedures were employed. Unless otherwise stated, method (*a*) was used for the preparation of extracts in which electron-transport enzymes were measured, and method (*b*) for phosphorylation studies.

(*a*) A suspension of washed organisms (50 to 100 mg. dry wt/ml.) in 0·1 M-potassium phosphate buffer, pH 7·6 was subjected to ultrasonic treatment (M.S.E. 100 W 20 KHz instrument) for three 45 sec. periods at 0° under a constant stream of nitrogen. Cell debris was removed by centrifugation at 10,000 *g* for 15 min. Cell breakage under oxygen-free nitrogen yielded extracts several times more active with regard to NADH and NADPH oxidases.

(*b*) A suspension of washed organisms (50 to 250 mg. dry wt/ml.) in sorbitol, 330 mM; HEPES, 10 mM; $MgCl_2$, 1 mM; EDTA, 1 mM; pH 7·6 (hereafter called 'HEPES buffer') was incubated with lysozyme (0·1 mg./ml.) at 20° for 20 min. and then extruded through a pre-cooled French pressure cell (Aminco Instrument Co. Inc., Silver Spring, Maryland, U.S.A.) at 500 lb./in.^{2} Debris was removed by centrifugation at 5000 *g* for 10 min. Material was further fractionated as described in Results section.

(*c*) A suspension of washed organisms (150 to 250 mg. dry wt/ml.) in HEPES buffer, pH 7·6, was added to an equal volume of washed sand and ground with a mortar and pestle for 15 min. at 0 to 4°. The sand was removed by centrifuging at 2000 *g* for 10 min. and the suspension of broken organisms was then centrifuged at 10,000 *g* for 15 min. to remove unbroken cells and cell-wall debris.

Fractionation of cell-free extracts of Anabaena variabilis. The fractionation of a suspension of disintegrated *Anabaena variabilis* by differential centrifugation is summarized in Fig. 1.

Conditions and extinction values employed in enzyme assays. The spectrophotometric determinations described below were carried out using an Optica CFR4 or a Unicam SP 800 recording spectrophotometer at 34° in silica cuvettes (d = 1 cm.). Owing to the relatively dense cell-free extracts employed it was sometimes necessary to use the maximum energy of incident light available in the spectrophotometer, which resulted in an increased background 'noise' level; slow chart speeds (1 cm./min.) were also necessary to measure enzymic rates. Ferrocytochrome *c* was oxidized by ferricyanide and reduced by dithionite and dialysed against four changes of water at 4°. Quinones (menadione and vitamin K_1) were dissolved in ethanol and diluted with water to the required concentration. Brief ultrasonic treatment assisted in the even suspension of these compounds. Extinction coefficients ($E_{1\,mM}^{1\,cm.}$) employed were: NADPH and NADH, 6·2 at 340 nm.; ferrocytochrome *c* red/ox, 19 at 550 nm. (Haas, 1955); vitamin K_1,

0·258 at 245 nm. The latter value was kindly supplied by Dr P. J. Dunphy of this department. Amytal, rotenone and antimycin A were dissolved in ethanol; control assays showed that the ethanol at the concentration employed did not affect the enzyme examined. Anaerobic measurements were carried out in a spectrophotometer cuvette, modified to act as a Thunberg tube, that had been evacuated and gassed three times with oxygen-free nitrogen.

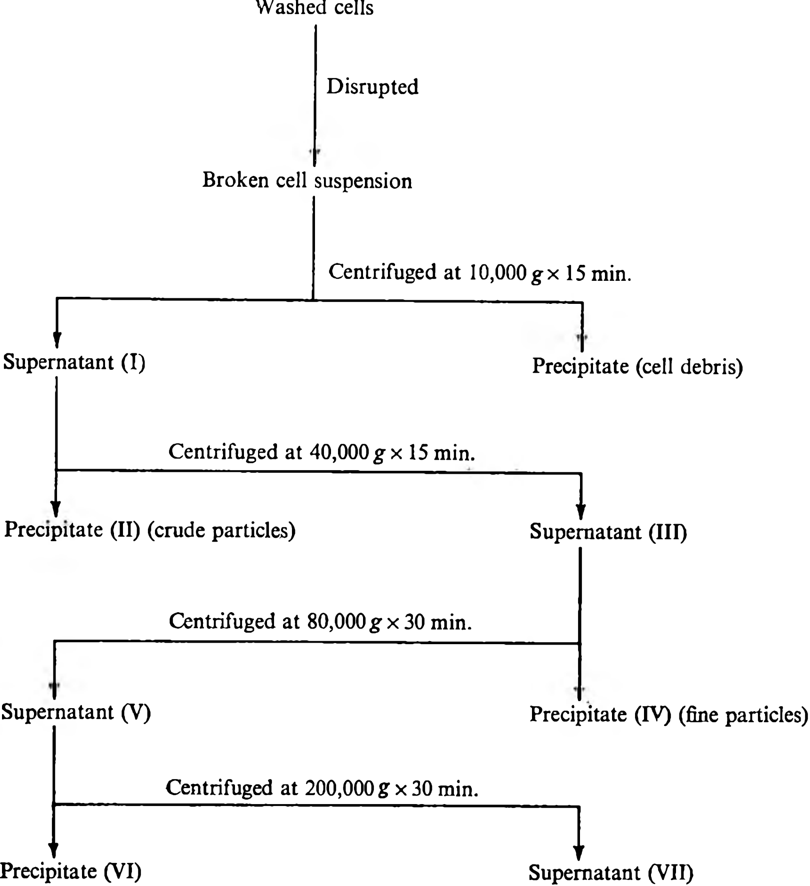

Fig. 1. Flow sheet of the subcellular fractionation of *Anabaena variabilis*.

Reduced pyridine nucleotide oxidase (NADPH: oxidoreductase)

Spectrophotometric measurement. Cuvettes contained in 3 ml.: potassium phosphate, pH 7·6, 200 μmoles; NADPH, 0·3 μmole and cell-free extract (0·5 to 5 mg. protein). The decrease in E_{340} in the experimental cuvette was measured against a boiled enzyme control.

Measurement by O_2 uptake. Oxygen consumption was measured using a Clark electrode (Yellow Springs Instrument Co. Inc.). The reaction mixture contained in 5 ml.: potassium phosphate, pH 7·6, 200 μmoles; extract containing 40 to 60 mg. microbial protein, and 10 mg. NADPH added to start the reaction. The rate of back-diffusion of oxygen into the electrode cell was corrected for, as were blank determinations without NADPH or with boiled enzyme.

Transhydrogenase (EC 1.6.1.1)

Acetyl pyridine analogue procedure (Kaplan, 1967). Potassium phosphate pH 7·6, 66 μmoles; NADPH, 0·5 μmoles and KCN, 20 μmoles were added to cell-free extract of *Anabaena variabilis* (5 to 10 mg. protein) in 1 ml. final volume. After the addition of acetyl pyridine NAD^+, the increase in extinction at 375 nm. was measured.

Coupling with pig heart isocitrate dehydrogenase (Kaplan, 1955). The reaction mixture containing, in 3 ml., $NADP^+$, 0·1 μmole; potassium phosphate, pH 7·6, 100 μmoles and isocitrate dehydrogenase (0·1 mg.; sp. act. 9·8 μmoles, min.$^{-1}$, mg.$^{-1}$) was allowed to proceed to completion after the addition of isocitrate (10 μmoles) with the small amount of $NADP^+$ present, and then NAD^+ (1 μmole) was added. After incubation for a few minutes (any transhydrogenase would then be detected) extract of *Anabaena variabilis* (2 to 5 mg. protein) was added. Increase in absorption at 340 nm. was assumed to be due to the transfer of hydrogen from NADPH to NAD^+ thus releasing $NADP^+$ for further reduction. Extracts of *A. variabilis* contain no detectable NAD^+-active isocitrate dehydrogenase (Pearce & Carr, 1967).

NADPH (NADH): Ferrocytochrome c oxidoreductase (EC 1.6.9.3). Cuvettes contained in 3 ml.: potassium phosphate, pH 7·6, 200 μmoles; KCN, 20 μmoles; NADPH or NADH, 0·3 μmoles and cell-free extract (0·5 to 2·0 mg. protein). The reaction was started by the addition of oxidized ferrocytochrome *c* (0·2 μmole) to each cuvette and the increase in E_{550} measured against the blank lacking NADPH.

NADPH (NADH): menadione oxidoreductase (EC 1.6.99.2). The oxidation of NADPH in the presence of menadione was observed at 340 nm. Cuvettes contained in 3 ml.: KCN, 20 μmoles; NADPH, 0·3 μmoles; potassium phosphate, pH 7·6, 200 μmoles, and cell-free extract (0·5 to 2·0 mg. protein). The reaction was started by the addition of menadione (0·5 μmole) to the experimental cuvette. The enzyme was also measured with vitamin K_1 used in place of menadione, in which case the increase of E_{245} due to vitamin K_1 reduction was measured as well as the decrease of E_{340} due to pyridine nucleotide oxidation.

Ferrocytochrome c: oxygen oxidoreductase (EC 1.9.3.1). Oxidation of reduced ferrocytochrome *c* was measured by decrease in E_{550}. Cuvettes contained in 3 ml.: potassium phosphate, pH 7·6 (200 μmoles) and 2 to 4 mg. algal protein. 50 nmoles reduced ferrocytochrome *c* were added to the experimental cuvettes.

Succinic dehydrogenase (EC 1.1.99.1). This assay was carried out as previously described (Pearce, Leach & Carr, 1969).

The oxidative synthesis of ATP by extracts of Anabaena variabilis

The synthesis of ATP coupled with the oxidation of reduced pyridine nucleotides and organic substrates was determined by a modification of a procedure of Avron (1960). The standard reaction mixture, in a total volume of 1 ml., contained in μmoles: sorbitol, 330; [^{32}P]sodium phosphate, 1 (specific radioactivity, 1·5 to 2·5 μCi/μmole); HEPES buffer pH 7·6, 10; $MgCl_2$, 1; EDTA, 1; ADP, 1; extract (1·5 to 4·0 mg. protein); and NADPH, NADH (1·5 μmole) or organic substrates (20 μmoles) and 1·5 μmole of $NADP^+$ and NAD^+. Appropriate controls were assayed, omitting the oxidized nucleotides or organic substrates. The reactions were allowed to proceed in the dark for 10 min. at 34° and were terminated by the addition of 0·1 ml. of 20 % (w/v) trichloroacetic acid. The precipitated protein was separated by centrifugation and

0·3 ml. samples of the supernatants were used for ATP measurement. Subsequent steps were carried out at 0 to 2°.

Acetone (1·2 ml.) was added to each sample, mixed, allowed to stand for 10 min., and the water content of each tube was then made up to 2·5 ml. with water saturated with isobutanol+benzene (1:1) mixture. Seven ml. of isobutanol+benzene (1:1) saturated with water was added and thoroughly mixed. After phase separation, 0·8 ml. of molybdate reagent (5 g. ammonium molybdate dissolved in 40 ml. 10N-H_2SO_4 and made up to 100 ml. with water) was run down the side of each tube and the water layer was mixed gently. After allowing to stand for 5 min., the contents were mixed thoroughly for 30 sec. and allowed to stand. After phase separation, the upper layer which contained the majority of [^{32}P]PO_4 molybdate complex was removed and 0·02 ml. of 0·02 M-KH_2PO_4 were added to the aqueous layer, followed by 7·0 ml. of the isobutanol:benzene mixture saturated with water. After re-extraction of the phosphate molybdate complex, the isobutanol:benzene phase was removed. Some of the aqueous phase was placed in a stainless steel planchet and the [^{32}P] content counted in a Nuclear Chicago gas flow counter at an efficiency of 33 % to an accuracy of at least 0·5 %. Samples of the aqueous phase were also used in chromatographic determinations.

The use of [^{32}*P*]*ATP in glucose-6-phosphate formation.* To link the [^{32}P]ATP synthesized to hexokinase activity, 15 i.u. of hexokinase (E.C. 2.7.11) and glucose (5 μmoles) were added to the standard phosphorylation mixture. After incubation, the reaction was stopped by the addition of 0·1 ml. of 20 % trichloroacetic acid and the remaining [^{32}P]PO_4 removed as a molybdate complex as described above. [^{32}P] present in the glucose-6-phosphate formed was assayed after chromatographic separation.

Chromatography of [^{32}*P*]*ATP and* [^{32}*P*]*glucose-6-phosphate.* Chromatographic separations were achieved on thin layer cellulose plates and on paper (Whatman no. 1) and radioactive peaks were measured on a Nuclear Chicago Actiograph III scanner. Solvents used for the separation of ATP were: isobutyric acid+water (7+5+3, by vol.); *n*-propanol+water+trichloroacetic acid+ammonia (75+20+5+0·3, by vol.) (see Block, Durrum & Zweig, 1968). Inorganic phosphate, AMP, ADP, and ATP were chromatographed as standards and were located by molybdate reagent or the unstained nucleotides were observed when viewed under u.v. light. Glucose-6-phosphate was chromatographed in methanol+ammonia+water (6+1+3, by vol.) and in methanol+formic acid+water (80+15+5, by vol.) (see Block *et al.* 1968).

Protein determination. A modified biuret reaction was used (Pearce & Carr, 1967).

Chlorophyll and phycocyanin determinations. Spectra of diluted extracts were measured between 400 nm. and 700 nm. and the extinction of chlorophyll was measured at 663 nm. and that of phycocyanin *c* at 620 nm. Extinction values were not converted to weight of material present.

Determination of ATP by a luciferin: luciferase assay system. The assay procedure was a simplification of the method of Lyman & DeVincenzo (1967). A modified single-sided Locarte fluorometer MK 11 was used in a dark room. The photomultiplier tube was dismantled from its housing and a plate with a small mirrored chamber and a 10 mm. × 80 mm. spectrophotometric tube attached was fitted. The standard reaction mixture in 1·55 ml. contained glycine buffer at pH 8·0 (100 μmoles) and $MgCl_2$ (2 μ-moles). Commercial ATP samples at a suitable dilution were added in 0·2 ml. of solution. Readings on the instrument's galvanometer were recorded and then 0·25 ml.

of firefly lantern extract (10 mg. firefly tails homogenized in 10 ml. of glycine buffer and filtered) were rapidly injected into the tube by means of a syringe. The maximum reading on the galvanometer for each ATP sample was recorded and a standard relationship between this and the ATP supplied was constructed. This procedure could be employed only with purified ATP samples, owing to quenching by other co-factors.

Chemicals and enzymes. Nucleotides, pig heart isocitrate dehydrogenase and hexokinase were purchased from Boehringer und Soehne G.m.b.H., Mannheim, Germany; horse ferrocytochrome *c* from Seravac Ltd, Maidenhead, Berkshire, [^{32}P]phosphate from The Radiochemical Centre, Amersham, Buckinghamshire; firefly tails and acetyl pyridine nucleotide from Sigma (London) Ltd; amytal and antimycin A from Koch-Light Ltd, Colnbrook, Buckinghamshire. Vitamin K_1 was kindly given by Dr P. J. Dunphy (of this department), and FCCP was a gift from Dr P. Scholes (Glyn Research Laboratories, Bodmin, Cornwall).

Abbreviations. Non-standard abbreviations used: EDTA, ethylene-diaminetetra-acetic acid disodium salt; HEPES, *N*-2-hydroxyethyl-piperazine-*N'*-2-ethanesulphonic acid; FCCP, carbonylcyanide *p*-tri-fluoromethoxyphenylhydrazone.

RESULTS

Oxidative activities of cell-free extracts. NADPH and NADH oxidases were detected in cell-free extracts of *Anabaena variabilis*, and after fractionation as described in Fig. 1 these activities were distributed throughout the supernatant fractions and were present at highest specific activity in fraction VII. After breaking *A. variabilis* by grinding with sand in HEPES buffer, the partial localization of NADPH oxidase in the particulate fractions II and IV was observed (Table 1). The supernatant fraction VII prepared from ultrasonic treated cells contained some NADPH oxidase activity but no chlorophyll, demonstrating a complete separation of chlorophyll from part of the oxidase activity. Most of the oxidase activity, however, sedimented with the chlorophyll-containing fractions and with extracts prepared from cells ground in sand the oxidase was confined to the heavy fractions along with the chlorophyll. The sedimentation characteristics of NADPH and NADH oxidase were similar, and the activity with both coenzymes present was not greater than with NADPH alone. NADH oxidase activity varied between 40 % and 65 % of the NADPH oxidase in all extracts assayed. Transhydrogenase activity could not be detected by the acetyl pyridine analogue procedure of Kaplan (1967) but a low rate (0·31 nmole/min./mg.) was detected using this method coupled with pig heart isocitrate dehydrogenase (Kaplan, 1955). In subcellular fractions prepared from ultrasonic treated extracts succinic dehydrogenase showed a broad distribution of activity, similar to that of NADPH oxidase (see Table 1). Confirmation that the oxidations of reduced pyridine nucleotides were by true oxidases was given by lack of activity under anaerobic conditions (Fig. 2*a*). After oxidation the $NADP^+$ was re-reduced by the action of isocitrate dehydrogenase when isocitrate (10 μmoles) was added to the cuvette (Fig. 2*b*), which indicated that the initial decrease in $E_{340\,nm}$ could be attributed to an oxidase activity and not to the destruction of the nucleotide. NADPH oxidase had a broad pH optimum around 7·6, although a limited activity could be detected at pH 9·0 (Fig. 3*a*). NADPH oxidation by cell-free extracts showed marked dependence on temperature, the rate at 34° being

Table 1. *Distribution of NADPH oxidase compared with that of succinic dehydrogenase in subcellular fractions of Anabaena variabilis*

Total activities = nmoles/min. Specific activities = nmoles/min./mg. protein.

Fraction (see Fig. 1)	NADPH oxidase extracts prepared by: Ultrasonic disintegration		French pressure cell		Ground with sand		Succinic dehydrogenase extract prepared by ultrasonic disintegration	
	Sp. act.	Total act.	Sp. act.	Total act.	Sp. act.	Total act.	Sp. act.	Total act.
I. Supernatant	2·0	210	9·4	1000	8·1	720	0·2	20
II. Precipitate	0·8	13	3·1	4·4	13	290	0	0
III. Supernatant	2·6	200	10	880	7·1	450	0·22	17
IV. Precipitate	1·1	44	4·2	130	10	330	0·09	3·7
V. Supernatant	3·5	120	13	650	2·1	61	0·34	12
VI. Precipitate	1·9	55	8·4	320	0·23	38	0·18	5·1
VII. Supernatant	9·7	21	21	110	0	0	0·89	4·8

several times faster than at room temperature. Maximal rates were obtained at 37 to 39° (Fig. 3*b*).

The electron-transfer pathway of NADPH oxidase was examined in extracts of *Anabaena variabilis* by the assay of component enzymes and by the effect of inhibitors

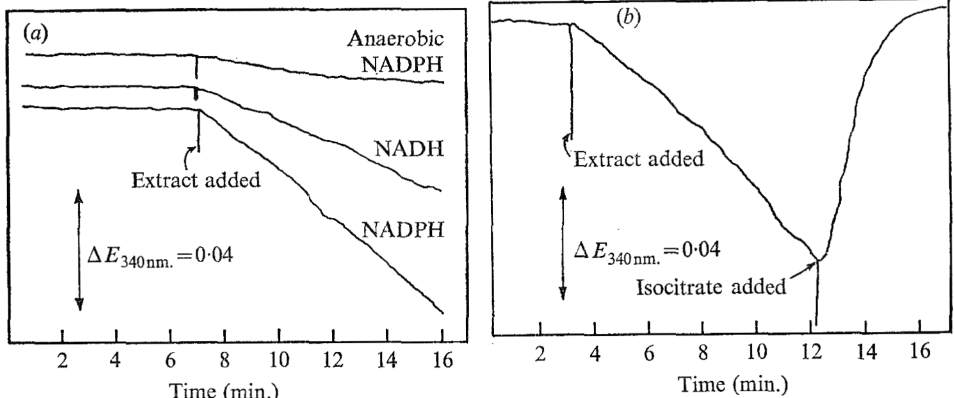

Fig. 2*a*. Spectrophotometric traces showing the aerobic oxidation of NADPH and NADH by ultrasonicated extracts of *Anabaena variabilis*. Cuvettes contained 2·8 mg. protein (for further details see Methods).

Fig. 2*b*. The regeneration of NADPH after its aerobic oxidation by an extract of *Anabaena variabilis* following the addition of isocitrate (10 μmoles). The cuvette contained 3·4 mg. protein (for further details see Methods). NADPH was excluded from the blank cuvette.

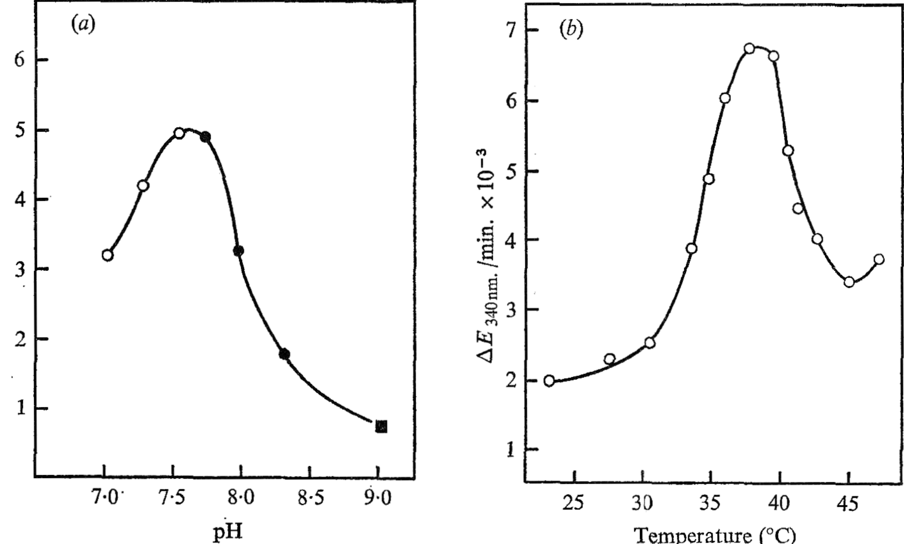

Fig. 3*a*. pH dependence of NADPH oxidase activity in ultrasonicated cell-free extracts of *Anabaena variabilis*. Cuvettes contained 2 mg. protein and buffer (100 μmoles). ○, Phosphate buffer; ●, tris + HCl buffer; and ■, borate + NaOH buffer (for further details see Methods).

Fig. 3*b*. The effect of temperature on the rate of NADPH oxidase from ultrasonicated cell-free extracts of *Anabaena variabilis*. Cuvettes contained 1·2 mg. protein and were pre-equilibrated for 5 to 6 min. before the addition of reduced pyridine nucleotide. (For further details see Methods.)

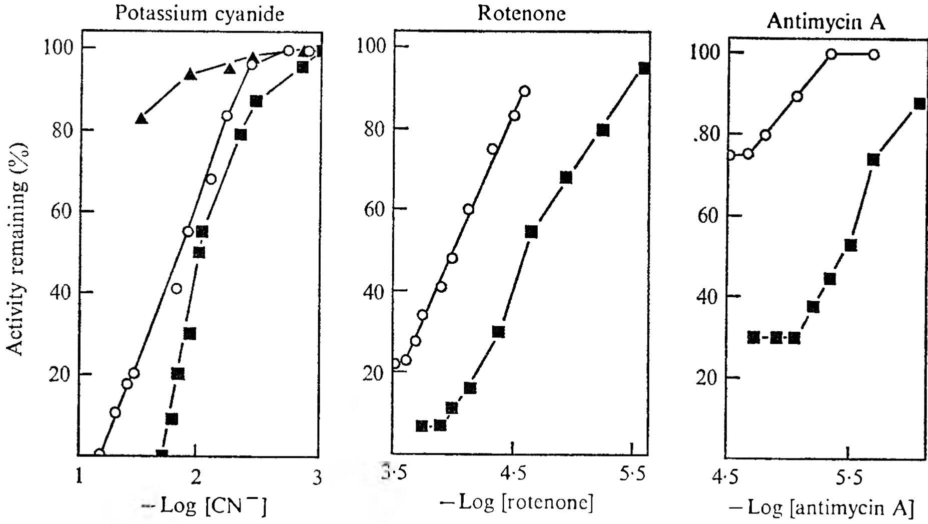

Fig. 4. The effect of inhibitors on electron transport activities of cell-free extracts of *Anabaena variabilis*. Reaction mixtures as described in Methods and the inhibitors were added in a small volume (0·2 ml.). The figures show the % of electron transport activities remaining against − log of molar concentration of the inhibitor; ○, NADPH oxidase; ■, NADH oxidase: ▲, NADPH+cytochrome *c* reductase.

Table 2. *Electron transport activities present in extracts of Anabaena variabilis prepared by ultrasonic treatment*

	Electron donor	
	NADPH (nmole/min./ mg. protein)	NADH (nmole/min./ mg. protein)
Reduced pyridine nucleotide: oxidase (by $E_{340nm.}$)	2·1	1·2
Reduced pyridine nucleotide: oxidase (by O_2 uptake)	2·3	1·3
*Reduced pyridine nucleotide: cytochrome *c* reductase (by $E_{340nm.}$)	4·5	1·2
*Reduced pyridine nucleotide: cytochrome *c* reductase (by $E_{550nm.}$)	4·7	1·6
Reduced pyridine nucleotide: menadione reductase (by $E_{340nm.}$)	23	1·1
Reduced pyridine nucleotide: vitamin K_1 reductase (by $E_{340nm.}$)	16	1·0
Reduced pyridine nucleotide: vitamin K_1 reductase (by $E_{340nm.}$)	15	1·1
Transhydrogenase	0·31	
*Cytochrome *c* oxidase (by $E_{550nm.}$)	2·3	
Succinic dehydrogenase	0·2	

Enzyme activities were measured as in Methods, the particular procedure employed being indicated in parentheses. Enzyme activities are expressed as nmoles/min./mg. protein and are the mean of at least three determinations.

* These rates are from extracts that had been dialysed against phosphate buffer (see Results).

on oxidation and reduction rates. The activities of respiratory enzymes were consistently higher with NADPH than with NADH, and only the former values will be discussed in detail.

Electron-transfer reactions that were detected in cell-free fractions are summarized in Table 2. The rates of NADPH-menadione reductase and NADPH-vitamin K_1

reductase showed a dependence on the concentration of quinone. Maximal rates were obtained with quinone concentrations greater than 2 mM. When oxidized ferrocytochrome *c* was added to extracts that had been stored at -18° for 3 days, it was immediately reduced without the addition of an electron donor; after dialysis of such extracts, the reduction of ferrocytochrome *c* required an electron donor. Thus dialysis permitted the measurement of both NADPH:ferrocytochrome *c* reductase and ferrocytochrome *c* oxidase in stored extracts.

The rates of reduction of ferrocytochrome *c*, menadione and vitamin K_1 by NADPH were all greater than the overall NADPH oxidase activity. The rate of activity of cytochrome *c* oxidase was similar to that of NADPH oxidase. With NADH as the electron donor, the rate of reduction of the quinones and ferrocytochrome *c* were of the same order as the NADH oxidase activity, which was about half that of NADPH oxidase.

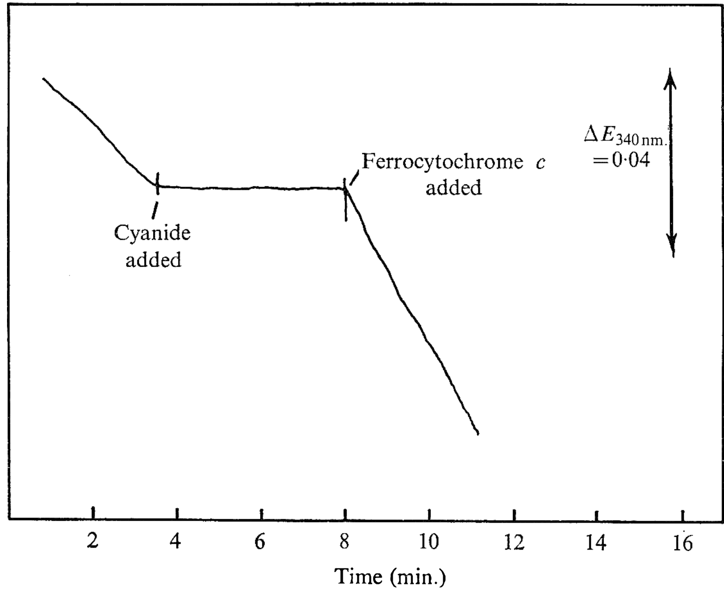

Fig. 5. The release of cyanide inhibition of NADPH oxidation in extracts of *Anabaena variabilis* following the addition of ferrocytochrome *c* (0·2 μmoles). The cuvette contained 3·4 mg. protein (further details as in Methods); NADPH was excluded from the blank cuvette.

Effects of inhibitors on electron transport activities (Fig. 4). Relatively high levels of inhibitors were necessary to demonstrate inhibition. Thus total inhibition of NADPH oxidase was obtained with 5×10^{-2} M-cyanide; NADH oxidase was completely inhibited by 2×10^{-2} M-cyanide. NADH oxidase activity was ten times more sensitive to inhibition by antimycin A than was NADPH oxidase; rotenone was several times more effective against NADH oxidase than NADPH oxidase. Amytal (10^{-3} M) inhibited the NADH oxidase activity by approximately 50%; sodium azide 10^{-3} M had little effect on either oxidase. NADPH: ferrocytochrome reductase was not affected by cyanide, but ferrocytochrome *c* oxidase was as sensitive to cyanide as was the reduced pyridine nucleotide oxidase. The release of the cyanide inhibition of NADPH oxidation by ferrocytochrome *c* is shown in Fig. 5.

When extracts were incubated with oxidized ferrocytochrome *c* and NADH, in the absence of cyanide, no net accumulation of reduced ferrocytochrome *c* was detected spectrophotometrically. However, with NADPH as electron donor, there was an accumulation of reduced cytochrome *c* which was detected as an increase in optical density at 550 nm. The redox dye, phenolindo-2,6-dichlorophenol was also rapidly reduced by NADPH (90 nmoles/min./mg. protein) but the rate with NADH was substantially less (1·02 nmoles/min./mg.).

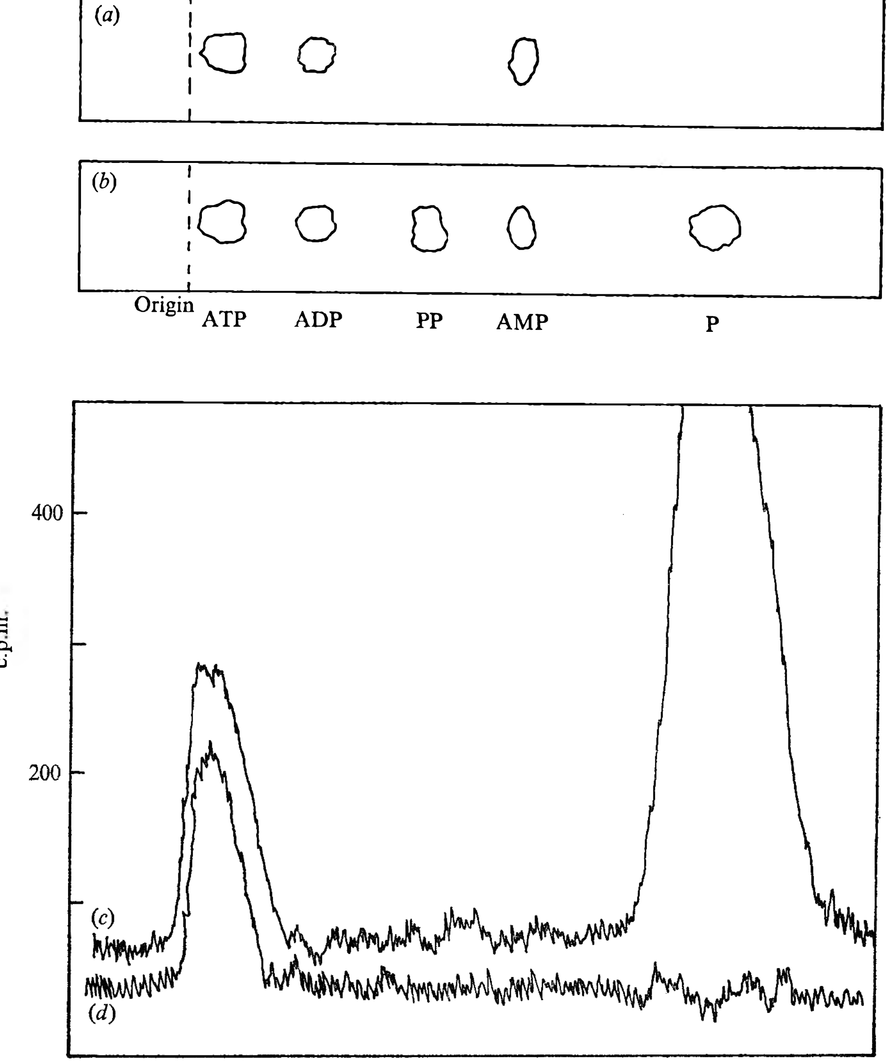

Fig. 6. The separation of [^{32}P]ATP and [^{32}P]inorganic phosphate by paper chromatography. The chromatographic separation of adenosine nucleotides and inorganic phosphate using *n*-propanol + H_2O + trichloroacetic acid + ammonia 22 (75 + 20 + 5 + 0·3) as the developing solvent. The R_F value for inorganic phosphate was 0·65. After developing the chromatogram (*a*) shows the spots seen under u.v. light and (*b*) shows the spots that develop with molybdate spray for phosphates. The radioactive traces were obtained, using an Actiograph III strip scanner (see Methods). Trace (*c*) shows the position of label before extraction of inorganic phosphate as a molybdate complex and trace (*d*) after extraction of inorganic phosphate.

Oxidative phosphorylation. When cell-free preparations of *Anabaena variabilis* were incubated in the dark under the conditions described in Methods for oxidative phosphorylation studies, there was a net accumulation of esterified [^{32}P]phosphate which co-chromatographed with ATP (Fig. 6). The initial rate of ATP synthesis, when extracts were incubated with NADPH, was linear with time (Fig. 7) and corresponded to a rate of $0{\cdot}43 \pm 0{\cdot}06$ nmoles of ATP formed/min./mg. of protein. NADPH was twice as effective as NADH as a source of electrons whose oxidation was linked to phosphorylation. When the potent uncoupling agent FCCP (5 μmoles) was included in the reac-

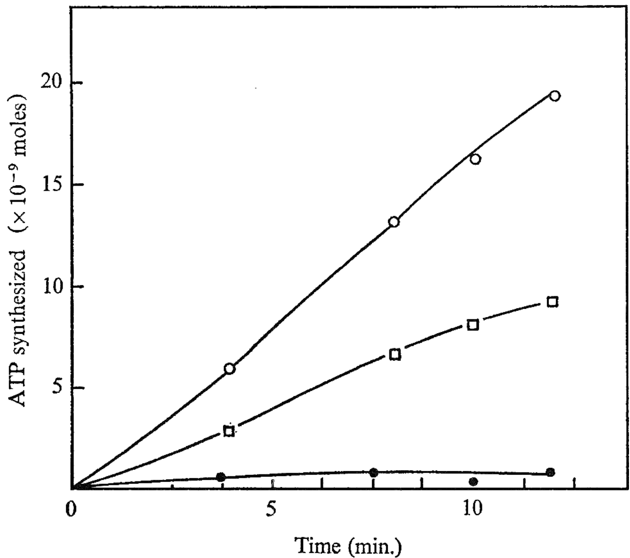

Fig. 7. The time course of synthesis of [^{32}P]ATP concomitant with the oxidation of reduced pyridine nucleotides by a cell-free extract of *Anabaena variabilis*. Reaction mixtures as described in Methods. ○, NADPH; □, NADH; ●, reduced pyridine nucleotides omitted.

tion mixture, phosphorylation was reduced by 25 %. Omission of reduced pyridine nucleotide, or its replacement with oxidized nucleotide, caused a marked decrease in the amount of ATP synthesized, as did the omission of ADP or its replacement with AMP. Anaerobic conditions reduced phosphorylation to less than 4 % of the amount obtained by incubating under aerobic conditions (Leach & Carr, 1969).

When hexokinase and glucose were added to the standard phosphorylation mixture, the total amount of esterified [^{32}P]phosphate remained the same, but chromatographically the radioactive material was recovered in glucose-6-phosphate although some remained as unconverted ATP.

When NADPH was replaced by the oxidized nucleotide and a substrate such as malate, which is known to lead to its reduction in extracts of *Anabaena variabilis*, ATP synthesis could again be demonstrated. However, the levels of phosphorylation were then substantially lower than those obtained with the reduced nucleotides (Table 3). As would be expected, succinate served as a poor source of electrons to support ATP synthesis and was independent of the addition of pyridine nucleotides. Isocitrate or malate, in the presence of $NADP^+$, was about half as effective as the commercial reduced coenzyme. The results recorded in Table 3 are the highest and lowest

of at least three determinations. They show quite a wide difference and this probably reflects the low rate of activity and fragility of the phosphorylating system.

The assay for ATP gave a linear response of light emitted to ATP added between the range of 2×10^{-6} and 10^{-3} μmoles of ATP. Unfortunately, the assay was found unsuitable for ATP estimations in supernatant obtained after arresting phosphorylation by adding trichloroacetic acid or perchlorate (see Methods).

The specific radioactivity of the biosynthesized ATP was $1{\cdot}28 \times 10^{6}$ c.p.m. μmole, compared to the specific activity of $1{\cdot}66 \times 10^{6}$ c.p.m. μmole for the inorganic [^{32}P]-phosphate added; the difference between these two values is probably a reflexion of the presence of a small amount of phosphate in the extract.

Table 3. *Phosphorylation supported by the oxidation of organic substrates in extracts of Anabaena variabilis*

Incubation additions	ATP formed (nmole/min./mg. protein)
Standard (see Methods)	0·35 to 0·48
NADPH replaced by:	
NADH	0·13 to 0·29
NADP	0·006 to 0·01
NADP+succinate	0·06 to 0·08
NAD+succinate	0·07 to 0·09
NADP+malate	0·16 to 0·26
NAD+malate	0·09 to 0·15
NADP+isocitrate	0·16 to 0·33
NAD+isocitrate	0·09 to 0·18
succinate	0·06 to 0·11
malate	0·08 to 0·1
isocitrate	0·05 to 0·09

The highest and lowest results of at least three separate determinations are given.

DISCUSSION

This communication describes some stages in the dark aerobic oxidation of NADPH and NADH and an associated phosphorylation in extracts of *Anabaena variabilis*. The sedimentation properties of NADH oxidase described here and the effects of various inhibitors are comparable to those of another blue-green alga, *Anacystis nidulans* (Horton, 1968). In extracts of the five species of blue-green algae tested, NADPH was oxidized more rapidly than NADH (Leach & Carr, 1968), and NADPH oxidation by extracts of *Anab. variabilis* was less sensitive to inhibitors acting prior to the terminal oxidase. In whole cells of *Anac. nidulans* in the dark, anaerobic conditions lead to an accumulation of NADPH and not NADH (Biggins, 1969).

Biggins (1969) has observed oxidation of reduced cytochrome *c* by extracts of *Anacystis nidulans*, although Horton (1968) did not observe any activity in extracts of *Anac. nidulans* nor *Anabaena variabilis*. Because of its relative insensitivity to cyanide and azide, the terminal oxidase in the respiratory electron chain of *Anab. variabilis* is probably of the cytochrome *o* type, as has been observed in the colourless Cyanophyta (Webster & Hackett, 1965; Biggins & Dietrich, 1968) and in the photosynthetic bacterium *Rhodospirillum rubrum* (Horio & Kamen, 1962). NADH oxidase is more sensitive to inhibitors of electron flow from pyridine nucleotides to quinones or cytochrome *c* than is NADPH oxidase. This suggests that NADH and NADPH are

oxidized by separate pathways up to the cytochrome *c* stage. However, the fact that the rates of the oxidation of NADPH and NADH are not additive indicates that they share some stages of their dark electron transport pathway. The pyridine nucleotide: quinone oxidoreductase was readily detected with NADPH, with either vitamin K_1 or menadione as electron acceptor. *Anab. variabilis* does not contain vitamin K_2 or ubiquinone (Carr *et al.* 1967), which are commonly involved in bacterial respiration. It may be that both photosynthetic electron flow and dark respiratory electron transfer in *Anab. variabilis* share some common components and that this may account for the photo-inhibition of respiration observed in the blue-green algae (Brown & Webster, 1953; Jones & Myers, 1963). This explanation has been advanced by Horio & Kamen (1962) to account for this phenomenon in *Rhodospirillum rubrum*. In an examination of the link between respiration and photosynthetic electron flow in *Anac. nidulans*, Jones & Myers (1963) concluded that competition for electrons exists between chlorophyll *a* and oxygen. The cytological association of the photosynthetic lamellae with reducing activity, detected by potassium tellurite and tetranitro-blue tetrazolium reduction, has been noted in *Nostoc sphaericum* (Bisalputra, Brown & Weier, 1969). Most of the NADPH oxidase activity in our preparations sedimented with the chlorophyll-bearing fragments, but a small portion was soluble. The latter activity, measured as a decrease in extinction at 340 nm., may be due to NADP:ferredoxin diaphorase (E.C. 1.6.99.4) which was easily separated from the photosynthetic lamellae of *Anab. variabilis* (Susor & Krogmann, 1966).

Perhaps the more important question is not whether dark NADH or NADPH oxidation occurs in blue-green algae, but whether this process is linked to phosphorylation. The aerobic formation of ATP in the dark by whole cells of *Anacystis nidulans* was sensitive to the uncoupling agent 2,4-dinitrophenol and to the electron-flow inhibitors amytal and cyanide, but was insensitive to fluorocitrate (Batterton & Van Baalen, 1968). These authors suggest that their observations exclude a fermentative synthesis of ATP in *Anac. nidulans*. Recently Biggins (1969) has shown a reduction in the cellular ATP level in *Anac. nidulans* in the dark when anaerobic conditions were introduced, and he has demonstrated an increase in respiratory rate when the uncoupling agents, FCCP and 2,4-dinitrophenol, were added to intact cells. In a brief communication (Leach & Carr, 1969) the formation of ATP by extracts of *Anabaena variabilis*, concomitant with reduced pyridine nucleotide oxidation, was reported and in this paper these results are extended and include the aerobic formation of ATP in the presence of several substrates and oxidized nucleotides, together with a report of inhibition by the uncoupling agent FCCP. The development of a proton gradient (as occurs in micro-organisms known to effect oxidative phosphorylation) has been observed to follow the oxygenation of a dark suspension of *Anab. variabilis* (Scholes, Mitchell & Moyle, 1969).

The data presented in this paper, together with the whole-cell studies cited above, show that a process of oxidative phosphorylation occurred in the obligately autotrophic *Anabaena variabilis* and suggests its operation in other species of blue-green algae. This phosphorylation was based on a respiratory activity which was low compared with other aerobic procaryotes (e.g. *Bacillus megaterium*, Q_{O_2} of 120 in the presence of glucose; Weibull, 1953) and could not be increased by either exogenous substrates or by growth in the presence of oxidizable substrates (Pearce & Carr, 1967). Possibly this low respiration rate limits the amount of ATP which could be formed.

We suggest that this low level of phosphorylation may have a role in providing the 'energy of maintenance' (Dawes & Ribbons, 1962) under non-photosynthetic conditions, but its contribution to a culture in the light is possibly insignificant if only because of the photoinhibition of respiration demonstrated in this organism.

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Calorimetric Determination of Free Energy Efficiency in *Nitrobacter winogradskyi*

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SUMMARY

The heat evolved by growing Nitrobacter is a measure of its efficiency of free energy utilization which is related to the apparent molar heat of substrate oxidation. The maximum free energy efficiency was about 50% of a total free energy change of -17.5 kcal./mole. The molar growth yield decreased rapidly with the age from 10^7 to 3×10^6 organisms/μmole substrate. The free energy needed for the synthesis of one Nitrobacter varied from 0·45 nanocalories at the onset of growth to 2·9 at later stages. From these values and the bacterial enthalpy content its negentropy content can be calculated.

INTRODUCTION

Calorimetric studies of the growth of heterotrophic micro-organisms have been summarized recently (Forrest, 1970). No calorimetric study of growth of chemo-autotrophic bacteria has been reported. There should be a distinct advantage in using these organisms since the free energy corresponding to the oxidation of 1 mole of substrate is accurately known; for heterotrophs the available free energy has to be estimated as the difference between the free energy of the substrate and that of metabolic products not incorporated into cell substance.

The present study reports a comparison of the calorimetrically measured efficiency of free energy utilization with the molar growth yield of the autotrophic bacterium *Nitrobacter winogradskyi* which uses as sole energy source the oxidation of nitrite to nitrate. Previous studies (Laudelout, Simonart & Van Droogenbroeck, 1968) on the utilization of free energy by that micro-organism were limited to resting suspensions; a difficulty being the high variability of the observed molar heat of substrate oxidation. A similar observation has been made by Poe & Estabrook (1968) while measuring the molar heat of substrate oxidation by mitochondria when the oxidation process occurred through NAD.

METHODS

Nitrobacter winogradskyi (a strain originally received from Professor Engel, Hamburg, West Germany) was grown at various nitrite concentrations in the following nutrient solution: $NaNO_2$, 20 to 100 mM; Na_2HPO_4, 18 mM; KH_2PO_4, 2 mM; $MgSO_4$, 0·04 mM; $ZnSO_4.7H_2O$, 20 μg.; $CuSO_4.7H_2O$, 20 μg.; $Na_2MoO_4.2H_2O$, 20 μg.; $FeSO_4.7H_2O$, 5 mg.; EDTA, 5 mg./l. This solution was sterilized by autoclaving at 121°. The final pH was 7·6. Seven ml. portions were inoculated in the cell of a Calvet microcalorimeter (Calvet & Prat, 1956) with about 1×10^6 bacteria. The lag of the

culture (a few hours) was always longer than the time necessary for thermal equilibration at the calorimeter temperature, i.e. 30°.

The culture was aerated by bubbling sterile air saturated with water vapour at the temperature of the calorimeter. The Calvet instrument is a differential calorimeter with two identical sets of thermocouples mounted around two cells in opposition, so a stream of air was bubbled through the control cell containing only distilled water. The two air streams were balanced with valves of the NUPRO (Cleveland, Ohio, U.S.A.) type before growth started. The heat evolved was recorded until the return of the trace to the base line indicated that the substrate had been completely oxidized. The calorimetric cell was then dismantled, the disappearance of nitrite checked and the number of bacteria counted, first by a direct microscopic method similar to that of Jones & Mollison (1948) and later with a Coulter counter. The model 'FN' of this instrument was used with a 30 nm. aperture, the electrolyte was 1 M-NaCl, the aperture current 1·43 mA. The calibration of the threshold values with respect to the volume of the particles was made with a monodisperse suspension of polystyrene beads with a mean diameter of $1 \cdot 099 \pm 0 \cdot 006$ μm.

To study effects of age without having to sample the growth vessel, cultures of initial nitrite concentrations from 5 to 100 mM were examined. Admittedly the results would not be strictly comparable if the initial substrate concentration influenced the growth process in any way other than by extending the number of possible generations. This, however, does not seem to be the case since the lag was not appreciably influenced by the initial substrate concentration within the limits studied here. Furthermore, end product accumulation slows down the rate of growth to a negligible value only in the upper range of concentrations used here, as evidenced by Fig. 2B. Unless the maintenance energy expended during the growth of Nitrobacter is considerably larger than for heterotrophic bacteria, slowing down the rate of growth due to nitrate accumulation will be without noticeable effect on the number of generations.

Non-bacterial organic matter excreted into the culture solution was estimated as follows: cylindrical culture vessels aerated through a fritted glass bottom plate and containing about 200 ml. culture solution with 30 mM nitrite were inoculated to $1 \cdot 5 \times 10^6$/ml. bacteria. When the initial nitrite had been oxidized, 20 mM more was added. When the nitrite had disappeared, the number of cells/unit volume was counted and a Millipore filtrate was prepared and freeze-dried. Its organic carbon content was determined with a CHN-analyser (Hewlett-Packard). To a parallel culture further additions of nitrite were made until the total amount oxidized was 100 mM. This pulse-feeding technique has the advantage of ensuring rapid and uniform growth of the parallel cultures. This was especially important for the cultures which were observed after the complete usage of 100 mM nitrite. It is unlikely that the pulse-feeding technique vitiates the comparison with the cultures in the calorimeter since determination of non-bacterial organic matter in calorimeter cultures at 100 mM initial concentration gave a value which was comparable to that observed in the larger scale cultures, albeit imprecise due to the small amount of culture solution which could be analysed.

RESULTS

Molar growth yield

The molar growth yield decreased with the initial nitrite concentration and became constant between 50 and 100 mM nitrite at 3×10^6 organisms/μmole nitrite (Fig. 1). The

molar growth yield observed at very low substrate concentrations was not accurate, but extrapolation of the curve of Fig. 1 gives a limiting value of about 10^7 bacteria/μmole. Thus, presumably, at the onset of growth the yield was more than three times higher than at later stages. The average dry weight was $0\cdot9 \times 10^{-13}$ g./bacterium as determined by a gravimetric method on suspensions containing known numbers.

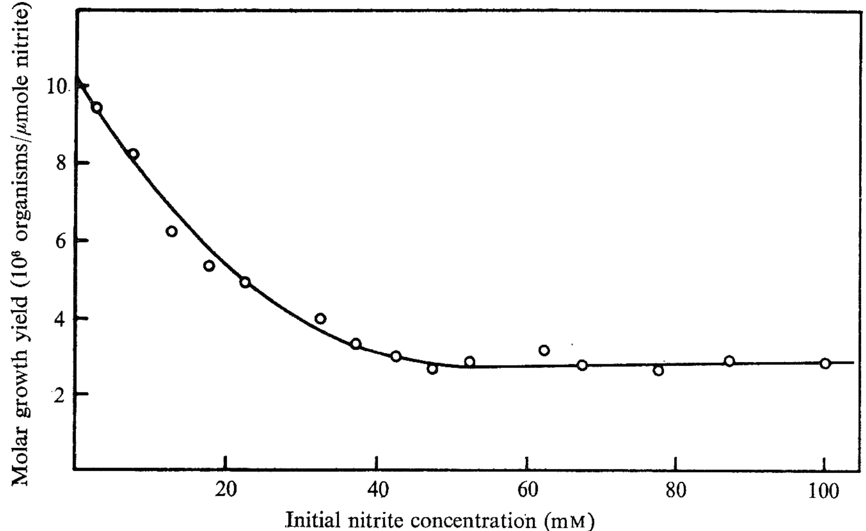

Fig. 1. Molar growth yield of Nitrobacter as a function of the initial substrate concentration in the nutrient solution.

The average volume of the bacteria according to the Coulter counter volume spectra determinations was $0\cdot25$ μm.^{3}, i.e. $2\cdot5 \times 10^{-13}$ g. for a wet bacterium of specific gravity 1. The volume spectrum changed but little during growth and so did presumably the average weight/bacterium. If a bacterium contains 75 to 80% water (Porter, 1946) an average volume of $0\cdot25$ μm.^{3} is equivalent to a dry weight of about $0\cdot7 \times 10^{-13}$ g./bacterium.

The molar growth yield of Nitrobacter cultures thus varied from 700 mg./mole of substrate at the onset of growth to 210 mg./mole at later stages.

Molar heat of substrate oxidation during growth

Fig. 2 shows two recordings of the heat flux for cultures with 24·5 mM and 96·0 mM nitrite. Integration of the curves gives the total heat evolved. At the higher concentration, the rate of heat evolution increased and then remained constant for a few hours. Since the calorimeter registers the rate of heat evolution, the constancy showed that growth had ceased or that the total amount of nitrite oxidase was constant and oxidized the substrate at a constant rate.

Apparent molar heats of nitrite oxidation were calculated from the amount of heat evolved and the amount of nitrite initially present in the calorimeter cell. To simplify discussion, these molar heats have been recalculated as efficiency of free energy utilization according to the relation:

$$\text{Efficiency of free energy utilization} = \frac{\Delta H_0 - \Delta Q}{\Delta G_0} \times 100\,\%,$$

where ΔQ is the observed molar heat of substrate oxidation expressed, by convention, as a negative value in kcal./mole, $\Delta H_0 = -24{\cdot}2$ kcal./mole and $\Delta G_0 = -17{\cdot}8$ kcal./mole for $NO_2' \rightarrow NO_3'$ (Fig. 3). No accurate estimate of the free energy utilization could be obtained below an initial substrate concentration of 20 mM. The extrapolated value to zero initial concentration, i.e. 26 %, is thus more unreliable than that obtained from Fig. 3.

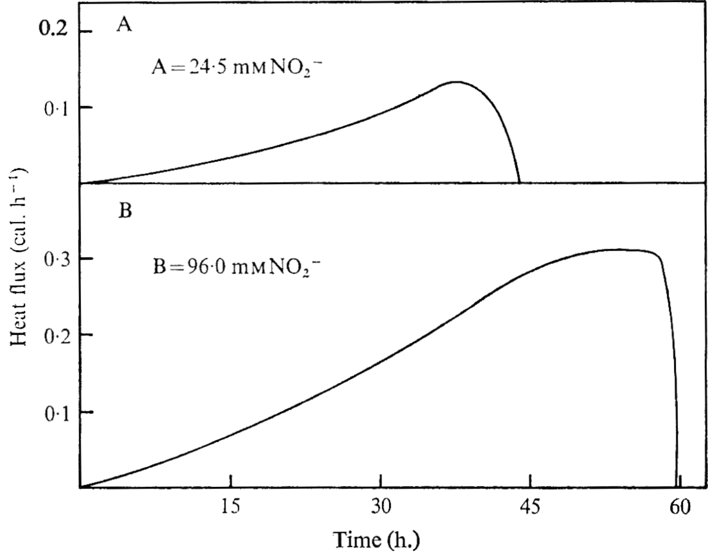

Fig. 2. Rate of heat flow from growing cultures of Nitrobacter for two cultures (7 ml.) at different initial nitrite concentrations.

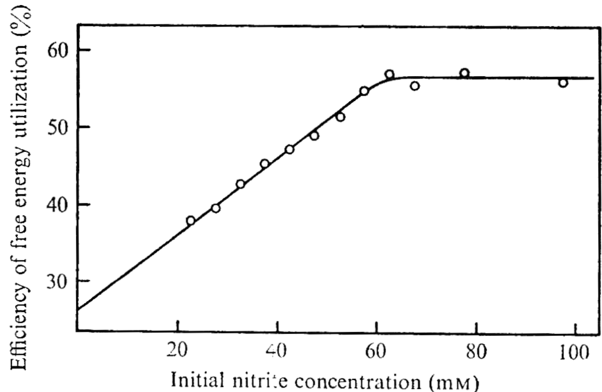

Fig. 3. Efficiency of free energy utilization calculated from the molar heat of substrate oxidation against initial concentration of nitrite.

Production of extracellular organic matter during growth

Table 1 shows that a large fraction of the CO_2 fixed by Nitrobacter appeared as extracellular carbon: 50 % after the oxidation of 50 mM nitrite and 59 % after the oxidation of 100 mM. The yield of extracellular C became practically constant after

50 mmoles/l. had been oxidized. This observation merely confirms the more detailed results shown in Fig. 3 on the constancy of the free energy utilization between 50 and 100 mM initial nitrite concentration.

Table 1. *CO_2 fixation by a Nitrobacter culture after oxidation of a given amount of nitrite*

Amount of nitrite oxidized µmole/ml.	Bacterial density in		Extracellular C (µg./ml.)	Total CO_2 reduced as C(µg./ml.)
	Numbers (10^6/ml.)	Weight (µg.C/ml.)		
0	1·5	—	0	—
50	177	6·2	6·3	12·5
100	308	10·8	15·8	26·6

DISCUSSION

In cultures which have oxidized 50 mM nitrite we may calculate a free energy balance as follows: free energy not dissipated as heat (from calorimetric determination of the molar heat of substrate oxidation) = 10 kcal.; free energy content of extracellular carbon = 1·3 kcal. For the latter figure we assume that the free energy of compounds excreted into the culture medium is about 100 kcal./mole of C. There remains synthesis of 3×10^{12} cells per mole of substrate 8·7 kcal., equivalent to $2{\cdot}9 \times 10^{-9}$ cal./bacterium for its free energy content. Since the enthalpy change for the combustion of the cell material is about -110 kcal./mole of carbon (the percentage of which is 50 %) the heat of combustion/cell will be $-0{\cdot}3 \times 10^{-9}$ cal. The changes corresponding to the synthesis of one bacterium from CO_2, water and minerals are: $\Delta G = 2{\cdot}9 \times 10^{-9}$ cal., $\Delta H = 0{\cdot}3 \times 10^{-9}$ cal., $T\Delta S = -2{\cdot}6 \times 10^{-9}$ cal. This endergonic reaction is made possible by being coupled to nitrite oxidation, the efficiency of the coupling process being about 50 % in the later stages of the culture.

The calculation above is not unlike that presented by Linschitz (1953) for the information content of a bacterium, the upper limit of which was 10^{13} bits. About half that value (4×10^{12}) may be calculated from the results presented above.

At the onset of growth, more free energy was dissipated as heat and three times as many bacteria were formed in comparison with the later stages of growth, the free energy content of a bacterium was then noticeably lower, i.e. $0{\cdot}45 \times 10^{-9}$ cal./cell. It is unlikely that the enthalpy content was much lower at the onset of growth since volume and composition showed no dramatic difference with respect to later stages. The lesser information content of cells at the onset of growth may be correlated with a smaller degree of differentiation of the organelles, especially the membrane system.

A comparison may also be made between the free energy content of a Nitrobacter and the expenditure of free energy necessary for obtaining a heterotrophic bacterium from preformed monomers. The so-called Y_{ATP} value affords the easiest way for making such a comparison. One mole of ATP leads to the synthesis of very nearly 10 g. bacteria (see Stouthamer, 1969). This is equivalent to about 0·1 nanocal./organism for the upper limit of the difference between the free energy of a bacterium and that of its monomers. The free energy of the monomers entering into the synthesis of a Nitrobacter cannot be very different from its enthalpy of combustion of 0·3 nanocal. (above). Consequently, the free energy content of one Nitrobacter is $0{\cdot}45 - 0{\cdot}3 = 0{\cdot}15$

nanocal. when the expenditure of free energy for synthesis of its monomers is disregarded. It is then in good agreement with that of a heterotrophic bacterium at the onset of its growth but becomes larger at later stages.

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Decomposition of Cysteic Acid and Taurine by Soil Micro-organisms

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SUMMARY

Streptomycetes and aerobic bacteria able to utilize cysteic acid and taurine as sole sources of energy, nitrogen and sulphur were isolated from soil materials. None of the isolated fungi did this. The bacterium which was used in most experiments grew on both compounds. The sulphur of the compounds was recovered as sulphate and most of the nitrogen as ammonia after breakdown by growing or pregrown organisms. A significant portion of the nitrogen was assimilated by the growing organisms. Sulphite appeared as a transitory sulphur product. Deamination preceded desulphuration in early periods of incubation; the reactions were brought about by adaptive enzyme systems. Dissimilation of cysteic acid by pregrown organisms was maximum at reactions close to neutrality. During development of pregrown organisms at pH 8·5 and 9·0 appreciable amounts of sulphite were detected. Acid reactions which inhibited development of the cultures were produced during decomposition of taurine.

INTRODUCTION

Cysteic acid and taurine both occur in animals and taurine has been detected in some plants (Jacobsen & Smith, 1968). One or both of these sulphonates can serve as the sole source of sulphur for some micro-organisms (Steinberg, 1941; Roberts, Abelson, Cowie, Bolton & Britton, 1955; Margolis & Block, 1958; Braun & Fromageot, 1962). Ikeda, Yamada & Tanaka (1963) reported that an Agrobacterium species could use taurine as its principal source of both carbon and nitrogen. Taurine was used as a source of nitrogen by five of 13 bacteria tested by Den Dooren de Jong (1926), but it did not support growth of any of the cultures. With complete breakdown of taurine by the Agrobacterium species most of the nitrogen and sulphur of the compounds was released as ammonia and inorganic sulphate (Ikeda *et al.* 1963). Braun & Fromageot (1962) reported that *Aspergillus niger* converted cysteic acid to taurine which was deaminated to form isethionic acid, which in turn was broken to yield sulphate. Garreau (1941) noted that ammonia and sulphate were produced from taurine, but not from cysteic acid by *A. niger*, whereas others (Obata & Ishikawa, 1954) stated that no sulphate was produced from either cysteic acid or taurine by this fungus or three other aspergilli.

Frederick, Starkey & Segal (1957) observed that in soil, 50% of the sulphur of

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taurine was oxidized to sulphate in one week, and in a medium, inoculated with soil suspension, which contained taurine as the only organic compound, 65 % of the taurine sulphur appeared as sulphate in 2 weeks and 100 % in 6 weeks. The conversion of cysteic acid in some animals was ascribed to its decarboxylation followed by release and oxidation of the sulphur of the resulting taurine to sulphate by micro-organisms inhabiting the intestinal tract (White, Lewis & White, 1937; Schram & Crokaert, 1957; Block & Henry, 1961; Henry & Block, 1961; Boquet & Fromageot, 1965). Another type of transformation is suggested by the results of Sumizu (1962), who found that cysteinesulphinic acid was desulphurated to alanine and sulphite by an extract of rat liver. The present report is concerned with the decomposition of cysteic acid and taurine by micro-organisms isolated from soil, the course of breakdown by growing cultures and pregrown organisms, and the effects of some cultural factors on the transformations.

METHODS

Cultural. Pure cultures of micro-organisms were isolated from the following soil-inoculated medium used in 100 ml. amounts in 250 ml. Erlenmeyer flasks: cysteic acid or taurine, 5 g.; K_2HPO_4, 2·0 g.; KH_2PO_4, 1·0 g.; $CaCl_2.2H_2O$, 0·05 g.; $MgCl_2.2H_2O$, 0·05 g.; $FeCl_3.6H_2O$, 0·01 g.; distilled water to 1000 ml. After adding the cysteic acid or taurine the media were adjusted generally to pH 7·0. In pure culture studies the taurine medium contained 0·0004 % bromthymol blue. Periodically during incubation sterile NaOH was added to the cultures to neutralize the reaction. The cultures were incubated at 28° on a rotary shaker (240 to 280 cycles/min.). Pregrown organisms consisted of organisms from a 24 h. culture which had been centrifuged and the deposit washed. The recovered organisms were added to test media to provide 1×10^{12} organisms/ml. Unadapted organisms were those grown on nutrient broth. Adapted organisms had been grown in media containing cysteic acid or taurine for at least 20 h.

Analytical. Sulphate was determined as benzidine sulphate (Kahn & Goodridge, 1926). Sulphite was determined by iodine titration, its identity being confirmed by the blocking effect of formaldehyde on the reaction with iodine. Ammonia was determined by Nesslerization, and amino nitrogen by the method of Danielson (1933) as modified by Hotchkiss (1956), with correction for ammonia nitrogen in the solutions.

RESULTS

Breakdown by mixed microbial populations

Flasks of the cysteic acid and taurine media with initial reactions of pH 4·0 and 7·0 were inoculated with dilutions of three soil materials and incubated for 10 days, after which the sulphate content and pH values were determined. Table 1 shows that the reaction of all the inoculated taurine culture solutions had become strongly acid, but that little or no sulphate was produced from taurine in the medium which was initially acid, and that about 30 % of the taurine sulphur was released as sulphate in the solutions which were initially at pH 7·0. There was little change in the reaction of the cysteic acid media and appreciable amounts of sulphate were produced in only one of the culture solutions.

Transformation by growing cultures

Many cultures of aerobic micro-organisms isolated from the enrichment media were tested for their ability to utilize cysteic acid or taurine as sole source of organic matter, sulphur and nitrogen. Several bacteria and streptomycetes but none of the isolated fungi were able to do this. More of the cultures survived in the cysteic acid medium than in the taurine medium. The destructive effect of the latter is ascribed to the acid product of taurine breakdown; even when the initial pH value was neutral it became below pH 3·0 in a few days. Three of the isolated cultures were selected for further study. One

Table 1. *Breakdown of taurine and cysteic acid by mixed soil populations*

The culture media containing cysteic acid or taurine and inoculated with soil suspensions were tested for changes in reaction and sulphate production after incubation for 10 days.

Inoculum	Organic compound	pH values: Initial	pH values: Final	Sulphate S, % of initial organic S
Compost	Cysteic acid	4·0	3·7	7
Barnyard soil	Cysteic acid	4·0	4·5	1
Pine forest soil	Cysteic acid	4·0	3·5	8
Compost	Cysteic acid	7·0	6·5	93
Barnyard soil	Cysteic acid	7·0	6·7	4
Pine forest soil	Cysteic acid	7·0	6·6	1
Compost	Taurine	4·0	2·8	3
Barnyard soil	Taurine	4·0	3·0	0
Pine forest soil	Taurine	4·0	2·7	7
Compost	Taurine	7·0	2·8	30
Barnyard soil	Taurine	7·0	3·8	25
Pine forest soil	Taurine	7·0	2·7	33

Table 2. *Deamination and desulphuration of cysteic acid and taurine by growing cultures*

The cultures were grown in media containing 0·5 % cysteic acid or taurine. Deamination and desulphuration were determined from amounts of ammonia and sulphate produced, respectively.

Days	Culture	Cysteic acid: Deamination (%)	Cysteic acid: Desulphuration (%)	Taurine: Deamination (%)	Taurine: Desulphuration (%)
1	B14	4	0	0	0
2	B14	21	7	5	1
3	B14	95	85	17	12
4	B14	97	99	41	41
6	B14	—	—	84	83
10	B14	—	—	86	90
3	S15	2	1	—	—
5	S15	23	17	—	—
6	S15	38	28	—	—
7	S15	92	82	—	—
9	S15	98	91	—	—
7	B17	—	—	10	2
12	B17	—	—	19	11
14	B17	—	—	54	54

of these, bacterium B 14 (*Pseudomonas* sp.), grew in media which contained either cysteic acid or taurine as sole organic constituent and source of sulphur and nitrogen. Bacterium B 17 grew at the expense of taurine but not of cysteic acid; streptomycete S 15 grew on cysteic acid but not on taurine. In the following experiments the reaction of the taurine medium was held close to neutrality by adding NaOH periodically. There was little change in pH value of the cysteic acid culture solutions.

Nearly all of the cysteic acid was deaminated and its sulphur converted to sulphite and sulphate by organisms B 14 and S 15 in 4 to 9 days (Table 2). As much as 10 % of the cysteic acid sulphur appeared as sulphite during the period of most rapid growth of the streptomycete (5 to 7 days) and it disappeared subsequently. The amount of sulphite even exceeded that of sulphate at one test period. Deamination and desulphuration of taurine by organisms B 14 were nearly complete in 10 days, but were considerably slower with organism B 17. Deamination was greater than desulphuration by all cultures during the first few days of incubation. Comparison of the values for amino nitrogen and ammonia showed that the amount of deamination exceeded that of the ammonia released during the periods of most rapid growth. The difference is ascribed to assimilation of part of the nitrogen.

Table 3. *Deamination and desulphuration of cysteic acid and taurine by pregrown organism B 14 (Pseudomonas sp.)*

100 ml. portions of media contained 0·5 % cysteic acid or taurine and 1×10^{12} washed organisms. After incubation the media were tested for ammonia and sulphate as indices of deamination and desulphuration, respectively.

		Percent of substrate deaminated by organisms pregrown on following media			Percent of substrate desulphurated by organisms pregrown on following media		
Hours	Substrate	Nutrient broth (%)	Cysteic acid (%)	Taurine (%)	Nutrient broth (%)	Cysteic acid (%)	Taurine (%)
4	Cysteic acid	—	12	26	—	0	0
7		—	21	34	—	4	1
19		35	41	48	2	27	24
28		38	51	55	6	39	44
43		39	68	89	15	61	77
67		93	84	98	92	87	100
4	Taurine	—	—	16	—	—	1
7		—	—	18	—	—	4
19		7	1	35	0	1	25
28		9	2	55	0	0	44
43		12	16	76	1	8	76
67		17	37	94	6	29	97
92		35	68	96	31	62	100

Transformation by pregrown organisms

In the following experiments, only organism B 14 was used. As in the growth experiments, cysteic acid and taurine were broken down, deamination was greater than desulphuration during early incubation periods (Table 3), and loss of amino nitrogen exceeded production of ammonia. Organisms pregrown on cysteic acid or taurine attacked cysteic acid rapidly, and those grown on taurine decomposed taurine at the same rate, but there was a delayed attack of taurine by organisms grown on cysteic

acid. Both compounds were decomposed more slowly by organisms pregrown on nutrient broth, and desulphuration by these organisms was slower than deamination.

The following observations provide additional evidence that the dissimilations were brought about by adaptive enzyme systems. Chloramphenicol at 10 μg./ml. and higher inhibited growth of organism B14 on cysteic acid and greatly decreased the deamination and desulphuration by pregrown non-adapted organisms (Table 4). Whereas dissimilation of cysteic acid by similarly prepared organisms was completely suppressed by 20 μg./ml. chloramphenicol in the medium at the time the organisms were added, there was no inhibitory effect when the chloramphenicol was added 3 h. after the organisms had been introduced into the medium (Table 5). Chloramphenicol had little effect on either deamination or desulphuration of cysteic acid by adapted organisms; at 100 μg./ml. deamination was decreased only 24% and desulphuration 59%. Cysteic acid-adapted organisms attacked not only cysteic acid but also taurine in the presence of 40 μg. chloramphenicol/ml.; taurine-adapted organisms decomposed taurine but not cysteic acid under the same conditions.

Table 4. *Inhibitory effect of chloramphenicol on dissimilation of cysteic acid by non-adapted organism B14 (Pseudomonas sp.)*

100 ml. portions of media contained 0·5% cysteic acid, 1×10^{12} washed organisms and varying amounts of chloramphenicol. After incubation for 48 h., the media were tested for ammonia and sulphate as indices of deamination and desulphuration, respectively.

Chloramphenicol (μg./ml.)	Deamination (%)	Desulphuration (%)
0	76	74
1	71	61
5	50	36
10	36	18
20	13	5

Table 5. *Effect on dissimilation of cysteic acid of chloramphenicol added at different periods after introducing unadapted organism B14 (Pseudomonas sp.) to the substrate*

100 ml. portions of media contained 0·5% cysteic acid and 1×10^{12} washed organisms unadapted to the substrate. Chloramphenicol to provide 20 μg./ml. was added immediately or 3 or 6 h. later. Determinations for ammonia and sulphate as indices of deamination and desulphuration respectively were made 50 h. after adding the chloramphenicol.

Time before addition of chloramphenicol (h.)	Deamination (%)	Desulphuration (%)
0	10	2
3	95	92
6	94	94

Deamination and desulphuration of cysteic acid by pregrown organisms were maximum in media with initial reactions close to neutrality (Fig. 1). After incubation for 5 and 10 h. there was more deamination than desulphuration, but after 32 h. both transformations were close to 100% in media which had initial values above pH 6·5. Sulphite was detected in media at all reactions except pH 5·0, and it occurred in appreciable amounts only in those with initial reactions above pH 8·0 at the 5 and 10 h. periods. Sulphite had disappeared from all media in 32 h. Organisms added to a

mineral salts solution containing sulphite partially protected the sulphite against oxidation at pH 9·0; 24 % of the sulphite remained in the agitated solution incubated at 28° for 5 h., whereas all sulphite was oxidized in the absence of organisms. Cysteic acid was broken down by organism B14 only under aerobic conditions. Neither sulphate nor ammonia was produced in 22 h. by pregrown organisms suspended in media which completely filled the culture flasks. Furthermore, only small amounts were produced in 50 ml. portions of medium in 250 ml. flasks incubated without shaking, whereas practically all of the nitrogen and sulphur was released in 100 ml. portions of mechanically shaken medium.

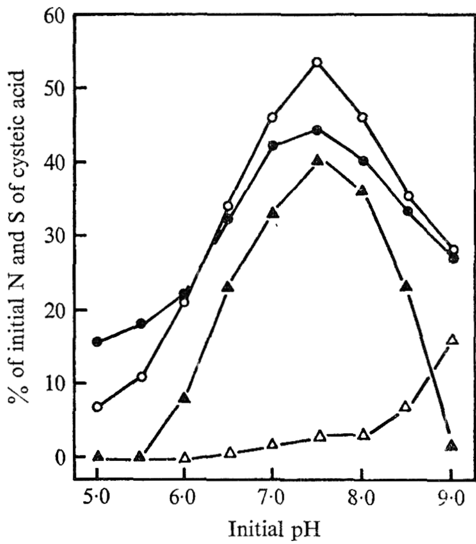

Fig. 1. Influence of pH value on the transformation of nitrogen and sulphur of cysteic acid by organism B14. Incubation period 10 h. Symbols: ○, loss of amino nitrogen; ●, nitrogen recovered as ammonia; △, sulphur recovered as sulphite; ▲, sulphur recovered as sulphate.

DISCUSSION

Bacteria and streptomycetes isolated from soil utilized cysteic acid and taurine as their sole sources of sulphur, nitrogen and energy for growth, but none of the fungi did so. Fungi studied by Braun & Fromageot (1962) did not use taurine as a carbon source but did use it as a source of sulphur. Deamination of both compounds exceeded the amount of desulphuration during the early periods of their breakdown. This indicates that deamination preceded desulphuration, a conclusion reached by Ikeda *et al.* (1963) for decomposition of taurine by *Achromobacter* sp. The results of Kearney & Singer (1953) led to the same conclusion for transformation of cysteic acid by washed *Proteus vulgaris*. The observation by Braun & Fromageot (1962) that isethionic acid is an intermediate in the transformation of cysteic acid and taurine could explain this effect. They noted that *Aspergillus niger* decarboxylated cysteic acid to taurine which was deaminated to isethionic acid, which, in turn, was desulphurated with release of sulphate. However, the bacterial extract of Ikeda *et al.* (1963) which deaminated and desulphurated taurine, did not liberate sulphate from isethionic acid, and this compound did not promote oxygen uptake by the extract.

Sulphite appeared as a transitory product of desulphuration of cysteic acid when the medium was distinctly alkaline, it was not detected as a breakdown product of taurine. However, sulphite is readily oxidized chemically at acid reactions, especially in the presence of iron (Heimberg, Fridovich & Handler, 1953; Fridovich & Handler, 1957); the transformation of taurine was not studied at alkaline reactions.

There is still uncertainty about the course of events in the dissimilation of cysteic acid by micro-organisms, and there is evidence that the events may not be the same in all cases. According to prevailing opinion the first step in breakdown of cysteic acid by animal tissues and micro-organisms is decarboxylation to taurine. Results with organism B 14 pregrown on cysteic acid, taurine and nutrient solution in the presence and absence of chloramphenicol indicate that this was the initial transformation of cysteic acid by this organism. However, taurine was an unlikely intermediate of breakdown of cysteic acid by Streptomyces S 15 which grew on cysteic acid but not on taurine. Instead of decarboxylation, the initial reaction by this Streptomyces S 15 may have been deamination by transamination. Such reactions have been noted for enzyme systems of muscle tissue whereby cysteic acid underwent transamination with α-ketoglutarate or oxaloacetate to produce sulphonylpyruvate and glutamate or aspartate (Braunstein, 1939; Cohen, 1940; Darling , 1952; Kearney & Singer, 1953). Whether ammonia was released by deamination of taurine or of the amino acids produced by the transamination reaction, the evidence indicates that deamination preceded desulphuration.

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The Genetic Relatedness of Proteolytic *Clostridium botulinum* Strains

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SUMMARY

DNA binding and competition tests indicate that the species *Clostridium botulinum* is composed of three groups with only 4 to 18 % DNA homology with each other. One group includes the proteolytic *C. botulinum* and *C. sporogenes* strains. The DNA isolated from seven proteolytic *C. botulinum* A, B, F, and six *C. sporogenes* strains was 50 to 100 % homologous to the *C. botulinum* A62 DNA. *Clostridium botulinum* C and D strains form another group. *Clostridium botulinum* C8613 and D8625 strains have 38 % homology with the *C. botulinum* C573 strain. The last group comprises the non-proteolytic *C. botulinum* B, E, and F strains reported previously. All the *C. botulinum* strains tested were related to only one of the three groups and no intermediate strain was detected.

INTRODUCTION

The relatedness of bacteria can be measured by phage infectivity, genetic transfer mechanisms and also by the degree of common DNA base sequence. Jones & Sneath (1970) reviewed all these criteria with regard to bacterial taxonomy. Until now *Clostridium botulinum* genetics has not been developed and the relatedness of various toxic and non-toxic Clostridium strains is open to question. Smith & Holdeman (1968) divided *C. botulinum* into three related groups, I, II, and III, on the basis of physiological and serological characteristics. Group I includes the proteolytic *C. botulinum* toxin types A, B and F strains. Group II includes the non-proteolytic *C. botulinum* toxin types B, E and F strains. Group III includes the *C. botulinum* toxin types C and D strains.

In our previous paper (Lee & Riemann, 1970) the relatedness of various toxic and non-toxic strains of the group II of *Clostridium botulinum* was studied. We found that the *C. botulinum* strains have very similar guanosine + cytosine (GC) content (28 to 29 %) but they could be distinguished by DNA homology and competition. Kindler, Mager & Grossowicz (1956) stated that the nutritional and biochemical requirements of *C. sporogenes* were indistinguishable from the proteolytic *C. botulinum* strains. In this paper the relatedness of toxic *C. botulinum* and *C. sporogenes* was tested. We also tested the relatedness of three *C. botulinum* C and D strains and the relatedness between the three groups of *C. botulinum*.

METHODS

Cultures. The sources of many of the cultures have been described (Lee & Riemann, 1970). Additional strains of *Clostridium botulinum* A79, B213, B13983, and *C. sporogenes* PA3679 were received from K. Ito (National Canners Association, Berkeley, California). *Clostridium botulinum* C8613 was obtained from W. P. Segner (Continental Can Co., Chicago, Illinois) and *C. botulinum* D (NCTC8265) was supplied by the National Collection of Type Cultures (Central Public Health Laboratory, Colindale, London, N.W. 9). *Clostridium sporogenes* 175 was obtained from L. S. McClung (Indiana University, Bloomington, Indiana) and *C. sporogenes* SC4131 and SC4679 were obtained from L. DS. Smith (Virginia Polytechnic Institute, Blacksburg, Virginia). *Clostridium sporogenes* 10-1026 was supplied by V. R. Dowell (Anaerobic Bacteriology Laboratory, National Communicable Disease Center, Atlanta, Georgia). All the cultures received were re-isolated by streaking on blood agar and all the toxic isolates typed with specific botulinum toxin antiserum before they were grown for DNA isolation.

Medium and growth. Cells for DNA isolation were grown in the proteose-peptone and yeast extract (Difco) medium under conditions described (Lee & Riemann, 1970). *Clostridium botulinum* C8613 and D8265 grew well in the above medium provided that the inoculum was grown in a fresh liver infusion (clear infusion from 500 g. liver boiled in 1000 ml. H_2O, pH 8·5 with NaOH plus 10 g. Tryptose (Difco), 1 g. soluble starch, 1 g. K_2HPO_4, 1·4 g. $NaHCO_3$ and 0·5 ml. mercaptoethanol). Radioactive DNA of *C. botulinum* A62 was prepared from cells grown in 450 ml. Edamin S medium without Triton X155 (Lee & Riemann, 1970) and with 1·75 mC of [^{3}H]thymidine added. *Clostridium botulinum* C573 did not grown in Edamin S medium. The radioactive DNA of *C. botulinum* C573 was obtained from cells grown in 200 ml. of trypticase medium (50 g./l. trypticase (BBL), 1 g./l. soluble starch, 1·4 g./l. $NaHCO_3$, 0·25 ml./l. Triton X155, 1 mg./l. resazurin, 0·5 ml. mercaptoethanol, pH 7·2 with KOH). Growth was initiated by adding 3 ml. of inoculum grown in fresh liver infusion for 16 h. at 37°. After 3 h. of visible growth, 1 mCi of [^{3}H]thymidine and 1 mCi of [^{3}H]-adenosine were added, and the cells harvested 2 h. later. Strict anaerobic technique (Cato *et al.* 1969), such as the use of inert gas to exclude air and prereduced medium, was necessary for the good growth of the C and D strains.

DNA isolation. Lysis and DNA isolation from *Clostridium botulinum* C and D strains were difficult and variable. *Clostridium botulinum* D8265 was treated with 50 μg./ml. of penicillin G and D-cycloserine 2 h. before harvest, and the cells were lysed in ice cold tris-SDS buffer (0·1 M-tris (hydroxymethyl) aminomethane, 1 % Na dodecyl sulphate, (SDS) 0·1 M-NaCl, pH 9·0 with HCl). The DNA of *C. botulinum* C8613 was obtained by grinding phenol-treated cells with alumina in tris-SDS buffer. The radioactive DNA of the *C. botulinum* C573 was obtained from cells treated with 1 mg./ml. of Pronase and lysozyme (Calbiochem, Los Angeles, California) in saline-EDTA buffer (NaCl 0·15 M, Na_2 ethylenediaminetetra-acetate (Na_2 EDTA) 0·1 M, pH 9·0) for 30 min. at 37° followed by addition of 1 % SDS. The proteolytic *C. botulinum* and *C. sporogenes* strains always lysed by the above Pronase and lysozyme treatment and the DNA recovery was good.

The DNA was purified by the procedure of Miura (1967) including the isopropanol precipitation step. *Clostridium perfringens* DNA was purchased from Worthington

Biochemical Corporation, Freehold, New Jersey. *Escherichia coli* DNA was a gift of P. Packnaud.

Analytical methods. DNA assay, binding, competition, T_m and thermal denaturation procedures have been described (Lee & Riemann, 1970) but certain modifications were introduced. DNA binding and competition were assayed by a modified method of Denhardt's (1966). Thirty μg. of DNA were applied to each 25 mm. Millipore HA filter. A length of 0·75 inch solid glass rod was inserted into the 1 ml. vial to reduce the liquid volume to 0·5 ml. and increase binding (Johnson & Ordal, 1968). In the binding experiment, 0·45 ml. of preincubation mixture (PM) (Denhardt, 1966) and 0·05 ml. of 0·1 × SSC (NaCl 0·15 M, sodium citrate 0·015 M, pH 7·2) containing the radioactive DNA were added to each vial. Two μg. (8000 c.p.m.) of *Clostridium botulinum* A62 DNA, or 2 μg. (8800 c.p.m.) of *C. botulinum* E MINN, or 0·3 μg. (25,000 c.p.m.) of *C. botulinum* C573 were added to each vial. In the competition experiment, 0·5 ml. of PM was removed after preincubation, and 0·28 ml. 0·1 × SSC containing 250 μg. unlabelled, 1 μg. homologous radioactive *C. botulinum* A62 DNA together with 0·12 ml. of 10 × SSC was added to each vial. *Clostridium botulinum* C573 competition was carried out with 250 μg. of unlabelled DNA and 0·3 μg. of homologous radioactive C573 DNA. Seven replicates were used in the binding experiments but each competition experiment was made only once or twice due to a limited amount of DNA available. Incubation was carried out at 66° (T_m 81°) for 12 h.

RESULTS

DNA binding and competition. The optimum conditions for DNA duplex formation on filters were tested. Maximum binding (c.p.m.) or saturation of the filter was reached when 15 μg. of *Clostridium botulinum* A62 or A78 DNA was applied to each filter. To be safe, 30 μg. of DNA was used. DNA duplex formation was directly proportional to the amount of radioactive *C. botulinum* A62 DNA added to the solution up to 3 μg. level. [^{3}H]thymidine uptake by *C. botulinum* A62 was very poor and was not enhanced by the addition of Triton X155 detergent. The best preparation contained only 4000 c.p.m./μg. of DNA. The DNA duplex of *C. botulinum* on filters was stable and showed a thermal denaturation curve similar to that of the native DNA. The T_m of the radioactive duplex on filters was 76·6° as compared to the T_m of 81° of the native DNA.

Results of percent DNA binding and competition of 28 strains relative to the *Clostridium botulinum* A62 DNA are presented in Table 1. The average of A62 homologous DNA binding was 4150 c.p.m., and the standard deviation of each set was 5 to 26%. In competition experiments the binding was reduced from 3940 to 1130 c.p.m. with 250 μg. of unlabelled homologous A62 DNA.

Maximum binding was achieved when 30 μg. of *Clostridium botulinum* C573 or D8265 DNA was applied to each filter, but 60 μg. of C8613 DNA was used. Very little (40 μg.) of radioactive *C. botulinum* C573 DNA was isolated because of extensive degradation of the DNA during lysis of the cells. The amount of DNA recovered was just adequate for the experiment presented in Table 2. The average count of seven replicates of C573 homologous DNA binding was 3633 ± 923 c.p.m. The binding was reduced from 5030 to 1060 c.p.m. with 250 μg. of unlabelled homologous C573 DNA in competition experiments.

Optimum conditions for binding with *Clostridium botulinum* E MINN were tested previously (Lee & Riemann, 1970). The relatedness of the C and D strains with the E MINN strains is presented in Table 3.

Table 1. *Percent binding and competition of DNA of various strains relative to [³H]DNA of Clostridium botulinum A62*

Group	Toxic strains		Source	Binding (%)	Competition (%)
I	*C. botulinum*	A62	Cow, Nevada, U.S.A.	100	100
I		A78	Spinach, U.S.A.	100	100
I		A79	Berlin, no. 95	74	—
I		B32	Horse	83	41
I		B213	Onions, U.S.A.	69	—
I		B13983	Asparagus, U.S.A.	85	—
II		B17	Pacific mud, U.S.A.	8	0
III		C573	Horse, France	5	0
III		C8613	Marine mud, Maryland, U.S.A.	12	0
III		D8265	South Africa	13	0
II		E DETROIT	Fish, Great Lakes, U.S.A.	9	0
II		E MINNESOTA	Fish, Great Lakes, U.S.A.	6	13
II		E3324/61	Fish, Baltic Sea	6	—
II		F202	Pacific mud, U.S.A.	15	0
I		F LANGELAND	Liver, Denmark	100	100
	Non-toxic strains				
I	*C. sporogenes*	PA3679	U.S.A.	100	90
I		175	U.S.A.	100	51
I		213	U.S.A.	86	80
I		SC4131	Goat, U.S.A.	52	—
I		SC4679	Chicken, U.S.A.	60	—
I		10-1026	Lucheon meat, U.S.A.	48	—
II		S5 (E BOTICIN)	Mud, Great Lakes, U.S.A.	4	0
II		28-2 (E BOTICIN)	Mud, Great Lakes, U.S.A.	5	—
II		24NT	North Sea	10	—
II		26NT	North Sea	4	—
		OS/EI	Toxic E culture	5	—
		OS/MINN	Toxic E culture	7	—
	C. perfringens		—	18	0
	E. coli		—	12	—

Table 2. *Percent binding and competition of DNA from various strains relative to [³H]DNA of Clostridium botulinum C573 and their GC contents*

Group	Strains		GC (%)	Binding (%)	Competition (%)
III	*C. botulinum*	C573	27·6	100	100
III		C8613	27·8	38	34
III		D8265	27·2	39	35
I		A62	28·5*	18	—
II		E MINN	27·9*	5	—
	C. perfringens		—	24	—

* Reported previously.

Table 3. *Percent binding of Clostridium botulinum* C *and* D *strains relative to* [^{3}*H*]*DNA of Clostridium botulinum* E MINN

Group	Strains		Binding (%)
II	*C. botulinum*	E MINN	100
III		C573	11
III		C8613	12
III		D8265	11

DISCUSSION

The above results confirm the observations of Kindler *et al.* (1956) that *Clostridium sporogenes* and the proteolytic group I or *C. botulinum* are genetically related. Three of the *C. sporogenes* strains tested have 86 to 100% common base sequence with the *C. botulinum* A62 strain and the results of competition experiments generally confirm the DNA binding data (Table 1). We will attempt to determine if there are measurable DNA binding error or differences between these highly related *C. sporogenes* and *C. botulinum* strains. Both group I and II (Lee & Riemann, 1970) of *C. botulinum* have 50 to 100% DNA homology within the group.

The three strains of group III of *Clostridium botulinum* showed limited (38%) homology with each other (Table 2). The cytosine plus guanine content of their DNA is very similar to the *C. botulinum* A62 and E MINN strains (Table 2). Many non-toxic variants of *C. botulinum* C and D strains have been reported (Smith & Holdeman 1968; Inoue & Iida, 1970) and most probably group III of *C. botulinum* also contains related toxic and non-toxic strains.

The relatedness between the groups, I, II, and III is very limited and all the strains tested were related to only one of the group (Tables 1 and 2) (Lee & Riemann, 1970). The DNA binding between groups was only 4 to 18%. By comparison the binding of *Clostridium botulinum* A62 DNA to the completely unrelated *Escherichia coli* DNA was 12% (Table 1) and that of the *C. botulinum* E MINN to *Micrococcus lysodeikticus* was 4% (Lee & Riemann, 1970). Walker & Batty (1967) found serological cross-reactions within the three groups of *C. botulinum* but not between the groups. It is apparent from our studies and the study of others that the three groups of *C. botulinum* are not related to each other as a single species and they have little common DNA base sequence.

The ribosomal RNA (rRNA) base sequence is conserved in bacterial evolution and rRNA/DNA homology is used as a broader measurement of bacterial relatedness (Doi & Igarashi, 1965). In view of the heterogeneity we have observed, the relatedness of various Clostridium species may be best determined by rRNA/DNA binding tests. The relatedness of Clostridium and Bacillus species has not been tested. It is possible that Clostridium is an oxygen-sensitive mutant of Bacillus, analogous to the oxygen-sensitive hemin-deficient *Escherichia coli* mutant isolated by Beljanski & Beljanski (1957). However, the GC content of *C. botulinum* DNA is 28% (Table 2), which is lower than the minimum Bacillus GC content of 32% (Hill, 1966). This difference in DNA composition makes it difficult to relate *C. botulinum* with any Bacillus species.

The relatedness of *Clostridium sporogenes* and *C. botulinum* group I strains naturally raises the question of possible transfer of toxigenesis from the toxic to non-toxic strains. Inoue & Iida (1970) reported the conversion of a non-toxic strain to a toxic *C. botulinum*

C strain by filterable phage-like particles. Lytic phages of *C. sporogenes* but not of the proteolytic *C. botulinum* have been reported (Betz & Anderson, 1967).

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Effects of Sodium Chloride on Steady-state Growth and Metabolism of *Saccharomyces cerevisiae*

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SUMMARY

Sodium chloride decreased the maximum specific growth rate of *Saccharomyces cerevisiae*. Chemostat experiments showed this to be largely due to an increased requirement for energy-yielding substrate, apparently linked to maintenance and leading to a decrease in the yield. The increased maintenance requirement is probably concerned with maintaining an intracellular Na^+ concentration ten times lower than the extracellular concentration. NaCl caused much higher concentrations of glucose to be required to maintain any particular glucose-uptake rate; it also increased the production of glycerol.

INTRODUCTION

Sodium chloride affects many parameters of yeast growth, including growth rate (Norkrans, 1966; Combs, Guarneri & Pisano, 1968), yield of biomass (Ross & Morris, 1962; Norkrans, 1966; Combs *et al.* 1968), lag phase of growth (Phaff, Mrak & Williams, 1952; Ross & Morris, 1962; Norkrans, 1966) and cell composition (Combs *et al.* 1968). The tolerance of various yeast species to NaCl varies greatly (Phaff *et al.* 1952; Ross & Morris, 1962; Norkrans, 1968; van Uden & Buckley, 1970; van Uden & Vidal-Leiria, 1970). The two most important factors appear to be the presence of a mechanism for retaining a low concentration of NaCl within the organism (Norkrans & Kylin, 1969) and the ability of enzymes to operate in the presence of high concentrations of NaCl (Ingram, 1957). The present paper is an attempt to interpret various effects of NaCl on growth of *Saccharomyces cerevisiae* in the chemostat through an analysis of growth kinetics and energetics.

METHODS

Organisms used were *Saccharomyces cerevisiae* IGC 3507 from the culture collection of this laboratory and a respiratory-deficient mutant of it. Experiments were carried out using the mutant unless otherwise indicated in the text. The mutant was chosen to eliminate effects due to variation in respiratory quotient with either change in dilution rate or presence of NaCl and thus to facilitate the computation of yield and maintenance parameters.

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Growth conditions. Cultures were grown in a chemostat at 20° (Watson, 1969). The culture medium was similar to that used by van Uden (1967*a*) except that 1 % (w/v) glucose and in certain experiments 1·0 M-NaCl were used. Under these conditions the cultures were carbon-limited. Samples of culture containing approximately 20 mg. dry wt organisms were filtered through preweighed filters (1·2 μm. pore size; Millipore Filter Corporation, Bedford, Massachusetts). The filters were washed with three volumes of distilled water and dried to constant weight at 80°.

Determination of glucose and glycerol. Samples of culture were rapidly filtered through a Millipore membrane. Glucose and glycerol in the filtrates were estimated by the glucose oxidase and glycerol kinase methods (Biochemica Test Combinations, Boehringer Mannheim GmbH).

Production of carbon dioxide by cultures was measured using an infrared carbon dioxide analyser (M.S.A. Lira Infrared Analyser Model 300; Mine Safety Appliances Company, Glasgow; Watson, 1969).

Sodium and potassium contents of organisms. A portion of culture containing approximately 40 mg. dry wt of organisms was rapidly filtered and washed with 5 × 10 ml. of 0·1 M-$MgCl_2$ (pH 6·0 to 6·5; Tempest, Dicks & Hunter, 1966). The filter was placed in approximately 7 ml. of de-ionized water and boiled for 10 min. The suspension was centrifuged, washed with a small volume of de-ionized water, recentrifuged and the combined extracts made up to 10 ml. with de-ionized water. Analyses of Na^+ and K^+ were carried out using an atomic absorption Spectrometer (Perkin-Elmer Model 290, Perkin-Elmer Corp., Norwalk, Connecticut).

RESULTS

Effect of NaCl on yeast growth

In batch cultures of the yeast, the maximum specific growth rate $(\mu)_{max}$ and the yield with respect to glucose (Y^G) but not the rate of glucose uptake decreased with 0·25 to 1·50 M-NaCl. An extension in the duration of the lag phase of growth was also observed. Subsequent experiments were carried out in the chemostat under conditions, of glucose limitation.

Pirt (1965) derived the following equation relating yield (Y), maximum yield (Y_{max}), specific uptake rate of energy-yielding substrate specifically for maintenance (k_m), and specific growth rate (μ)

$$\frac{1}{Y} = \frac{1}{Y_{max}} + \frac{k_m}{\mu}. \quad (1)$$

If Y_{max} and k_m are invariant with μ, a plot of $1/Y$ against $1/\mu$ will give a straight line with slope k_m and intercept on the 'y' axis of $1/Y_{max}$. Fig. 1 shows such a plot for chemostat cultures grown in the presence and absence of NaCl. A glucose-limited culture without NaCl showed a small increase in yield with increase in dilution rate as predicted by equation (1). Deviation from linearity occurred at high dilution rates; a k_m value of approximately 0·2 μmole glucose/mg. dry wt/h. was calculated from the linear part. A glucose-limited culture grown in the presence of 1·0 M-NaCl showed still less linearity in this plot, but a very pronounced decrease was obtained in the yield following a decrease in dilution rate, indicating a high maintenance requirement of approximately 2 μmoles glucose/mg./h.

Effect of NaCl on energy metabolism

As pointed out by van Uden (1968), concepts of maintenance and yield may also be applied to the products of energy metabolism. Fig. 2 shows results for the evolution of CO_2. The approximate k_m values obtained were, without NaCl, 0·4 μmole CO_2/mg./h., and with NaCl, 2·0 μmoles CO_2/mg./h. Since the ratio of the k_m values gives the direct stoichiometry for the net conversion of energy substrate during maintenance without growth, the ratio k_m glucose:k_m CO_2 of 1:2 obtained for the culture lacking NaCl suggests the conversion of 1 mole of glucose to 2 moles of CO_2. The stoichiometry for the culture containing NaCl was 1 mole of glucose producing 1 mole of CO_2 and suggested incomplete fermentation of substrate for maintenance, due to the production of end products in addition to CO_2 and ethanol.

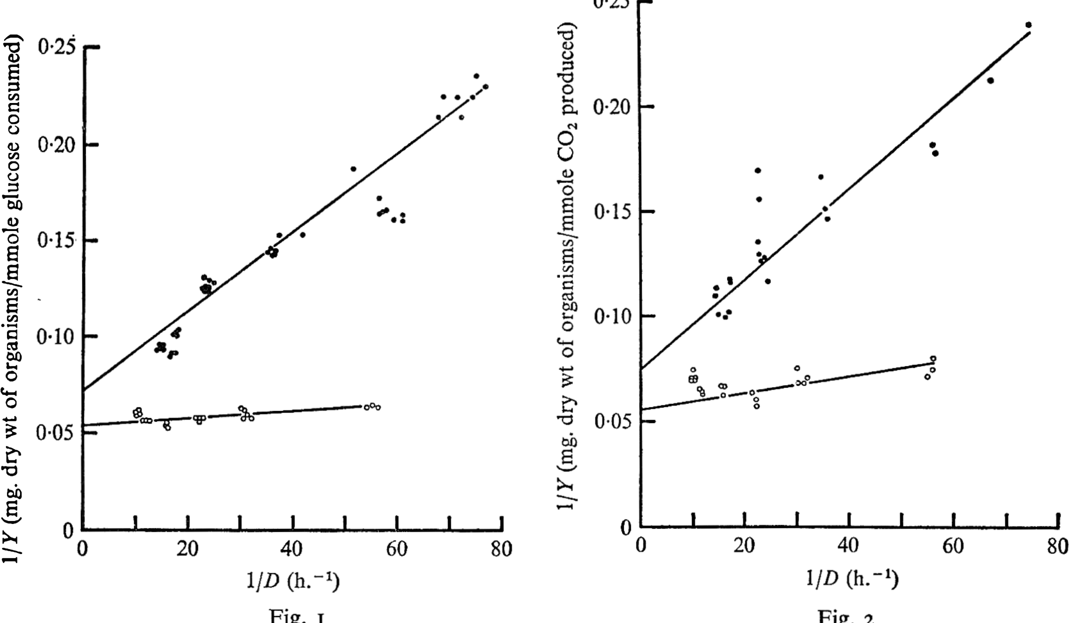

Fig. 1. Reciprocal plots relating yield (based on glucose consumed) to dilution rate in carbon-limited continuous cultures of a respiratory-deficient mutant of *Saccharomyces cerevisiae* IGC 3507. ●. Data for culture with 1·0 M-NaCl; ○, data for culture without NaCl.

Fig. 2. Reciprocal plots relating yield (based on CO_2 produced) to dilution rate in carbon-limited continuous cultures of a respiratory-deficient mutant of *Saccharomyces cerevisiae* IGC 3507. ●, Data for culture with 1·0 M-NaCl; ○, data for culture without NaCl.

The culture grown without NaCl produced glycerol. *Saccharomyces cerevisiae* grown anaerobically produces glycerol (Nordström, 1966, 1968) probably due to the generation of excess $NADH_2$ which is removed through the conversion of dihydroxyacetone phosphate to glycerol phosphate. The yield with respect to glycerol production in this culture was constant for most dilution rates studied and suggested a surplus of $NADH_2$ directly proportional to biomass production (Fig. 3). The culture containing NaCl also produced glycerol. However, the presence of NaCl resulted in a lowering of the yield of organisms with respect to glycerol (Fig. 3). Similar results were found in

batch culture (Table 1). Respiratory-sufficient *S. cerevisiae* grown aerobically does not normally produce glycerol. Glycerol production was, however, induced when the wild-type was grown in the presence of NaCl (Table 1). Similar results have been recorded for other yeasts (Ônishi, 1963). It would appear, then, that enhanced glycerol production under the influence of NaCl cannot be explained directly in terms of the maintenance of a certain intracellular $NAD/NADH_2$ ratio.

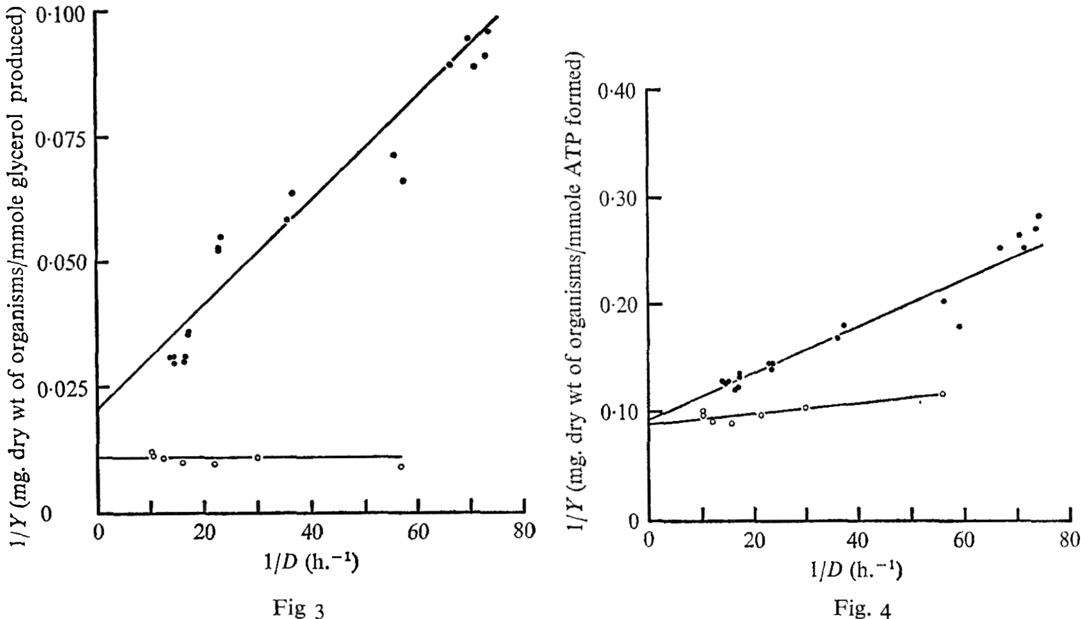

Fig. 3. Reciprocal plots relating yield (based on glycerol produced) to dilution rate in carbon-limited continuous cultures of a respiratory-deficient mutant of *Saccharomyces cerevisiae* IGC 3507. ●, Data for culture with 1·0 M-NaCl; ○, data for culture without NaCl.

Fig. 4. Reciprocal plots relating calculated yield (based on ATP formed) to dilution rate in carbon-limited continuous cultures of a respiratory-deficient mutant of *Saccharomyces cerevisiae* IGC 3507. ●, Data for culture with 1·0 M-NaCl; ○, data for culture without NaCl.

Table 1. *Production of glycerol by wild-type and mutant strains of Saccharomyces cerevisiae IGC 3507 grown batchwise in media containing sodium chloride*

Strain	NaCl concentration in medium (M)						
	0	0·25	0·50	0·75	1·00	1·25	1·50
	Yield (g. dry wt organisms/mole glycerol produced)						
Wild-type strain	> 100,000	64,000	850	330	140	89	55
Mutant strain	66	56	43	34	27	24	18

Organisms grown continuously in the presence of NaCl showed greatly increased production of glycerol per unit of biomass formed as the dilution rate was decreased. The plot of $1/Y$ (glycerol) against $1/D$ was not linear; glycerol production is apparently not a simple function of energy metabolism and variations in Y_{max} (glycerol) may therefore be expected to occur with dilution rate. The value of k_m with respect to

glycerol was 1 μmole/mg./h. (Fig. 3). In the NaCl-free culture there was no production of glycerol linked to maintenance.

Since any deviations from linearity in the plot of $1/Y$ (glycerol) against $1/D$ will cause deviations in the plot for yield based on glucose, and since there was production of glycerol during maintenance in the NaCl-containing culture, a more satisfactory analysis of the results would be obtained in terms of ATP utilized for biomass production (Y^{ATP}) eliminating completely the 'glycerol effect'. Fig. 4 shows a reciprocal plot for Y^{ATP} assuming that each mole of glucose utilized produces 2 moles of ATP, unless it is converted to glycerol when it consumes 2 moles of ATP (or 1 mole of ATP/mole of glycerol formed). The calculated values are probably low with respect to Y^{ATP}_{max} as some glucose will certainly not enter the Embden–Meyerhof pathway but may enter biosynthetic pathways. This, however, should not affect the slope of the line as it is reasonable to assume that under carbon-limitation the partial contributions of processes leading to biomass production are not affected too greatly by growth rate. Hence an evaluation of the ATP turnover for maintenance may be obtained and was 0·52 μmole of ATP/mg. dry wt/h. for organisms grown without NaCl, and 2·2 μmoles of ATP/mg./h., with NaCl. Virtually the same Y^{ATP}_{max} of 11 mg. dry wt organisms/mmole of ATP was obtained for both cultures, suggesting that the effect of NaCl on the ATP yield is solely due to an increased requirement of maintenance energy.

Cation contents of organisms

Organisms grown with and without NaCl contained the same K^+ content. Some penetration of Na^+ occurred in the yeast grown with NaCl (approximately 0·1 M intracellular Na^+ concentration) but an extracellular:intracelluar Na^+ ratio of 10:1 was maintained (Table 2).

Table 2. *Sodium and potassium contents of a respiratory-deficient mutant of Saccharomyces cerevisiae* IGC *3507 grown in carbon-limited continuous culture*

Dilution rate	Ion content (m-equiv./g. dry wt)			
	Without NaCl		With NaCl (1·0 M)	
	Na^+	K^+	Na^+	K^+
< 0·02	0·012 ± 0·001(2)	0·58 ± 0·07(2)	0·25 ± 0·06(3)	0·67 ± 0·06(3)
0·06–0·07	—	—	0·29 ± 0·05(3)	0·65 ± 0·02(3)
> 0·09	0·012 ± 0·001(2)	0·76 ± 0·10(2)	—	—

Means ± standard errors are quoted with the number of observations in parenthesis. Since yeast contains approximately twice its dry wt as intracellular water, the above results may be expressed as molar concentrations upon multiplication by 0·5.

Kinetics of glucose uptake

The kinetics of glucose uptake appeared to be changed by NaCl. The yeast is transport-limited in glucose-limited continuous culture (van Uden, 1967*b*) as shown by the unidirectional Michaelis–Menten kinetics of the rate of sugar uptake without NaCl (Fig. 5*a*). The culture containing NaCl did not show unidirectional uptake kinetics (Fig. 5*b*); in addition, much higher concentrations of glucose were required to maintain a particular rate of glucose uptake.

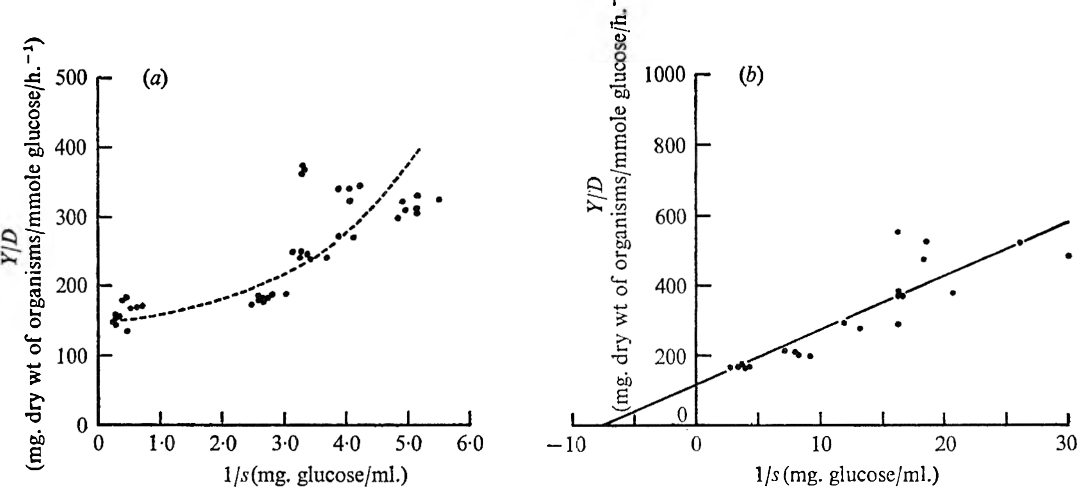

Fig. 5. Reciprocal plots relating specific glucose uptake rate to glucose concentration in carbon-limited continuous cultures of a respiratory-deficient mutant of *Saccharomyces cerevisiae* IGC 3507. (*a*) Shows data for a culture containing 1·0 M-NaCl; (*b*) for a NaCl-free culture.

DISCUSSION

The decrease in the maximum specific growth rate in the presence of NaCl can be largely explained in terms of an increased requirement for energy-yielding substrate, apparently linked to maintenance, and leading to a decrease in the yield. It is probable that the increased maintenance requirement associated with growth in the presence of higher NaCl concentrations was concerned with maintaining an electrochemical gradient of sodium ions, the extracellular Na^+ concentration being ten times greater than the intracellular concentration. There is probably a passive or facilitated diffusion into the organisms (i.e. independent of metabolic energy), balanced by an active outward transport of Na^+ (see Stein, 1967). If the chloride ions move in both directions by either simple or facilitated diffusion then, in order to maintain equilibrium with respect to Cl^-, a membrane potential, E, must be generated (negative to the inside) sufficient to balance the opposing chemical potential difference (Nernst equation)

$$E = \frac{RT}{F} \ln \frac{[\text{Cl}]_0}{[\text{Cl}]_i}, \tag{2}$$

where F is the Faraday, R is the gas constant, $[\text{Cl}]_0$ and $[\text{Cl}]_i$ are the extracellular and intracellular concentrations (activities) of chloride. The sodium ions, however, will be subjected to both an adverse chemical and electrical potential gradient. The free energy change for the movement of 1 mole of sodium across the membrane will therefore be

$$\Delta G_{\text{Na}^+} = RT \ln \frac{[\text{Na}]_0}{[\text{Na}]_i} + EF, \tag{3}$$

where $[\text{Na}]_0$ and $[\text{Na}]_i$ are extracellular and intracellular concentrations (activities) of

sodium. Assuming $[Cl]_i$ and $[Cl]_0$ to be equal to $[Na]_i$ and $[Na]_0$ respectively, equation (3) reduces to

$$\Delta G_{Na+} = 2RT \ln \frac{[Na]_0}{[Na]_i}. \quad (4)$$

To maintain active transport of sodium ions, a metabolic free energy decrease (ΔG_{met}) must be linked to the overall process in such a way that

$$\Delta G_{Total} = (\Delta G_{Na+} + \Delta G_{met}) < 0. \quad (5)$$

In the present example, ΔG_{Total} must be sufficiently negative to assure an active Na^+ efflux that will compensate for the passive influx of Na^+, thus maintaining a steady-state. If the increase of ATP turnover for maintenance, in the culture with NaCl, is concerned with Na^+ transport, the decrease of metabolic free energy for transport may be estimated. The difference between the k_m^{ATP} of organisms grown with and without NaCl is 1·7 μmoles ATP/mg./h. Taking the standard free energy change of ATP hydrolysis as a measure, the metabolic free energy decrease linked to Na^+ transport would be -5×10^{-2} J/mg. organisms/h.

Substitution of $[Na]_0 = 1{\cdot}0$ and $[Na]_i = 0{\cdot}1$ into equation (4) shows that the rate of active transport of Na^+ out of the cell (or the diffusion rate into the cell) must be less than 5 μmoles/mg. dry wt of organisms/h. (equation 5). If, further, a direct stoichiometric relationship exists between the sodium transport rate and the turnover rate of ATP, e.g. through an ATPase (see Glynn, 1968), the above result only allows two possible values, 1 or 2 Na^+/ATP molecule hydrolysed or a Na^+ transport rate of 1·7 or 3·4 μmoles Na^+/mg./h. Measurement of the intial uptake rate of labelled Na^+ into *Saccharomyces cerevisiae* from 1·35 M-NaCl by Norkrans & Kylin (1969) gave a value of approximately 1 μmole Na^+/mg./h. showing a reasonable agreement.

The present study reveals no information about the mechanism of the active transport process or whether it is linked directly to the metabolic energy of ATP. The significance of increased glycerol production is also obscure. Its production could be a result of the activation of certain enzymes on the pathway leading to glycerol by NaCl (Schoeffeniels, 1969); alternatively, it might be a byproduct of increased organic acid production necessary for $NAD/NADH_2$ regulation (a fall in pH value would be advantageous as it decreases the velocity of Na^+ uptake; Armstrong & Rothstein, 1964). It is, however, difficult to explain why the increased production of glycerol expressed itself primarily as a maintenance effect.

The present finding may be significant in a marine environment containing low concentrations of energy source (Duursma, 1960; Vaccaro, Hicks, Jannasch & Carey, 1968). Not only will the yield of organisms, with similar Na^+ requirements, be severely decreased at the low growth rates imposed but, in some cases, the concentration of assimilable carbohydrate may not be high enough to sustain growth, or to allow competition with better adapted organisms using lower concentrations for an equal growth rate (Jannasch, 1968; van Uden & Fell, 1968).

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Genetic Control of Fertility in *Streptomyces coelicolor* A3(2): the IF Fertility Type

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SUMMARY

Wild-type *Streptomyces coelicolor* A3(2), and many mutant and recombinant derivatives of it, are of the IF (Initial Fertility) type. At an early step in the production of recombinant strains from some of the first derivatives of A3 (2), a variant fertility type arose (NF: Normal Fertility), and subsequently IF and NF segregated within the pedigree of stock cultures. IF × IF crosses are about 100-fold less fertile than NF × NF or IF × NF crosses, but the clearest distinction between IF and NF is achieved by crossing with a strain of the previously described UF (Ultra-Fertile) type, when the difference in fertility approaches 1000-fold.

The IF strains give rise to UF strains with a high spontaneous frequency, and the frequency is increased by ultraviolet or X-irradiation but not appreciably by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. NF strains do not give rise to UF variants with a high frequency.

The difference between IF and NF is determined by a chromosomal locus near the 9 o'clock position on the linkage map. There is no evidence for the infectious conversion of one type of strain to the other in a mixed culture.

In crosses with an NF strain, both IF and UF strains contribute the whole chromosome to the effective merozygotes, and the NF strain contributes the fragment, which obligatorily includes the 9 o'clock region. However, whereas in UF × NF crosses there is obligate inheritance of the 9 o'clock region of the NF genome by all sexually produced progeny, this is not true of IF × NF crosses.

INTRODUCTION

All the strains of *Streptomyces coelicolor* used recently in genetic studies were derived from a single spore isolate; this wild-type is called A3(2) (Hopwood, 1959). Mixtures of the first mutant and recombinant derivatives of A3(2) were characterized by a rather low level of recombinant production, or 'fertility'. Later, with the introduction of further genetic markers into the stocks and the enlargement of the culture collection by the isolation of more and more recombinant descendants of the earlier strains, the range of fertility levels encountered in the crosses increased (Hopwood, 1958). However, no recognizable pattern of fertility variation was seen, and it fell to Sermonti & Casciano (1963) to group the strains into two classes, which they called R^+ and R^- (R = recombination). $R^+ \times R^+$ and $R^+ \times R^-$ crosses yielded recombinants, whereas $R^- \times R^-$ mixtures were sterile, or nearly so, under their cultural conditions.

Although the R^+ and R^- strains of Sermonti & Casciano (1963) derived directly, or after further recombinational steps, from the Hopwood stock culture collection, Hopwood (1967) found no sterile combinations amongst his strains. It was possible

that some of the strains had undergone a heritable change during their subculture for a few generations under the possibly different environmental conditions employed by Sermonti & Casciano.

In the hope of throwing further light on the nature of any variation in sexual capabilities that existed amongst the *Streptomyces coelicolor* strains, Hopwood, Harold, Vivian & Ferguson (1968, 1969) attempted to isolate strains of altered fertility after mutagenic treatments, rather than relying on spontaneous variations that might arise by chance in the stock culture collection. They were rewarded by the isolation of a class of 'Ultra-Fertile' (UF) variants displaying remarkable properties when crossed with each of eight strains chosen at random from the stock culture collection. Since all eight of these strains behaved identically, they, and the strain from which the UF variants had been isolated, which had likewise been chosen at random, were regarded as belonging to the same fertility type, called NF (Normal Fertility). The characteristics of NF × UF crosses were described by Hopwood *et al.* (1969). Almost all the viable spores produced by the mixed culture were recombinant. Moreover, all recombinants were of NF fertility, and all inherited a particular region of the NF parental genome, corresponding to 9 o'clock on the linkage map. The NF parent was deduced to be a 'donor' in the sense that it obligatorily contributed a chromosome fragment to each merozygote, while the UF was a 'recipient', contributing its entire genome to all merozygotes. (Throughout this paper we shall refer to the constitution of merozygotes in particular crosses. It is understood that we are discussing those merozygotes effective in yielding progeny, since only these are at present open to study. It is possible that in certain cases other classes of zygote arise, but fail to yield viable progeny.)

The results described in the present paper arose from experiments to test the fertility of UF × UF crosses, which might have been expected to be sterile if UF strains were obligate recipients. They showed that the UF strains were powerful indicators of the sexual capability of other strains, and that the stock culture collection contains strains of at least two fertility types, NF and IF. The IF (Initial Fertility) type, which is that of the wild-type A3(2), is the subject of this paper. The relationship of our NF and IF types with the R^+ and R^- strains of Sermonti & Casciano (1963) is discussed.

METHODS

General. Complete (CM) and minimal (MM) media and standard cultural techniques were those described by Hopwood (1967).

Strains. The strains, which are all mutational and recombinational derivatives of *Streptomyces coelicolor* A3(2) (Hopwood, 1959), are listed in Table 1. The linkage relations of their genetic markers are shown in Fig. 1.

Mutagenesis. Ultraviolet (u.v.) irradiation was carried out as described by Harold & Hopwood (1970). Treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) was by the schedule of Delić, Hopwood & Friend (1970).

Testing of fertility by 'plate crossing'. This is an indirect selection procedure, originally described by Sermonti & Casciano (1963). For the isolation of UF variants it was performed as described by Hopwood *et al.* (1969). To test their fertility, existing strains were inoculated in defined areas of a 'master plate' and tested in the same way as presumptive UF variants (Hopwood *et al.* 1969), but this time against UF as well as NF tester strains.

Genetic analysis. Haploid recombinant selection was done in the way described by Hopwood (1967). Non-selective analysis was performed by the method of Hopwood *et al.* (1969).

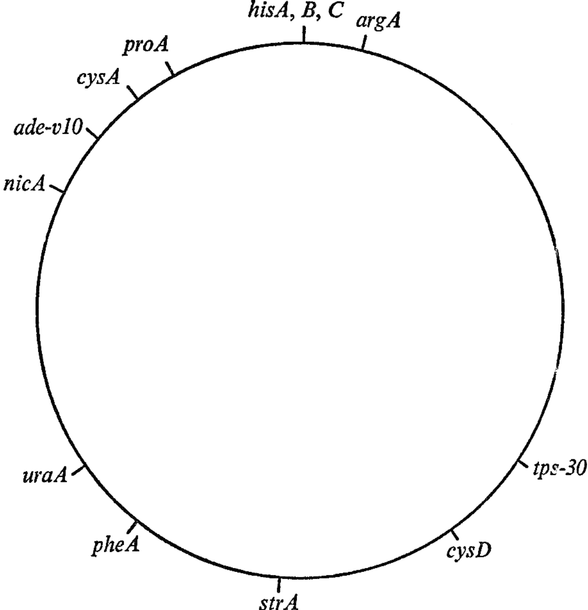

Fig. 1. Linkage map showing the locations of markers referred to in the paper. Marker abbreviations have conventional meanings (Hopwood, 1967); *tps* = temperature-sensitive (Hopwood, 1966*a*).

RESULTS

Heterogeneity of the culture collection in respect of fertility

The recognition of the IF type arose from experiments to test the fertility (or sterility) of mixtures involving only UF strains. All the UF strains obtained by Hopwood *et al.* (1969) derived from a single strain (12) and all were therefore isogenic in respect of standard markers, carrying only the mutation *pheA1*. To select recombinants from UF × UF crosses we had to introduce other markers into one of the UF strains by two steps of mutagenesis:

12 IF ——→ 1098 UF ——→ 1143 UF ——→ A200 UF
pheA1 u.v. *pheA1* NTG *pheA1 str-5* NTG *pheA1 str-5 ade-v10.*

The final strain (A200) could now be crossed with strain 1098, and recombinants inheriting *ade*$^+$ and *str* selected. A similar cross was made between strain A200 and the progenitor of 1098, strain 12.

The results (Table 2) show that the UF × UF cross had a low but easily detected fertility. Unexpectedly strain 12, which had been assumed to be NF, was almost as infertile with strain A200 as was strain 1098, whereas if 12 had been NF the fertility should have been about 1000-fold higher. (These low levels of recombination, only some ten times higher than the frequency of *ade*$^+$ reverse mutations in this experiment, have subsequently been shown unequivocally to involve recombination rather than

Table 1. *Characteristics of strains*

Strain	Relevant phenotype													Fertility
	tps-30	*cysD18*	*strA1*	*pheA1*	*uraA1*	*nicA1*	*ade-v10*	*cysA15*	*proA1*	*hisA1*	*hisB12*	*hisC9*	*argA1*	
A3(2)*	+	+	S	+	+	+	+	+	+	+	+	+	+	IF
11	+	+	S	−	+	+	+	+	+	+	+	+	+	IF
12	+	+	S	−	+	+	+	+	+	+	+	+	+	IF
28	+	+	S	−	+	+	+	+	+	−	+	+	+	NF
29	+	+	S	+	−	+	+	+	+	−	+	+	+	NF
32	+	+	S	+	−	+	+	+	+	+	+	+	−	NF
104	+	+	R	+	−	+	+	+	+	−	+	+	+	IF
424	+	+	S	+	+	+	+	+	+	+	−	+	+	IF
749	+	−	S	+	−	+	+	+	−	+	+	+	−	NF
923	+	−	S	+	−	+	+	+	+	−	+	+	+	NF
949	−	+	S	+	−	−	+	−	−	+	+	+	−	NF
1098	+	+	S	−	+	+	+	+	+	+	+	+	+	UF
1100	+	+	S	−	+	+	+	+	−	+	+	+	−	NF
1143	+	+	R†	−	+	+	+	+	+	+	+	+	+	UF
1258	+	−	R	+	−	+	+	+	−	+	+	−	−	NF
1190	+	+	R	+	−	+	+	+	+	−	+	+	+	UF
A200	+	+	R†	−	+	+	−	+	+	+	+	+	+	UF
A317	+	+	R	+	−	+	+	+	+	−	+	+	+	NF
A332	+	+	S	−	+	+	+	+	+	+	+	+	+	NF

* The wild-type. † *str-5*, assumed to be allelic with *strA1*.

mutation in crosses in which non-selected markers were available.) Therefore strain 12 was of a fertility type quite distinct from NF (as well as UF). This fertility type was designated IF (Initial Fertility). The main criterion for distinguishing it from NF strains is that in a plate-test (Pl. 1) with a UF tester it gives few or no recombinants, whereas an NF strain gives a confluent patch of recombinants.

Table 2. *Frequency of selected recombinants in crosses of strains 12 and 1098 with a* UF *tester, strain* A 200

			Number of colonies on media selective for			
			Parentals		Recombinants or mutants	
	Strains	Fertility	*pheA1*	*ade-v10 pheA1 str-5*	*pheA1 str-5*	Recombinants or mutants* (%)
Controls	12	IF	$1{\cdot}24 \times 10^8$	—	0	$< 8 \times 10^{-7}$
	1098	UF	$2{\cdot}74 \times 10^8$	—	0	$< 3 \times 10^{-7}$
	A 200	UF	—	$3{\cdot}6 \times 10^8$	37	$1{\cdot}0 \times 10^{-5}$
Crosses	12 × A 200	IF × UF	$3{\cdot}7 \times 10^6$	$2{\cdot}1 \times 10^7$	79	$3{\cdot}2 \times 10^{-4}$
	1098 × A 200	UF × UF	$5{\cdot}6 \times 10^6$	$1{\cdot}2 \times 10^7$	22	$1{\cdot}3 \times 10^{-4}$

* Per total parental colonies.

Diagnosis of fertility of some key strains within the descendants of the wild-type A 3(2), using the plate-test (Pl. 1) incorporating both NF and UF tester strains, revealed that the culture collection contained both NF and IF strains. Strain A 3(2) and all its mutational derivatives so far tested, together with a few recombinants, are of the IF type (Fig. 2). Unfortunately some of the strains in early parts of the pedigree, which were isolated some 15 years ago, have since died or been discarded and their fertility cannot be determined; nevertheless the origin of the NF character from IF has been traced to strain 4 or one of its immediate ancestors.

Segregation of fertility in IF × NF *crosses*

Crosses were made between suitably marked NF and IF parents and the progeny, either selected or non-selected, were characterized in respect of fertility. An example of a cross of strains 949 NF and 12 IF (Fig. 3), analysed non-selectively (Hopwood *et al.* 1969) is given in Tables 3 and 4. The fertility of all the progeny was tested against a UF tester, strain A 200, selecting *ade*$^+$ and *str*.

In this analysis (Table 3), 13 % of the progeny were recombinants, the remainder being parental *in respect both of standard markers and of fertility*. The proportions of the two parental genotypes were equal within less than a factor 2, indicating a well-balanced mixture of the parents. There is clearly no evidence for the infectious conversion within this intimate mixture of one parental fertility type into the other.

Amongst the 132 recombinants there was also a clear classification of the IF/NF phenotypic difference. The segregation indicated a chromosomal location for the IF/NF determinants in the 9 o'clock region of the genome between *nicA* and *uraA*. The simplest evidence for this conclusion derives from three-point analysis of the data (Table 4). This location is compatible with the allele ratios at the various loci (Fig. 3);

the explanation of the particular pattern of allele ratios observed will be considered in the final section of the results.

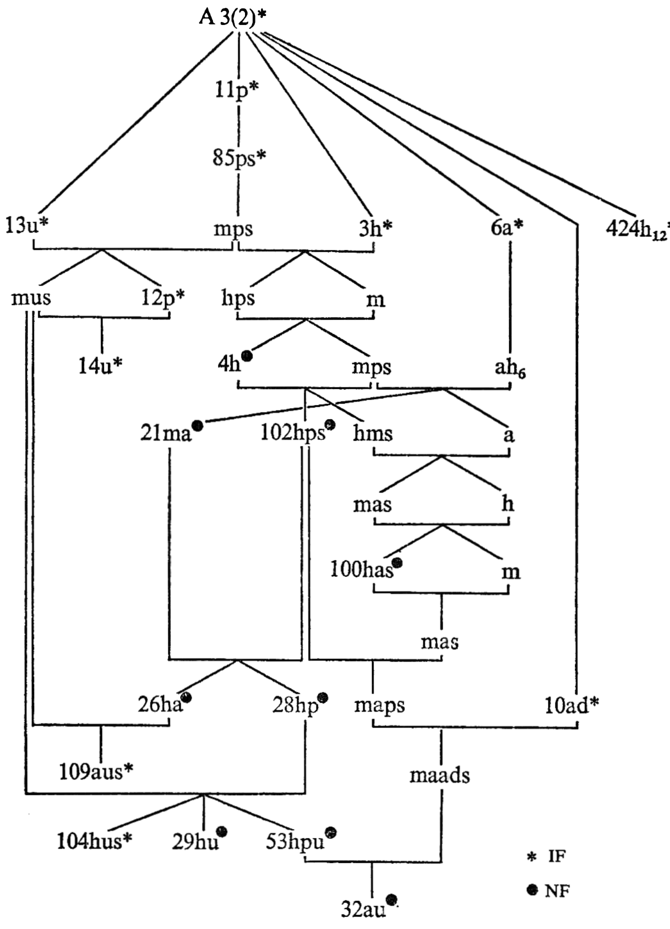

Fig. 2. Interrelationships and fertility type of some mutant and recombinant derivatives of the wild-type A 3(2). Numbers are stock collection numbers; strains without numbers were dead and unavailable for fertility testing. Lower case letters refer to genetic markers, as follows: a = *argA1*; ad = *adeA3*; h = *hisA1*; h_6 = *his-6*; h_{12} = *hisB12*; m = *metA2*; p = *pheA1*; s = *strA1*; u = *uraA1*.

Production of UF *from* IF *strains*

Table 5 summarizes quantitative data on the spontaneous and induced production of UF variants from two well-characterized IF strains. An example of the test is shown in Pl. 2; the relative fertility of UF and IF colonies under the conditions of this test was such that UF variants could be identified rather objectively. The spontaneous frequency of UF variants within IF strains varied, not unexpectedly, between different clones, ranging in these experiments from 0·03 to 0·4 %. Ultraviolet and X-rays induced UF variants with frequencies of up to at least 1 to 2 %. There is evidence for an increase in UF frequency with u.v. dose (decreasing survival). On the other hand NTG had only a marginal effect on UF frequencies, even at survival levels at which u.v. and X-rays

were very effective, and under conditions of very effective general mutagenesis (Delić *et al.* 1970).

UF strains have also been obtained, with comparable frequencies, from IF strains 11, 13 and 14, in addition to 12 and 104; the interrelations of these strains are in Fig. 2. Table 6 (top two lines) shows the negative results of attempts to isolate UF variants from two strains (28, 29; Fig. 2) classified by the plate test as NF. We conclude that, if UF variants arise from NF strains, their induced frequency is at least 100-fold lower than from an IF strain.

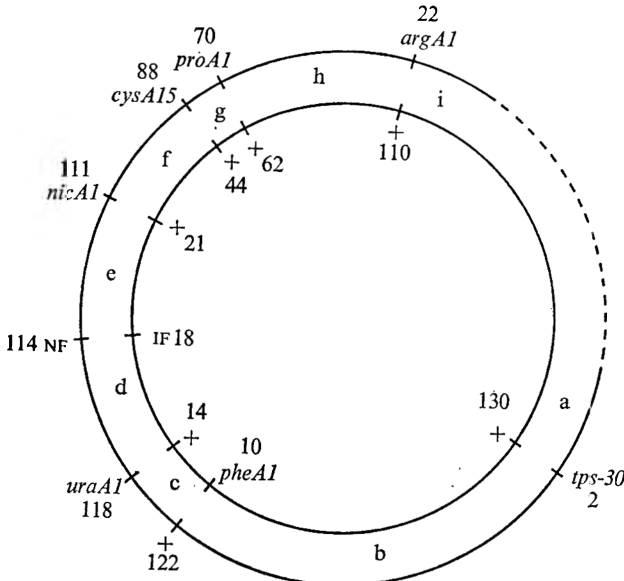

Fig. 3. Representation of a cross between strains 12 IF (inner circle) and 949 NF (outer circle). Letters indicate map intervals. Numbers are the frequencies of each allele in a sample of non-selected recombinants (Table 3).

Also in Table 6 are the frequencies of UF production from the *uraA1* recombinant progeny of the NF × IF cross (Table 3). Three of these had been classified as NF by the plate test and six as IF. All six IF strains produced UF variants with frequencies, after u.v. treatment, within the previously determined range, but no UFs were obtained from the three NF recombinants. These results thus confirm the original fertility typing of progeny by the plate test. They also indicate that the IF character had segregated from an NF × IF cross unchanged in phenotype in regard to UF production.

Frequency of recombination in crosses homologous and heterologous in respect of fertility type

We made two sets of strains, one set carrying the marker *pheA1* and the other the markers *hisA1 uraA1 strA1*, each set consisting of IF, NF and UF types. The UF strains derived by mutation from the corresponding IF strains. However, because we have not been able to derive NF by mutation from IF or UF strains, the corresponding NF strains were derived by appropriate marker selections from crosses involving only

strains known to be NF. These sets of strains have allowed the levels of recombination between different fertility types to be studied systematically.

The experiment consisted of plating the crosses on three media, selecting respectively recombinants and the two parental phenotypes. To minimize the effects of an inconstant parental imbalance in the crosses the results (Table 7) are expressed in terms of recombinant frequencies per *minority* parental phenotype. We see that the IF × IF, IF × UF and UF × UF crosses had a low, and similar, recombinant frequency, rather greater than one in 10^4 of the minority parent. In contrast, NF × NF and NF × IF crosses had

Table 3. *Non-selective analysis of a cross between strains 12* IF *and 949* NF

Fertility was tested against strain A200. The marker arrangements, map intervals and allele ratios amongst the recombinants are in Fig. 3.

Genotype	Fertility	Crossover in interval	Number
tps + *ura nic cys pro* +	NF	a, h	1
+ + + + + + +	IF	b, c	7
+ + *ura* + + + +	IF	b, d	6
+ + *ura* + + + +	NF	b, e	4
+ + *ura nic* + + +	NF	b, f	21
+ + *ura nic cys* + +	NF	b, g	18
+ + *ura nic cys pro* +	NF	b, h	45
+ + *ura nic cys pro arg*	NF	b, i	17
+ *phe ura nic* + + +	NF	c, f	1
+ *phe ura nic cys* + +	NF	c, g	1
+ *phe ura nic cys pro* +	NF	c, h	1
+ *phe ura nic cys pro arg*	NF	c, i	1
+ *phe* + *nic cys pro* +	NF	d, h	1
+ *phe* + *nic cys pro arg*	NF	d, i	1
+ *phe* + *nic* + + +	IF	e, f	1
+ *phe* + + + *pro* +	IF	g, h	1
+ *phe* + + + + *arg*	IF	h, i	2
tps + *ura nic* + *pro arg*	NF	a, b, h, i	1
+ + + *nic cys* + +	IF	b, c, e, g	1
+ + *ura* + *cys pro* +	NF	b, e, f, h	1
	Total recombinants		132 (13%)
tps + *ura nic cys pro arg*	NF	Parental	359 (34%)
+ *phe* + + + + +	IF	Parental	564 (53%)
	Total colonies		1055

Table 4. *Ordering of* IF/NF *between nicA and uraA*

Data from Table 3. The marker arrangements, map intervals and allele ratios amongst the recombinants are in Fig. 3. Crossovers in intervals outside d and e are ignored.

Marker combination	Crossover in intervals		Number	
uraA1 NF *nicA1*	—	No crossover	107	117
+ IF +	—		10	
uraA1 IF +	d	Single crossover	6	8
+ NF *nicA1*	d		2	
uraA1 NF +	e	Single crossover	5	7
+ IF *nicA1*	e		2	
uraA1 IF *nicA1*	d, e	Double crossover	0	0
+ NF +	d, e		0	

Table 5. *Isolation of* UF *variants from two* IF *strains after treatment with ultraviolet light* (*u.v.*), *N-methyl-N′-nitro-N-nitrosoguanidine* (*NTG*) *or X-rays*

The test strain was 923 for strain 12 and 1100 for strain 104.

Strain	Treatment	Survival (%)	Number of colonies examined: Total	Number of colonies examined: UF	% UF: Total	% UF: Induced*
12†	Control	100	3600	1	0·03	—
	u.v.	3	5250	18	0·34	0·31
12	Control	100	5057	3	0·06	—
	u.v.	3	3682	12	0·33	0·27
104	Control	100	5255	13	0·25	—
	u.v.	0·1	2918	40	1·37	1·12
104	Control	100	5309	22	0·41	—
	u.v.	0·3	1219	18	1·48	1·07
12†	Control	100	3800	1	0·03	—
	NTG	8	2550	3	0·11	0·08
104	Control	100	1608	2	0·12	—
	NTG	5	1483	1	0·07	−0·05
104	Control	100	1685	1	0·06	—
	NTG	6	1344	1	0·07	0·01
104	Control	100	988	1	0·10	—
	NTG	3	2850	3	0·10	0
104	Control	100	2401	0	< 0·04	—
	NTG	2	2436	3	0·12	> 0·08
104‡	Control	100	2893	8	0·28	—
	X-rays	4	2763	72	2·61	2·33

* Total minus control; a genuine induction, rather than a selection, is indicated by the finding that the ultraviolet sensitivity of strains 104 IF and 1190 UF were the same (R. Baumann, personal communication).

† Data from Hopwood *et al.* (1969).

‡ Data kindly supplied by Mr T. Schupp.

Table 6. *Attempts to isolate* UF *variants from* NF *strains, and segregation of ability to yield* UF *variants*

The strains were irradiated with u.v. to the survival levels indicated.
The test strain was 749 for strain 28 and 1100 for the remaining strains.

Strain	Fertility	Survival (%)	Number of colonies examined: Total	Number of colonies examined: UF	% UF
28	NF	2	11731	0	< 0·009
29	NF	0·5	4255	0	< 0·02
A321*	NF	0·4	1458	0	< 0·07
A323*	NF	6·8	2101	0	< 0·05
A329*	NF	0·8	900	0	< 0·1
A322*	IF	0·4	751	10	1·3
A324*	IF	0·1	1392	32	2·3
A326*	IF	0·2	4009	44	1·1
A327*	IF	0·2	3773	42	1·1
A328*	IF	0·3	3941	50	1·3
A330*	IF	0·4	6523	62	1·0

* These strains are *uraA1* recombinants from a cross of strain 949 NF × 12 IF (Table 3).

frequencies between 2 and 17 %, the NF × IF crosses being rather more fertile than the NF × NF. This greater fertility of NF × IF was confirmed in crosses analysed non-selectively (Table 8: see next section), in which NF × IF crosses gave an average total recombinant frequency per total progeny some sixfold higher than a corresponding NF × NF cross. The determination of fertility by *selective* plating, as in Table 7, while clearly differentiating between grossly different fertility levels, gives only semiquantitative results, since the fertility indicated depends on the particular selection applied, in relation to the pattern of merozygote polarization and asymmetry (see next section); this is doubtless the explanation for the different recombinant frequencies in the two crosses involving NF and IF strains in Table 7.

Table 7. *Frequency of selected recombinants in crosses involving strains of various fertility types*

Strains				
pheA1		*hisA1 uraA1 strA1*	Fertility	Recombinant frequency*
A332	×	A317	NF × NF	$1 \cdot 6 \times 10^{-2}$
A332	×	104	NF × IF	$4 \cdot 0 \times 10^{-2}$
12	×	A317	IF × NF	$1 \cdot 7 \times 10^{-1}$
12	×	104	IF × IF	$1 \cdot 5 \times 10^{-4}$
1098	×	104	UF × IF	$3 \cdot 9 \times 10^{-4}$
1098	×	1190	UF × UF	$2 \cdot 9 \times 10^{-4}$

* Ratio of selected recombinants (*his*$^+$ *str* or *ura*$^+$ *str*) to minority parental type (*phe* or *his ura str*)

Merozygote polarization and asymmetry in crosses heterologous in respect of fertility

We shall use the terms polarization and asymmetry in the following senses. Polarization (Hopwood, 1967) refers to the roles of the two parents in merozygote formation, a polarized cross being one in which the chromosome fragment comes preferentially (or exclusively) from one particular parent and the complete chromosome from the other. Asymmetry refers to non-randomness of the chromosome fragments in the zygotes, such that they preferentially include some particular region or regions of the genome. The zygotes in an NF × UF cross (Hopwood *et al.* 1969) are polarized, since the chromosome fragment comes exclusively from the NF parent, and asymmetric, since the fragment always includes the 9 o'clock region of the genome.

The allele frequencies at different loci in the non-selective analysis of the NF × IF cross (Fig. 3) were very unequal: in particular there was a shortage of NF markers at loci in the right-hand half of the map (*argA1* and *tps-30*) and an excess of NF markers in the left-hand half. This result suggested a polarization and asymmetry of the merozygotes, possibly resembling that in NF × UF crosses (Hopwood *et al.* 1969). In order to investigate this merozygote polarization and asymmetry more specifically, two sets of crosses were made, each involving strains isogenic for standard markers and having a common NF parent, the other parent being in turn of the three fertility types, NF, IF and UF. The first set of crosses was analysed non-selectively (Table 8) and the second selectively (Fig. 4).

In Table 8, data for several replicates of the NF × NF and NF × IF crosses indicate the relatively small effects of clonal fluctuation between crosses. The data are presented in summary form, with omission of the many individual recombinant classes from which the frequencies of the various markers of the common NF parent (strain 749) were

Table 8. *Non-selective analysis of* NF × NF, IF × NF *and* UF × NF *crosses*

pheA1 strains of different fertility (IF, NF or UF) were crossed with NF strain 749 *argA1 cysD18 proA1 uraA1*.

pheA1 parent		Numbers of progeny									
		Parental					Allele frequencies of strain 749 amongst recombinants				
Stock no.	Fertility	*pheA1*	*argA1 cysD18 proA1 uraA1*	Recombinants†	Total	Recombinants (%)	*cysD18*	*pheA1*+	*uraA1*	*proA1*	*argA1*
A 332	NF	282	443	4	729	0·5	2	2	3	1	3
		73	304	3	380	0·8	3	2	1	2	3
		66	296	9	371	2·4	2	8	8	3	3
		67	290	6	363	1·7	3	6	4	4	2
		33	339	5	377	1·3	2	4	4	2	3
		521	1672	27	2220	1·2	44 %	81 %	74 %	44 %	52 %
11	IF	275	34	60	369	16·2	2	55	38	6	2
		212	149	35	396	8·7	1	32	30	18	4
12	IF	63	18	6	87	6·9	0	5	5	3	1
		164	59	20	243	8·2	2	15	10	5	2
		352	14	12	378	3·2	2	11	10	6	3
		226	178	9	413	2·2	2	8	8	4	2
		1292	452	142	1886	7·5	5 %	89 %	71 %	30 %	10 %
1098*	UF	330	13	647	990	67	< 0·2 %	73 %	95 %	13 %	1 %

* Data recalculated from Hopwood *et al.* (1969).

† Includes only recombinants in respect of standard markers; in the case of the UF × NF cross, the large class of progeny parental in respect of standard markers but recombinant in respect of fertility have been tabulated under 'parentals'.

calculated. Considering these frequencies first for the NF × NF cross, we see that they are not markedly different from one another or from 50 %. This agrees with previous conclusions (Hopwood, 1967, 1969) that in normal crosses, now known to have involved NF strains, the mixture of merozygotes is non-polarized and symmetrical. (The moderate excess of the marker *pheA1*$^+$, and to a lesser extent of the nearby marker *uraA1*, in these data probably reflects a selective disadvantage of the allele *pheA1* compared with *pheA1*$^+$, which is consistently observed.)

In marked contrast to these data for the NF × NF cross, those for the NF × IF cross (Table 8) show a very significant shortage of the NF markers *cysD18* and *argA1* reminiscent of, although less extreme than, the comparable figures for the NF × UF cross (Table 8). As discussed in detail by Hopwood *et al.* (1969), the NF × UF data are compatible with the hypothesis that the merozygotes are completely polarized, and also asymmetrical in that all NF fragments include the 9 o'clock region. Moreover, in such NF × UF crosses this region of the NF genome is obligatorily inherited by the progeny derived from the merozygotes. The low frequencies amongst the recombinants of such markers as *cysD18* and *argA1*, far from 9 o'clock, follow from their poor chances of being carried on the NF fragment. The data in Table 8 suggest that the merozygotes in an NF × IF cross resemble those in an NF × UF cross. However, the data of these crosses and those analysed selectively (Fig. 4) indicate that the resemblance is not complete.

In Fig. 4 the data are again reduced to the allele frequencies at each locus. Considering the NF × NF cross (Fig. 4*a*) as a basis of comparison for the heterologous crosses, we see the effects of merozygote symmetry expressed in the allele ratios at the four non-selected loci, which are considered in pairs, each pair flanking one of the selected loci. The frequencies of crossing-over in the intervals on either side of these selected loci, a and f for *hisC* and c and d for *strA*, are reflected by the frequencies of the alleles *argA1* and *proA1*, and *cysD18*$^+$ and *uraA1*$^+$ respectively. Interval a is known (Hopwood, 1966*a*, *b*) to be only some two-thirds of the length of interval f, and this difference is reflected in the frequencies of *argA1* and *proA1*; on the other hand the lengths of intervals c and d are not very different (Hopwood, 1966*a*, *b*), and the allele frequencies of *cysD18*$^+$ and *uraA1*$^+$ are in agreement. Thus there is no evidence for asymmetry in the zygotes, although there is a slight polarization of this particular cross in favour of strain 1258 (represented by the outer circle in Fig. 4*a*) contributing the complete genome; this is indicated by the frequencies of crossing-over per unit length in intervals a and f being somewhat higher than in c and d.

The data for the NF × IF cross (Fig. 4*b*) differ strikingly from those of the NF × NF cross in respect of the non-selected markers in the right-hand half of the map. There is a conspicuous shortage of crossing-over in interval a (a low frequency of *argA1*) and a large excess of crossing-over in interval c (a high frequency of *cysD18*$^+$). From this result we deduce that the effective zygotes in this NF × IF cross have the constitution shown in Fig. 4*d*, with the 9 o'clock region of the NF fragment obligatorily represented and the duplicated region extending, in different zygotes, for varying distances on either side of this region. Owing to the pattern of selection in this cross, the NF fragment in all zygotes contributing selectable progeny had to include the selected marker *strA1*; crossing-over in interval c was very frequent since the strand carrying *strA1* would very often not have extended beyond the locus *cysD* into interval b. The low likelihood of crossing-over in interval a reflects two factors: many zygotes will have

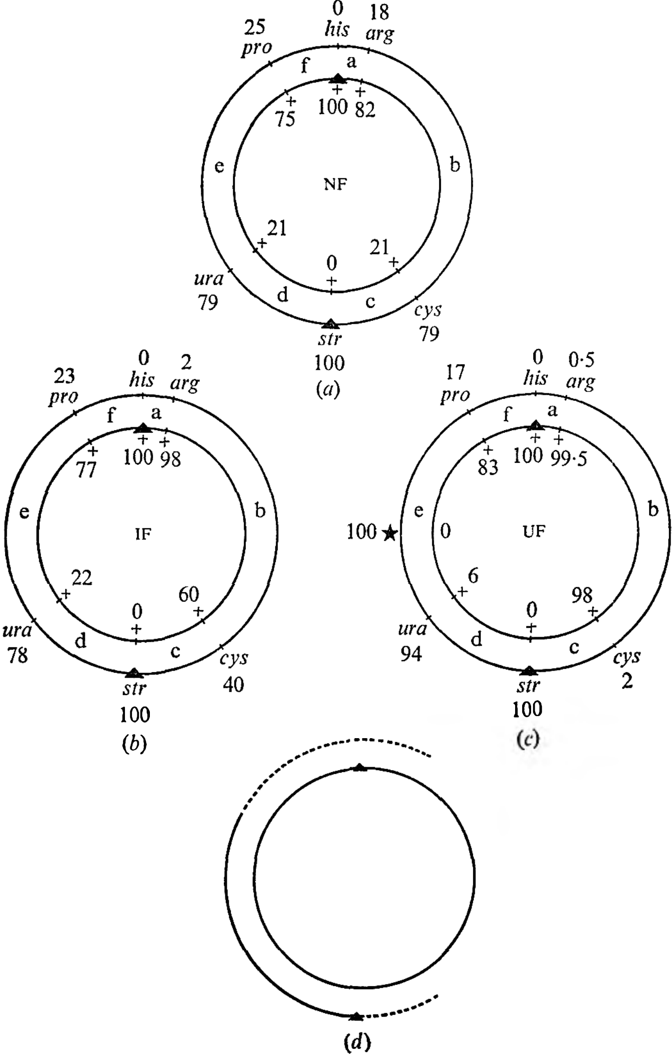

Fig. 4. Selective analysis of (*a*) NF × NF, (*b*) IF × NF and (*c*) UF × NF crosses. Strains carrying the markers *pheA1* and of varying fertility (NF = A332, IF = 12, UF = 1098) were crossed with a common NF strain (1258, *argA1 hisC9 proA1 uraA1 strA1 cysD18*). Recombinants inheriting the alleles $hisC9^+$ and *strA1* (indicated by triangles) were selected and classified in respect of the non-selected markers. Numbers are the percentage frequencies of each non-selected allele among the selected recombinants (sample sizes 191–199); only two markers flanking each selected locus have been considered for simplicity. The asterisk denotes the 'required region' of the NF genome inherited by all recombinants in a UF × NF cross (Hopwood *et al.* 1969).

(*d*) Postulated constitution of merozygotes in an IF × NF cross. The IF strain contributes the complete genome (inner circle) and the NF strain contributes a fragment (outer arc) obligatorily including the 9 o'clock region and extending for varying distances on either side (broken lines). Triangles indicate the alleles selected in the cross in (*b*); thus in all zygotes leading to selected recombinants in this cross the NF fragment must have included the region between 9 o'clock and *str*.

contained fragments that did not extend into this interval; and from those that did, *argA1* could have been inherited by recombinant progeny only when two non-selected crossovers occurred, one in interval a and one in b.

Fig. 4 shows very clearly the difference between NF × IF (Fig. 4*b*) and NF × UF (Fig. 4*c*) crosses. First, the low frequency of the allele $uraA1^{+}$ in the UF cross follows from the obligate inheritance of the 9 o'clock region (the 'required' region: Hopwood *et al.* 1969) of the NF fragment by all the progeny; thus any segregant inheriting $uraA1^{+}$ must have arisen by two crossovers, one in interval d and one in that region of e between the *uraA* locus and the 'required' region. The much higher frequency of $uraA1^{+}$ in the NF × IF cross tells us that the 'required' region is not obligatorily inherited by the progeny, although it is obligate in the zygotes, in such a cross. Second, the frequencies of the alleles *argA1* and *cysD18* are even lower in the NF × UF than in the NF × IF cross. This finding might indicate the average NF fragment length in NF × UF crosses to be shorter than in NF × IF crosses.

DISCUSSION

The results described in this paper provide an explanation for some hitherto puzzling observations made early in the study of the genetics of *Streptomyces coelicolor* A3(2). The earliest crosses (Hopwood, 1957, 1959) were characterized by the production of complementary recombinant genotypes with statistically equal frequencies. This finding indicated an equal contribution of genetic material by the two parents to the progeny, although not necessarily to each zygote (Hopwood, 1959). In later crosses, involving further recombinant descendants of the early strains, complementary genotypes in general had unequal frequencies (Hopwood, 1961; Hopwood & Sermonti, 1962), and some crosses were also considerably more fertile than the early crosses (Hopwood, 1958).

It is now clear that this changed behaviour of the crosses reflected the origin of the variant fertility type, NF, from one of the IF descendants of the wild-type, and its inheritance by other strains in the culture collection (Fig. 2); some crosses were thus heterologous in respect of fertility (NF × IF). Whereas IF × IF crosses, like the NF × NF crosses described in the present paper and previously (Hopwood, 1967), are characterized by random merozygote formation, each parent contributing markers to the zygotes with statistically equal frequencies, in NF × IF crosses the roles of the parents differ: the IF parent contributes the complete genome to all merozygotes effective in recombinant formation, and moreover the fragments contributed by the NF parent are non-random. The result is that markers of the IF parent tend to be inherited preferentially by the progeny, particularly at loci far from the 9 o'clock region of the genome, and of course complementary recombinant genotypes must therefore have unequal frequencies.

We have deliberately designated the fertility types in *Streptomyces coelicolor* A3(2) by terms (IF, NF, UF) that do not recall the plasmid-determined mating types of Gram-negative bacteria, such as F^{+}/F^{-} (Hayes, 1968), R^{+}/R^{-} (Meynell, Meynell & Datta, 1968) or Col^{+}/Col^{-} (Nomura, 1967) in *Escherichia coli*, FP^{+}/FP^{-} in *Pseudomonas aeruginosa* (Holloway, 1969), or P^{+}/P^{-} in *Vibrio cholerae* (Bhaskharan, 1960) since, as we have seen in this and a previous paper (Hopwood *et al.* 1969), there appear to be no close analogies between *S. coelicolor* and these eubacteria in respect of the known fertility types. In particular we have so far implicated exclusively chromosomal,

rather than plasmid, determinants of fertility, with no evidence of infectious conversion of one fertility type to another, although an interaction between chromosomal alleles and one or more plasmids may of course remain to be recognized.

At least some of the strains designated R^- and R^+ by Sermonti & Casciano (1963) appear to correspond with our IF and NF types respectively. It is not possible to identify the derivation from our strains of all the cultures classified by Sermonti & Casciano (1963), but their strains numbered 8R^-, 109R^+ and 118R^- must have been derived by subculture from the Hopwood stock cultures 424, 32 and 109 respectively. The derivations of these strains are in Fig. 3 and their fertility types are IF, NF and IF respectively. Moreover the patterns of marker inheritance observed in $R^+ \times R^-$ crosses (Sermonti & Casciano, 1963), which were difficult to interpret in the absence of the later acquired knowledge of the location of the markers on the circular linkage map (Hopwood, 1965, 1966*a*, *b*), seem compatible with those we observe in NF × IF crosses. The only important discrepancy appears to be the conclusion by Sermonti & Casciano (1963) of infectious conversion of an R^- strain to R^+ by mixed growth with an R^+ strain, on the basis of which an analogy was claimed between R^+ and R^- *Streptomyces coelicolor* and F^+ and F^- *Escherichia coli* strains (Spada-Sermonti & Sermonti, 1970). As we have shown in the present paper, there is no evidence for conversion of IF to NF in our mixed cultures (Table 3); instead there is a clear segregation of fertility attributable to a pair of chromosomal 'alleles'. The explanation of Sermonti & Casciano's result is conceivably that the R^+ strains emerging from their $R^+ \times R^-$ cross with the standard markers of the R^- parent were in fact recombinants which, because of the particular pattern of crossing-over, inherited a parental combination of standard markers. It was stated (Spada-Sermonti & Sermonti, 1970) that the frequency of recombinant production in the cross was only 1×10^{-4} per total progeny, but if the parental balance had been very unequal, with the R^- parent in the minority, the frequency of the 'converted' R^+ class could have been comparable with those of other, overtly recombinant, classes.

We feel that the R^+/R^- nomenclature for the NF/IF strains of *Streptomyces coelicolor* is inappropriate. Historically, NF arose from IF (Fig. 3), rather than the reverse, indicating no close analogy between NF and IF *S. coelicolor* and F^+ and F^- *Escherichia coli*. Furthermore, the designation R^- (recombination-less: Sermonti, 1969) for the IF strains could cause confusion with the very different *rec*$^-$ (recombinationless) mutations of *E. coli* (Clark & Margulies, 1965) and other microbes. In any case, IF × IF (and even UF × UF) crosses are far from sterile, at least under our conditions: the early crosses must have involved exclusively such strains, and they allowed quite effective recombinant selection (Hopwood, 1959). The levels of fertility observed by Sermonti & Casciano (1963) in $R^+ \times R^-$ and $R^- \times R^-$ crosses were some 100-fold lower than those we observe in NF × IF and IF × IF crosses, perhaps because of a difference in media. Thus their $R^- \times R^-$ crosses yielded recombinants at barely detectable frequencies and were described as sterile. However, this has always been a relative term (Sermonti & Casciano, 1963; Sermonti, 1969).

In this paper we have merely defined in genetic terms some of the fertility variation in *Streptomyces coelicolor* A3(2). An understanding of the mechanisms involved in the change of one fertility type to another, in determining the polarization and asymmetry of crosses, the exclusion of the 9 o'clock region of the UF genome in NF × UF crosses (Hopwood *et al.* 1969), and the abolition of asexual reproduction in such crosses

(Hopwood *et al.* 1968) must await further work. It seems likely that the control of sexual reproduction in this morphologically complex bacterium, particularly in relation to morphological differentiation (Hopwood *et al.* 1969), may provide an insight into biological problems that do not arise in the much simpler eubacterial systems.

It is a pleasure to acknowledge the able assistance, during successive periods of this investigation, of Mrs Anne Rush and Mrs Helen Tovell. We should particularly like to thank Mrs Helen M. Wright (née Ferguson) for her skilled performance of the experiments in Table 8 and Fig. 4, and for the preparation of the specimens in Pl. 1. One of us (A. V.) gratefully acknowledges a post-doctoral stipend from Science Research Council grant number B/SR/4060.

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NF
IF UF
NF tester
UF tester
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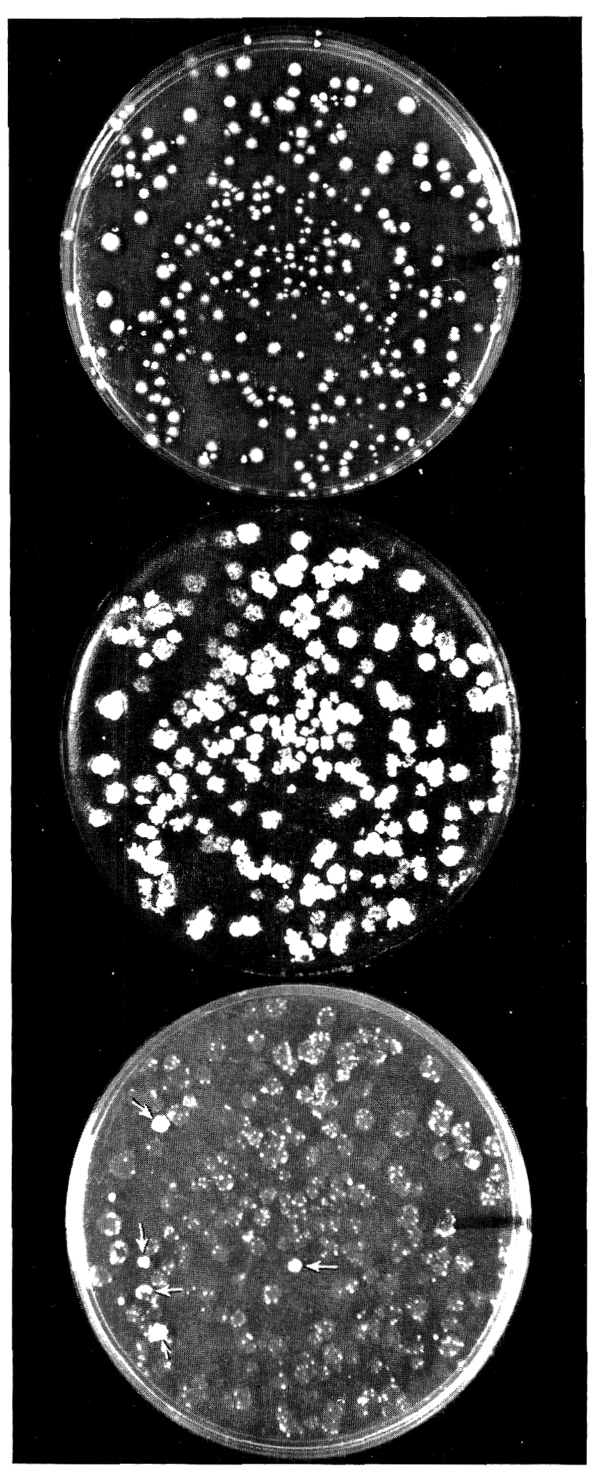

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EXPLANATION OF PLATES

PLATE 1

Diagnosis of the fertility of NF, IF and UF strains by a plate test involving NF and UF tester strains. The top plate is a non-selective streaking of the strains to be tested. The middle two plates are non-selective 'plate crosses' prepared by replicating the top plate to 'lawns' of spores of the tester strains. The bottom two plates are selective for recombinants and were prepared by replicating the plate crosses to selective media.

PLATE 2

Isolation of UF variants from an IF strain. The top plate bears the survivors of u.v. irradiation of strain A 327 (see Table 6). The middle plate is a plate cross involving NF tester strain 1100. The bottom plate is selective for recombinants; five UF variants have given rise to dense patches of recombinants (arrows), whereas each IF colony produced only a few recombinants on a background of slight parental growth.

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SHORT COMMUNICATIONS

Origin of the D-1-Aminopropan-2-ol Fragment of Vitamin B_{12}

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1-Aminopropan-2-ol is a component of vitamin B_{12} (Cooley, Ellis & Petrow, 1950) possessing the Dg-configuration (Wolf, Jones, Valiant & Folkers, 1950). X-Ray crystallography confirmed that the amino alcohol linked the substituted corrin and nucleotide-like ring systems (Hodgkin *et al.* 1956; Hodgkin, 1958).

The metabolic origin of the nitrogen atom of D-1-aminopropan-2-ol in vitamin B_{12} was established using [^{15}N]-L-threonine, added to the growth medium of *Streptomyces griseus* (Krasna, Rosenblum & Sprinson, 1957). It was suggested that the carbon skeleton of the aminopropanol moiety was also derived from threonine by decarboxylation, although this was not investigated. Direct decarboxylation of the amino acid has never been demonstrated, and neither the threonine nor threonine-*O*-phosphate analogues of vitamin B_{12} serve as precursors of cobinamide in either *Escherichia coli* or *Propionibacterium shermanii* (Bernhauer & Wagner, 1961, 1962). The discovery of L-threonine dehydrogenase (Neuberger & Tait, 1960, 1962) and D-1-aminopropan-2-ol dehydrogenase activity (Turner, 1966; Dekker & Swain, 1968; Lowe & Turner, 1968) revived interest in the possibility that the free amino alcohol, formed from L-threonine via aminoacetone, served as a precursor. The roles of L-threonine and D-1-aminopropan-2-ol in vitamin B_{12} biosynthesis by a Streptomyces strain were therefore investigated using ^{14}C-labelled compounds.

EXPERIMENTAL

The actinomycete used by Krasna *et al.* (1957) was not available and the alternative *Streptomyces olivaceus* NCIB 8238 was chosen in view of its previous use in studies of vitamin B_{12} production (Hall, Benedict, Wiesen, Smith & Jackson, 1953; Weygand, Klebe & Trebst, 1954). Growth under conditions described by Hall *et al.* (1953). gave a yield of 0·5 mg. vitamin B_{12}/l. of entire culture in our hands. The vitamin was assayed microbiologically with the auxotrophic mutant *Escherichia coli* NCIB 8134 (Davis & Mingioli, 1950; Bessell, Harrison & Lees, 1950). L-Threonine (5·4 and 208 mCi/mmole) was obtained from the Radiochemical Centre, Amersham, Buckinghamshire. [3-^{14}C]-DL-1-Aminopropan-2-ol hydrochloride (0·085 mCi/mmole) was kindly donated by Pfizer Ltd, Sandwich, Kent. [U-^{14}C]-D-1-Aminopropan-2-ol hydrochloride was made from [U-^{14}C]-L-threonine (208 mCi/mmole) by a modification of the thermal decarboxylation method of Chatelus (1964) (see Lowe & Turner, 1970). Radioactive D-1-aminopropan-2-ol was separated from unreacted L-threonine and non-radioactive phenylethylamine by thin-layer chromatography on cellulose using butan-1-ol + glacial acetic acid + water (12 + 3 + 5, by vol.). The R_F values were 0·39, 0·55 and 0·76

respectively. The radioactive amino alcohol also co-chromatographed with authentic D-1-aminopropan-2-ol as a single spot in ethanol + aqNH_3(sp. gr. 0·880) + water (18 + 1 + 1, by vol.). The yield was about 16 %.

RESULTS

In initial experiments the uptake of radioactivity from [U-^{14}C]-L-threonine (0·55 mg./l.; sp. act. 5·4 mCi/mmole) in growing cultures of *Streptomyces olivaceus* was complete and concomitant with the growth phase. Radioactivity within the micro-organism was mainly accounted for as threonine in cellular protein as shown by extraction and hydrolysis. Labelled 1-aminopropan-2-ol was not identified in any of the subcellular fractions, i.e. cold TCA, hot ethanol, hot TCA and hydrolysed protein (see Roberts, Abelson, Cowie, Bolton & Britten, 1957). In similar experiments radioactivity from [3-^{14}C]-DL-1-aminopropan-2-ol (2·0 mg./l.; sp. act. 0·085 mCi/mmole) was found to be taken up only during the stationary phase, coinciding with the production of vitamin B_{12}. Chromatographic analysis of culture supernatants indicated complete uptake of DL-1-aminopropan-2-ol with the simultaneous excretion of several acidic compounds, the major one being identified as lactate. Analysis of radioactivity in subcellular fractions (see Roberts *et al.* 1957) did not show the presence of free labelled 1-aminopropan-2-ol or aminoacetone. Differences in the metabolism of L-threonine and DL-1-aminopropan-2-ol by *Streptomyces olivaceus* were further evident in studies using washed bacteria suspended in 0·1 M-phosphate buffer, pH 7. L-Threonine uptake (measured as the incorporation of radioactivity) occurred more rapidly when the actinomycete was harvested during the growth rather than stationary phase. Conversely, radioactivity from labelled DL-1-aminopropan-2-ol was only taken up by the micro-organism harvested in the stationary phase, and after a lag period of several hours.

To investigate the incorporation of radioactivity into the synthesized vitamin B_{12} [U-^{14}C]-L-threonine (0·5 μmole; 100 μCi) was added to 6 l. of growth medium and the actinomycete cultured in 1000 ml. aliquots in 2 l. Ehrlenmeyer flasks at 28° for 5 days. Parallel experiments were carried out with an addition of [U-^{14}C]-D-1-aminopropan-2-ol (0·1 μmole; 16 μCi). Vitamin B_{12} was extracted from the cultures as outlined in Table 1, the purified cyanocobalamin was hydrolysed and 1-aminopropan-2-ol isolated as described by Cooley, Ellis & Petrow (1950) and purified by thin-layer chromatography. From the data shown in Table 1 radioactivity from [U-^{14}C]-L-threonine was specifically incorporated into the D-1-aminopropan-2-ol moiety of vitamin B_{12}. The ratio of radioactivity/carbon atom of D-1-aminopropan-2-ol to radioactivity/carbon atom of the remainder of the vitamin molecule was 12:1. Although the vitamin B_{12} synthesized in the presence of [U-^{14}C]-D-1-aminopropan-2-ol was radioactive, the amino alcohol released on hydrolysis had no measurable radioactivity and hence no specific incorporation of the amino alcohol had occurred. The analysis of radioactivity in other subcellular fractions (Roberts *et al.* 1957) of the micro-organism showed that D-1-aminopropan-2-ol had been incorporated into a wide range of substances, including lipids and proteins. Solvent extraction and chromatography, followed by hydrolysis of lipids and rechromatography, failed to show that the amino alcohol had been incorporated specifically into lipids, e.g. as a phosphatidyl base analogous to ethanolamine.

Table 1. *Incorporation of radioactivity from [U-^{14}C]-L-threonine and [U-^{14}C]-D-1-aminopropan-2-ol into the amino alcohol moiety of vitamin B_{12}*

The freeze-dried material from 6 l. of entire culture medium was suspended in 400 ml. water containing 2 mmoles KCN and autoclaved. The vitamin was extracted into 150 ml. *p*-cresol + carbon tetrachloride (1+4, wt/vol.) and back-extracted into 50 ml. of butan-1-ol + water (1+1, by vol.). Butan-1-ol was removed under reduced pressure and vitamin B_{12} isolated from the aqueous solution by chromatography on the weak cation exchange resin Bio-Rex 70 as described by Strohecker & Henning (1965), followed by chromatography on DEAE cellulose from which it was eluted by 0·2 M-NaCl. The vitamin was finally purified by thin-layer chromatography on cellulose in the lower phase of butan-2-ol + methanol + sodium acetate buffer 0·2 M, pH 4·6 (4+1+12, by vol.), R_F 0·85. Vitamin B_{12} was measured microbiologically as described in the text, and radioactivity was determined with a Nuclear Chicago gas flow counter.

	U-[^{14}C]-L-Threonine		[U-^{14}C]-D-1-Aminopropan-2-ol	
Treatment	Total vitamin B_{12} (mg.)	Radioactivity (d.p.m.)	Total vitamin B_{12} (mg.)	Radioactivity (d.p.m.)
Culture medium	2·16	$2·22 \times 10^8$	1·62	$3·56 \times 10^7$
p-Cresol/carbon tetrachloride	1·97	—	1·46	—
Aqueous butan-1-ol	1·85	$9·70 \times 10^5$	1·39	$4·20 \times 10^5$
Bio-Rex 70	0·95	$2·03 \times 10^5$	0·70	$1·09 \times 10^5$
DEAE cellulose	0·65	$1·07 \times 10^5$	0·53	$2·40 \times 10^4$
Thin-layer chromatography	0·44	$5·64 \times 10^4$	0·36	$1·23 \times 10^4$
Hydrolyzed vitamin B_{12}	0·31	$3·96 \times 10^4$	0·25	$8·66 \times 10^3$
D-1-Aminopropan-2-ol isolated from B_{12}	—	$1·36 \times 10^4$	—	$< 1·0 \times 10^2$

DISCUSSION

The incorporation of isotope from [U-^{14}C]-L-threonine into the 1-aminopropan-2-ol fragment of vitamin B_{12} shows that the amino acid contributes the carbon skeleton, as well as the nitrogen atom (Krasna *et al.* 1957), of the amino alcohol. The failure to detect specific incorporation of isotope from [U-^{14}C]-D-1-aminopropan-2-ol suggests that the free amino alcohol may not be involved in vitamin B_{12} biosynthesis. The incorporation of radioactivity into a wide range of other substances, including lipids and proteins, confirmed that the D-enantiomorph is readily metabolized by *Streptomyces olivaceus* probably by way of aminoacetone (Lowe & Turner, 1970).

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Peptostreptococcus elsdenii from the Caecum of Pigs

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(*Accepted for publication 1 October 1970*)

Large, Gram-negative, anaerobic cocci (LC) fermenting lactate were first isolated by enrichment culture from the sheep rumen (Elsden, Volcani, Gilchrist & Lewis, 1956). High counts (about 10^9/ml.) were obtained from young calves but not from adult ruminants by Hobson, Mann & Oxford (1958). The cocci were cultured in numbers of 10^6 to 10^8/ml. from the rumen of cattle fed high-grain rations and named *Peptostreptococcus elsdenii* by Gutierrez, Davis, Lindahl & Warwick (1959). They can be important fermenters of lactate in the rumen and are associated with amylolytic lactate producers such as *Streptococcus bovis* when large amounts of starch are consumed by ruminants. The production of propionic acid by *P. elsdenii* via the acrylate pathway (Ladd, 1959) has attracted the particular interest of biochemists and nutritionists. The types of bacteria active in the rumen are not found only in this organ (Giesecke, 1970), and recent experiments indicate that *P. elsdenii* is also a prominent lactate fermenter in the large intestine of pigs fed on diets containing potatoes.

METHODS

Media. Reinforced clostridial agar (RCM, Oxoid Ltd) in roll-tube cultures with a gas phase of 5 % (v/v) CO_2+95 % (v/v) N_2 was used for initial isolation, and a semisolid lactate medium similar to that of Hobson *et al.* (1958), but incubated under pure N_2, was used for subculturing, preserving and testing isolates.

Isolation. Samples of caecum contents were obtained from six pigs with caecum cannulae, three of which were fed on raw potatoes and three on steamed potatoes (Ledinek, 1970). Amounts of 10 g. were vigorously shaken with 90 ml. of anaerobic mineral buffer solution (Bryant & Burkey, 1953), and diluted through 10^{-11} before inoculation into RCM.

Biochemical reactions and pH. Biochemical tests were performed according to Hobson *et al.* (1958) with 1 % (w/v) of substrates replacing lactate in the semisolid medium. For growth at different pH values the lactate medium was buffered with tris-maleate (Geigy, 1968). All pH values were measured with a glass electrode, and fermentation acids determined by gas chromatography (Ledinek, 1970).

RESULTS

After 2 to 3 days of incubation in RCM tubes the large Gram-negative cocci formed greenish yellow, circular colonies 1 to 2 mm. in diameter, in numbers corresponding to viable counts of 10^9 to 10^{10}/g. caecum contents. The properties of 13 isolates are listed in Table 1. Viable bacteria observed after incubation (for 24 h.) in semisolid medium

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Table 1. *Properties of Peptostreptococcus elsdenii from the caecum of pigs*

	Isolates from pigs fed raw potatoes									Isolates from pigs fed steamed potatoes				
	R11	R12	R13	R14	R21	R24	R25	R26	R31	G11	G12	G13	G32	
Dilution per ml. at isolation ...	10^{-9}	10^{-9}	10^{-8}	10^{-9}	10^{-9}	10^{-8}	10^{-8}	10^{-8}	10^{-9}	10^{-8}	10^{-8}	10^{-8}	10^{-8}	
Cell dimensions (μm.)...	1·9 × 1·1		1·8 × 1·4		2·0 × 1·4		2·0 × 1·5		2·0 × 1·5		1·8 × 1·3		1·9 × 1·2	
	Fermentation reactions*													Mean final pH†
Xylose	+	+	+	+	+	−	−	+	+	+	+	+	−	5·7
Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	5·9
Fructose	+	+	+	+	+	+	+	+	+	+	+	+	+	4·8
Maltose	−	+	−	+	−	+	+	+	+	−	+	−	+	5·7
Sucrose	+	+	+	+	+	+	+	+	+	+	+	+	+	5·0
Raffinose	+	+	+	+	+	+	+	+	+	+	+	+	+	5·3
Mannitol	+	+	+	+	+	+	+	+	+	+	+	+	+	5·5
DL-Lactate	+	+	+	+	+	+	+	+	+	+	+	+	+	7·0

All isolates were Gram-negative cells arranged in diplococci or short chains; all isolates produced H_2S. Gelatine liquefaction, indole production and nitrate reduction were always negative.

* Arabinose, rhamnose, galactose, mannose, trehalose, cellobiose, lactose, starch, inulin, salicin, aesculin, sorbitol, dulcitol, inositol, glycerol, and succinate were not fermented.

† Initial pH 6·7 to 6·8.

showed pronounced flattening of adjacent cell walls and occurred in pairs and short chains of up to 10 cells. In older cultures bacteria of variable size were observed, sometimes arranged in long chains of more than 50 units.

In addition to lactate a limited number of substrates were fermented with the production of gas, which was not analysed. Preliminary observations on five isolates indicated that acetic, propionic, butyric and valeric acids were the fermentation acids from lactate, and caproic acid was formed in addition from glucose. Of seven strains tested at different pH values, all grew at pH 5·2 to 7·2, three at pH 7·8, but none at pH 5·0 or below or pH 8·1 or above. Sodium citrate, which was first tried as a buffer, inhibited growth even at concentrations of 0·01 M. Of five isolates tested, all grew at 37° and 42°, two at 45°, and three at 30°, but no growth was observed at 25° or 50°.

On the basis of these characters it is concluded that the strains of large Gram-negative cocci from the caecum of pigs belong to the species *Peptostreptococcus elsdenii.*

DISCUSSION

Dimensions, Gram-negative staining, anaerobiosis, utilization of lactate, production of H_2S and of fatty acids with 2 to 6 carbon atoms are distinctive features of *Peptostreptococcus elsdenii* (Elsden *et al.* 1956; Gutierrez *et al.* 1959). The isolates from pigs are similar to rumen strains, though some differences appear to exist. The size is smaller than that given for isolates from the rumen (2·4 × 2·6 μm.), but the ages of the latter cultures were not reported. The pig strains differed from those of Elsden *et al.* (1956) and Hobson *et al.* (1958) in fermenting sucrose and raffinose but not sorbitol, and in variable fermentation of maltose and xylose. The strains of Gutierrez *et al.* (1959) failed to attack sorbitol and mannitol but some fermented sucrose and glycerol. *Peptostreptococcus elsdenii* from calves produced growth at a broader range of pH (4·8 to 8·1) than pig strains and seemed less sensitive to citrate (Hobson *et al.* 1958).

Gram-negative, anaerobic cocci resembling *Peptostreptococcus elsdenii* were cultured only occasionally and in very low counts from the gut of young pigs by Fuller & Lev (1964). In the present experiments the pigs were reared, housed, and cared for separately and had thus no contact with ruminants. Microscopic observations showed that Gram-negative cocci accounted for about 25% of the total bacteria in their caecum contents, and large Gram-negative cocci were 17·4% of the total anaerobic bacteria (10^{10} to 10^{11}/g. caecum contents) cultured on RCM by Ledinek (1970). On feeding potatoes, considerable amounts of carbohydrate escape pancreatic digestion and are fermented in the large intestine where lactic acid appears to be an important intermediate. It is thus obvious that *P. elsdenii* may occupy an ecological niche in digestive tract habitats other than the rumen.

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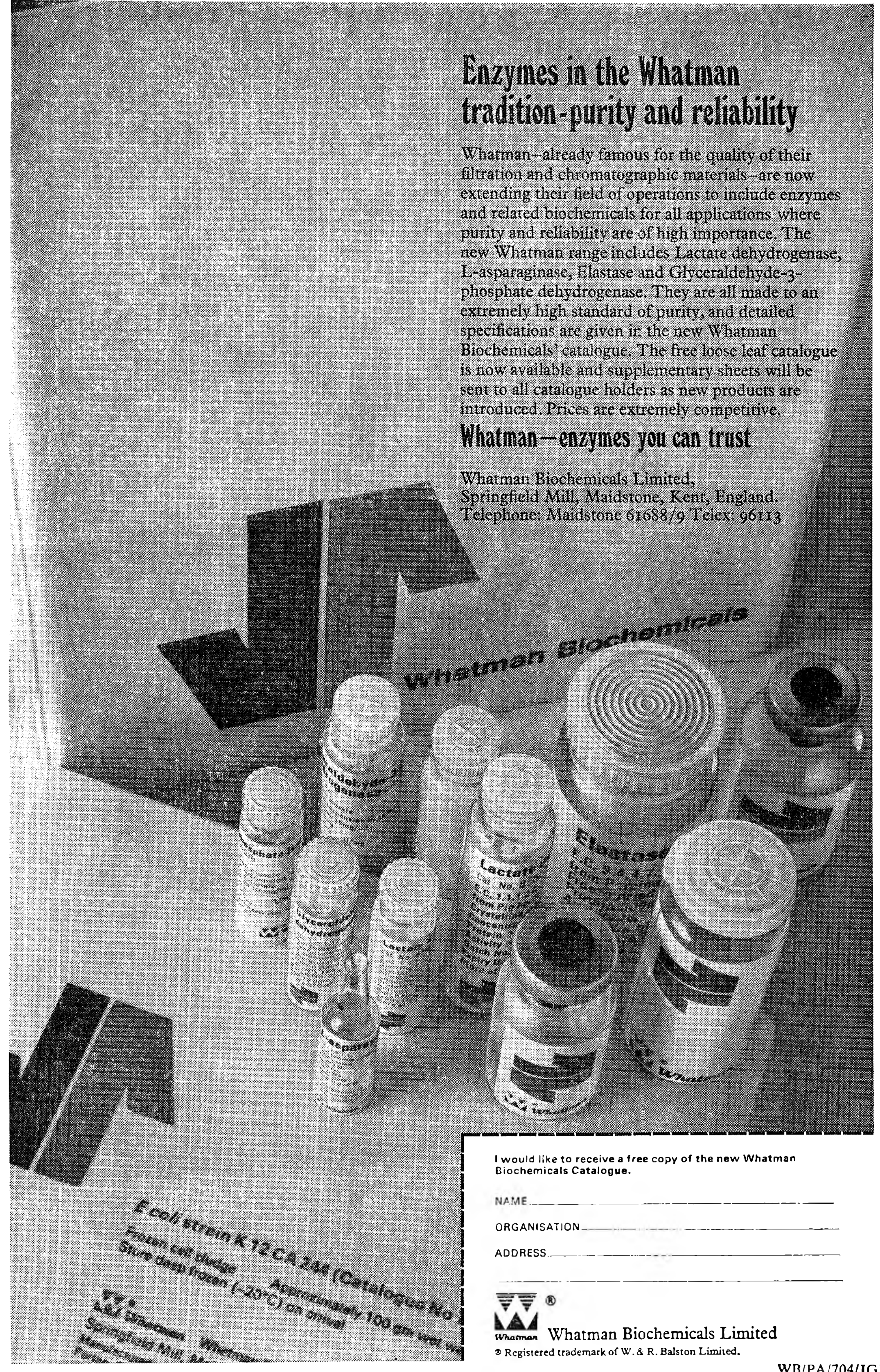
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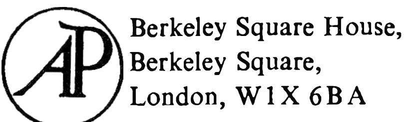

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