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EDITOR: L. E. GLYNN

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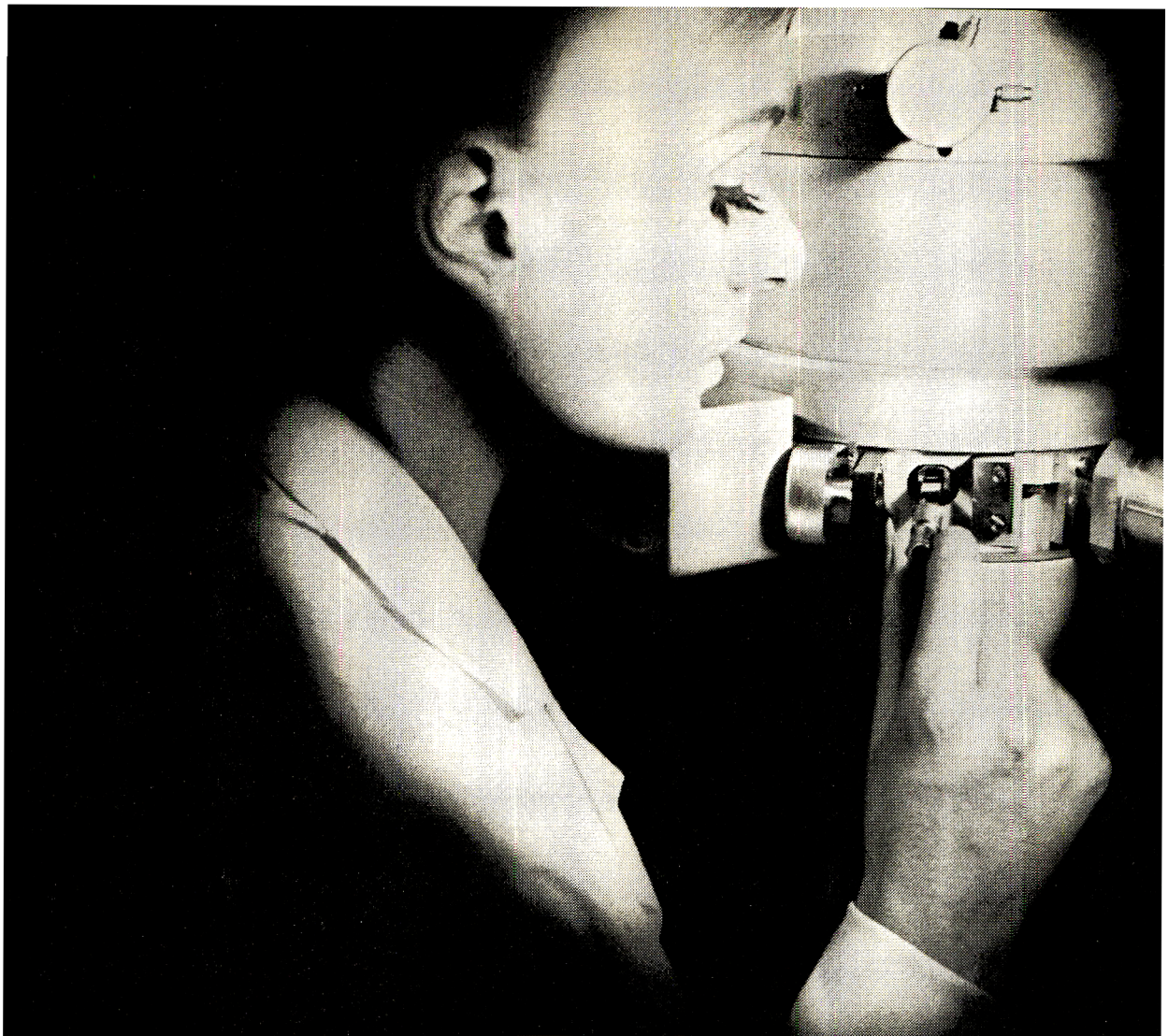

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THE JOURNAL OF GENERAL MICROBIOLOGY

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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 *J. gen. Microbiol.* (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 ed. (1957), edited by R. S. Breed, E. G. D. Murray and A. P. Hitchens. London: Ballière, Tindall and Cox.

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

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

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

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Studies on Lipids of Soil Micro-organisms with Particular Reference to Hydrocarbons

By J. G. JONES*

Department of Microbiology, University College of South Wales and Monmouthshire, Cathays Park, Cardiff, CF1 3NR

(*Accepted for publication 18 August 1969*)

SUMMARY

The lipids of soil micro-organisms harvested from simple and complex media varied from 2 to 20% in bacteria, 10 to 20% in fungi, 2·5 to 15% (w/w) in algae. The bulk of the lipid usually consisted of polar compounds; paraffinic hydrocarbons comprised 0·008 to 2·7% in bacteria, 0·04 to 0·7% in fungi, 0·08 to 2·9% (w/w) in algae. Lipid contents of algae were more affected by growth medium composition than were those of the bacteria and fungi. Gas–liquid chromatography showed that the hydrocarbons were paraffins in the range C_{16-36}. The hydrocarbon patterns varied with species and growth medium. A peak in the range C_{27-31} was usual in bacteria with sometimes a minor peak in the range C_{18-22}. The fungi exhibited slightly more stable hydrocarbon patterns (except *Trichoderma viride*) than bacteria and most showed major and minor paraffin peaks in similar regions.

The algae showed a peak at C_{17} regardless of the growth medium but *Tetraspora gelatinosa* showed an increase in C_{25} and C_{27} paraffin content when grown with acetate. The ratio of paraffin chains consisting of odd numbers of carbon atoms to those containing even numbers of carbon atoms was around unity for bacteria and fungi, also for *T. gelatinosa* when it was grown on CO_2 as sole carbon source. The *Nostoc* sp. and *T. gelatinosa* (grown with CO_2 plus acetate) contained predominantly C_{odd} paraffins.

INTRODUCTION

The first report of the isolation of hydrocarbons from microbes was by Jankowskii & ZoBell (1944). Oppenheimer (1965) reported the production of 'hydrocarbon-like' materials in *Desulfovibrio desulfuricans* and in mixed cultures of micro-organisms. Other investigations of microbial hydrocarbons have been reported by Albro & Huston, 1964; Oró, Tornabene, Nooner & Gelpi, 1967; Tornabene, Gelpi & Oró, 1967; Tornabene, Bennet & Oró, 1967; Tornabene & Oró, 1967; Han *et al.*, 1968.

The range of *n*-paraffins isolated from fungi is very similar to that in bacteria although, as with bacteria, the composition of the mixture varied with the species investigated. Merdinger & Devine (1965) and Merdinger & Frye (1966) demonstrated *n*-alkanes in the range C_{16-39} with a peak at C_{22} in *Debaryomyces hansenii*; glucose carbon was incorporated into these hydrocarbons. Oró, Laseter & Weber (1966) isolated a similar range of hydrocarbons from fungal spores and noted a C_{odd} predominance with peaks at C_{27}, C_{29} and C_{35}. Most investigations of algal paraffins

* Present address: Freshwater Biological Association, Windermere Laboratory, The Ferry House, Far Sawrey, Ambleside, Westmorland.

indicate a carbon preference index (ratio of C_{odd} to C_{even} compounds) of unity. Patterson (1967), however, noted an increase in concentration of the C_{25} and C_{27} paraffins in *Chlorella vulgaris* when it was grown chemo-organotrophically. Other workers have noted a lower boiling point range (C_{15-25}) of *n*-paraffins in certain algae (Oró *et al.* 1967; Han *et al.* 1968), sometimes with peaks around C_{17}. The present study reports patterns of alkanes produced by soil micro-organisms to determine what role if any these alkanes played in the hydrocarbon spectrum of the soil.

METHODS

Bacteria. These were dominant species isolated from an upland moorland soil. Methods of isolation and details of the site have been reported previously (Jones & Edington, 1968). The species were *Arthrobacter* sp. NCIB 10407, *Micrococcus* sp. NCIB 10405, *Corynebacterium* sp. NCIB 10406, *Bacillus* sp. NCIB 10404, *Mycobacterium* sp. NCIB 10403, and a Gram-negative rod, NCIB 10408.

Fungi and algae were isolated from the same soil samples and so were *Penicillium* sp., *Aspergillus* sp., *Trichoderma viride*, *Saccharomyces* sp., *Tetraspora gelatinosa* and *Nostoc* sp.

Preparation of material. The basal succinate medium (BSM) for the fungi or bacteria contained (g./l. distilled H_2O): Na succinate, 10·0; K_2HPO_4, 1·5; KH_2PO_4, 0·5; $MgSO_4.7H_2O$, 0·2; $(NH_4)_2SO_4$, 2·0; KNO_3, 2·0; NaCl, 0·1; $FeCl_3$, 0·01; yeast extract (oxoid), 1·0; pH 7·2 for bacteria, 5·0 for fungi. KNO_3 was omitted for *Mycobacterium* sp. The complex medium used was tryptone soya broth (oxoid) (TSB) adjusted to pH 5·0 for fungi. Slope cultures of bacteria and fungi were subcultured into 20 ml. starter cultures of BSM which were inoculated into 500 ml. BSM or TSB and subsequently into 9 l. batches of the respective media. The micro-organisms were harvested when they entered the stationary phase. The basal medium for *Tetraspora gelatinosa* was Knop solution (g./l. dist. H_2O): KNO_3, 1·0; $Ca(NO_3)_2$, 0·1; K_2HPO_4, 0·2; $MgSO_4.7H_2O$, 0·1; $FeCl_3$, 0·001, pH 7·0 (code 1 in Tables). The *Nostoc* sp. was grown on the medium (code 3 in Tables) of van Baalen (1965), pH 7·0, which also contained 6 ml. of van Baalen's 'H_5' trace element solution. Na_2CO_3 (0·020 g./l.) was added to both media. The algae were also grown with 10^{-2} M-Na acetate (Hoare, Hoare & Moore, 1967) added (code 2 and 4 in Tables).

The cultures were grown under conditions of forced aeration, bacteria and fungi at 25° in the dark and algae at *c.* 20°, 6 in. from 3-ft. fluorescent lamps (*c.* 800 ft. candles). Bacteria were harvested in the 6 l. swing-out head of the Mistral 6 L centrifuge (MSE Ltd.) at 1570*g* for 30 min., the filamentous fungi were harvested by filtration, and the algae by using the continuous head attachment on the MSE 17 centrifuge at 3000 rev./min. They were washed twice and dried overnight under vacuum over P_2O_5. The dried material was ground in a pestle and mortar.

Extraction procedures. The ground samples in fat-free thimbles were extracted with a 2+1 (v/v) chloroform+methanol mixture for 8 hr in a Soxhlet-type extractor (ASTM D297, Gallenkamp & Co. Ltd.). Solvent was removed *in vacuo* at 40° to give the 'total lipid fraction'.

Column chromatography. The methods used were based on those of Evans, Kenny, Meinschein & Bray (1957), Meinschein & Kenny (1957), Kolattukudy (1966), Oró *et al.* (1967). The total lipid fraction on a silica gel column (prewet with *n*-hexane) was

successively eluted with *n*-hexane, benzene and methanol to give paraffinic, aromatic and polar lipid fractions respectively. The adsorbents were washed with chloroform + methanol (2 + 1), before activation at 110° overnight for silica gel (100 to 200 mesh) and 48 hr at 165° for alumina. If it was not used immediately the adsorbent was stored in the vacuum oven over P_2O_5 at 50°.

The adsorbent: total lipid ratio was never less than 300: 1 (w/w) and the adsorbent: paraffin ratio never less than 1000: 1. The paraffins were recovered from the eluate, then purified by further elution with *n*-hexane through a column of alumina. The column height: diameter ratios were never less than 10: 1. All columns were surrounded by water jackets at 40°, and the rate of elution was always 8 ml./min.

The solvents were partially removed in a rotary evaporator at 40°. and completely in a vacuum oven at the same temperature over a dessicant.

Gas chromatography. A Pye 'Series 104' Chromatograph (Model 24) was used fitted with stainless steel columns packed with Phasecarb L on Universal support B, 85–100 mesh (Phase Separations Ltd., Cheshire). Columns were purged with argon at 325° for 48 hr before use. Samples were run at 300° isothermally also programmed from 230° to 280° at 8°/min., then maintained at the higher temperature. Carrier gas flowed at 50 ml./min. (45 lb./in.^{2}), hydrogen, 50 ml./min. (18 lb./in.^{2}) and air, 500 ml./min. (30 lb./in.^{2}) were supplied to a flame ionization detector. Samples, dissolved in *n*-hexane, were injected with a 1 μl. syringe (S.G.E. Melbourne) fitted with a Chaney adapter. All columns were plugged with glass wool, since stainless steel column plugs caused spread of the sample. The recorder was a Kent Mark 3 Instrument (George Kent Ltd., Beds.; range −0·1 to 1·0 mV).

The retention volumes of the *n*-alkanes were compared with that of *n*-eicosane which was added to the samples on their second test run. At regular intervals a mixture of known *n*-alkanes (C_{16-36}) was injected for further comparisons. The relative concentrations of the hydrocarbons were calculated from the peak areas.

Reagents. Solvents used in extraction and chromatographic procedures were of Analar grade or of higher purity. Solvents of insufficient purity were redistilled. Storage was over anhydrous Na_2SO_4.

RESULTS

Analysis of lipid fractions. Table 1 shows that the average fungal lipid contents were generally higher than those in bacteria. The fungi, *Aspergillus* sp., *Trichoderma viride*, and the bacteria, *Bacillus* sp., *Mycobacterium* sp. and the Gram-negative rod, had higher lipid contents when grown on TSB. The algae, however, contained less lipid when acetate was added to the salts media. In nearly all the microbial lipids the smallest fraction was paraffinic and the largest contained polar compounds.

Gas chromatographic analyses of microbial hydrocarbons. These are presented in Table 2; unidentified peaks bear the symbol '?'. The carbon preference indices of the paraffins are given in Table 3; in the fungi and bacteria they tended to unity. The *Trichoderma viride* paraffins showed a slight C_{even} predominance especially when the fungus was grown on TSB. Algal paraffins showed a strong C_{odd} predominance except *Tetraspora gelatinosa* grown on Knop's medium with no added acetate. There was a slight tendency for bacterial paraffins from BSM to exhibit greater C_{odd} predominance, but this was not considered significant. *Arthrobacter* sp. contained the highest proportion of *n*-alkanes in the range C_{16-22}, especially if grown on TSB. The most even distri-

bution of *n*-alkanes (C_{16-32}) was in the Gram-negative rod, grown on both media. and in *Corynebacterium* sp. grown on TSB. The simplest pattern was in the *Micrococcus* sp., which consisted almost entirely of *n*-alkanes in the range C_{25-29}. There was no clear trend in the patterns of bacterial paraffins but most species showed a peak in the region C_{26-31} which was most evident in the *Bacillus* sp. and the *Mycobacterium* sp. regardless of growth medium. A minor peak was sometimes noted in the region C_{18-22}.

Table 1. *Microbial lipids*

Lipids were extracted from micro-organisms (for media see 'Methods') with chloroform + methanol (2 + 1) for 8 hr and eluted through silica gel column with *n*-hexane, benzene and methanol. All solvents removed under vacuum at 40°. Contents expressed as mg. hydrocarbon/g. dry wt organism.

	Medium	Lipid	Paraffins	Aromatics	Polar compounds
Arthrobacter sp.	BSM	41	9·3	1·9	10·4
	TSB	22	3·5	0·8	8·3
Micrococcus sp.	BSM	100	6·8	33·4	45·4
	TSB	30	3·8	0·9	10·1
Gram-negative rod	BSM	28	0·08	2·7	13·9
	TSB	126	2·4	1·2	105·4
Corynebacterium sp.	BSM	178	1·7	6·2	62·1
	TSB	49	10·9	2·5	22·3
Bacillus sp.	BSM	64	3·3	32·0	18·9
	TSB	92	0·5	0·9	35·4
Mycobacterium sp.	BSM	151	26·9	10·4	61·5
	TSB	193	21·4	3·2	97·1
Penicillium sp.	BSM	145	0·8	1·6	140
	TSB	158	0·5	1·9	95·7
Aspergillus sp.	BSM	101	0·6	1·1	46·2
	TSB	190	0·4	9·9	87·7
Trichoderma viride	BSM	43·5	7·0	3·7	29·3
	TSB	112	0·6	9·8	84·4
Saccharomyces sp.	BSM	178	0·4	0·8	3·5
	TSB	170	1·1	3·9	54·7
Tetraspora gelatinosa	1	144	28·8	23·8	81·1
	2	96	6·6	8·3	48·7
Nostoc sp.	3	104	5·9	30·7	88·4
	4	26	0·8	4·8	29·1

Fungal hydrocarbons showed a more stable pattern than bacteria, with a major peak at around C_{29} and a minor one in the range C_{17-20}. The exception was *Trichoderma viride* grown on BSM, which had peaks at C_{30} and C_{34}. These disappeared on TSB and an increase of an unidentified component around C_{16} and a slight peak at C_{24} were noted. These components make a major contribution towards the slight C_{even} predominance of the paraffins of this species when grown on TSB.

The algal paraffins showed a large peak at C_{17} regardless of the growth medium used. Acetate in the medium increased the C_{25} and C_{27} paraffins in *Tetraspora gelatinosa* but not *Nostoc* sp. The blue-green alga contained a simpler pattern of hydrocarbons than any of the other micro-organisms, and this did not include a peak at C_{29}.

A few hydrocarbons remained unidentified and other *n*-alkane peaks were thought, because of their shape, to be impure. These are considered in more detail in the discussion.

Table 2. *Hydrocarbon contents of micro-organisms*

Extracts of micro-organisms were examined by g.l.c. (for details of culture media, extraction and chromatography see text); hydrocarbon contents as % (w/w) total paraffin from peak areas; 't' indicates 'trace' detected, * indicates impure material, ? signifies unidentified hydrocarbon peak.

Percentage of total paraffins

	Gram-negative rod		*Arthrobacter* sp.		*Micrococcus* sp.		*Corynebacterium* sp.		*Bacillus* sp.		*Mycobacterium* sp.		*Penicillium* sp.		*Aspergillus* sp.		*Saccharomyces* sp.		*Trichoderma viride*		*Tetraspora gelatinosa*		*Nostoc* sp.	
	BSM	TSB	BSM	TSB	BSM	TSB	BSM	TSB	BSM	TSB	BSM	TSB	BSM	TSB	BSM	TSB	BSM	TSB	BSM	TSC	1	2	3	4
n-C_{14}	—	—	—	—	—	—	—	—	4·82	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
n-C_{15}	—	9·30	0·09	t	—	—	t	0·17	—	0·17	—	—	t	—	0·14	0·74	—	0·38	0·51	0·84	—	—	t	—
?	—	—	—	—	—	—	0·09	0·83	—	—	—	—	0·07	—	—	—	—	t	—	—	—	—	—	t
n-C_{16}	0·38	4·44	21·39	0·02	—	—	0·09	1·33	—	*1·57	t	0·05	t	0·10	0·13	0·37	—	0·28	—	t	—	t	—	0·04
?	—	—	—	—	—	—	—	—	—	—	—	—	—	t	0·06	t	—	0·13	6·23	36·8	t	—	1·57	—
n-C_{17}	3·85	10·69	1·30	0·07	0·09	—	*9·66	7·33	t	9·13	1·46	1·04	1·61	2·5	*1·7	4·98	0·10	3·88	t	—	*19·61	*30·05	71·39	75·65
?	—	—	0·26	—	—	3·77	—	—	—	—	—	—	3·89	—	—	—	—	4·28	—	—	—	—	—	—
n-C_{18}	5·77	6·94	0·57	0·10	0·04	1·88	*9·14	10·00	0·04	4·43	2·59	1·56	7·32	*3·06	*0·75	2·60	0·33	1·75	2·08	0·41	0·17	t	—	—
?	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	t	—	0·88	—	—	—	—	—	—
n-C_{19}	4·62	3·47	0·39	0·78	0·01	—	6·90	6·00	0·10	0·87	5·56	1·82	1·14	*1·11	*0·16	1·08	0·42	1·72	0·51	0·16	0·13	*0·81	0·06	0·09
?	—	—	—	—	—	—	—	t	—	—	—	—	—	t	—	—	—	—	—	—	—	—	0·41	—
n-C_{20}	5·38	1·39	4·52	0·10	0·14	—	3·45	4·00	0·11	1·30	2·22	2·86	1·14	1·04	0·18	0·91	0·50	1·31	1·56	0·94	4·7	2·30	3·92	0·20
?	—	—	—	—	—	—	0·52	0·17	—	—	—	—	—	t	—	—	—	—	—	—	—	—	t	—
n-C_{21}	3·46	1·94	16·87	37·90	0·20	—	3·10	3·33	—	1·30	1·46	2·86	1·43	0·97	0·20	0·69	0·50	1·63	1·29	1·56	1·87	0·53	2·94	0·43
?	—	—	—	3·48	—	—	0·52	—	—	—	—	—	—	t	t	t	—	0·08	—	—	—	t	—	—
n-C_{22}	3·46	3·33	15·30	30·98	0·03	—	3·45	2·67	0·27	1·26	1·46	3·65	1·39	1·60	0·44	1·15	0·63	0·56	0·78	12·00	0·09	0·30	0·06	0·04
?	—	—	—	—	—	—	5·69	—	—	—	—	—	—	—	t	t	—	—	1·55	—	—	—	1·24	—
n-C_{23}	3·08	3·89	2·15	3·40	0·18	—	0·60	6·33	0·50	1·43	4·44	0·05	1·25	1·60	0·72	1·40	0·75	3·28	2·58	7·88	4·09	1·40	4·22	0·59
?	—	—	—	2·80	—	—	2·41	—	—	—	—	—	—	—	t	t	—	—	—	7·35	—	—	—	—
n-C_{24}	3·85	3·89	3·13	4·45	0·21	—	0·52	3·33	2·46	2·96	4·44	2·60	1·71	2·50	2·00	2·20	1·21	2·84	1·69	16·34	0·57	0·15	0·08	0·30
?	—	—	0·21	3·27	—	—	0·43	1·17	—	—	—	—	—	—	—	—	—	—	—	—	t	—	—	—
n-C_{25}	7·31	5·00	1·04	—	0·90	—	1·38	6·33	4·71	5·09	9·63	4·17	4·86	5·35	5·13	4·43	3·75	6·75	2·47	1·25	3·35	20·4	8·31	13·28
?	—	—	0·33	4·9	—	—	0·86	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
?	—	—	—	—	16·83	—	3·10	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
n-C_{26}	6·54	6·67	2·09	0·65	2·89	13·21	5·52	2·50	7·71	8·70	10·37	10·94	8·21	8·89	9·40	8·15	9·17	12·16	5·19	1·09	5·22	0·70	0·39	0·70
?	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	3·35	0·02	—
n-C_{27}	9·62	7·36	7·65	1·67	45·70	11·32	17·93	6·67	32·57	13·39	17·78	10·94	12·96	14·58	15·12	13·29	15·50	11·25	3·12	1·97	13·30	28·37	4·29	4·57
n-C_{28}	7·69	7·50	6·26	1·33	32·40	39·62	7·24	8·00	15·71	14·87	20·74	28·13	13·07	16·25	16·52	16·49	20·00	11·78	5·45	2·50	11·74	2·12	0·37	0·91
n-C_{29}	10·77	8·89	6·26	1·20	0·94	30·19	7·24	6·00	13·00	13·04	11·85	15·62	14·86	15·56	17·64	17·23	19·50	15·19	8·31	3·06	15·65	5·63	0·41	1·39
?	—	—	—	—	0·01	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
?	—	—	—	—	0·06	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
n-C_{30}	3·85	5·00	2·78	1·05	0·01	—	4·14	6·00	8·36	8·87	2·96	6·25	11·25	10·00	13·20	10·83	13·75	8·13	12·47	1·75	10·43	1·28	0·08	0·78
n-C_{31}	19·62	2·50	2·61	0·60	—	—	3·45	10·00	6·07	6·09	2·96	6·25	9·29	10·00	9·52	7·39	9·00	6·75	2·34	1·13	5·65	2·23	—	0·43
n-C_{32}	—	7·22	1·04	0·40	—	—	0·86	8·00	1·57	3·13	—	—	3·00	3·06	3·84	3·46	3·75	2·44	5·06	0·88	0·96	0·60	—	0·30
n-C_{33}	—	—	1·04	0·12	—	—	0·86	—	1·71	1·65	—	—	1·39	2·08	2·80	1·97	1·50	2·13	12·99	0·44	1·39	—	—	0·17
n-C_{34}	—	—	—	0·53	—	—	—	—	0·54	—	—	—	—	—	1·00	0·49	0·63	—	14·55	0·50	—	—	—	—
n-C_{35}	—	—	—	—	—	—	—	—	—	—	—	—	—	—	0·56	—	—	—	4·16	3·88	—	—	—	—
n-C_{36}	—	—	—	—	—	—	—	—	—	—	—	—	—	—	0·60	—	—	—	5·45	—	—	—	—	—

Table 3. *Carbon preference indices derived from chromatographic analysis of microbial hydrocarbons*

	Growth medium	Carbon preference index
Gram-negative rod	BSM	1·38
	TSB	1·20
Arthrobacter sp.	BSM	1·17
	TSB	0·67
Micrococcus sp.	BSM	1·33
	TSB	0·79
Corynebacterium sp.	BSM	1·34
	TSB	1·24
Bacillus sp.	BSM	1·60
	TSB	1·09
Mycobacterium sp.	BSM	0·70
	TSB	1·18
Penicillium sp.	BSM	1·02
	TSB	1·11
Aspergillus sp.	BSM	1·12
	TSB	1·12
Trichoderma viride	BSM	0·76
	TSB	0·54
Saccharomyces sp.	BSM	1·03
	TSB	1·25
Tetraspora gelatinosa	1	1·61
	2	10·70
Nostoc sp.	3	11·56
	4	18·17

DISCUSSION

Lipids made up between 2 and 20% of the bacterial dry weights, the highest levels being found in *Mycobacterium* sp. The levels were similar to those reported by Albro & Huston (1964), Davis (1967) and Davis (1968). There was good correlation between published data and the paraffin contents of the bacteria (Han *et al.* 1968; Tornabene, Bennet & Oró, 1967).

A variety of bacterial hydrocarbon patterns was noted and only certain general trends will be discussed here. The range of *n*-alkanes (C_{16-36}) was similar to those reported by authors mentioned below. The minimum at C_{18-21} reported by Clarke (1966) was only evident in the *Bacillus* sp. grown on BSM; indeed a minor peak at this range was noted in some species. A modest maximum was almost always present at C_{27-30} (Albro & Huston, 1964; Davis, 1968). Tornabene, Bennet & Oró (1967) reported a more even hydrocarbon distribution when *Sarcina lutea* was grown on complex rather than simple medium and although this was true of the Gram-negative rod in this study, it was not observed in the other species. The same authors reported a C_{odd} predominance on a simple medium but not on Trypticase Soy Broth; this trend was not observed in the organisms used in this investigation.

Higher carbon preference indices were sometimes obtained when bacteria were grown on BSM but the values were not considered to be significantly different from those on TSB.

The lipid and hydrocarbon contents of the fungal and algal species were similar to those reported by Han *et al.* (1968) and Davis (1967) and so was the range of paraffins.

The patterns were less variable than those in bacteria but were not always similar to those reported by these authors. The *Saccharomyces* sp. did not have a peak at C_{22} unlike *Debaryomyces hansenii* (Merdinger & Devine, 1965). The patterns of hydrocarbons in three of the fungi (peaks at C_{27-29}) were similar to those reported in the spores of *Ustilago* sp. and *Sphacelotheca* sp. (Oró *et al.* 1966).

The increase in C_{25} and C_{27} components of *Tetraspora gelatinosa* in the presence of acetate was similar to that reported by Patterson (1967) in *Chlorella vulgaris*, grown chemo-organotrophically, although in the latter organism the hydrocarbons were monoenes. The predominant C_{17} paraffin was present in both algae, being thus in agreement with Han *et al.* (1968) and Oró *et al.* (1967). The suspect purity of the C_{17} paraffin in *T. gelatinosa* suggests the presence of the corresponding alkenes.

The carbon preference indices in fungi agreed with those in the literature. A value of around unity was also obtained when *Tetraspora gelatinosa* was grown on CO_2 as sole carbon source as with *Chlorella vulgaris* (Patterson, 1967) but not when acetate was added to the medium. *Nostoc* sp. hydrocarbons exhibited a strong C_{odd} predominance on both media.

The hydrocarbons labelled '?' in the tables were thought to be impure. From the retention volume data (Albro & Huston, 1964) with a similar column and published results (Oró *et al.* 1967; Han *et al.* 1968; Patterson, 1967; Tornabene, Gelpi & Oró, 1967), some components detected immediately before or with the *n*-alkane may be the corresponding *n*-alkanes.

The analyses of microbial hydrocarbons are in general agreement with those of Han *et al.* (1968). In algae the major component is a C_{17} hydrocarbon and the majority have chain lengths shorter than C_{20}. In bacteria and fungi the greater proportion have chain lengths greater than C_{20}. This does not, however, agree with the results of Oró *et al.* (1967) and Tornabene, Gelpi & Oró (1967), who reported lower hydrocarbons in certain bacteria (*Vibrio marinus*) and unsaturated, methylbranched paraffins in *Sarcina lutea* ATCC 535. Albro & Huston (1964) and Tornabene, Gelpi & Oró (1967) obtained differing results with *S. lutea*. Obviously microbial hydrocarbon patterns depend on growth media, growth conditions and analytical methods. Standardized procedures for growth, extraction and analysis of microbial hydrocarbons should be adopted. Since the growth medium used influences the hydrocarbons produced any attempt to correlate such results with possible geological roles of micro-organisms (Han *et al.* 1968) should take account of the substrates available in the field.

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Physiological Ecology of *Leucothrix mucor*

By M. T. KELLY AND T. D. BROCK
Department of Microbiology, Indiana University, Bloomington, Indiana 47401, U.S.A.

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SUMMARY

Temperature, salinity and pH optima of the marine bacterium *Leucothrix mucor* were determined directly in nature by use of tritiated thymidine autoradiography and compared with the same characteristics of laboratory cultures. Field studies were done in Puget Sound, Washington, U.S.A., and Loch Ewe, Scotland. The temperature optima of cultures isolated from various sea-coast areas around the world were 28°, irrespective of the temperature of the habitat from which the culture was derived. In contrast, the temperature optima in the natural environment were significantly lower, ranging from 6·5° to 25°, depending on the habitat studied. Attempts to obtain physiological adaptation of laboratory cultures to low temperature failed. The results emphasize the danger of inferring the response to temperature of natural populations from the characteristics of laboratory cultures. In contrast, responses to salinity and pH optima in natural environments were the same as those of laboratory cultures.

INTRODUCTION

The development of an autoradiographic technique using tritiated thymidine for measuring growth rates of the marine bacterium *Leucothrix mucor* directly in its natural environment (Brock, 1967) opened up the possibility of studying the physiological ecology of natural populations of this organism. In previous work in marine bacteriology, the behaviour of natural populations was inferred from the characteristics of laboratory cultures. Because of the widespread ability of bacteria to adapt to changing environments, it is possible that the characteristics of laboratory cultures do not reflect those of the same organism in nature. We were especially interested in studying the temperature optima of *L. mucor* directly in the sea because this organism was generally found in cool temperate waters, but the temperature optima of all pure cultures isolated were considerably higher than the temperatures of the habitats in which the organism lives.

The present paper reports studies carried out in two temperate sea-coast regions which are geographically distant from each other but which are ecologically similar, namely the west coast of Scotland and Puget Sound in the region of Friday Harbor, Washington, U.S.A. *Leucothrix mucor* is a bacterial epiphyte of seaweeds and is especially common on filamentous red and green algae living in habitats where water flow and aeration are good due to extensive wave action or tidal current (Brock, 1966). In such habitats, *L. mucor* is often virtually the only epiphyte present, and because of its large size and distinctive morphology it can easily be recognized microscopically. The plan of the experiments was to remove algal fronds containing *L. mucor* from a variety of habitats of known characteristics, place them at a number of different

temperatures, salinities and pH values and without preadaptation measure the growth rates by tritiated thymidine autoradiography. At the same time, a number of pure cultures of *L. mucor* were isolated and the temperature, salinity and pH optima of these cultures determined in the laboratory. The results showed that the temperature optima of the natural material varies markedly but was usually lower than the optima of pure cultures. The manner in which ecological adaptation to low temperatures might occur is discussed, especially in relation to the absence of similar adaptation to low temperatures by laboratory cultures. On the other hand, salinity and pH optima determined in the natural environment are quite similar to those of laboratory cultures.

METHODS

Habitats. Most of the experiments were done in the waters around San Juan Island, Puget Sound, Washington, U.S.A. The experiments were done in the summer of 1967, and the water temperatures of the habitats selected remained relatively constant throughout this time (except as noted). The area is characterized by generally cold waters (12 to 13°) and rocky shorelines with abundant algal growth and ample wave action. There are however shallow-water areas where the water, warmed by sunlight, reaches temperatures as high as 20 to 22°. There are other areas where a sandy shoreline with moderate wave action exists. Therefore the area where these studies were done presented a wide variety of habitats, and the temperature adaptation of *Leucothrix mucor* could be studied in several different types of environment. The habitats and their characteristics are described below, and for clarity the habitats will be referred to henceforth by their designated letters. Habitat A: South Beach; temperature, 12°; tidal range, low intertidal; terrain, pebble beach with large boulders; aeration, strong wave action; algal substrate, a branched rhodophyte (*Pterosiphonia* and *Polysiphonia* spp.); habitat rarely exposed to drying. Habitat B: location, Eagle Cove; temperature, 12·5°; tidal range, low intertidal; terrain, sandy beach with large boulders; aeration, moderate wave action; algal substrate, branched rhodophyte (*Pterosiphonia* and *Polysiphonia* spp.); habitat rarely exposed to drying. Habitat C: location, Argyle Lagoon Channel; temperature, 13 to 20°; tidal conditions, tidal creek connecting open water (13° water) with a lagoon (20° water) (at high tide water flows into the lagoon while at low tide water flows out of the lagoon, and therefore the temperature fluctuates between 13° and 20° due to changes in the direction of flow of the tidal creek); terrain, pebbles and rocks up to 12 in. diameter; aeration, moderate due to flowing water; algal substrate, branched rhodophyte (*Pterosiphonia* and *Polysiphonia* spp.). Habitat D: location, shoreline north of Friday Harbor, Washington, U.S.A.; temperature, 13°; tidal range, middle intertidal; terrain, large boulders on shelf rock; aeration, moderate wave action; algal substrate, unbranched rhodophyte (*Bangia fuscopurpurea*); habitat sometimes exposed to drying at low tide. Habitat E: location, shoreline north of Friday Harbor, Washington, U.S.A.; temperature, 15°; tidal range, high intertidal; terrain, flat shelf rock; aeration, moderate wave action; algal substrate, unbranched rhodophyte (*Bangia fuscopurpurea*); habitat frequently exposed to drying at low tide. Habitat F: location, South Beach; temperature, 13°; tidal range, middle intertidal; terrain, pebble beach with large boulders; aeration, moderate wave action; algal substrate, unbranched chlorophyte; habitat sometimes exposed to drying at low tide.

One series of experiments was done at Firemore, Loch Ewe, Scotland, an area of this large sea loch which is relatively exposed to the open ocean. The oceanographic characteristics of this area were presented by Steele & Baird (1968). The present work was done with material collected at low tide from a rocky headland adjacent to the sandy bay studied by Steele & Baird (1968), and the incubations were done within one half-hour of collection at a small field laboratory made available at the site by the Marine Laboratory, Department of Agriculture and Fisheries, Aberdeen, Scotland. The samples were collected in locations which were submerged even at low tide. The experiments were done in April 1966 when the water temperature was 6·5°. The temperature of this same habitat in midsummer rarely exceeds 14°.

Experimental techniques. The relative rate of incorporation of tritiated thymidine at various temperatures, salinities and pH values was used to determine the optima for these factors for DNA synthesis in *Leucothrix mucor* taken directly from nature. The assumptions and justification for the use of tritiated thymidine incorporation to measure the growth rate of *L. mucor* in nature were presented by Brock (1967).

In a typical temperature adaptation experiment, samples of algae known to contain *Leucothrix mucor* were taken from the sea and placed in large plastic bags. The samples were then returned to the laboratory within one hour, and were kept as close to the environmental temperature as possible. Small samples of algal fronds were transferred to 5 ml. screw-capped vials containing 0·9 ml. of seawater from the area of sample collection. The vials were then equilibrated in water baths and incubators at six or seven temperatures for 15 min. Tritiated thymidine was added by injecting 0·1 ml. of a 10 μC./ml. stock solution without removing the vials from the incubators. After 1 hr, 0·1 ml. of formalin was injected into each vial to terminate the experiment. For studies on the effect of salinity and pH value, a temperature of 25° was used. The experiments were done using samples taken from Habitat B. pH Values ranging from 2·4 to 10·7 were obtained by addition of concentrated HCl or NaOH to natural seawater. A range of salinities was obtained by use of seawater concentrated by evaporation.

A detailed description of the preparation of autoradiograms has been presented by Brock & Brock (1968), and the procedure will be described only briefly here. An algal frond from each incubation vial was placed on a microscope slide which had been coated with Ullrich's adhesive. The frond was spread out and allowed to dry overnight at 40°. The slides were then washed five times for 1 min. in distilled water and dried. They were then coated with emulsion by dipping into Eastman Kodak NBT-2 nuclear track emulsion (diluted 1/2·5) and dried. The slides were left in the dark for 5 days and then developed. The number of labelled and unlabelled cells was determined using a 40× (N.A. 0·75) phase-contrast water-immersion lens on a Zeiss microscope equipped with 12·5× eyepieces.

In counting the autoradiograms, ten filaments were chosen at random and the number of labelled and unlabelled cells determined. A minimum of 250 cells was counted for each autoradiogram, and the results are expressed as per cent labelled cells.

Laboratory studies. The strains of *Leucothrix mucor* used in these studies were isolated from various areas around the world, and their sources were given by Brock & Mandel (1966) and by Kelly (1969). One strain (no. 19) isolated from warmer waters in the Florida Keys had temperature relations different from the others (Kelly & Brock, 1969). Temperature optima of laboratory cultures were determined

roughly by estimating the amount of growth of liquid cultures incubated at different temperatures for a standard period of time. More detailed temperature optima were determined for some strains either by measuring the rate of increase of turbidity and protein content in shaken liquid cultures or by measuring the rate of increase of diameter of colonies growing on agar plates incubated at various temperatures. The latter procedure permitted measurement of growth rates on a number of strains simultaneously. The agar plates were inoculated with appropriate dilutions of suspensions of gonidia (obtained by the filtration method of Harold & Stanier, 1955) so that about 100 colonies developed on each plate. The colony diameters were measured daily or less frequently depending on growth rate and, from these measurements, the relative growth rates at various temperatures were estimated. The defined medium of Brock (1966) was used for these studies, with 1·0% (w/v) or 0·1% (w/v) monosodium glutamate as sole source of carbon, nitrogen and energy.

To determine temperature optima for laboratory cultures previously grown for at least 15 generations at 2°, 10°, 25° or 30°, 5·0 ml. samples of early exponential-phase cultures were removed and incubated with aeration at temperatures ranging from 0° to 35° for 5 min. to allow equilibration. Tritiated thymidine (1·0 μc./ml.) was then added, and the incorporation was terminated by addition of formalin (final concentration 4%) after 1 hr. This technique made it possible to determine whether there was any transitory adaptation to various temperatures. In a preliminary experiment, it was shown that incorporation of tritiated thymidine as followed by autoradiography closely paralleled that measured by liquid scintillation counting (Fig. 1). Therefore, the samples were supplemented with cold 5% trichloroacetic acid, filtered, and the precipitate washed and counted in a Packard Tri-Carb liquid scintillation system using a toluene–PPO–POPOP scintillation liquid. The strains were grown at various pH values and salinities in a synthetic seawater-salts medium supplemented with 0·1% (w/v) mono-sodium glutamate (Brock & Mandel, 1966), and growth was estimated visually. Although the synthetic seawater had a salinity less than natural seawater, addition of 0·1% mono-sodium glutamate brought the medium to a salinity the same as natural seawater.

RESULTS

Response to temperature

Temperature optima of laboratory cultures. The temperature optima of all strains tested were around 25 to 28°, irrespective of the habitat from which the strain was isolated. The temperature range over which growth occurred was wide, all except three strains growing at 2 to 3°, and most strains growing at 30 to 32°. One strain isolated from warmer waters in the Florida Keys was more stenothermal, and was unable to grow at temperatures below 12 to 13°, although still showing an optimum at about 28° and a maximum between 32° and 35°. The characteristics of this strain are reported in greater detail elsewhere (Kelly & Brock, 1969). For strain no. 1, generation times have been determined in liquid cultures of 0·1% glutamate medium, and the doubling time at the optimum temperature of 28° was about 205 min. At 18°, this strain had a doubling time of 230 min., and at 10° it was 410 min. In a medium with 0·1% tryptone+0·1% yeast extract instead of glutamate, strain no. 1 had a generation time of 185 min. at 28°.

Temperature optima in natural environments. Figures 2 to 4 give representative data

from the extensive series of experiments done at Friday Harbor, Washington, U.S.A. In most experiments, a relatively sharp temperature optimum was found, although the optimum varied from one habitat to another. In Fig. 2 data are presented for two experiments where temperature optima of 13° were obtained, which in both cases was also the habitat temperature. Note that, although the temperature optima were the same, the growth rate (as indicated by the percentage of labelled cells) was more

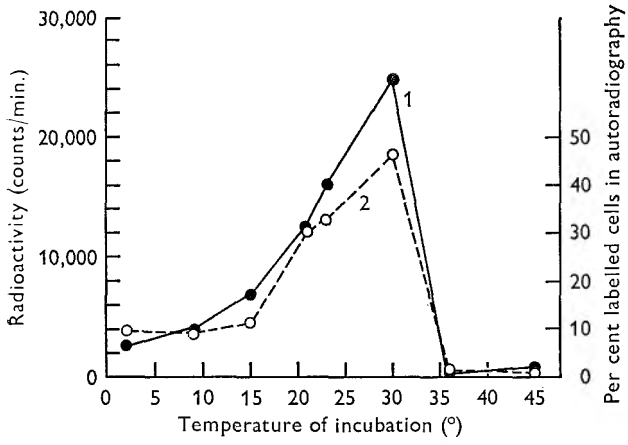

Fig. 1

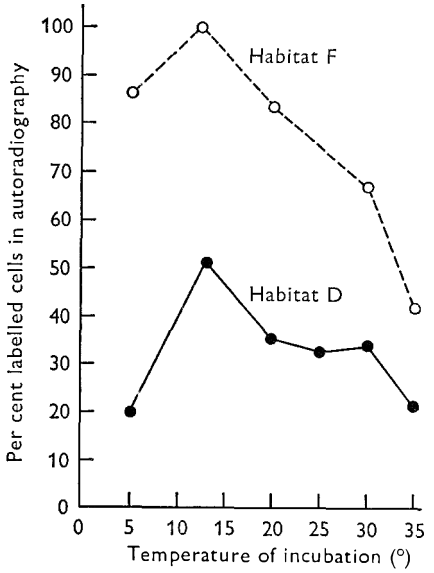

Fig. 2

Fig. 1. Radioactivity of *Leucothrix mucor* as measured by liquid scintillation counting and autoradiography. Samples of a suspension of organisms which had been cultured at 10° were incubated at various temperatures in a solution containing 1 μc. tritiated thymidine per ml. for 1 hr and then assayed. Curve 1 shows results for scintillation counting; curve 2 for autoradiography (per cent labelled cells per 6 min. incubation).

Fig. 2. Temperature optima for growth of natural populations of *Leucothrix mucor* from habitats D and F as measured by autoradiography. Temperatures of habitats: D, 13°; F, 13°.

rapid at one of these habitats than at the other. In contrast, Fig. 3 presents data from two separate experiments at a habitat where the temperature optimum was 25°. Note also that the optimum in Fig. 3 is much sharper than that of Fig. 2. The seawater temperature at the habitat where the experiments shown in Fig. 3 were done was only slightly higher than those of Fig. 2, but a major difference was that the habitat of the experiments in Fig. 3 was in the high intertidal range and the algae were frequently exposed to drying at low tide. Under such conditions, the seaweeds became considerably warmed by sunlight and hence the *L. mucor* populations were exposed to higher temperatures.

Figure 4 presents results of two experiments done in the tidal creek connecting Argyle Lagoon with the sea. As noted in Materials and Methods, the temperature of this creek fluctuated through the tidal cycle from 13° to 20°. In one experiment, two distinct temperature optima at 10° and 25° were found, whereas in the second experiment there was an optimum at 25° and a shoulder at 10°.

Five experiments were done with samples from habitats A and B where the water temperature was about 12°, and the algae were rarely exposed to drying. All these experiments showed optima of about 20°. The data for the one experiment done at Loch Ewe, Scotland, are presented in Fig. 5. The maximum growth rate was found at the lowest temperature used, 6·5°, which was the temperature of the habitat. However, the growth rate was not much slower at temperatures of 13·5° and 18°, suggesting that at this habitat there was a fairly broad temperature optimum. The experiment at Loch Ewe was done in April, and the seaweeds even at low tide would have been

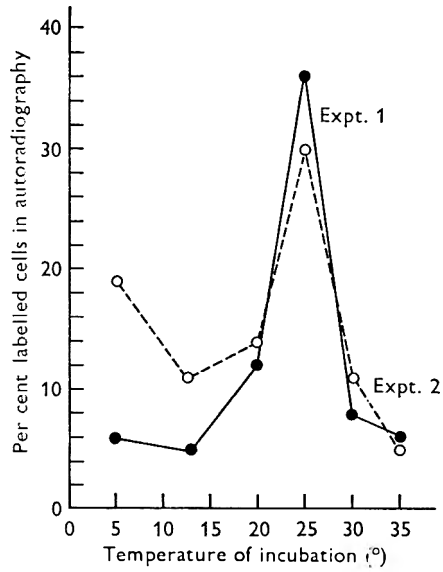

Fig. 3

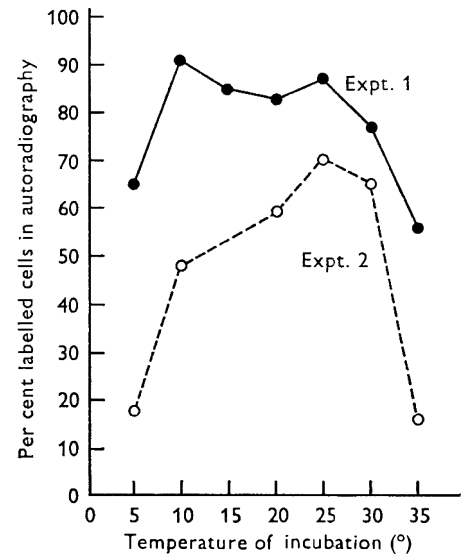

Fig. 4

Fig. 3. Temperature optimum for growth of natural populations of *Leucothrix mucor* from habitat E as measured by autoradiography. Experiments 1 and 2 were done with different samples of algae. Temperature of habitat: 15° but warming during drying at low tide.
Fig. 4. Temperature optimum for growth of natural populations of *Leucothrix mucor* from habitat C as measured by autoradiography. Experiment 1 was done on 8 July 1967 and Expt. 2 done on 10 August 1967. Temperature of habitat: variable from 13° to 20°.

subjected to only fairly low temperatures because of the cool cloudy weather over the previous winter months. Another experiment was done at Loch Ewe in late July, 1967, but unfortunately the *L. mucor* populations on the algae were too low.

Temperature adaptation in laboratory cultures. Because many of the temperature optima found in natural populations were considerably lower than those of laboratory cultures, it was considered of interest to see if *L. mucor* cultures would show short-term adaptation to low temperatures. Consequently a series of experiments was done in which *L. mucor* was grown for at least 15 generations at low temperatures, and the immediate response of these cultures to warming was studied. To make these studies analogous to those done in nature, and to make possible the measurement of only transient adaptation to low temperature, the rate of incorporation of tritiated thymidine was measured by liquid scintillation counting. In these studies the samples were incubated at the various temperatures for only 5 min. before tritiated thymidine was added. The data from one experiment are summarized in Fig. 6.

It can be seen that the optimum temperature for tritiated thymidine incorporation was about 26° regardless of whether the cultures were grown at 2, 10, 25 or 30°. In three other experiments in which cultures were grown at 10° and in two in which cultures were grown at 2°, optima of 25 to 30° were found. Thus, there was no adaptation to growth at low temperatures under the conditions used in the laboratory.

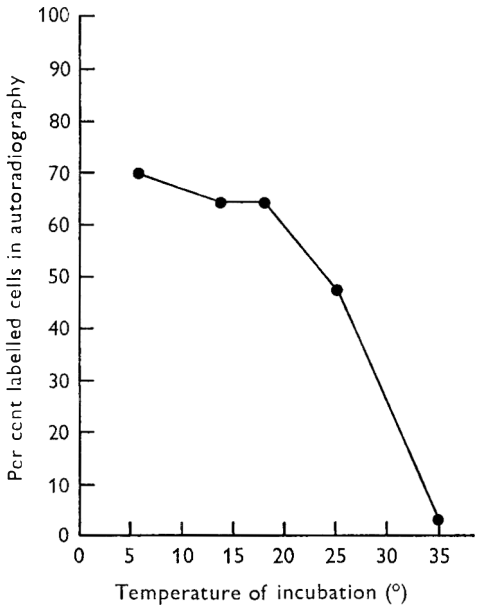

Fig. 5

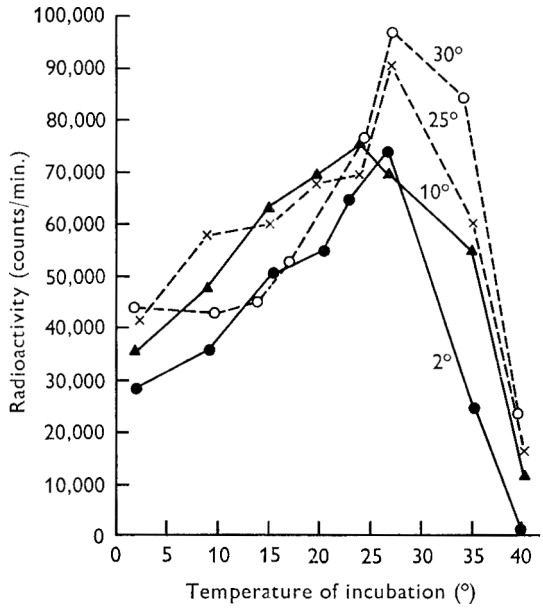

Fig. 6

Fig. 5. Effect of temperature on incorporation of tritiated thymidine as measured by autoradiography for populations of *Leucothrix mucor* from Loch Ewe, Scotland. Temperature of habitat: 6·5°.

Fig. 6. Effect of temperature on incorporation of tritiated thymidine as measured by liquid scintillation counting for cultures of *Leucothrix mucor* strain no. 1 grown for many generations at the stated temperatures.

Response to pH value and salinity

The optimum pH value for *L. mucor* was 7·6 in culture, and the results of an experiment done under natural conditions are presented in Fig. 7. The optimum pH value of *L. mucor* in the natural environment was between 7·0 and 8·0 and thus corresponds well with the pH optimum in culture. Results obtained in the salinity studies are presented in Fig. 8. The salinity optimum in culture was found to be around 31 p.p.t. for the 27 strains analysed, and the optimum in the natural environment was almost exactly the same.

DISCUSSION

The difficulty which existed in attempting to determine the characteristics of an organism in nature from its characteristics in culture was pointed out by ZoBell (1946). He noted that marine bacteria often grew best in the laboratory at temperatures 10 to 20° higher than those of their natural habitats. Similarly, Haight & Morita (1966) stressed the importance of correlating the results of laboratory and field studies

involving marine bacteria. They demonstrated that *Vibrio marinus* differed physiologically when grown under the conditions of the natural environment or under conditions which were optimal for its growth in the laboratory.

In the present study a comparison of the temperature optima of *Leucothrix mucor* in nature with those obtained in culture emphasized this problem. The temperature optimum of *L. mucor* in culture was found to be about 28°, and all the strains had the same temperature characteristics regardless of their original locations. In contrast,

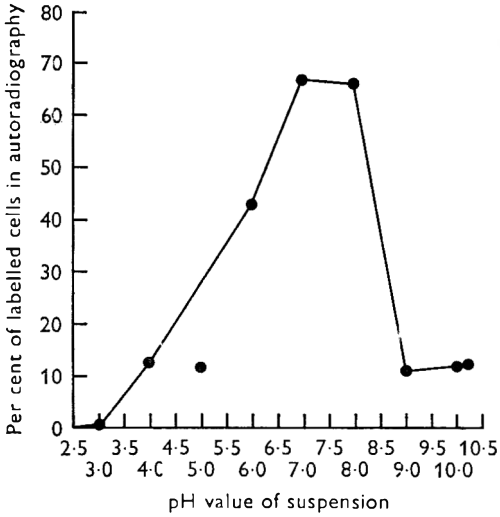

Fig. 7

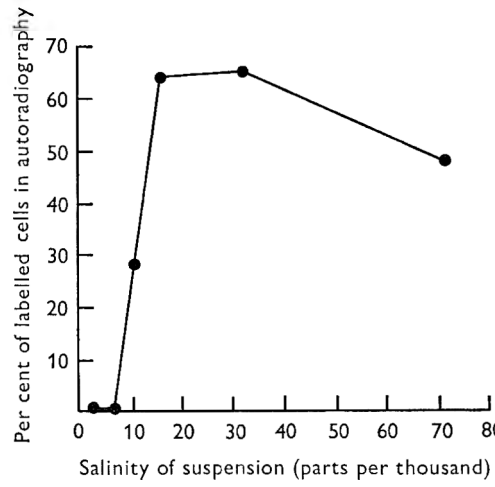

Fig. 8

Fig. 7. Effect of pH value on the incorporation of tritiated thymidine in natural populations of *Leucothrix mucor*. An algal sample was removed from the sea and incubated with 1·0 μg. of tritiated thymidine/ml. seawater at various pH values. The incorporation was measured by autoradiography.

Fig. 8. Effect of salinity on incorporation of tritiated thymidine in natural populations of *Leucothrix mucor*. An algal sample was removed from the sea and incubated with 1·0 μg. of tritiated thymidine/ml. sea water at various salinities. The incorporation was measured by autoradiography.

the temperature optima determined for *L. mucor* in its natural environment by autoradiography ranged from 6·5 to 25° depending on the habitat used, and the optima in all the experiments were lower than the optima in culture. In general, optima in nature were higher in warmer habitats, although this relationship did not always hold.

This discrepancy could be due to a physiological adaptation to the low temperatures of the natural environment. However, efforts to duplicate this adaptation in culture were not successful, suggesting that there may be differences between the conditions in culture and in nature. It must be remembered that *L. mucor* grows in nature as an algal epiphyte and thus probably has a very complex nutritional supply in addition to interactions with other micro-organisms, and such conditions are not duplicated in laboratory cultures.

An alternative explanation for the different optima in culture and in nature could be that since the strains were isolated at temperatures of 20 to 25°, only strains with high temperature optima were obtained. These organisms could be strains which occur in very small numbers in the low-temperature environments but are able to

grow better than the majority of the organisms at higher temperatures. Evidence that such strains exist has been presented by Sieburth (1967), who was able to show that the temperature optima of the various types of bacteria in Narragansett Bay change as a function of the water temperature. The temperature optima lag two months behind the fluctuations in water temperature suggesting a selection of different temperature strains of each type of organism rather than a physiological adaptation which would require only a few generations to achieve. These strains could arise by random mutation, since it has been shown by Olsen & Metcalf (1968) that a small number of genetic loci may control the temperature characteristics of an organism. A factor which argued in favour of this interpretation was that only a small percentage of the *L. mucor* filaments on the plates during isolation actually formed colonies. However, since most of the filaments in each autoradiogram were labelled to the same extent, the number of organisms in the population with optima at 25 to 30° would have to be quite small.

In contrast to the temperature studies, a good correlation was found between the optima determined in culture and those determined in the natural environment for salinity and pH value. However, it should be noted that, although the optimum in terms of total salinity was the same in culture and in nature, the concentrations of individual ions were not the same. For example the medium which gave optimal growth had a salinity of 31 p.p.t. and contained 0·42 M-Na^+ (0·28 M as NaCl and 0·14 M as monosodium glutamate), 0·027 M-Mg^{2+}, and 0·005 M-Ca^{2+} while seawater has a salinity of 32 p.p.t. and 0·45 M-Na^+, 0·05 M-Mg^{2+}, 0·01 M-K^+ and 0·01 M-Ca^{2+}. It appears therefore that the total salinity may be more important for the growth of *L. mucor* than the concentrations of individual ions.

The effects of individual ions on marine bacteria have been analysed by MacLeod (1965) and by Korngold & Kushner (1968), but little work has been done on the effects of the over-all salinity on these organisms. Korngold & Kushner (1968) noted the stabilizing effect of divalent cations on a marine bacterium, and MacLeod emphasized the importance of Na^+ concentration for growth of marine bacteria. He found that the optimum Na^+ concentration for several marine bacteria was 0·2 to 0·3 M, and the results presented here for the cultural studies of *L. mucor* were in agreement with these findings. Although these results indicate that cultural studies may correlate with studies of an organism in its natural environment, it is clear that micro-organisms should be analysed under natural conditions before any conclusions about their activities in the natural environment are made. It should be possible in many cases to carry out these studies through the use of the tritiated thymidine autoradiography technique.

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Energetics of Growth of *Azotobacter vinelandii* in a Glucose-limited Chemostat Culture

By S. NAGAI, Y. NISHIZAWA and S. AIBA
Institute of Applied Microbiology, University of Tokyo, Tokyo, Japan

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SUMMARY

With a glucose-limited chemostat culture of *Azotobacter vinelandii* IAM 1078 the yield factor, Y_S^X, for glucose increased from 0·3 to 0·12 following an increase in the dilution rate from 0·1 to 0·35 hr^{-1}. However, even when growing at the faster rate the value of Y_S^X still was extremely small compared to those found with other micro-organisms. This low yield value was not due to an incomplete oxidation of glucose, since most of the glucose carbon utilized (83 to 97%) could be accounted for as carbon dioxide, the percentage conversion depending on the dilution rate. The specific respiration rate (Q_{O_2}) of the growing culture was determined *in situ* and found to remain nearly constant over the range of dilution rates. However, the value of Y_S^X diminished progressively from 0·12 to 0·03 when the dissolved oxygen concentration in a chemostat culture was increased from 3·3 to 5·7 p.p.m. The yield factor, Y_{ATP} (evaluated by making certain assumptions) was much smaller than the value of 10 found with other microbial culture, even though the value of Y_{ATP} increased appreciably with the increase of dilution rate. These results are discussed with reference to some of the known physiological characteristics of *A. vinelandii*.

INTRODUCTION

With several species of bacteria, a linear relationship has been found between the reciprocal of the overall yield factor Y_S^X and the reciprocal of the specific growth rate μ_X (Pirt, 1965). Similarly, a glucose-limited chemostat culture of *Azotobacter vinelandii* (dilution rate = 0·1 to 0·25 hr^{-1}) showed the same general pattern (Aiba, Nagai, Nishizawa & Onodera, 1967). In order to understand the physiological implications of this variation in Y_S^X with growth rate, we have examined the effects of varying the dilution rate on the fate of the glucose substrate.

With various microbial cultures, many workers have observed an 'ATP-yield value' (Y_{ATP} = mg. bacteria synthesized/mmole ATP consumed) of about 10 (see Bauchop & Elsden, 1960; Stouthamer, 1962; Hadjipetrou *et al.* 1964). But since all these workers grew their organisms in uncontrolled environments (i.e. batch cultures) the influence of specific nutritional conditions, as realized in a chemostat culture, remained to be investigated. The effect of growth rate on the Y_{ATP} value of a glucose-limited culture of *Azotobacter vinelandii* is also reported here.

METHODS

Organism and cultural conditions. The bacterium used was *Azotobacter vinelandii* IAM 1078 (ATCC 9046). The medium composition was (g./l.): glucose, 5; KH_2PO_4, 0·2; K_2HPO_4, 0·8; $MgSO_4.7H_2O$, 0·2; NaCl, 0·2; sodium citrate, 0·05; $FeSO_4.7H_2O$, 0·005; $Fe_2(SO_4)_3.3H_2O$, 0·005; $Na_2MoO_4.2H_2O$, 0·001; $CaCl_2.2H_2O$, 0·01; pH 7·0–7·2. It was confirmed by preliminary experiments that sodium citrate in the medium did not serve as a carbon source; glucose was the sole utilizable carbon source.

The reactor vessel (working volume = 13–18 l., nominal volume = 29 l.) was equipped with a standard flat-blade turbine (780 r.p.m.). The aeration from a ring sparger below the impeller was kept at 0·5 to 1·5 v.v.m. Each continuous culture run (dilution rate = 0·05–0·35 hr^{-1}) was assumed to be in a steady state if the optical density value (at 610 mμ) for the culture remained unchanged for about 24 hr. The residual glucose concentration in the effluent from the vessel was of the order of 2 μg./ml. The reactor was run at 30° ± 0·5°.

Determination of cell mass and glucose concentration. Bacterial mass was measured as a freeze-dried powder (Aiba *et al.* 1967). Water content of this material was around 15%. Residual glucose was measured either by the method of Somogyi (1952) or with a Glucostat reagent (Worthington Biochemical Corporation).

Measurement of the oxygen uptake rate. Aeration rate was carefully controlled to determine the oxygen uptake of bacteria in the chemostat. A reducing valve and a rotameter were installed on the air line. Oxygen in the air flow, before entering and after leaving the chemostat, was determined using a Beckman oxygen electrode (Beckman-Toshiba Type 777).

Measurement of rate of carbon dioxide evolution. Water, 5·5 l., in a conical flask was replaced by the exhaust air from the chemostat. An aqueous solution (100 ml.) of 0·2-N barium hydroxide was then carefully added and the flask shaken mechanically for about 30 min. After standing for 3 hr, the solution was back titrated with oxalic acid (0·1 N) and the concentration of carbon dioxide in the exhaust air calculated from these data. The concentration of carbon dioxide in the ambient air was measured, as control, by the same procedure. The rate of carbon dioxide evolution in the chemostat was calculated from the difference in CO_2 content between the ambient and exhaust air (duplicate measurement), and the air flow rate. Temperatures of the air entering and leaving the fermentor were measured and the rates of oxygen uptake and carbon dioxide evolution corrected to 0°, 1 atm.

RESULTS

Experimental data from a typical chemostat culture experiment are shown in Table 1. Clearly the maximum dilution rate under these conditions was about 0·34 hr^{-1}. Since the glucose concentration in the extracellular fluid was insignificant compared with that of fresh medium ($S_0 = 5 \pm 0{\cdot}2$ mg./ml.), the value Y_S^X could be determined merely from the concentration ratio of cultured bacterial mass to glucose in the input medium.

Assimilation-dissimilation ratio of glucose

The metabolic activities, as represented by the specific rates of glucose consumption, assimilation to cell carbon and dissimilation to CO_2, are plotted against the dilution

rate in Fig. 1. An average of carbon content (45%) determined by elemental analysis of the bacteria from the chemostat culture was used to assess the specific rate of the assimilation.

The recovery ratio, defined by the ratio of a sum of the assimilated and dissimilated carbon to the glucose carbon consumed, is also shown in this figure. Despite the data

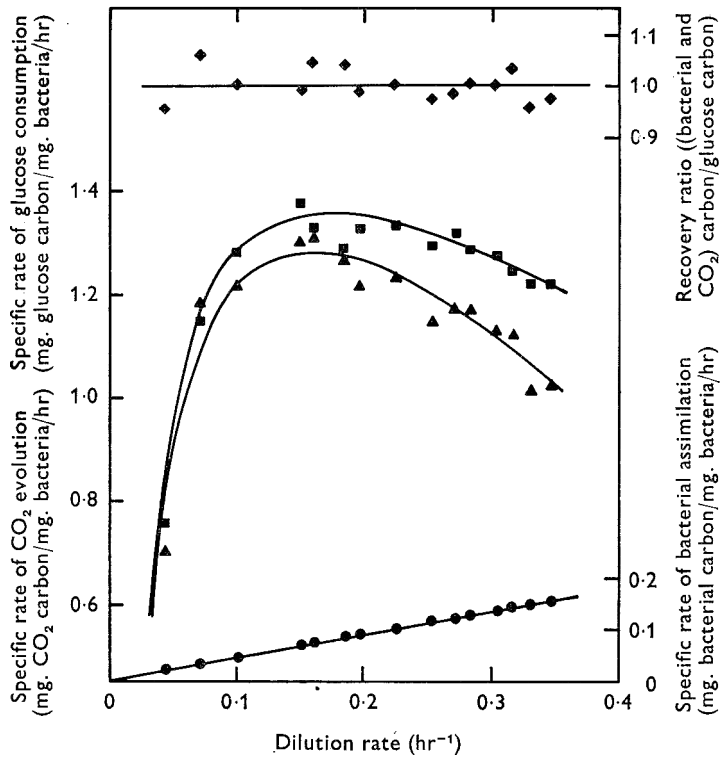

Fig. 1. Specific rates (carbon basis) of cellular assimilation, CO_2 evolution and glucose consumption of *Azotobacter vinelandii* as varied with dilution rate of glucose-limited chemostat culture. Glucose concentration in fresh medium, $S_0 = 5 \pm 0{\cdot}2$ mg./ml. ●—●, cellular assimilation; ▲—▲, CO_2 evolution; ■—■, glucose consumption; ◆—◆, recovery ratio.

Table 1. *Bacterial mass and glucose concentrations in a glucose-limited chemostat culture of Azotobacter vinelandii*

Glucose concentration in fresh medium = 5 ± 0·2 (mg/ml.); agitation speed = 780 r.p.m.; aeration rate = 0·7 ~ 1·0 v.v.m.

Dilution rate (hr^{-1})	Bacterial mass concentration (mg. cell/ml.)	Glucose concentration (μg. glucose/ml.)
0·100	0·150	0·8
0·149	0·192	1·2
0·224	0·331	1·0
0·253	0·384	2·0
0·304	0·470	1·5
0·316	0·509	3·0
0·340	0·536	27·0

scattering, the recovery ratio was invariably nearly one. Therefore in this chemostat culture, *Azotobacter vinelandii* obviously produced no metabolites other than bacterial cells and carbon dioxide.

Experimental data of yield factor for glucose (Y_S^X), the ratio of assimilation to dissimilation ($\Delta X/\Delta CO_2$), and the ratio of dissimilation to glucose consumption ($\Delta CO_2/-\Delta S$) are shown in Fig. 2. Clearly growth of this micro-organism in a chemostat culture was characterized by a very small value of Y_S^X compared with the data reported by other workers (for example, Y_S^X is 0·5 for *Aerobacter cloacae* (Herbert,

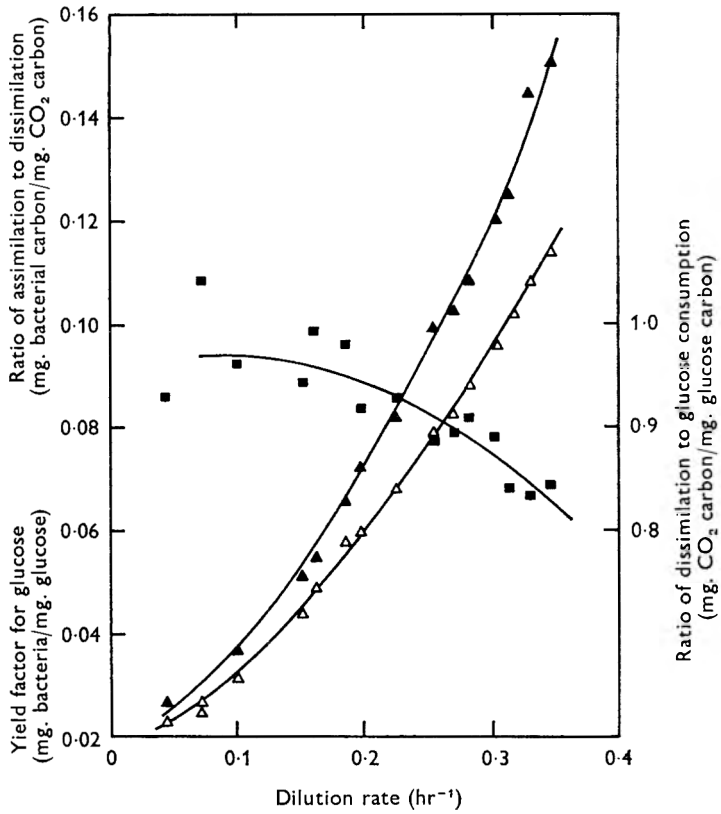

Fig. 2. Yield factor for glucose, ratio of assimilation to dissimilation and that of glucose consumption for *Azotobacter vinelandii* (glucose-limited chemostat culture). These ratios are on the carbon bases. △—△, yield factor for glucose; ■—■, ratio of dissimilation to glucose consumption; ▲—▲, ratio of assimilation to dissimilation.

Elsworth & Telling, 1956), *Escherichia coli* (Schulze & Lipe, 1964) and *Aerobacter aerogenes* (Baidya, Webb & Lilly, 1967), and 0·3 to 0·4 in *Aerobacter cloacae* (Pirt, 1965) in carbohydrate-limited chemostat cultures). Furthermore, as this dilution was increased progressively, so Y_S^X and $\Delta X/\Delta CO_2$ increased appreciably and $\Delta CO_2/-\Delta S$ diminished.

Oxygen uptake rate

The influence of dilution rate on the specific rate of oxygen uptake (Q_{O_2}), glucose consumption, the dissolved oxygen concentration and yield factor for oxygen (Y_O^X)

are plotted in Fig. 3. Values of the respiratory quotient (R.Q.) were approximately unity, and the specific rate of oxygen uptake was nearly 0·1 m-mole O_2/mg. bacterium/hr ($\simeq$ 2,000 μl. O_2/mg. bacterium/hr), irrespective of the dilution rate. This large Q_{O_2} value is in agreement with that published by Tissières, Hovenkamp & Slater (1957) on the same species. Indeed, *Azotobacter vinelandii* is characterized by the large value of Q_{O_2}.

The dissolved oxygen concentration decreased with an increase in dilution rate (see Fig. 3), whereas the values of Y_O^X increased appreciably. The same trend was

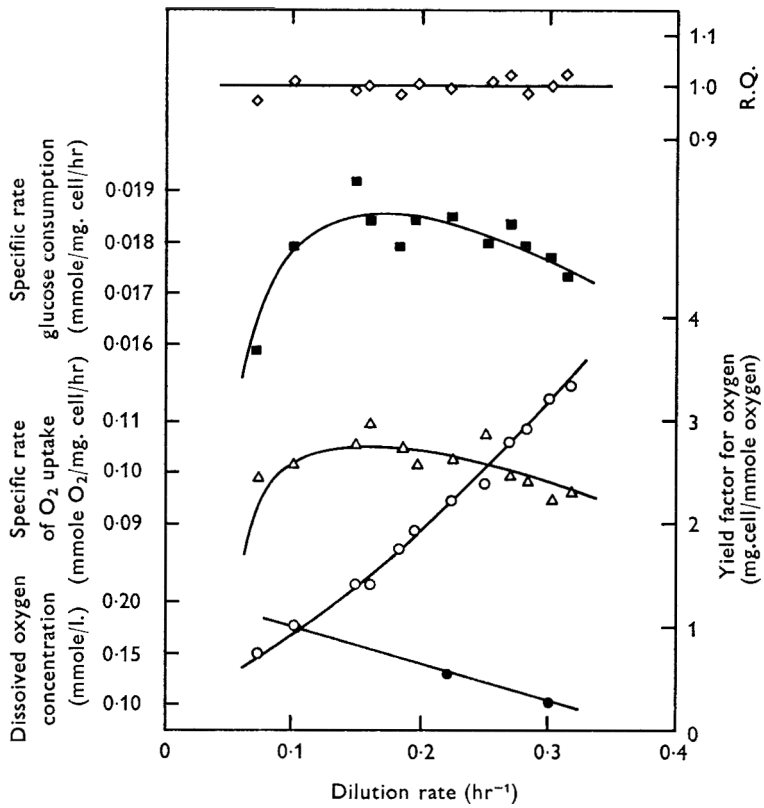

Fig. 3. Yield factor for oxygen, specific rate of O_2 uptake and dissolved oxygen concentration in glucose-limited chemostat culture of *Azotobacter vinelandii*. Specific rate of glucose consumption reproduced from Fig. 1 and R.Q. values are included. Specific rate in glucose-carbon basis formerly is converted here to molar unit. R.Q. values are duly calculated referring to Fig. 1. Dissolved oxygen concentration is measured at agitation speed = 780 r.p.m. and aeration rate = 0·6 v.v.m. O—O, yield factor for oxygen; △—△, specific rate of O_2 uptake; ●—●, dissolved oxygen concentration; ■—■, specific rate of glucose consumption; ◇—◇, respiratory quotient, R.Q.

apparent between Y_S^X (see Fig. 2) and dissolved oxygen. This fact suggests the possibility that high levels of dissolved oxygen inhibit the cell synthesis and energy metabolism. In this connection, Parker & Scutt (1960) found an inhibitory effect of oxygen on the growth of Azotobacter.

DISCUSSION

Y_{ATP}, defined by the ratio of the bacterial mass synthesis to ATP, can be represented by the following equation,

$$Y_{ATP} = \frac{D}{2(P/O)Q_{O_2} + (\alpha - 1)\nu'_X}, \quad (1)$$

where Y_{ATP} = (mg. dry mass/mmole ATP), D = dilution rate (specific growth rate, hr^{-1}), α = ATP synthesized by the substrate level phosphorylation per glucose consumed (mmole ATP/mmole glucose), ν'_X = specific glucose consumption rate (mmole glucose/mg. bacterium hr).

The term $2(P/O)Q_{O_2}$ implies the specific rate of production of ATP by the oxidative phosphorylation, whereas another term, $(\alpha - 1)\nu'_X$ signifies the specific rate of production of ATP by the substrate level phosphorylation. The second term $(\alpha - 1)\nu'_X$ generally is insignificant compared with the first term, particularly in this strictly aerobic organism.

Table 2. *Values of Y_{ATP} (from equation (2)) for a glucose-limited chemostat culture of Azotobacter vinelandii*

It was assumed that the ratio P/O = 1:. Y_{ATP} from equation (2) was multiplied by 0·85 to be consistent with dry mass basis (see the text).

Dilution rate (hr^{-1})	Y_{ATP} (mg. dry wt/ mmole ATP)	Y_O^X (mg. bacteria/ mmole O_2)	Y_S^X (mg. bacteria/ mg. glucose)
0·1	0·425	1·0	0·03
0·2	0·77	1·8	0·06
0·3	1·27	3·0	0·10

The P/O ratio of *Azotobacter vinelandii* changes most likely if the branched electron transport pathway (Jones & Redfearn, 1967) is acceptable. Here the P/O ratio was assumed unity, referring to the data (P/O = 0·2 to 0·8) published by Tissières *et al.* (1957) and Hovenkamp (1959), because the previous data *in vitro* seemed to justify the assumption.

Thus, the value of Y_{ATP} is estimated approximately by the following equation,

$$Y_{ATP} = \frac{D}{2Q_{O_2}}. \quad (2)$$

The result of this calculation is shown in Table 2, in which the values of Y_S^X and Y_O^X are cited from Fig. 2 and 3.

Although with many organisms the Y_{ATP} value was found to be approximately 10 (Bauchop & Elsden, 1960; Stouthamer, 1962; Hadjipetrou *et al.* 1964), Hernandez & Johnson (1967) pointed out the possibility that the value of Y_{ATP} may become smaller than 10 if some substances other than ATP are limiting the synthesis of cellular material. We therefore conclude that the value of Y_{ATP} for *Azotobacter vinelandii* was remarkably small (Table 2) since, although they had an abundance of ATP, they lacked materials for growth in this glucose-limited environment. Thus the increase of Y_S^X found when the dilution rate was increased was accompanied by the increase of the value of Y_{ATP} (and Y_O^X).

Thanks are due to M. Onodera, for his technical assistance.

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The Stability and Cell Content of Penicillinase Messenger RNA in *Bacillus licheniformis*

By J. W. DAVIES

Department of Molecular Biology, University of Edinburgh, King's Buildings, Edinburgh EH9 3JR

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SUMMARY

The stability of penicillinase messenger RNA has been assessed from the kinetics of enzyme production after inhibition of RNA synthesis with actinomycin or rifampicin. An average messenger half-life of 4·7 min. is indicated, both for induced *Bacillus licheniformis* 749, and the constitutive mutant 749/C. Although this value is twice that estimated for average total m-RNA by similar techniques, there is no indication of a 'long-lived messenger' as previously implicated in other reports. An attempt was made to estimate the content of penicillinase messenger from data on the level of penicillinase made per bacterium, the polysome size, the messenger half-life and the total level of unstable RNA per bacterium. It is suggested that at the maximum induced rate of enzyme formation there are approximately 110 penicillinase messengers per bacterium, and in the constitutive about twice this number.

INTRODUCTION

The concept of unstable bacterial messenger RNA (m-RNA) is generally accepted and is implicated in the inducer-repressor enzyme control model as suggested by Jacob & Monod (1961). While it is feasible that some structural proteins such as flagellin (see Martinez, 1966) might be exceptions, and that higher organisms may have relatively stable messengers (Revel & Hiatt, 1964; Gayler & Glasziou, 1968), it is not easy to accept, without strong evidence, that inducible bacterial enzymes have relatively stable messengers. Penicillinase is sometimes quoted as an example of inducible bacterial enzyme with a relatively stable messenger (for example, Forchhammer & Kjeldgaard, 1967; Richmond, 1969) referring to the work of Pollock (1963) and Yudkin (1966). Yudkin (1966) reported that penicillinase messenger in the constitutive strain 749/C is more stable than in the inducible strain. Several other constitutive strains were later investigated (Yudkin, 1968) but similar results were not obtained, and quantitative measurement of messenger half-life was not presented.

In order to determine whether penicillinase is more stable than other bacterial messengers an attempt is made to estimate the half-life of *in vivo* messenger decay. An alternative explanation of high ('magno') levels of enzyme production is that more m-RNA is transcribed. A direct method for measuring intracellular levels of penicillinase m-RNA is not yet available, but some preliminary indirect estimations can be made.

METHODS

Organisms. Bacillus licheniformis strain 749 inducible for penicillinase, and the constitutive mutant 749/C (see Pollock, 1963), were used in the experiments.

Growth conditions. Spores (see Collins, 1964) were germinated, and incubated for 12 hr, when the exponentially growing culture contained about 0·75 mg. dry weight organism/ml. A portion 1 ml. of this seed culture was used to inoculate the experimental culture, in medium consisting of sodium L-glutamate (10 mg./ml.), casein hydrolysate (1 mg./ml.), maltose (0·25 mg./ml.), thiamine (1 μg./ml.), $FeCl_2.6H_2O$ (0·1 mg./ml.), $MnSO_4.H_2O$ (2 mg./ml.) and minimal basal salts ('MBS') consisting finally of $(NH_4)_2SO_4$ (2 mg./ml.), KH_2PO_4 (5·5 mg./ml.), K_2HPO_4 (14·65 mg./ml.), sodium citrate (3 mg./ml.) and $MgSO_47H_2O$ (0·22 mg./ml.). Usually, 50 ml. cultures were incubated in 250 ml. conical flasks, aerated on a rotary shaker (at 280 revs/min.) at 37°. Under these conditions, a doubling time of about 70 min. was obtained. Growth was measured by optical density at 650 nm ($O.D._{650}$ (1 cm. path) 1·0 $\equiv$ 0·3 mg. dry weight/ml. $\equiv 3 \times 10^8$ bacteria).

Induction. Strain 749 was induced with either 1 unit (0·6 μg.) benzylpenicillin or 1 μg. cephalosporin C (Glaxo Laboratories Ltd) /ml. unless otherwise stated. Penicillinase synthesis was still being induced 60 min. after addition of inducer, the maximum differential rate not being reached until after one doubling time after induction (Fig. 1). In the experiments designed to determine messenger half-life, inducer was added 40 or 60 min. before sampling or resuspending organisms. Bacteria for resuspension were collected by centrifugation at room temperature and resuspended in fresh warm medium, also containing the inducer; 10 min. shaking was allowed before sampling. Growth continued at the same rate as before harvesting.

Sampling and enzyme assay. Samples (2 ml.) were transferred at intervals of 2 or 5 min. to tubes containing 0·1 ml. (1 mg./ml.) of chloramphenicol solution (Parke Davis and Co.), kept at 0°. Except when total enzyme was to be assayed, bacteria were pelleted and resuspended in 2 ml. of 10 mM potassium or sodium phosphate buffer, pH 6·9. Usually, these samples were frozen and thawed before assay. Triplicate portions were taken for penicillinase assay by the method of Perret (1954). One unit of enzyme is that amount of enzyme which hydrolyses 1 μmole of benzylpenicillin in one hour at pH 7·0 and 30°, with an enzyme-saturating concentration of substrate. One unit of enzyme is equivalent to $5{\cdot}5 \times 10^{10}$ molecules (based on 440 units/μg. protein, the specific activity of purified 749/C exoenzyme: R. J. Meadway, personal communication).

Incorporation of amino acid and nucleotide. Samples from cultures containing ^{14}C-L-phenylalanine for ^{14}C-uracil were added direct to an equal volume of 20% (w/v) trichloroacetic acid containing unlabelled phenylalanine or uracil accordingly. These suspensions were left overnight at 4°. Precipitates were collected on glass-fibre filters (Whatman GF/A), and washed with cold 10% trichloroacetic acid containing uracil (1 mg./ml.), or hot 10% trichloroacetic acid containing phenylalanine (1 mg./ml.). The filters were subsequently washed with 95% (v/v) ethanol containing 1% (w/v) potassium acetate, absolute ethanol, ethanol + ether (1 + 1) and twice with ethyl ether. Dried filters were counted by liquid scintillation techniques (Davies & Hall, 1969).

Cell lysates and polysome preparations. Washed bacteria were resuspended in cold 10 mM-tris-HCl (pH 7·8) containing 10 mM-magnesium acetate, 50 mM-KCl and 5 mM-dithiothreitol, and treated with lysozyme (150–400 μg./ml.) for 20 min. at 10–15°,

before cooling again in ice. Lysis was promoted by addition of the nonionic detergent Brij 58 (final concentration 0·25%) and sodium deoxycholate (final concentration 0·02%). Deoxyribonuclease (Sigma: ribonuclease free) 1–3 μg./ml. was also added. Polysomes were obtained from this crude lysate by differential centrifugation (see Davies, 1969). Chloramphenicol (100 μg./ml.) was added to cultures before harvesting, and to buffer solutions to prevent translation 'run off' of ribosomes from polysomes.

Sucrose gradient analysis. Lysozyme cell lysates or isolated polysome/ribosome preparations were analysed on linear 5%–20% sucrose gradients, containing 10 mM-tris-HCl (pH 7·8; 5°), 10 mM-magnesium acetate, 50 mM-KCl, 5 mM-dithiothreitol and 0·02% (w/v) sodium deoxycholate. Samples (1 ml.) were applied and centrifuged for $3\frac{1}{2}$–5 hr at 30,000 revs/min. in a 3 × 23 ml. swing-out rotor (MSE superspeed 50). Fractions (1 ml.) were collected from the bottom of the tube through a hypodermic needle. Most gradients had a 1 ml. 20% sucrose 'cushion' at the bottom. Absorption at 260 or 280 nm was recorded for each fraction.

RNA and protein determinations. RNA and protein were precipitated with cold 10% trichloroacetic acid. In some assays cell wall material was partially removed by lysozyme digestion. The precipitates were washed with ethanol and ethyl ether (see above) by centrifugation and resuspension. Washed precipitates were dissolved in 0·1 N-NaOH at 60°. Samples in which the RNA was extracted with hot perchloric acid were also assayed. RNA was estimated by an orcinol method, based on that of Schneider (1957), using yeast total RNA as standard. Protein was assayed by the method of Lowry, Rosebrough, Farr & Randall (1951) using ovalbumin or bovine serum albumin as standards. Results given for RNA and protein per cell are mean values from several separate determinations.

RESULTS

Inhibition of growth by inhibitors of RNA synthesis

Concentrations of actinomycin D (Merck, Sharp and Dohme Ltd) and rifampicin (CIBA Laboratories Ltd) were required which would prevent synthesis of the majority of the proteins of the cell and therefore growth. Both antibiotics (1 mg./ml.) rapidly inhibited exponential growth (Fig. 2), there being no multiplication for the next 10–15 min. Some recovery of growth was then evident for a few minutes, resulting in slight stepwise growth. The critical period of m-RNA decay after inhibition of RNA synthesis occurred during the 10–15 min. when there was no growth.

Inhibition of RNA synthesis, average cell messenger half-life, and total messenger content

Both actinomycin D and rifampicin inhibited RNA synthesis almost immediately, as measured by incorporation of ^{14}C uracil into cold trichloroacetic acid-insoluble precipitates. The average cell messenger half-life was estimated from the kinetics of labelled RNA decay, and the bacterial content by the amount of rapidly decaying RNA compared with the concentration of stable RNA (Levinthal, Fan, Higa & Zimmermann, 1963; Zimmermann & Levinthal, 1967). Initially, decay kinetics were measured after pulse-labelling for 1 min but little difference in the decay rate was observed even after twenty minutes of labelling. The half-life was also calculated from

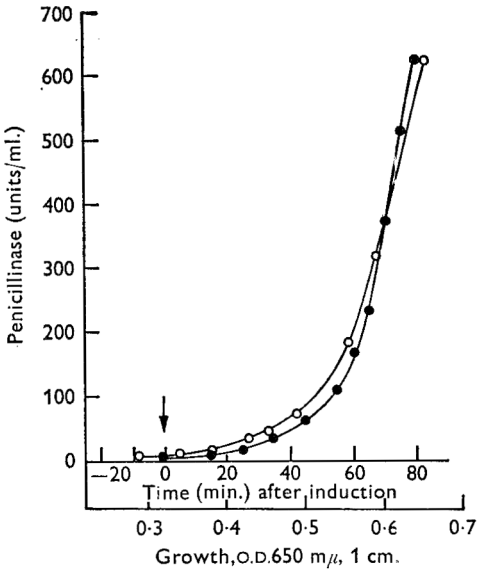

Fig. 1

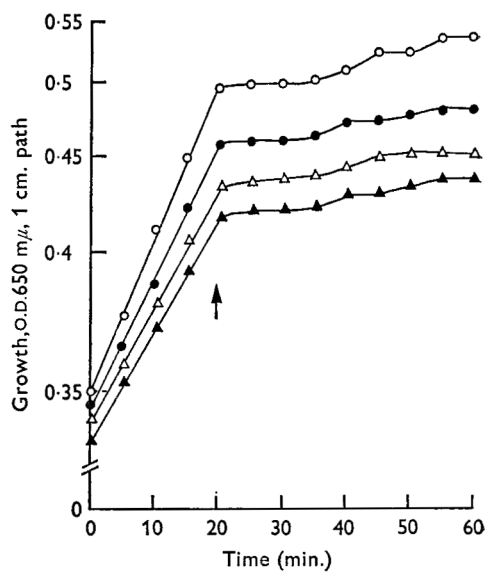

Fig. 2

Fig. 1. Kinetics of induction and differential rate of synthesis of penicillinase by *Bacillus licheniformis* 749. Inducer (5 units benzylpenicillin/ml.) was added when the culture had reached 0·1 mg dry weight/ml. (doubling time 75 min.). Total enzyme was assayed although most enzyme was cell-bound during this time. ● shows kinetics of induction units of (enzyme/ml. vs. time after induction); ○ differential rate of synthesis (units of enzyme/ml. vs. growth).

Fig. 2. Effect of actinomycin D and rifampicin on growth of *Bacillus licheniformis* 749. ○ indicates 5 μg. actinomycin D/ml.; △, 1 μg. actinomycin D/ml.; ●, 5 μg. rifampicin/ml.; ▲, 1 μg. rifampicin/ml. The arrow indicates the time of addition.

the protein-synthesizing capacity, measured by ^{14}C amino acid incorporation. Both methods gave half-lives between 2 and 3 min., averaging 2·5 min. from eight determinations. Some results are given in Fig. 3. The m-RNA content, measured by the type of experiment shown in Fig. 3(*a*) but allowing two generations of labelling first, was estimated to be around 7–10% of the cellular RNA. Slightly higher contents were apparent in rifampicin experiments (see Fig. 3(*e*)).

Penicillinase mRNA half-life

Enzyme-forming capacity after actinomycin D treatment. The increase in induced penicillinase synthesis was measured after addition of actinomycin D to exponentially growing cultures. Results were obtained for cephalosporin and benzylpenicillin induced organisms in fresh media and also without a medium change. Initially, bound, exo- and total enzyme was measured (Fig. 4). 'Enzyme forming capacity' was calculated as the final (maximum) yield of enzyme minus the enzyme level at time *t*. Semi-log plots gave the half-life of the decay rate. Similar experiments were carried out with strain 749/C (Fig. 5). The estimated half-life was comparable to that for induced 749 (about 4·5 min.). Occasionally, a decrease in enzyme activity was observed. This atypical phenomenon is not yet understood, but may involve a reversible inhibition of enzyme activity by actinomycin D. In some experiments, therefore, the antibiotic was also added to the pre-20 min. samples such that all samples assayed contained the same concentration of actinomycin.

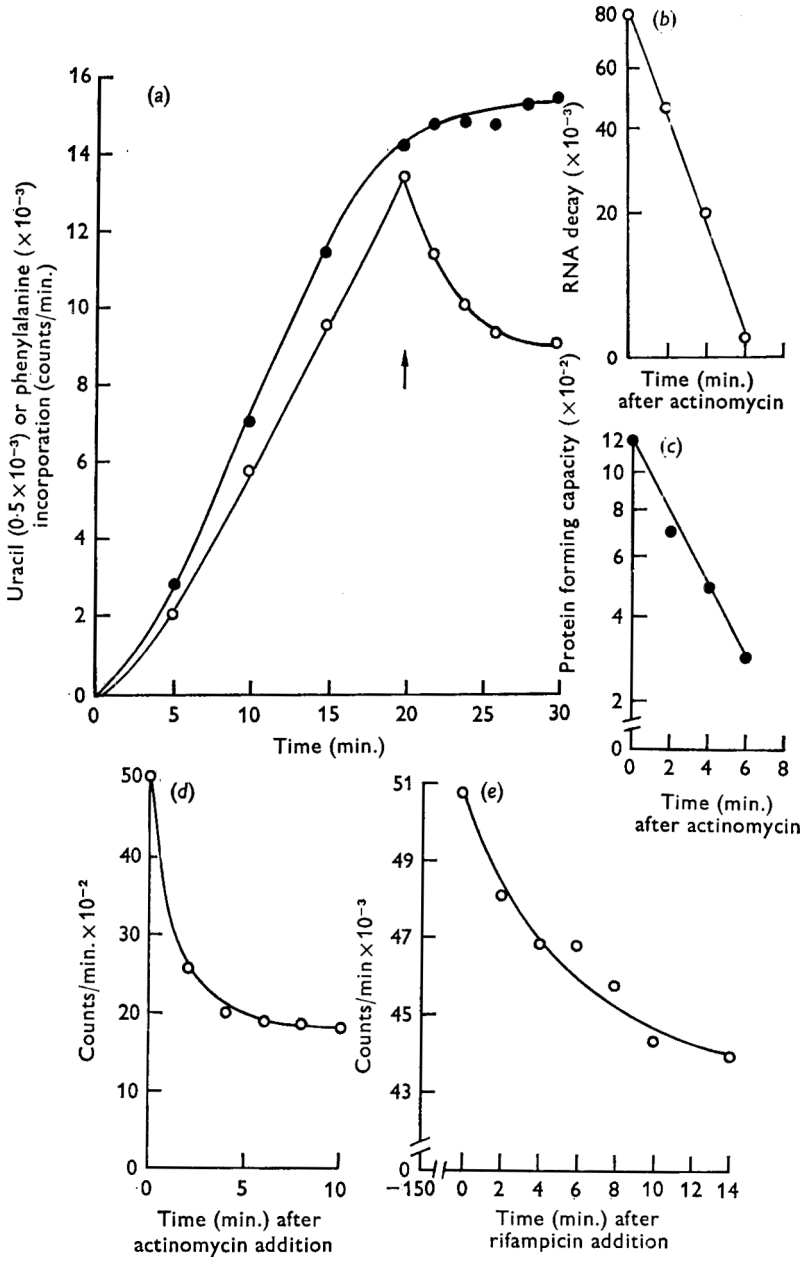

Fig. 3. Incorporation of ^{14}C uracil and ^{14}C phenylalanine into trichloroacetic acid-insoluble material during exponential growth, and the decay of total protein-forming capacity and unstable RNA in *Bacillus licheniformis*. ^{14}C Uracil (50 μc/μmole) and ^{14}C-L-phenylalanine (25 μc/μmole) were added to give 0·5 μc/ml. (*a*). ● indicates amino-acid incorporation ('total protein' synthesis); ○, uracil incorporation (RNA synthesis). Actinomycin (1 μg./ml.) added when indicated (arrow). (*b*) Semi-log plot of total m-RNA decay (^{14}C uracil counts), calculated as counts/min. at time *t* minus counts/min. finally. (*c*) Semi-log plot of total protein-forming capacity, calculated as counts/min. finally minus counts/min. at time *t*. (*d*) and (*e*) show decay of unstable RNA after long-period and short pulse labelling. In (*d*) organisms were pulse-labelled with ^{14}C uracil for 1 min.; actinomycin 5 μg./ml. In (*e*) cells were labelled for 2 generations (^{14}C uracil 1 μc (5 μmoles/ml.) rifampicin 5 μg./ml.). Curve asymptote 4400 counts/min.

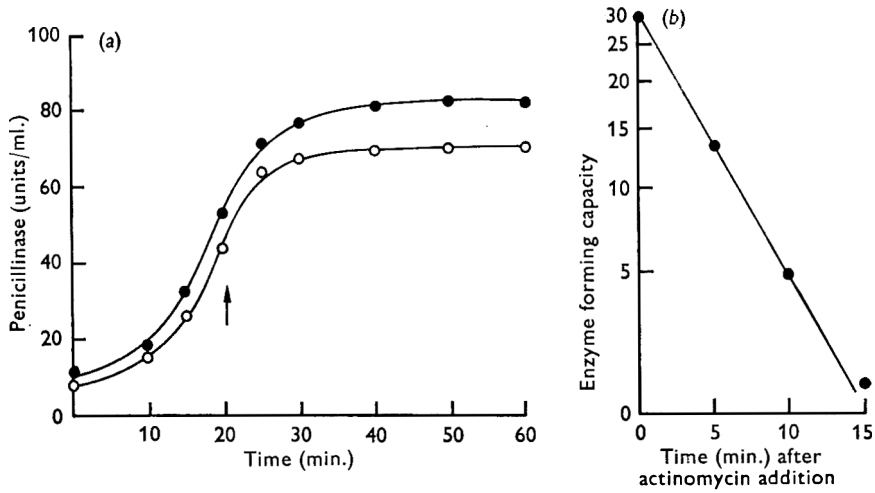

Fig. 4. Actinomycin inhibition of induced penicillinase synthesis, and decay of the enzyme-forming capacity by *Bacillus licheniformis* 749. (*a*) Bacteria were not resuspended (optical density at 650 nm at zero time = 0·15), but were induced with 1 μg. cephalosporin/ml. 35 min. before assaying. Actinomycin D 1 μg./ml. finally (arrow). ●, indicates total enzyme; ○, cell-bound enzyme. The arrow indicates the time of addition of actinomycin D to give a final concentration of 1 μg./ml. (*b*) Semi-log plot of enzyme-forming capacity after addition of actinomycin. Similar results were obtained for penicillin-induced cells resuspended in fresh medium.

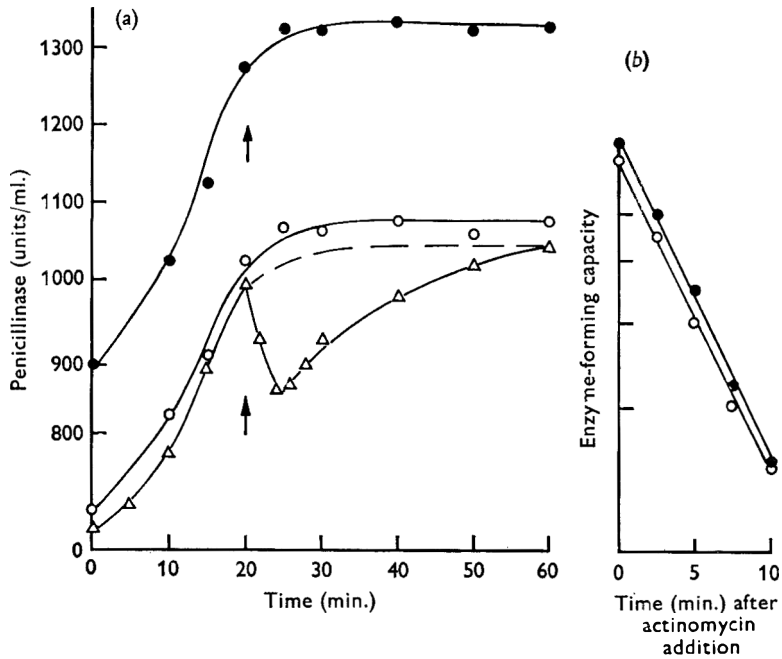

Fig. 5. Actinomycin inhibition of constitutive penicillinase formation, and the decay of enzyme forming capacity by *Bacillus licheniformis* 749/C. Bacteria were resuspended in fresh medium 10 min. before the zero-time sample (optical density at 650 nm = 1·0). (*a*) ● total enzyme; ○ cell bound enzyme. The arrow indicates the time of addition of actinomycin D, to give a final concentration of 1 μg./ml. △, indicates an atypical effect obtained in a separate experiment in which maltose was omitted and actinomycin was added to give a final concentration of 5 μg./ml. (*b*) Semi-log plots of enzyme-forming capacity.

Enzyme-forming capacity after rifampicin treatment. In view of the possibility that actinomycin D may have a preferential effect on some cistrons, or that it could influence messenger decay, experiments were performed with rifampicin, which inhibits

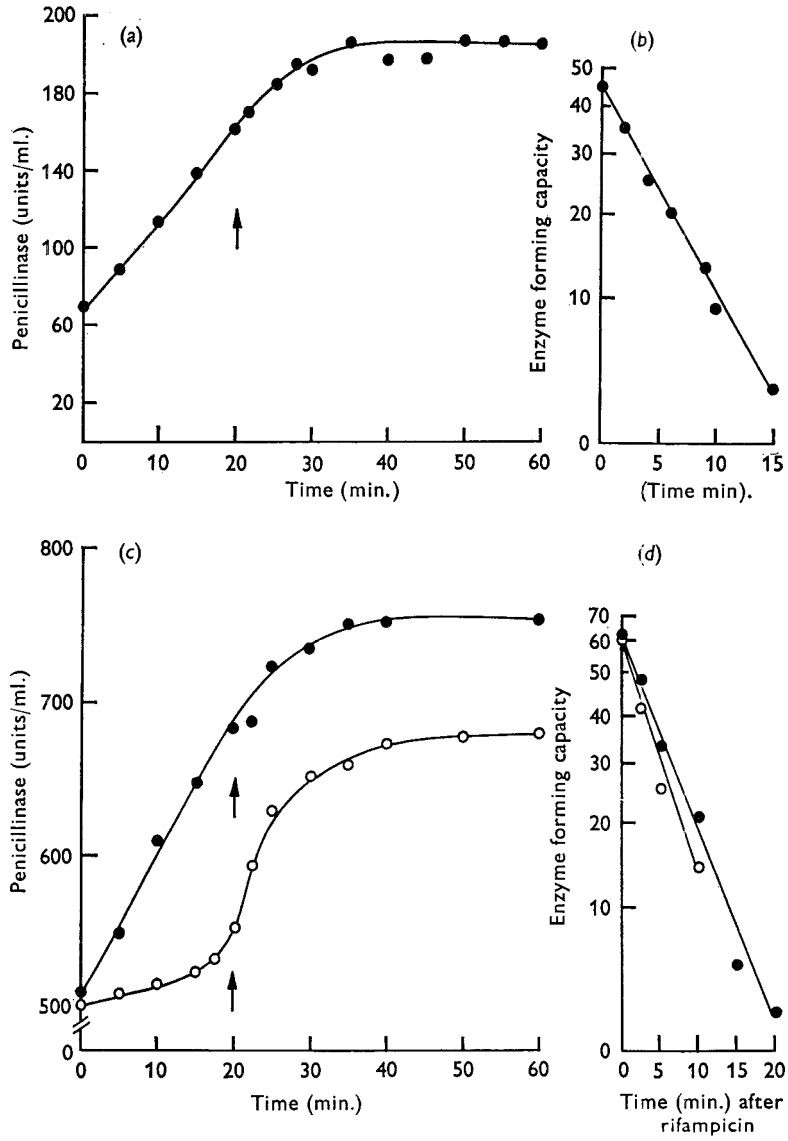

Fig. 6. Rifampicin inhibition of induced and constitutive penicillinase formation by *Bacillus licheniformis*, and decay of the enzyme-forming capacity. (*a*) Strain 749, induced with 1 μg. cephalosporin/ml. 60 min. before resuspending in fresh medium zero time 10 min. before. Optical density at 650 nm = 0·15. The arrow indicates the time of addition of rifampicin to give a final concentration of 1 μg./ml. (*b*) Semi-log plot of enzyme-forming capacity by strain 749. (*c*) Strain 749/C. The initial optical density at 650 nm = 0·4. ● indicates sampling started immediately (not resuspended); ○, cells resuspended in fresh medium. Results are for cell-bound enzyme only. Rifampicin 1 μg./ml. (*d*) Semi-log plot of enzyme forming capacity by strain 749/C.

action of RNA polymerase, thus stopping transcription generally. The kinetics of enzyme-forming capacity after rifampicin addition suggested messenger decay with a half-life around 4–5 min., both for the inducible and constitutive strains (Fig. 6). Results of actinomycin and rifampicin experiments are summarized in Table 1.

Cell contents of RNA, protein and penicillinase

Cell contents of protein and RNA were estimated, and the average cell content during exponential growth was calculated (Table 2). The level of penicillinase was also estimated from a large number of determinations, made at least 80–120 min. after induction, when the bacteria were close to or at the maximum differential rate of synthesis. It is emphasised that these results are a population average during exponential growth only. The figure of 107,000 molecules of penicillinase is the average cell content and corresponds to 2340 units of enzyme per mg. dry weight of organism. The amount of penicillinase to be synthesized by a single bacterium per division cycle, is in 2 × average

Table 1. *Stability of penicillinase messenger RNA in Bacillus licheniformis*

Penicillinase messenger half-life was estimated from semi-log plots of the enzyme-forming capacity, from several experiments of the type shown in Fig. 4, 5 and 6

	Strain *749*		Strain *749*/C	
Inhibitor of RNA synthesis	Mean half-life (min.)	No. of determinations	Mean half-life (min.)	No. of determinations
Actinomycin D (1 μg./ml.)	5·0 ± 1·0	4	4·2 ± 0·25	2
Actinomycin D (2 μg./ml.)	—	—	4·0 ± 0	2
Actinomycin D (5 μg./ml.)	5·0 ± 0	1	5·0 ± 0	1
Rifampicin (1 μg./ml.)	4·5 ± 0·5	2	5·9 ± 0·5	5
Rifampicin (5 μg./ml.)	4·4 ± 0·4	2	4·0 ± 1·0	2
Mean half-life*	4·7	—	4·7	—

* The mean half-life was calculated from an equal number of determinations for each concentration of the inhibitors.

Table 2. *Cell content of total protein, RNA, unstable RNA and penicillinase in Bacillus licheniformis*

Component	Average cell content	% of dry weight of bacterium
Protein (total)	$6{\cdot}5(\pm 1{\cdot}0) \times 10^{-7}$ μg.	55–75
RNA (total)	$1{\cdot}5(\pm 0{\cdot}5) \times 10^{-7}$ μg.	10–20
m-RNA*	$0{\cdot}12(\pm 0{\cdot}04) \times 10^{-7}$ μg.	0·2–1·6
Penicillinase† (molecules/generation)	107,000 ± 16,000	0·44–0·60

Bacillus licheniformis 749 was induced with 1 μg./ml. (approx. 1 unit/ml.) cephalosporin C. Similar values, within the variation range shown, are obtained for cultures induced with up to 5 μg. cephalosporin C or penicillin/ml. Growth conditions and medium as described in Methods. Generation time (doubling time) varied from 65 to 75 min.

* m-RNA is assumed to be that which is rapidly labelled, and which decays quickly after addition of actinomycin D. Total average contents are calculated from experiments of the type shown in Fig. 3.

† Total enzyme: strictly speaking not 'cell content' since about 20% is released as exoenzyme.

cell content of penicillinase (107,000 × 0·693 = 74,150 molecules/bacteria/division cycle). The constitutive strain (749/C) produced about twice that amount of enzyme under the same conditions.

Number of nascent polypeptide chains per bacterium

The average number of nascent chains per bacterium (equivalent to the numbers of ribosomes synthesizing penicillinase) can be calculated directly from the number of molecules synthesized per bacterium per generation, if the rate of peptide bond synthesis is known (J. Imsande, personal communication):

$$\text{nascent chains/bacterium} = \frac{\text{molecules/bacterium/division cycle} \times \text{amino acids/molecule}}{\text{generation time (sec.)} \times \text{peptide bond rate}}$$

Kepes & Beguin (1966) suggest an average 'step time' for peptide bond formation of 65 msec. at 37°. This corresponds to a rate of 15–16 amino acids/sec., the rate quoted by Maaløe & Kjeldgaard (1966) for *Escherichia coli* growing at a rate of 1–2 generations/hour at 37°. This figure is assumed for *B. licheniformis*. Exopenicillinase has 265 amino acids/molecule (Ambler & Meadway, 1969); the newly synthesized cell-bound enzyme is assumed in this work to have 270 amino acids (it is thought that during release of the enzyme, some terminal amino acids are removed). Thus, if 74,150 molecules are synthesized in a generation time of 70 min., at a rate of 16 amino acids/sec. and 270 amino acids/molecule, then there are about 300 nascent chains of penicillinase per bacterium when induced to the maximum rate of synthesis. The constitutive strain has about 700 nascent chains per bacterium.

Penicillinase polysome size

Attempts were made to measure the size of the penicillinase polysome on sucrose gradients. That the enzyme activity peaks in the polysome region mainly represented nascent chains, rather than free enzyme absorbed or otherwise associated with other ribosomes, was suggested by the following evidence. Polysome-ribosome preparations incubated *in vitro* (see Davies, 1969) showed penicillinase activity associated with the labelled polysome region, and not associated or bound to inactive monosomes, although these were in excess (Fig. 7*a*). A separate peak was identified in the polysome region for non-induced bacteria, high levels of enzyme which might obscure the true picture being absent (Fig. 7*c*). Uracil pulse-labelling and electron microscopy were used to ascertain that the fractions were polysomes. Experiments were also carried out only 60 min. after induction, when the enzyme was not released as free exoenzyme and could not therefore absorb onto ribosomes. The majority of cell-bound enzyme is associated with particles of large molecular weight (see Lampen, 1967; Davies, 1969) thought to be bacterial membrane, and therefore probably not free to absorb easily onto ribosomes. Penicillinase released from these washed polysome preparations was found to be about the same size and charge as the main exoenzyme fraction. Penicillinase peaks were routinely identified, and corresponded to a polysome size of 2–3 ribosomes (estimated from distance moved from the meniscus relative to 70s ribosomes; see Martin & Ames, 1961). Some results are shown in Fig. 7. Polysome sizes ranging from 2 to 5 ribosomes were obtained, but mostly a size of 2 to 3 ribosomes was indicated. The figure of 3 ribosomes per message (for a doubling time of 70 min.) was assumed for further calculations.

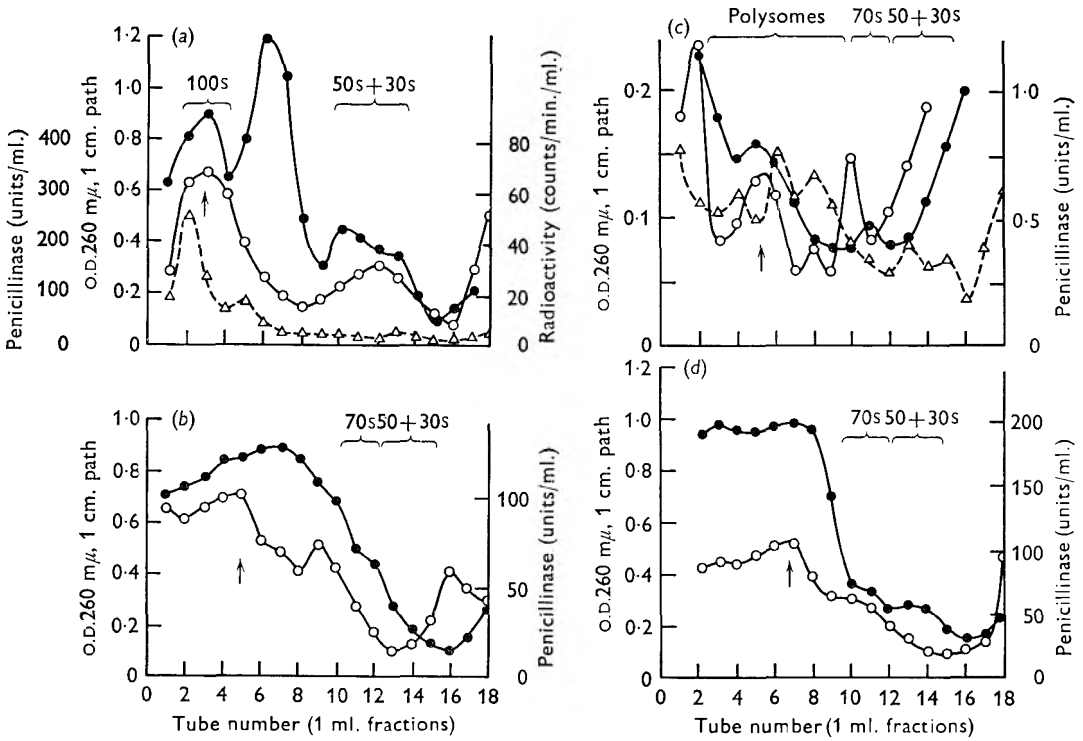

Fig. 7. Sucrose-gradient analysis of cell lysates and polysome preparations from *Bacillus licheniformis*. (*a*) Analysis of polysomes from strain 749/C incubated for 20 min. under cell-free protein synthesizing conditions (Davies, 1969) to incorporate ^{14}C-L-leucine into nascent polypeptide chains. Re-isolated polysomes and ribosomes were then centrifuged for 5 hr on a sucrose gradient. Penicillinase (arrow) and radioactivity are associated with polysomes (100 s), not with the large 70–80 s monosome peak. ● indicates optical density 280 nm; ○, penicillinase activity; △, radioactivity. (*b*) Polysomes isolated from strain 749/C pre-treated with chloramphenicol (100 μg./ml.): note absence of the 70 s peak. Bacteria were grown in nutrient broth (doubling time approximately 60 min.). Gradient was centrifuged for 3 hr. ● indicates optical density 280 nm.; ○, penicillinase activity. The main penicillinase peak around tubes 4 and 5 (arrow) corresponds to a polysome of about 3–5 ribosomes. The smaller peak, corresponding to about 80–100 s is thought to be due to 'monomers' and 'dimers' from breakdown of the larger polysomes. (*c*) Crude lysates from strain 749 non-induced. Doubling time was 70 min. Approximately 90% lysis was obtained. Tubes 1–2 contained some cells and cell-wall debris. A 40-sec. pulse of ^{14}C uracil was given immediately before harvesting. Gradient was centrifuged for 3·5 hr. A large proportion of the uracil label, representing m-RNA, was in the polysome region. The penicillinase peak in the polysome region (arrow) corresponds to 3 to 4 ribosomes. Penicillinase activity (basal) was assayed by the Pardee assay (see Imsande & Gerber, 1967). △ indicates radioactivity; ○, penicillinase activity; ●, optical density at 260 nm. (*d*) Isolated polysomes from strain 749 induced for 60 min. and chloramphenicol treated. Doubling time was 75 min. Gradient centrifuged for 3·5 hr. The suggested penicillinase polysome position (see Fig. 7*c*) corresponding to 2–3 ribosomes is indicated. ●, indicates optical density at 260 nm.; ○, penicillinase activity. Polysome sizes were calculated from $d_1/d_2 = (N_1/N_2)^{\frac{2}{3}}$ where d is the distance moved (approximated by tube number) and N is the number of ribosomes. Calculations were relative to $N_1 = 1$ for 70 s ribosomes (for theory and discussion, see Martin & Ames, 1961).

Cell content of penicillinase messenger

Three approaches have been taken to estimate the average cell content of penicillinase messenger RNA.

From the numbers of nascent chains and polysome size. The number of nascent chains per bacterium must be:

polysome size (ribosomes/message) × messages/bacterium.

Thus, under the growth conditions used in these experiments when the number of nascent chains per bacterium is about 300 for induced strain 749, the numbers of messages per bacterium would be 100, taking a polysome size of 3 ribosomes/message. The polysome size in the constitutive is the same as in the inducible but the number of nascent chains is higher, thus the number of messages is also higher (230).

From messenger stability. The cell content of penicillinase messenger can be estimated from the average life-time (T) of the molecules, and the amount of enzyme made in a generation. If $t\frac{1}{2}$ is the half-life of a population of molecules, then since $T = 1/\lambda$ where λ is the decay constant, then $T = 1{\cdot}443t\frac{1}{2}$. Thus, the half-life of 4·7 min. (Table 1) corresponds to an average life-time for individual molecules of 6·78 min. The rate of synthesis of penicillinase is about 3·5 molecules per ribosome per minute (equivalent to 270 amino acids polymerized at 16 amino acids/sec.). The number of times a messenger is read (protein molecules made per message) = messenger life × ribosomes/message × rate of synthesis, giving a value of 71 ($6{\cdot}78 \times 3{\cdot}0 \times 3{\cdot}5$). This, divided into the number of enzyme molecules made by a single bacterium in a generation (74,150) gives the number of messages used in a generation. For a 70 min. generation time, this is 1059. If the messenger life time is 6·64 min., then at any one time there are $1059 \times 6{\cdot}64/70 = 100$ penicillinase messenger molecules/cell.

From the level of penicillinase protein and total messenger RNA per bacterium. Since one unit of enzyme is equivalent to $2{\cdot}27 \times 10^{-3}$ μg. of protein, the specific activity of 2340 units/mg. dry weight is equivalent to 5·3 μg. of penicillinase protein/mg. dry weight. However, the average total protein content is about 65% of the dry weight (Table 2) or 650 μg. total protein/mg. dry weight. Thus penicillinase is equivalent to 0·8% of the total protein (in 749/C, 1·6–2%). The average total messenger RNA content is 1·2% of the dry weight (Table 2).

Thus, if this quantity of messenger is responsible for the total cell protein, then 0·0096% (m-RNA% of dry weight) would be required to make 0·8% of the total protein. Penicillinase messenger could therefore account for 0·0096% dry weight, or $0{\cdot}96 \times 10^{-10}$ μg. (dry weight of one bacterium approximately 10^{-6} μg.; 1 mg. $\equiv 10^9$ bacteria). Penicillinase m-RNA is assumed to have 810 nucleotides (triplets for 270 amino acids). From the genetic code and amino-acid composition of penicillinase, an approximation of the likely GC content of the messenger can be made; this is estimated to be 50%, which indeed might be expected as an 'average' composition. Thus, with equal amounts of the four bases, an average nucleotide molecular weight (340) can be used to estimate the molecular weight of penicillinase messenger as $340 \times 810 = 275{,}400$. The level of $0{\cdot}96 \times 10^{-10}$ μg. penicillinase m-RNA $= 6{\cdot}03 \times 10^{23} \times 0{\cdot}96 \times 10^{-10}/2{\cdot}75 \times 10^5 \times 10^6 = 210$ molecules/induced bacterium. This figure represents the average number of penicillinase messengers per bacterium in the exponential growing population. The number of messengers a single bacterium synthesizes during its division cycle is

210 × ln 2, which is 145. Taking into account the errors indicated in Table 2, there is an overall error in this figure of about ±40%. A figure of 145 penicillinase messenger molecules/bacterium agrees closely with the values calculated by the first two methods, the overall average being approximately 110 penicillinase messages/bacterium for induced 749, and approximately twice this number for the constitutive strain.

DISCUSSION

Recent work has provided good arguments for the reliability of the techniques used in this work. First the results of Fan (1966) indicate that, after repression of m-RNA synthesis, subsequent formation of enzymes is largely due to translation of intact messengers, not completion of partially translated messengers. Second, although Acs, Reich & Valanju (1963) suggested that actinomycin D caused some breakdown of RNA, it is now considered that this effect is negligible (see Leive, 1965; Salser, Janin & Levinthal, 1968). Results for enzyme messenger stability using actinomycin D (Hartwell & Magasanik, 1963, 1964; Leive & Kollin, 1967) are comparable to results obtained by inducer removal (see Kepes, 1963; Nakada & Magasanik, 1964). Third, an independent technique involving the effects of turnover rate and quantity of unstable RNA on the labelling kinetics of the GTP pool has been developed (Salser *et al.* 1968), which gives results comparable with those observed for actinomycin experiments.

Rifampicin, unlike actinomycin, binds to RNA polymerase (Wehrli, Knüsel, Schmid & Staehelin, 1968), and only to polymerase free from the transcription complex (see von Hartmann, Behr, Beissner, Honikel & Sippel, 1968). For messengers of average size around 1000 bases, like penicillinase, transcription takes 20–30 sec. During this time, rifampicin may not exert its full effect, but this does not alter the decay rate significantly. The gene for a very large protein, or more so, a polycistronic messenger, may have several or many RNA polymerase molecules transcribing at once. Thus, after addition of rifampicin, a considerable number of intact messengers can be formed. For penicillinase, however, the rifampicin results confirmed those obtained with actinomycin (Table 1).

The value for the penicillinase messenger RNA half-life of 4·7 min. seems contradictory to previous reports (Pollock, 1963; Yudkin, 1966, 1968). However, several points should be taken into consideration. Firstly, messenger decay is a function of the rate of translation and can be protected by lower temperatures or decreased aeration conditions (Levinthal *et al.* 1963). The rapid rotary shaking used in the experiments reported here may afford higher aeration compared to the conditions employed by Yudkin, who also performed experiments at 30° and 25°, not 37°. Secondly, samples taken at, say, 10 min. intervals miss the early kinetics, and show results for recovered growing cells (see Fig. 2). If bacteria are still growing (as in the experiments of Pollock, 1963), the proteins required for growth are being synthesized; thus continued synthesis of penicillinase need not imply a messenger any more stable than that of the average cell protein.

A short-lived penicillinase messenger ($t\frac{1}{2}$ = 2 min.) has been suggested by similar actinomycin and rifampicin experiments with *Bacillus cereus* 569 (J. Imsande and J. W. Davies, unpublished results).

Two of the approaches taken to estimate contents of penicillinase m-RNA in bacteria involved the polysome size. A recent model for *tryp* m-RNA translation in

Escherichia coli at 37° is that ribosomes occupy 20–40% of the outstretched *tryp* polycistronic messenger (Morse, Baker & Yanofsky, 1968). For the penicillinase messenger of only 5000 Å, 5 ribosomes per message (20%, assuming that one ribosome occupied 200 Å) would still be consistent with the model. Zimmermann & Levinthal (1967) calculate that, in *Bacillus subtilis* (in which unstable RNA accounts for 8% of the total RNA), there may be about 500 nucleotides of m-RNA per 70s ribosome. On this reckoning, penicillinase messenger would be expected to have about 2 ribosomes. The size of 3 ribosomes per message used in the calculations of contents of penicillinase messenger in bacteria is therefore thought to be of the right order, but with a possible error of about ±60%. Thus, the estimation of the content of penicillinase messenger in bacteria also cannot be expected to be better than ±60% on overall average. That all the data used in the three approaches to estimate messenger content including the messenger half-life can be integrated and show consistency, is to some extent a test of the validity of the figures.

The technique of hybridization of specific bacteriophage RNA may afford a more direct approach, although assumptions concerning the specific activity and percentage of hybridization are necessary. Using that technique, Stubbs & Hall (1968) estimate that wild-type *Escherichia coli* has about 2 *tryp* m-RNA molecules per bacterium, while a constitutive mutant has 25. Edlin, Stent, Baker & Yanofsky (1968) estimate 4–8 *tryp* m-RNA molecules per bacterium (corrected values) when fully derepressed. It seems likely that the levels of penicillinase messenger in non-induced and constitutive bacteria also vary, rather than stabilization of the messenger (Harris & Sabath, 1964; Yudkin, 1966) accounting for higher levels of enzyme.

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The Effects of Ultra-violet Irradiation on the Fertility of F$^+$ and Hfr Strains of *Escherichia coli* K12 Defective for the Repair of Damaged DNA

By K. A. STACEY

School of Biological Sciences, University of Sussex, Brighton, Sussex, BN1 9QG

AND Z. EVENCHIK AND W. HAYES

M.R.C. Microbial Genetics Research Unit, Hammersmith Hospital, London W. 12

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SUMMARY

The effect of irradiating donor cells, immediately before mating, upon the yield of recombinants has been investigated for male (F$^+$ + Hfr) strains of *Escherichia coli*, which, owing to a mutational block (*uvr*), are unable to excise pyrimidine photoproducts. Despite the extreme sensitivity of the *uvr*$^-$ strains employed, as judged by colony formation, the yield of recombinants was surprisingly little affected by u.v. In particular, using an Hfr uvr_B^- strain it was found that, after an initial fall to about 30–40% of that given by the unirradiated control, the yield of recombinants for both early and late markers declined with increasing dose at about the same rate as for the parent *uvr*$^+$ strain. There was evidence of damage in the DNA transferred from irradiated males in that normal linkage of unselected markers was reduced, but the decline in linkage with increasing dose was the same for both *uvr*$^+$ and *uvr*$^-$ strains. The yield of recombinants was nearly independent of the *uvr* phenotype of the *F*$^-$ parent. Thus although fertility and survival are closely correlated in the *uvr*$^+$ Hfr, this correlation disappears in the *uvr*$^-$ male. Instead the u.v. sensitivity of the processes involved in chromosome transfer appears only slightly altered in these mutants despite the considerable change in sensitivity as judged by colony-formation.

INTRODUCTION

Irradiation of F$^+$ cultures of *Escherichia coli* K12 with small doses of u.v. light stimulates the transfer of chromosomal material to F$^-$ recipients, increasing the yield of recombinants 10 to 100 fold (Hayes, 1952). In the course of experiments exploring the requirements for this stimulation of fertility we examined the effect of u.v. upon F$^+$ donors which carried mutational defects (*uvr*$^-$) in the repair mechanisms for u.v. and other forms of damage to DNA (Evenchik, Stacey & Hayes, 1969). Surprisingly, the yield of recombinants remained similar to that of the unirradiated control even when the survival of the donor population, as judged by colony formation, had fallen to 10^{-3}. Because a number of observations concerning the effects of u.v. on the fertility of Hfr strains had shown that fertility and the capacity to form colonies are lost more or less in parallel (Joset & Wood, 1966; Doudney & Bruce, 1966; Evenchik, Stacey & Hayes, 1969), we investigated the fertility of Hfr strains defective in one of the genes governing the excision repair process, in particular an Hfr H strain carrying

an uvr_B^- mutation. These experiments showed that, as for the F^+ strains, fertility was lost much more slowly than viability; the yield of recombinants fell only to 40% of the unirradiated control for a colony-forming fraction less than 10^{-3}.

This paper reports our preliminary observations on this system. They are very similar to the results obtained by Wilkins & Howard-Flanders (1968).

EXPERIMENTAL

Strains. All strains were derivatives of *Escherichia coli* K12.

W1655 F^+ *met*$^-$ *Sm*^{5} λ^- λ^R (Scaife & Gross, 1963).

W1655 F^+ *uvr*$^-$ isolated by us using the technique of Howard-Flanders & Theriot (1962).

KMBL 49 F^- *thr*$^-$ *leu*$^-$ *thi*$^-$ *lac*$^-$ *thy*$^-$ *ura*$^-$ *Sm*^{R} (van de Putte *et al.* 1965).

KMBL 49 F^+ isolated by us after conjugation with W1655.

KMBL90 F^- uvr_B^- mutant of KMBL49 (van de Putte *et al.* 1965).

KMBL90 F^+ isolated by us after conjugation with W1655.

Hfr H *Sm*^{5} (Evenchik *et al.* 1969).

Hfr Z48 (Hfr H *gal*$^+$ uvr_B^-, made by transduction of Hfr H *gal*$^-$) using phage P1 grown upon AB1885 *gal*$^+$ uvr_B^- and selected from among the *gal*$^+$ recombinants for the co-transduction of uvr_B^-.

F^- W945 *pro*$^-$ *Sm*^{R} (Evenchik *et al.* 1969).

F^- AB1157 *thr*$^-$ *leu*$^-$ *pro*$^-$ *his*$^-$ *arg*$^-$ *thi*$^-$ *ara*$^-$ *Sm*^{R} (Adelberg & Burns, 1960).

F^- AB1884 uvr_A^-, AB1885 uvr_B^-, AB1886 uvr_C^- } derivatives of AB1157, isolated by Howard-Flanders, Boyce & Theriot (1966).

Irradiations. Broth cultures of F^+ and Hfr strains were grown, from 1 in 20 dilutions of saturated over-night cultures in minimal media, to 1×10^8 bacteria/ml., filtered and resuspended in buffer at 5×10^8 bacteria/ml., and irradiated 50 cm. from a Hanovia lamp at dose rates of approximately 2 ergs/mm.^{2}/sec.

Crosses. After irradiation the bacteria were diluted with broth, mixed with an equal volume of a culture of the appropriate F^- strain and incubated for 45 min. Recombinants were, in most cases, scored on minimal media containing all but one of the requirements of the F^- strain and 200 μg./ml. of streptomycin. In the crosses involving the F^+ KMBL strains which are streptomycin resistant, the males were counter-selected by omission of their several requirements. In the analysis of the unselected markers recombinants were picked onto the same selective medium and these plates used as master plates for replica plating. The inheritance of arabinose fermentation was scored on EMB-arabinose plates.

RESULTS

$F^+ \times F^-$ crosses

Comparisons of recombinants by the F^+ derivatives of strains KMBL49 and 90 before and after doses of u.v. which reduced their survival to between 1 and 0·1%, showed little diminution in the yield of recombinants. Since this yield was in any case small, we isolated a *uvr*$^-$ strain of the relatively fertile F^+ used in previous studies (Evenchik *et al.* 1969). The loss with increasing u.v. dose of the capacity of this mutant, W1655 F^+ *uvr*$^-$, to form colonies, and its ability to reactivate u.v. irradiated phage were com-

parable with those of the most sensitive of the known *uvr*⁻ strains available to us for comparison. Again, after u.v. irradiation its capacity to transfer chromosomal material was still 40% of the control value even when its colony forming ability had been reduced to 10^{-3}. There was, however, none of the stimulation of chromosomal transfer at low doses (50% survival) which is shown by the parent, *uvr*⁺, strain (Evenchik *et al.* 1969). This apparent u.v. resistance of the transfer capacity prompted us to examine the effect of u.v. on the transfer of chromosomal material by a *uvr*⁻ mutant of an Hfr strain.

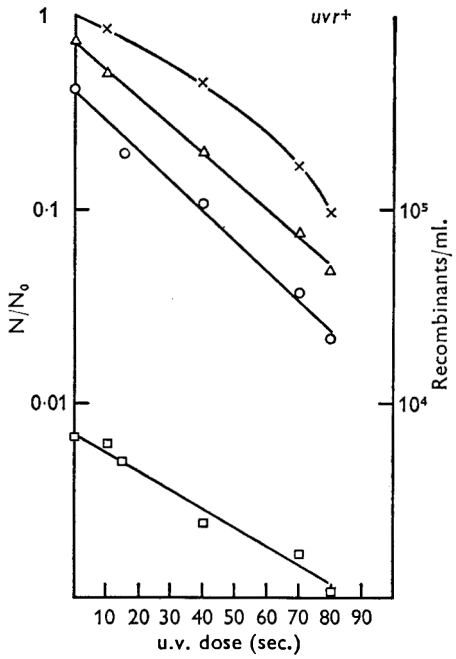

Fig. 1

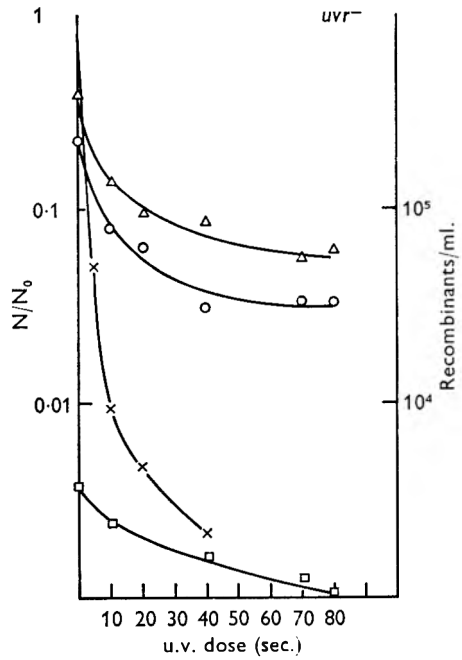

Fig. 2

Fig. 1. The effect of u.v. light on the survival of Hfr H *uvr*⁺ × — ×, and on the yield of recombinants in a standard cross (see Methods) with AB 1157 F⁻ selected for the inheritance of the donor markers threonine and leucine △—△, proline ○—○, and arginine □—□.

Fig. 2. The effect of u.v. light on the survival of Hfr H *uvr*⁻ (z48) × — × and on the yield of recombinants in a standard cross (see Methods) with AB 1157 F⁻ selected for the inheritance of the donor markers threonine and leucine △—△, proline ○—○, and arginine □—□.

Hfr × *F*⁻ *crosses*

We had already repeated some of the observations of Joset & Wood (1966), which showed that survival and the transfer of early markers by an Hfr *uvr*⁺ strain were affected by u.v. to about the same extent, in the course of the earlier experiments on the effect of u.v. on chromosomal transfer (Evenchik *et al.* 1969). These experiments were repeated over a wider range of doses and for a greater number of markers using as F⁻ recipients AB 1157 and three *uvr*⁻ strains derived from it (AB 1884 A⁻, AB 1885 B⁻ and AB 1886 C⁻) all kindly given by Dr P. Howard-Flanders. The results in Fig. 1 for the crosses with AB 1157 (confirming our earlier results) show that u.v. irradiation had a very similar effect on both viability and fertility even for the late

marker (*arg*). These results differ in detail from those of Joset & Wood, who reported that the yield of recombinants was more sensitive to irradiation, the further the selected marker from the origin. It is possible that this discrepancy may be explained by differences between the strains used, since Jacob & Wollman (1958) obtained results very similar to ours. The F^- *uvr*$^+$ parent and its three *uvr*$^-$ derivatives were equally good recipients irrespective of the irradiation dosage to the Hfr strain.

Table 1. *Effect of u.v. irradiation of Hfr H uvr*$^-$ *upon the yield of recombinants*

u.v. dose (sec.)	Survival (%)	Recombinants					
		F^- *thr*$^+$ *leu*$^+$	% of control	*pro*$^+$	% of control	*arg*$^+$	% of control
		10^5/ml.		10^5/ml.		10^4/ml.	
0	100	1884 uvr_A^- 17	100	3·7	100	4·0	100
5	12·3	12	69	3·2	86	1·8	45
10	1·2	6·1	36	1·6	43	1·4	35
0	100	1885 uvr_B^- 25	100	7·4	100	17	100
5	14·6	13·6	54	4·9	66	—	—
10	2·1	13	52	4·8	65	10	59
0	100	1886 uvr_C^- 26	100	8·2	100	7·4	100
5	11	23	88	5·7	70	6·1	82
10	0·94	12	46	3·1	58	—	—
20	0·4	5·2	20	1·5	18	—	—

Crosses under the same conditions with the same four recipients were then made with the u.v. sensitive strain Hfr Z48 carrying the uvr_B^- mutation, transferred by P1 transduction from AB 1885. Some of the results with AB 1157 as recipient are shown in Fig. 2: comparable results were obtained with the *uvr*$^-$ recipients (Table 1). Despite the great difference in sensitivity between the two Hfr strains, the only striking difference between the recombination data in Figs. 1 and 2 is that quite low doses cause about a halving in the number of recombinant given by the *uvr*$^-$ donor. It seems possible that there might be two populations, one very sensitive to u.v. and the other no more so, with respect to chromosome transfer, than a *uvr*$^+$ strain, but this is not reflected in the survival curve. In many *uvr*$^+$ strains there is a 3- to 20-fold increase in survival if, after irradiation, bacteria are incubated in a growth medium before plating. (Barner & Cohen, 1956 and Stacey & Atkinson, unpublished observations). To check that the 45 min. incubation with the F^- did not 'rescue' any substantial fraction of the irradiated donor bacteria, the survival of the Hfr *uvr*$^-$ strain was measured in control cultures both before and after a period of incubation equivalent to those used for mating. There was no increase in viable count for periods up to an hour.

The most obvious explanation of these results is that damaged DNA can be transferred and integrated into the chromosome of the F^- although neither donor nor recipient carry the full set of functional genes necessary for excision repair. By analogy with 'marker rescue' one might anticipate that crossing-over is more frequent in recombination involving damaged chromosomes. Such an effect has been observed in the region of the chromosome carrying the closely linked loci *thr*, *ara*, and *leu*. The recombinants selected for the distal marker, *pro*, were examined for the inheritance of both *thr*$^+$ and *leu*$^+$ and among these the inheritance of the interjacent *ara*$^+$ locus was about 98% in the control crosses; in a cross after a dose of 40 ergs/mm.^{2} which reduced the survival of Hfr Z48 to 0·1%, the percentage of *thr*$^+$ *leu*$^+$ recombinants that were

also *ara*$^+$ had been reduced to 78%. The linkage between *pro* and the *thr* and *leu* markers was also drastically reduced (Table 2). However, just such a breakdown in linkage was reported by Jacob & Wollman (1958) and confirmed by Joset & Wood (1966) in normal, *uvr*$^+$ Hfr strains. We therefore compared the linkage of unselected markers in the two types of cross at the same absolute doses. Table 2 shows that the difference between the two strains was very small.

These observations confirm a more extensive study by Wilkins & Howard-Flanders (1968) who propose that the explanation lies in the transfer of DNA which has been replicated with gaps opposite some of the DNA lesions, which in a *uvr*$^+$ strain would have been repaired by excision (Rupp & Howard-Flanders, 1968). Although our data do not permit any very direct comment on this idea, we have not observed any marked differences in the time of entry of early markers, which implies that if DNA synthesis is impeded under these conditions then this is not the rate-limiting step. Comparable experiments in which the *uvr*$^-$ F$^-$ parent has been irradiated, have, in a limited number of observations, shown that zygotes are more resistant than the survival curve would lead one to expect.

Table 2. *Effect of u.v. irradiation of the donor upon linkage*

Cross	u.v. dose (sec.)	Survival (%)	% Linkage of *leu*$^+$ to *pro*$^+$	% Linkage of *ara*$^+$ to *thr*$^-$ *leu*$^+$
Hfr H *uvr*$^+$ × AB 1157	0	100	73	96
	10	80	47	88
	80	5	19	78
Hfr H *uvr*$^-$ × AB 1157	0	100	70	96
	10	1	69	89
	80	< 0·1	36	74
Hfr H *uvr*$^-$ × AB 1886	0	100	86	98
	10	1	22	63

DISCUSSION

Irradiation of normal Hfr strains with ultra-violet light reduces their fertility to much the same extent as it renders them incapable of forming colonies. In contrast an Hfr strain which is incapable of excision-repair as a result of a mutation in the uvr_B locus, continues to yield recombinants at a high rate at doses of u.v. which have reduced viability to a low level, even when the recipient is also *uvr*$^-$. However, it is clear from analysis of the unselected markers in these recombinants that the transferred chromosome fragment is damaged because it appears tc be involved in more frequent recombination, but the magnitude of this effect is the same among recombinants from crosses with the *uvr*$^+$ Hfr strain at the same dose.

There seem to be two tenable hypotheses to explain these effects. The first is that DNA synthesis is possible even when there are many pyrimidine dimers present in the chromosome involved in transfer. This is the suggestion of Wilkins & Howard-Flanders (1968) based on the observations of Rupp & Howard-Flanders (1968) that DNA synthesis in irradiated *uvr*$^-$ bacteria leads to the formation of DNA which, when examined by the alkaline sucrose gradient technique of McGrath & Williams (1966), appears to contain many single strand breaks. This, they suggest, is due to interruptions in the newly synthesized strand caused by pyrimidine dimers in the template strand.

If this view is correct the transferred DNA contains gaps opposite dimers: since this damage cannot be repaired by the excision mechanism it would explain why the presence of *uvr*$^+$ genes in the recipient is irrelevant. It is then not clear why the DNA transferred by the Hfr *uvr*$^+$ seems to contain an equal amount of damage as judged by the linkage data.

The second hypothesis is that after irradiation DNA can be transferred without synthesis. If this occurred by transfer of the double strand then the capacity of the female to repair should be manifest, but if the transfer were of only one strand then again excision repair would be impossible. This idea, too, does not explain why the excision repair system does not reduce the degree of damage to DNA before transfer by the *uvr*$^+$ donor, and one is led to enquire whether conjugation in some way interferes with the normal process of repair. Experiments on this point are being made.

An attempt is also being made to investigate the nature of the DNA transferred by irradiated Hfr bacteria, but the experiments of Vielmetter, Bonhoeffer & Shütte (1968) appear to support very strongly the idea that only one DNA strand is transferred in the normal course of bacterial conjugation. They interpret their observations on the segregation of recombinants containing mutations generated in the Hfr parent immediately prior to transfer as being most consistent with single strand transfer (see also Kunicki-Goldfinger, Piekarowicz & Wlodarczyk, 1968). It is clear from the results of Rupp & Ihler (1968) that a particular strand in the Hfr chromosome is transferred, although their technique does not permit them to determine whether a complementary strand must be synthesized in the donor for transfer to take place.

One test of the hypothesis that u.v. damage is not repaired in the female because the DNA is transferred as a single strand, would seem at first sight to be a study of the effect of the *uvr* loci on transduction by u.v.-irradiated phage, because there is good evidence that the DNA of the transducing particle is double-stranded (Ikeda & Tomizawa, 1965). However the effect of u.v. on generalized transduction by phages P22 (Garen & Zinder, 1955) and P1 (Arber, 1960), and on specialized transduction by phage λ (Arber, 1958) is first to increase the efficiency of transduction, presumably by increasing the efficiency of recombination, well above the level obtained with unirradiated phage. In an *uvr*$^-$ recipient the efficiency of transduction by phage P22 falls with increasing irradiation (Takebe, 1968), so that a direct comparison is impossible. One can perhaps draw a parallel between the effects of u.v. on chromosomal mobilization by the F episome and transduction, for in both cases in *uvr*$^+$ strains there is an increase in recombination which is not seen in *uvr*$^-$ strains, and argue as we have previously (Evenchik *et al.* 1969), that the increase in recombination is the result of excision giving rise to single-stranded regions, which facilitate the pairing necessary to initiate recombination. This seems to be different from the situation studied here, where the recovery of recombinants fell with increasing u.v. dose, regardless of the possession by donor or recipient of the excision-repair system.

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Serological Specificity of Yeast Mannan

By I. CAMPBELL AND R. H. GILMOUR
Heriot-Watt University, Edinburgh, EH1 1HX

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SUMMARY

Soluble serologically active preparations extracted by various methods from *Saccharomyces* spp. showed the group specificity previously detected by agglutination tests with whole cells. The antigenic properties of the four serological groups of the genus *Saccharomyces* are probably determined by the cell wall mannan.

INTRODUCTION

Antigenic analyses of yeast species have depended mainly on agglutination tests with whole cells (Tsuchiya, Fukazawa & Kawakita, 1961; Campbell, 1968) but water-soluble polysaccharide material, presumably from the cell wall, has also shown antigenic differences between species of the genera *Saccharomyces* and *Candida* (Summers, Grollman & Hasenclever, 1964; Suzuki, Suzuki, Yokota, Sunayama & Sakaguchi, 1967). Gel-diffusion methods have been used successfully with saline extracts (Sandula, Kockova-Kratochvilova & Zamecnikova, 1964), phenol extracts (Campbell, Robson & Hough, 1968) and mannan preparations (Summers *et al.* 1964) against Saccharomyces or Candida sera prepared by immunisation of rabbits with whole cells.

Campbell (1968), by means of four suitably absorbed antisera, classified 19 species of the genus *Saccharomyces* into four antigenic groups A, B, C and D. These results obtained by agglutination tests were consistent with the observations of Sandula *et al.* (1964) in gel-diffusion experiments with various *Saccharomyces* species. Yeast mannan oligosaccharides were characteristic of species (Stewart & Ballou, 1968) and reacted with yeast antisera, blocking the immune reaction against whole yeasts (Suzuki & Sunayama, 1968; Suzuki, Sunayama & Saito, 1968). With the exception of Snider & Miller's (1966) report suggesting protein as the major cell wall antigen of yeasts, all previous work has indicated that the polysaccharide fraction, and probably the mannan, is the determinant of antigenic activity of Saccharomyces species.

Cell-wall material was isolated from typical strains of the four groups of our antigenic analysis scheme and the antigenic properties of these fractions are described below.

METHODS

Strains. Strains and routine methods of culture were as described by Campbell (1968); cultures were identified and classified according to the system of Lodder & Kreger-van Rij (1952). Two strains were chosen of each of the antigenic groups A, B, C and D (Campbell, 1968) for serological tests. *Saccharomyces microellipsodes* 698, of group D (unpublished results), was chosen as the second strain of that group. Large quantities of yeasts were grown in 15 litres of Sabouraud glucose broth in 20-litre bottles for 3 days at 25°. Aeration at 0·8 litres/min. through a sintered-glass diffuser at

the base of the bottle also served to agitate the culture. Yeasts were washed thrice with water, and the final centrifuged deposit was stored at $-15°$.

Extracts. Isolated cell walls were prepared by the method of Trevelyan (1966). Phenol extracts were prepared by the method of Westphal, Lüderitz & Bister (1952); only the water phase was retained as antigen (Campbell *et al.* 1968). Hot water extracts were prepared by shaking approx. 10^9 yeasts/ml. at 68–70° for 1 hr. Mannans were prepared by the method of Peat, Whelan & Edwards (1961). Also, antigenic material was recovered from the cell-free supernatant fluid of a 3-day culture at 25° in Difco yeast carbon medium plus glucose. All preparations were dialysed overnight against running tap water and freeze-dried.

Serological methods. Antisera were prepared and agglutination and gel-diffusion tests performed as described previously (Campbell, 1968; Campbell *et al.* 1968). Absorbed antisera used for gel-diffusion tests were freeze-dried and reconstituted, with distilled water, to the volume required to give homologous agglutination titres equal to those of the unabsorbed sera used in gel-diffusion tests with similar extracts.

Chromatography. Approximately 500 μg. of each extract were hydrolysed with 3 ml. of 2 N-H_2SO_4 for 2 hr at 100°. The hydrolysates were neutralised with $BaCO_3$, deionised with mixed resin (Amberlite IR 120 and Amberlite IR 45, B.D.H. Ltd.), reduced in volume and chromatographed on Whatman no. 1 paper. Descending chromatography was used with ethyl acetate + pyridine + water (10 + 4 + 3 v/v) as the solvent. Reducing sugars were detected with alkaline $AgNO_3$ (Trevelyan, Procter & Harrison, 1950) or aniline oxalate (Partridge, 1949). Glucose, mannose and ribose were used as standards.

RESULTS

Reducing sugars. The cell-wall preparations on hydrolysis contained glucose, mannose and traces of ribose. Similar chromatograms were obtained from hydrolysed phenol extracts and hot water extracts of whole yeasts, but the minimal medium preparations showed only glucose and mannose. Mannan preparations yielded mannose with only traces of glucose.

Protein content of extracts. All freeze-dried mannan preparations and phenol extracts contained less than 0·1 % protein, measured by the method of Opienska-Blauth, Charezinski & Berbec (1963) against a standard of bovine serum albumin (B.D.H. Ltd.). Hot water extracts contained 1–2 % protein, depending on the yeast species, and minimal medium extracts, up to 1 %.

Comparison of gel-diffusion and slide agglutination tests with unabsorbed antisera. Since previous reports from various authors have dealt with soluble antigens prepared in different ways, gel-diffusion tests with mannan, hot water and phenol extracts and antigenic material recovered from minimal medium were compared with our previous results by agglutination tests. The two strains of each antigenic group were tested with unabsorbed antisera to the following strains: group A—*Saccharomyces carlsbergensis* 1116; group B—*S. fragilis* 100 and *S. marxianus* 111; group C—*S. willianus* 106; group D—*S. fermentati* 161. *Saccharomyces marxianus* antiserum was added to the series as a result of preliminary experiments in which certain discrepancies were noted between the standard group B *S. fragilis* antiserum and the serum against *S. marxianus*, apparently group B but which on occasions reacted weakly in addition as a group C serum.

Gel-diffusion tests of the various extracts against unabsorbed antisera showed general agreement with the agglutination tests as shown in Tables 1–5. However, unabsorbed antiserum to *Saccharomyces carlsbergensis* strain 1116 agglutinated *S ellipsoideus* Y4 and *S. willianus* 106 (Table 1) but produced no precipitin reaction with any of the extracts of these two strains (Tables 2–5). The same serum agglutinated *S. fragilis* 100 and *S. marxianus* 111 (Table 1), reacted with the mannans (Table 2), minimal medium extracts (Table 4) and hot water extracts (Table 5) of these strains but showed no precipitin lines with their phenol extracts (Table 3). Antiserum to

Table 1. *Agglutination of Saccharomyces suspensions by unabsorbed anti-Saccharomyces sera*

		Agglutination by antiserum to:				
Agglutinable suspensions		*S. carlsbergensis* 1116	*S. fragilis* 100	*S. marxianus* 111	*S. willianus* 106	*S. fermentati* 161
Group A:						
S. carlsbergensis	396	+	+	+	−	+
S. rouxii	170	+	+	+	−	+
Group B:						
S. fragilis	100	+	+	+	±	−
S. marxianus	111	+	+	+	+	+
Group C:						
S. cerevisiae var. *ellipsoideus*	Y4	+	−	+	+	−
S. willianus	106	+	−	+	+	−
Group D:						
S. fermentati	161	−	−	−	−	+
S. microellipsodes	698	−	−	−	−	+

+ = agglutination; − = no reaction; ± = variable.

Table 2. *Precipitin tests of Saccharomyces mannans by unabsorbed anti-Saccharomyces sera*

		Precipitin bands formed by antiserum to:				
Mannan solutions		*S. carlsbergensis* 1116	*S. fragilis* 100	*S. marxianus* 111	*S. willianus* 106	*S. fermentati* 161
Group A:						
S. carlsbergensis	396	+	+	+	−	−
S. rouxii	170	+	+	+	−	+
Group B:						
S. fragilis	100	+	+	+	+	−
S. marxianus	111	+	+	+	+	−
Group C:						
S. cerevisiae var. *ellipsoideus*	Y4	−	+	+	+	−
S. willianus	106	−	+	+	+	−
Group D:						
S. fermentati	161	−	−	−	−	+
S. microellipsodes	698	−	−	−	−	+

+ = band(s) formed; − = no reaction.

S. fragilis 100 agglutinated *S. carlsbergensis* 396 and *S. rouxii* 170 (Table 1) and produced precipitin bands with all except phenol extracts (Table 3). This antiserum failed to agglutinate *S. ellipsoideus* Y4 or *S. willianus* 106 (Table 1) but gave precipitin lines with all extracts of these strains (Tables 2–5). Antiserum to the other group B yeast, *S. marxianus* strain 111, differed from that against *S. fragilis* 100 in agglutinating *S. willianus* 106 and *S. ellipsoideus* Y4 (Table 1). Antiserum to *S. willianus* 106 did not react with the minimal medium extract of *S. fragilis* 100 (Table 4) or with hot water extracts of 100 or *S. marxianus* 111 (Table 5), but agglutinated these yeasts (Table 1)

Table 3. *Precipitin tests of Saccharomyces phenol extracts by unabsorbed anti-Saccharomyces sera*

Phenol extract solutions		Precipitin bands formed by antiserum to: *S. carlsbergensis* 1116	*S. fragilis* 100	*S. marxianus* 111	*S. willianus* 106	*S. fermentati* 161
Group A:						
S. carlsbergensis	396	+	−	−	−	−
S. rouxii	170	+	−	−	−	+
Group B:						
S. fragilis	100	−	+	+	+	−
S. marxianus	111	−	+	+	+	−
Group C:						
S. cerevisiae var. *ellipsoideus*	Y4	−	+	+	+	−
S. willianus	106	−	+	+	+	−
Group D:						
S. fermentati	161	−	−	−	−	+
S. microellipsodes	698	−	−	−	−	+

+ = band(s) formed; − = no reaction.

Table 4. *Precipitin tests of Saccharomyces minimal media by unabsorbed anti-Saccharomyces sera*

Minimal medium extract solutions		Precipitin bands formed by antiserum to: *S. carlsbergensis* 1116	*S. fragilis* 100	*S. marxianus* 111	*S. willianus* 106	*S. fermentati* 161
Group A:						
S. carlsbergensis	396	+	+	+	−	+
S. rouxii	170	+	+	+	−	−
Group B:						
S. fragilis	100	+	+	+	−	−
S. marxianus	111	+	+	+	+	−
Group C:						
S. cerevisiae var. *ellipsoideus*	Y4	−	+	+	+	−
S. willianus	106	−	+	+	+	−
Group D:						
S. fermentati	161	−	−	−	−	+
S. microellipsodes	698	−	−	−	−	+

+ = band(s) formed; − = no reaction.

and gave precipitin bands with mannan and phenol extracts (Tables 2 and 3). Unabsorbed antiserum to *S. fermentati* 161 agglutinated *S. carlsbergensis* 396, *S. rouxii* 170 and *S. marxianus* 111 (Table 1), but gave consistent precipitin bands with only the mannan and phenol extracts of *S. rouxii* 170 (Tables 2, 3) and the minimal medium preparation of *S. carlsbergensis* 396 (Table 4).

Since the mannan-containing enzyme invertase occurs in the yeast cell wall (Neumann & Lampen, 1967), it was possibly implicated in these reactions. A sample of yeast invertase (B.D.H. Ltd.) was dialysed against distilled water at 2° to remove glycerol, and lyophilised. The invertase was reconstituted at various concentrations for gel-diffusion tests against antisera to *Saccharomyces willianus* 106 and *S. fragilis* 100, but no reaction was observed.

Table 5. *Precipitin tests of Saccharomyces hot water extracts by unabsorbed anti-Saccharomyces sera*

		Precipitin bands formed by antiserum to:				
Hot water extract solutions		*S. carlsbergensis* 1116	*S. fragilis* 100	*S. marxianus* 111	*S. willianus* 106	*S. fermentati* 161
Group A:						
S. carlsbergensis	396	+	+	+	—	—
S. rouxii	170	+	+	+	—	—
Group B:						
S. fragilis	100	+	+	+	—	—
S. marxianus	111	+	+	+	—	—
Group C:						
S. cerevisiae var. *ellipsoideus*	Y4	—	+	+	+	—
S. willianus	106	—	+	+	+	—
Group D:						
S. fermentati	161	—	—	—	—	+
S. microellipsodes	698	—	—	—	—	+

+ = band(s) formed; — = no reaction.

Gel-diffusion and slide agglutination tests with absorbed antisera. Specific antisera were prepared by absorption with the species recommended previously (Campbell, 1968). In addition, *Saccharomyces marxianus* antiserum 111 was absorbed by *S. rouxii* 170 to produce an alternative group B antiserum, but despite extensive absorption retained a significant activity in agglutination tests against the group C strains *S. ellipsoideus* Y4 and *S. willianus* 106. A further absorption of *S. marxianus* 111 antiserum by *S. willianus* 106 produced a group B-specific antiserum suitable for agglutination tests but too weak for gel-diffusion, even after concentration. The four standard group A, B, C and D antisera showed the expected specific agglutination of their appropriate groups (Table 6).

In gel-diffusion tests the group A and D antisera and *Saccharomyces fragilis* antiserum absorbed by *S. rouxii* produced single or double bands of identity strictly in accordance with group specificity with all four types of extract, but the group B antiserum prepared with *S. marxianus* antiserum absorbed by *S. rouxii* 170 consistently reacted with both group B and group C extracts, and the group antiserum reacted with both group B and group C extracts (Table 7). The amount of extract required for best

Table 6. *Agglutination of Saccharomyces suspensions by absorbed anti-Saccharomyces sera*

Agglutinable suspensions		Agglutination by antiserum to: *S. carlsbergensis* 1116 absorbed by *S. willianus* 106	*S. fragilis* 100 absorbed by *S. rouxii* 170	*S. marxianus* 111 absorbed by *S. rouxii* 170	*S. marxianus* 111 absorbed by *S. rouxii* 170 & *S. willianus* 106	*S. willianus* 106 absorbed by *S. cerevisiae* 1006	*S. willianus* 106 absorbed by *S. cerevisiae* 1006 & *S. marxianus* 111	*S. fermentati* 161 absorbed by *S. cerevisiae* 1006
Group A:								
S. carlsbergensis	396	+	−	−	−	−	−	−
S. rouxii	170	+	−	−	−	−	−	−
Group B:								
S. fragilis	100	−	+	+	+	±	−	−
S. marxianus	111	−	+	+	+	+	−	−
Group C:								
S. cerevisiae var. *ellipsoideus*	Y4	−	−	+	−	+	+	−
S. willianus	106	−	−	+	−	+	+	−
Group D:								
S. fermentati	161	−	−	−	−	−	−	+
S. microellipsodes	698	−	−	−	−	−	−	+

+ = agglutination; − = no reaction; ± = variable.

precipitin reaction with an antiserum varied with the type of extract. Generally, with antiserum of agglutination titre 1 in 100, the required concentrations of the homologous mannans, minimal medium or hot water extracts, and phenol extracts were 0·1 mg./ml., 2 mg./ml. and 5 mg./ml. respectively. At these concentrations heterologous extracts failed to form precipitin lines and the antisera were therefore group-specific.

Table 7. *Precipitin tests* of *Saccharomyces mannans, phenol extracts, minimal media and hot water extracts by absorbed anti-Saccharomyces sera*

		Precipitin bands formed by antiserum to:				
Extract solutions		*S. carlsbergensis* 1116 absorbed by *S. willianus* 106	*S. fragilis* 100 absorbed by *S. rouxii* 170	*S. marxianus* 111 absorbed by *S. rouxii* 170	*S. willianus* 106 absorbed by *S. cerevisiae* 1006	*S. fermentati* 161 absorbed by *S. cerevisiae* 1006
Group A:						
S. carlsbergensis	396	+	−	−	−	−
S. rouxii	170	+	−	−	−	−
Group B:						
S. fragilis	100	−	+	+	+	−
S. marxianus	111	−	+	+	+	−
Group C:						
S. cerevisiae var. *ellipsoideus*	Y4	−	−	+	+	−
S. willianus	106	−	−	+	+	−
Group D:						
S. fermentati	161	−	−	−	−	+
S. microellipsodes	698	−	−	−	−	+

+ = band(s) formed; − = no reaction.

DISCUSSION

Mannans extracted from yeasts are serologically active and give broad bands on immunodiffusion (Summers *et al.* 1964; Sandula *et al.* 1964). Suzuki *et al.* (1967) used yeast mannans prepared by autoclaving yeasts in citrate buffer to absorb yeast antisera. These preparations contained less than 0·5% nitrogen, insufficient to account for the observed inhibition of antibody activity. Suzuki *et al.* (1968) showed that oligosaccharides prepared from mannan of *Saccharomyces cerevisiae* caused 100% inhibition of the homologous antiserum. Thus, mannan alone can account for the observed antigenic activity.

Not all mannan of the cell wall is structural. Invertase (Neumann & Lampen, 1967) and acid phosphatase (Boer & Steyn-Parvé, 1966), both mannan-proteins, occurred in yeast cell walls. Neumann & Lampen (1967) found that antiserum against *Saccharomyces cerevisiae* invertase formed a precipitin band with invertases from *S. cerevisiae* and a Saccharomyces hybrid strain, but no precipitin reaction was observed with cell wall mannan from *S. cerevisiae*. This agrees with the failure of commercial invertase, shown to contain mannan, to precipitate with our antisera to *S. fragilis* and *S. willianus*. Invertase may not be present in sufficient amount on the yeast cell surface to cause significant production of antibody.

The present paper shows that mannans, and preparations containing mannan, of

yeasts of different serological groups, contain the antigenic group determinants. Extracts prepared by autoclaving in citrate buffer, shaking in hot water, shaking with phenol and directly from minimal medium gave similar results in immunodiffusion tests. Although phenol, hot water and minimal media extracts contained glucose, which may indicate the presence of a soluble glucan in the outer layers of the yeast cell wall, antigenic activity was approximately proportional to mannan content. Phenol extracts were used in the strongest concentrations and gave the least number of positive reactions (Table 3). Apart from their lower content of mannan, phenol extracts may be less specific due to their severe method of preparation. Traces of ribose detected by chromatography are thought to be due to ribonucleic acid since phenol and hot water extracts gave absorption spectra with peaks at 260 mμ (unpublished results) as do purine/pyrimidine bases. These absorption peaks could be removed by treatment of the extracts with trichloracetic acid; the modified extracts gave the same immunodiffusion results as untreated material.

Agglutination tests were much more sensitive than gel-diffusion tests and it was not surprising that some gel-diffusion tests failed to show precipitin bands corresponding to positive agglutination results. This was shown by unabsorbed antiserum to *Saccharomyces carlsbergensis* 1116 in gel-diffusion tests with all extracts of the group C yeasts and the phenol extracts of the group B yeasts. Similarly, unabsorbed antiserum to either of the group B yeasts failed to give precipitin reactions with group A phenol extracts and unabsorbed antiserum to *S. willianus* 106 failed to show a precipitin reaction with group B hot-water extracts.

It was more surprising to find positive gel-diffusion results for corresponding negative agglutination tests as with unabsorbed *Saccharomyces fragilis* 100 antiserum and all group C extracts. These results suggested that both group B and C yeasts share an antigen but that *S. fragilis* has less of this antigen in its outer layer than *S. marxianus*. This suggestion is supported by the absorbed *S. marxianus* 111 and *S. willianus* 106 antisera giving positive agglutination and gel-diffusion results with both group B and C yeasts and extracts (Tables 6 and 7). The failure of absorbed *S. fragilis* 100 antiserum to react with group C extracts (Table 7) may be due to the small amount of C-specific antibody present originally being further depleted by the absorptions; other absorbed antisera had to be concentrated to obtain a visible precipitin reaction. It was possible to make absorbed *S. marxianus* 111 antiserum group-specific for agglutination tests by absorbing with a group C yeast plus *S. rouxii* (Table 6), but it is more convenient to use *S. fragilis* 100 antiserum absorbed by *S. rouxii* 170 as described previously (Campbell, 1968). Similarly, the absorbed *S. willianus* 106 antiserum could be made group-specific for agglutination tests by absorbing with a mixed suspension of *S. marxianus* and *S. cerevisiae* 1006 (Table 6).

Campbell's group C antiserum was specific without this further absorption, partly due to the fact that for agglutination tests a standard titre was specified. Undiluted absorbed group C antiserum agglutinated *Saccharomyces marxianus* strongly and *S. fragilis* weakly, but at the specified dilution, viz. homologous agglutination at 1 in 5 but not at 1 in 10, did not agglutinate either of these suspensions.

The activity shown by the minimal medium preparation suggests that mannan is dissolved from the cell surface as it is produced. This could have some role in yeast flocculence as the period of deflocculence coincides with the synthesis of mannan (Masschelein, Jeunehomme-Ramos, Castiau & Devreux, 1963) and inhibition of man-

nan synthesis by sodium fluoride (Chung & Nickerson, 1954) results in very flocculent yeasts. If, as Masschelein & Devreux (1957) suggest, an external mannan 'masks' the active groups it is possible that, in fermentation, the balance between production and removal of the 'masking' mannan could be critical.

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The Action of Streptomycins on the Chloroplast System of *Euglena gracilis*

By L. EBRINGER, J. L. MEGO, A. JURÁŠEK AND R. KADA

Department of Microbiology, Komensky University and The Department of Organic Chemistry, Slovak Polytechnic University, Bratislava, Czechoslovakia

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SUMMARY

Five streptomycins bleached the chloroplast system of *Euglena gracilis*; the most effective were those which contained an aldehyde group in the streptose moiety: streptomycin and hydroxystreptomycin. Derivatives containing a secondary alcohol group in place of the aldehyde (dihydro-, dihydrodesoxy-, dihydrohydroxystreptomycin) require higher concentrations to elicit the same effect. Hydroxylamine reversed the bleaching effects of streptomycin and hydroxystreptomycin but not of dihydro-, dihydrodesoxy- or dihydrohydroxystreptomycin. These observations suggest that the aldehyde group of the streptomycins contributes to the action of these antibiotics on Euglena chloroplasts; the action of hydroxylamine would be to either remove the antibiotic from its site of action or to render it ineffective *in vivo* by reaction with the aldehyde group.

INTRODUCTION

An interesting and as yet inadequately explained action of streptomycin is the ability of this antibiotic to eliminate chloroplasts from Euglena without significantly inhibiting cell division or viability of the cell. In bacteria, streptomycin binds to ribosomes causing miscoding of messenger RNA and production of faulty protein (Davies, Gilbert & Gorini, 1964). Accordingly, Schiff & Epstein (1965) suggested that bleaching agents may act by inhibiting protein synthesis in Euglena chloroplasts. However, Stern, Barner & Cohen (1966) demonstrated that, in *Escherichia coli*, streptomycin lethality occurred in the absence of protein synthesis. Since the Euglena chloroplast represents a different system in which to study the mechanism of action of antibiotics, further light may be shed on this important subject by the use of this organism.

Aronson, Meyer & Brock (1964) proposed a mechanism of action for the streptomycins based on the ability of the antibiotics to cross-link nucleic acid. Streptomycin is a more effective cross-linking agent *in vitro* than dihydrostreptomycin, and the presence of an aldehyde group in the streptose moiety may be responsible for the increased effectiveness. According to Aronson *et al.* (1964), this aldehyde is capable of forming a second cationic attachment site by reacting with the secondary amino group at the N-methyl glucosamine moiety, resulting in a cyclic iminium salt. Since dihydrostreptomycin cannot form this cationic cyclic structure, it would be expected to be a less effective agent for precipitating nucleic acids. Streptomycin is known to be more effective as a bleaching agent in Euglena than dihydrostreptomycin (Zahalsky,

Hutner, Keane & Burger, 1962), which supports the model of Aronson *et al.* (1964) and suggests that the aldehyde group may play a role in chloroplast elimination. We have observed that hydroxylamine reverses the action of streptomycin in Euglena chloroplasts if added before about 6 to 8 divisions have taken place in the presence of the antibiotic (Ebringer, Mego & Podová, 1967). In the studies reported in the present paper we compared the actions of five streptomycins as bleaching agents in Euglena. These antibiotics differ only in the streptose moiety: some contain aldehyde groups and others have alcoholic groups. We also considered it of interest to compare the effect of hydroxylamine as a reversing agent on the action of these streptomycins particularly since hydroxylamine may react with the aldehyde to form an oxime.

METHODS

Euglena gracilis, strain Z, was grown in a proteose peptone-tryptone medium (Mego, 1964). Cultivation conditions were described by Ebringer (1966). Antibiotics were dissolved in distilled water, sterilized by filtration and then added to the liquid media. After a period of growth with antibiotic, the organisms were washed, harvested by centrifugation and then plated. On the ninth day after plating about 300 to 600 colonies were counted and the numbers of white and green colonies were recorded. Individual counts were made microscopically in a Burker counting chamber.

The final concentration of hydroxylamine sulphate was always 10^{-1} M. Hydroxylamine was present for only 20 min. and was then removed by washing on the centrifuge. The chlorophyll content was determined according to the method of Arnon (1949). Sulphates of the streptomycins were obtained by the courtesy of the following: dihydrodesoxystreptomycin sulphate from Dr Kazuo Kagiwada, Kaken Chemical Co. Ltd., Tokyo, Japan; hydroxy- and dihydrohydroxystreptomycin sulphate from Dr J. C. Sylvester, Abbott Laboratories, North Chicago, Illinois, U.S.A; dihydrostreptomycin sulphate, from Dr Alvaro Zugaza, Antibiotics, S.A. Madrid, Spain.

The following abbreviations will be used: SM, streptomycin; OHSM, hydroxystreptomycin; DHSM, dihydrostreptomycin; DHOHSM, dihydrohydroxystreptomycin; DHDSM, dihydrodesoxystreptomycin; HA, hydroxylamine.

RESULTS

Figure 1 shows that bleaching action of the various streptomycins depended on the concentration and duration of action. The most effective were SM and OHSM, which produced 100% bleached colonies in 24 hr at 500 μg./ml., in 3 days at 100 μg./ml., only after 5 days at 10 μg./ml. DHSM, DHOHSM and DHDSM, however, required 4 days to produce 100 % bleached colonies at 500 μg./ml. Even after 6 or more days these antibiotics did not produce total bleaching at 10 μg./ml.

Table 1 shows that OHSM inhibited growth slightly more than the other antibiotics. In order to diminish this toxicity effect, we used 100 μg./ml. in further experiments since this concentration produced total bleached colonies. SM was used in this concentration, while DHSM, DHOHSM and DHDSM were used at 500 μg./ml.

Hydroxylamine reversed only the effects of SM and OHSM (Table 2). The reversal was nearly complete when this agent was added before 4·5 divisions. After about 5 divisions only a small percentage of the organisms were reversed. Reversal effectiveness

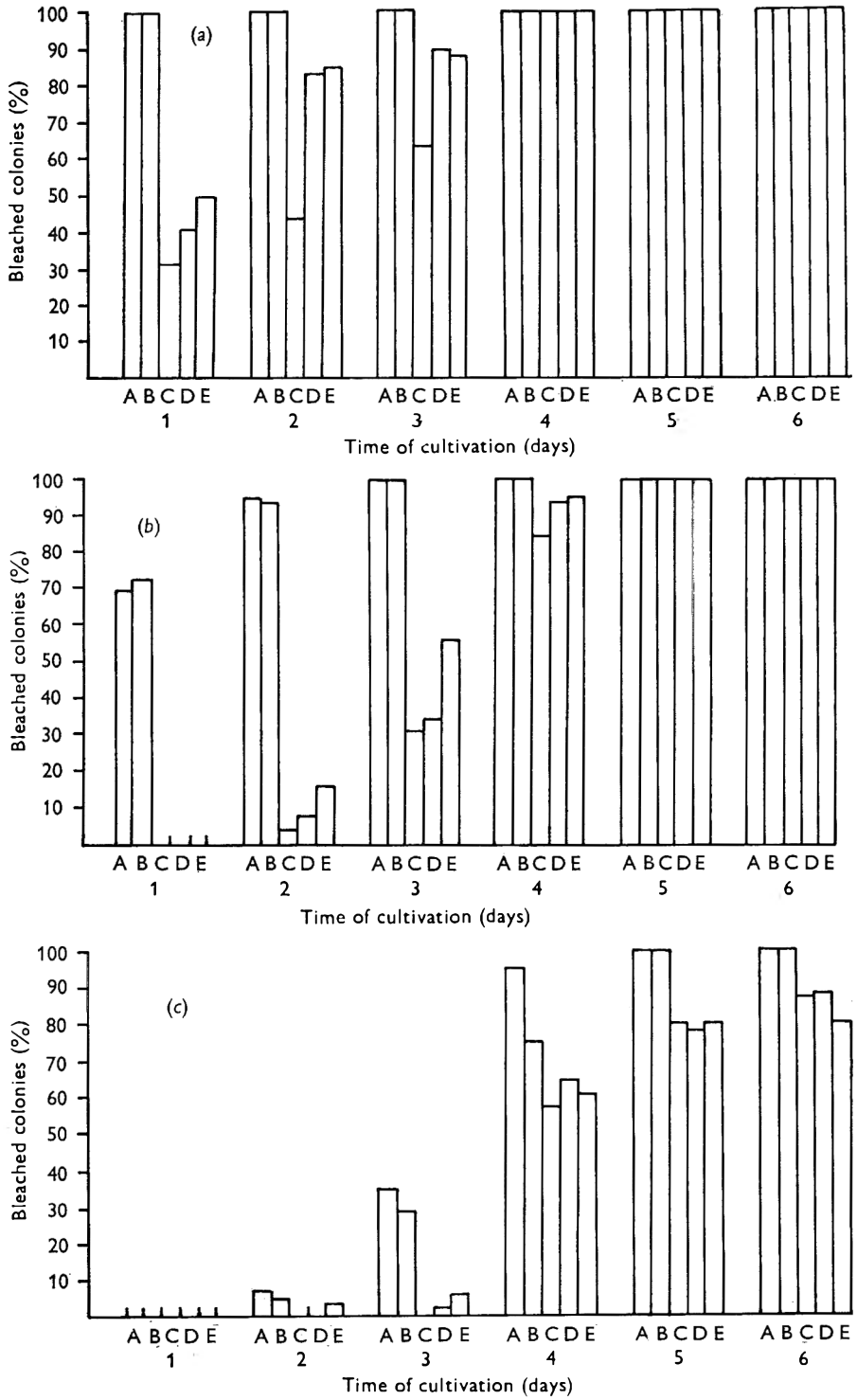

Fig. 1. Proportions of bleached colonies obtained after growth of *Euglena gracilis* in various concentrations of streptomycins for varying lengths of time. (*a*) 500 μg./ml. (*b*) 100 μg./ml. (*c*) 10 μg./ml. After the indicated period of cultivation with the streptomycins, the organisms were washed, plated on antibiotic-free media and bleached and green colonies counted after 9 days. A, SM; B, OHSM; C, DHSM; D, DHOHSM; E, DHDSM (for abbreviations, see text).

was approximately the same for SM and OHSM but completely ineffective for the other antibiotics.

Table 3 shows the chlorophyll content of the cultures during growth in the presence of the various antibiotics. Although 100% of bleached colonies were obtained after plating in many cases, the cultures contained considerable quantities of chlorophyll. These results suggest that chlorophyll content of the cultures does not represent a true measure of the bleaching effectiveness of these agents.

Table 1. *Effects of various streptomycins on growth of Euglena gracilis*

Antibiotic (μg./ml.)...	500	200	100	10
SM†	438*	440	530	572
OHSM	355	398	446	485
DHSM	418	470	520	536
DHOHSM	460	497	510	560
DHDSM	400	468	550	575

* The numbers represent thousands of organisms/ml. medium after 6 days at 24°. † For abbreviations see text.

Table 2. *Prevention of streptomycin-bleaching of Euglena gracilis by hydroxylamine*

Organisms in media containing streptomycins were allowed to grow for approximately the numbers of divisions in column 1. Samples were removed, treated with HA for 20 min., washed and plated on agar (column '+HA'). The column '−HA' indicates the results without hydroxylamine treatment. Colonies were counted after 9 days growth on agar at 24°.

	% permanently bleached colonies									
	SM		OHSM		DHSM		DHOHSM		DHDSM	
Numbers of divisions	−HA	+HA	−HA	+HA	−HA	+HA	−HA	+HA	−HA	+HA
1·0	87	8	93	4	25	26	40	48	52	49
2·5	100	1	100	7	40	39	64	63	57	62
3·9	100	1	100	2	63	59	80	80	85	89
4·5	100	2	100	2	84	86	91	92	91	95
4·9	100	40	100	50	100	100	100	100	100	100
5·4	100	93	100	98	100	100	100	100	100	100
5·9	100	100	100	100	100	100	100	100	100	100

For abbreviations and concentrations see text.

Table 3. *Chlorophyll content (as % of a control culture without added antibiotic) of Euglena gracilis and the percent streptomycin-bleached colonies obtained 9 days after plating on agar*

	500		200		100		10	
Antibiotic (μg./ml.)	Chloro-phyll	Colonies	Chloro-phyll	Colonies	Chloro-phyll	Colonies	Chloro-phyll	Colonies
SM	0	100	0	100	0	100	29	100
OHSM	0	100	0	100	0	100	69	100
DHSM	21	100	30	100	36	100	86	87
DHOHSM	26	100	35	100	43	100	55	85
DHDSM	31	100	32	100	33	100	61	79

For details and abbreviations see text.

DISCUSSION

The results presented in this paper establish that those streptomycins containing an aldehyde group on the streptose moiety were the most effective bleaching agents (see Fig. 2) and support the model proposed by Aronson *et al.* (1964) discussed in the introduction. There is no direct evidence yet to suggest that these antibiotics bind directly to chloroplast DNA; however, some mechanism must be proposed to account for the ability of streptomycin permanently to eliminate chloroplasts from Euglena. Zimmer, Triebel & Thrum (1967) have shown that certain antibiotics, including streptomycin and dihydrostreptomycin, interact with DNA *in vitro* to increase the melting

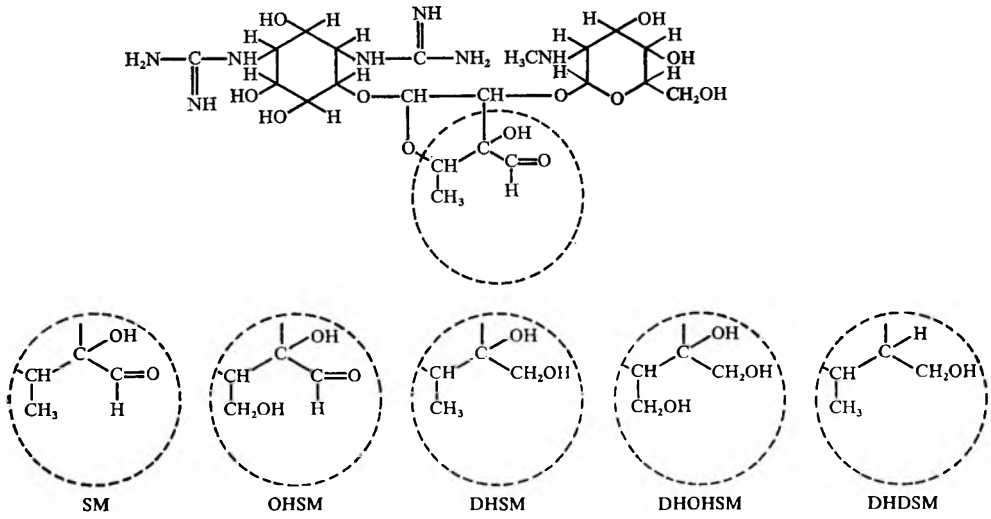

Fig. 2. The structures of streptomycins.

point of this nucleic acid. Their evidence suggests that the antibiotics cross-link DNA by electrostatic interactions. It does not seem unreasonable, therefore, to postulate that the antibiotics may prevent the separation of DNA strands after replication in the chloroplast. Hydroxylamine may act by releasing the antibiotics from electrostatic interaction with chloroplast DNA perhaps by reacting with the aldehyde group. This hypothesis would require either a different mechanism of action for the non-aldehyde-containing antibiotics or a less efficient binding to DNA.

Approximately six divisions were required before treatment with hydroxylamine became ineffective as a reversing agent. This correlates with the observation of Mego & Buetow (1967), who found that approximately this number of divisions was required for irreversible chloroplast loss induced by growth at elevated temperature. Again, it does not seem unreasonable to postulate that inhibition of DNA replication may require this number of divisions to 'dilute out' the relevant nucleic acid.

Other inhibitors of DNA synthesis are also effective bleaching agents in Euglena. These include nalidixic acid, porfiromycin, streptonigrin, novobiocin, rubiflavin, sarkomycin (Ebringer & Mego, unpublished observations), some mutagens (McCalla, 1967) and ultraviolet light (Lyman, Epstein & Schiff, 1961; Gibor & Granick, 1962).

An argument against the theory that bleaching agents act by inhibition of protein

synthesis in chloroplasts is the effect of chloramphenicol. This antibiotic is effective only in organisms containing 70S ribosomes (Vazquez & Monro, 1967) including Euglena chloroplasts. Chloramphenicol has no effect on protein synthesis in the cytoplasm of Euglena which contains 80S ribosomes. This substance, therefore, should be an ideal bleaching agent, since rapid cell division can proceed while chloroplast protein synthesis is inhibited. Chloramphenicol is not a bleaching agent, however, and only produces a temporary inhibition of chloroplast protein formation (Anderson & Smillie, 1966; Pogo & Pogo, 1965). Other inhibitors of protein synthesis such as the tetracyclines and puromycin are also not effective bleaching agents (Selsky, 1967). Although aureomycin has been reported to cause bleaching of Euglena (Robbins, Hervey & Stebbins, 1953), we have not been able to demonstrate more than a temporary inhibition of chloroplast formation under a variety of conditions.

We have also presented evidence that measurement of chlorophyll may not always be a valid index of permanent bleaching effectiveness of antibiotics. This may account for the observations of Linnane & Steward (1967) in which these authors reported that macrolidic antibiotics were not bleaching agents in Euglena.

Dr J. L. Mego is a participant in the U.S.A. and Czechoslovak Academies of Sciences Exchange Programme. Present address: Department of Biology, The University of Alabama, Alabama 35486.

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The Regulation of Some Enzymes Involved in Ammonia Assimilation by *Rhizobium japonicum*

By P. F. FOTTRELL AND PHYLLIS MOONEY
Department of Biochemistry, University College, Galway, Irish Republic

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SUMMARY

The concentrations of three enzymes active in ammonia assimilation by *Rhizobium japonicum* (glutamate dehydrogenase, aspartate aminotransferase, alanine aminotransferase) were influenced considerably by the N-source in the culture medium. Relatively high concentrations of these enzymes were present when *R. japonicum* was grown in a medium containing NH_4Cl + 2-oxoglutarate. Relatively low amounts of these enzymes were found in bacteria grown in the presence of various amino acids. Active protein and RNA synthesis were necessary for the induction of the enzymes by NH_4Cl + 2-oxoglutarate.

INTRODUCTION

It has been known for some time that ammonia formed during symbiotic nitrogen fixation is rapidly incorporated into amino acids (e.g. glutamate, aspartate, alanine; Aprison, Magee & Burris, 1954), but relatively little is known about the enzymes which catalyse these reactions in soybean nodules. We showed (Fottrell & Montgomery, 1968) that several enzymes active in ammonia assimilation, including glutamate dehydrogenase, aspartate aminotransferase and alanine aminotransferase were present in the bacteroids of soybean nodules. In the present report the mechanism involved in the regulation of these enzymes was investigated in *Rhizobium japonicum* (symbiont of soybeans).

METHODS

Rhizobium japonicum strain 392 (obtained from C. L. Masterson, Agricultural Institute, Wexford, Irish Republic), which is a slow-growing strain, was grown in a defined medium (Evans & Lowe, 1964) with different N-sources as indicated in Table 1. Cultures were grown in 4-l. Erlenmeyer flasks, from an inoculum of standard size, on an orbital shaker at 28°. Growth was estimated by measuring the extinction at 562 mμ.

At a suitable stage of growth, bacteria were harvested by centrifugation, washed with 0·9% (w/v) NaCl and suspended (1 g. bacteria/1·5 ml. buffer) in 0·06 M-phosphate buffer (pH 7·2) containing 1 mM-glutathione (reduced) + 0·1% (w/v) Triton X-100. The bacteria were disrupted with an MSE 60 W ultrasonic disintegrator for 1·5 min. at less than 5°, then centrifuged at 34,000 *g* for 90 min. and the clear supernatant fluid used for the enzyme assays. Enzymes were assayed spectrophotometrically at 340 mμ as described by Bergmeyer (1965). Assays were also made for glutamine synthetase and carbamyl phosphate synthetase by the methods of Woolfolk, Shapiro & Stadtman (1966) and Metzenberg *et al.* (1957), respectively. Specific enzyme activity

was expressed in International Units/g. protein (Bergmeyer, 1965). Protein was assayed by a modified biuret reaction (Gornall, Bardawill & David, 1949).

Net synthesis of glutamate and aspartate was shown by using ^{14}C-2-oxoglutarate (3·2 μc/μM) (Radiochemical Centre, Amersham, Buckinghamshire, England) as described by Weiss (1967). Glutamate and aspartate were separated by thin-layer chromatography on cellulose with isopropanol + formic acid + water (40 + 2 + 10, by vol.) for 3 hr. Autoradiographs were prepared with Kodak Industrex X-ray Film.

Table 1. *The effect of various nitrogen sources in the medium on enzymes in Rhizobium japonicum*

Nitrogen source	Conc. (mM)	Enzyme: Glutamate dehydrogenase	Enzyme: Aspartate amino-transferase	Enzyme: Alanine amino-transferase
		Specific activity (I. units/g. protein)		
KNO_3	7·0	20·1	22·0	18·6
	35·0	22·4	32·3	20·7
	70·0	35·5	48·7	40·2
NH_4Cl	14·0	15·3	40·8	28·1
$(NH_4)_2SO_4$	4·0	19·3	30·6	26·4
NH_4Cl + 2-oxoglutarate	14·0 + 2·5	96·8	230·5	142·2
L-Glutamate	3·0	0·0	98·2	37·0
L-Glutamine	3·0	12·0	27·2	7·2
L-Ornithine	5·0	8·2	15·8	5·6
L-Alanine	9·0	26·4	58·2	1·0
L-Aspartate	3·0	0·0	0·0	22·4

RESULTS

The amounts of glutamate dehydrogenase, aspartate aminotransferase and alanine aminotransferase were influenced by the N-source (Table 1). One of the most interesting findings was the relatively high amounts of enzyme in bacteria grown with NH_4Cl + 2-oxoglutarate. In contrast, glutamate dehydrogenase was not detected in bacteria grown with glutamate or aspartate. Aspartate aminotransferase was absent when the medium contained aspartate and alanine aminotransferase activity was not detected in bacteria grown with alanine. Further measurements showed that glutamine synthetase activity was very low in bacteria grown with KNO_3 (7 mM) or NH_4Cl (0·014 M), but was increased over 100-fold in bacteria grown with glutamate (3 mM). Carbamyl phosphate synthetase activity, in contrast, was relatively high in bacteria grown with nitrate, but was absent from bacteria grown with ornithine (5 mM) or alanine (9 mM).

To determine the mechanism involved in the regulation of glutamate dehydrogenase and aspartate aminotransferase, bacteria were grown in a medium containing aspartate (3 mM) for 48 hr. The bacteria were harvested under sterile conditions, washed with sterile 0·9% (w/v) NaCl and inoculated into new medium containing 2-oxoglutarate (2·5 mM) + NH_4Cl (0·014 M), in place of aspartate. Various concentrations of actinomycin D, puromycin or cycloheximide were added to replicate cultures in the new medium; control cultures contained no antibiotic. The results (Table 2) showed that enzyme induction was inhibited when either antibiotic was included in the medium.

With the exception of actinomycin D, which inhibited growth by 22% at 1 μg./ml. medium, there was no inhibition of growth by puromycin, cycloheximide or actinomycin D at the concentrations given in Table 2. Alanine amino-transferase activity was investigated only with actinomycin D; the results were very like those obtained for the other two enzymes. On the basis of these results we concluded that active protein and RNA synthesis were necessary for the induction of all three enzymes in *Rhizobium japonicum*.

Table 2. *Effects of inhibitors of RNA and protein synthesis on enzyme induction in Rhizobium japonicum*

Inhibitor	Concentration (μg./ml.)	Glutamate dehydrogenase	Aspartate aminotransferase
		Enzyme activity: % of control (without antibiotic)	
Actinomycin D	0·10	83·6	98·7
	0·25	61·4	70·3
	0·50	43·2	58·5
	0·75	0·0	31·0
	1·0	0·0	0·0
Puromycin	1·00	91·5	102·2
	2·00	49·0	21·7
	2·50	0·0	0·0
Cycloheximide	1·00	20·8	97·8
	2·00	0·0	73·6
	3·00	0·0	27·3
	5·00	0·0	0·0

DISCUSSION

The results show that enzymes probably active in the assimilation of ammonia in soybean nodules, were influenced to a considerable degree in *Rhizobium japonicum* by the N-source in the growth medium. As regards assimilation of fixed nitrogen by legume nodules it was interesting that glutamate dehydrogenase, aspartate aminotransferase and alanine aminotransferase were induced in cultures of *R. japonicum* by NH_4Cl + 2-oxoglutarate. Ammonia is the first stable intermediate formed in soybean bacteroids during nitrogen fixation (Bergersen, 1965) and 2-oxoglutarate would probably be supplied by the host legume since very little isocitrate dehydrogenase activity was detected in the bacteroids in our experiments. It seems that in bacteroids of soybean nodules, glutamate, aspartate and alanine are important intermediates in the assimilation of fixed nitrogen.

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The Fatty Acid Composition of Sporangiospores and Vegetative Mycelium of Temperature-adapted Fungi in the Order Mucorales

By J. L. SUMNER* AND E. D. MORGAN

Biology and Chemistry Departments, University of Keele, Staffordshire, ST5 5BG

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SUMMARY

The lipid content and fatty acid composition of sporangiospores and vegetative mycelium of mesophilic, thermotolerant and thermophilic fungi in the Mucorales were examined. In each fungus the spores contained less lipid than the vegetative mycelium. The mesophiles accumulated less lipid in spores and mycelium than did thermotolerants and thermophiles. No unusual fatty acids were detected by gas-liquid chromatography in the lipids of spores or mycelium. The fatty acid compositions of spores and vegetative mycelium were qualitatively very similar, but spore lipids were always more highly saturated than mycelial lipids. Lowering growth temperature from 48 to 25° increased the synthesis of unsaturated fatty acids in the spores and the mycelium of the thermotolerant and thermophilic fungi examined.

INTRODUCTION

There have been several investigations of the fatty acid composition of the lipids of fungal spores, in a number of which a significant proportion of the spore lipid has been shown to comprise 'unusual' fatty acids. In the order Uredinales (rusts) *cis*-9, 10-epoxyoctadecanoic acid formed up to 40% of the spore oil (Tulloch, Craig & Ledingham, 1959; Tulloch & Ledingham, 1962). In the order Erysiphales (powdery mildews) the spore lipid of *Sphaerotheca humuli* was shown to contain 42% behenic acid, which is rarely found in fungal lipids and then only in trace quantities, while in *Erysiphe graminis* the spore oil contained 45% of an unidentified fatty acid, thought to have a branched chain, or cyclic system (Tulloch & Ledingham, 1960). Sclerotia of *Claviceps purpurea* contained 34% ricinoleic acid (12-hydroxystearic acid) but this acid could not be demonstrated in other members of the Hypocreales (Shaw, 1965).

Linoleic acid has been found as a major component of the lipids of spores of widely different groups of fungi. It comprised as much as 60% of the total fatty acids of sclerotia of *Sclerotium rolfsii* (Howell & Fergus, 1964) and 65% of the spore fatty acids of *Penicillium atrovenetum* (Van Etten & Gottlieb, 1965). In the fruiting bodies of some Basidiomycetes it accounted for over 70% of the total fatty acids (Hughes, 1962; Bentley, Lavate & Sweeley, 1964; Shaw, 1966*a*), while spore lipid of the smut *Tilletia oetens* contained 63% of it (Tulloch & Ledingham, 1960).

The class Phycomycetes is conspicuously absent from the groups so far examined. The fatty acid composition of the spores of phycomycete fungi was therefore examined

* Present address: Department of Food Science, University of Alberta, Edmonton, Alberta, Canada.

firstly to determine whether it was similar to that of related mycleium, and secondly to determine whether spore lipids were influenced by the incubation temperature in the same way as were mycelial lipids. There is abundant experimental evidence that micro-organisms, in common with other poikilotherms, synthesize increased proportions of unsaturated fatty acids, at the expense of saturated fatty acids, as the incubation temperature is lowered (see Kates, 1964, 1966; and Farrell & Rose, 1967*a*, *b*; for reviews). Curiously, Long & Williams (1960) found that the spore lipids of the thermophile *Bacillus stearothermophilus* became more unsaturated as the temperature was raised.

A number of mesophilic, thermotolerant and thermophilic fungi in the order Mucorales were therefore grown at different temperatures, and the lipid content and fatty acid composition of sporangiospores and mycelium determined. The definition of Cooney & Emerson (1964) that a thermophilic fungus has a maximum temperature for growth at or above 50° and a minimum temperature for growth at or above 20°, was used to distinguish thermophilic from thermotolerant fungi, the latter having a minimum growth temperature below 20°.

METHODS

Organisms. The mesophilic fungi used in this investigation were obtained from the Commonwealth Mycological Institute, Kew, England. They were: *Mucor mucedo* Auct. (IMI 103731), *M. racemosus* Fresen. (IMI 103730), *M. ramanniaaus* Moller. (IMI 35044*a*) and *M. hiemalis* (+) Wehm. (IMI 21216). The thermotolerant and thermophilic fungi were obtained from Dr H. C. Evans, Biology Department, University of Keele. They were: *Rhizopus* sp. (thermotolerant), *Mucor miehei* and *M. pusillus* (thermophilic).

Composition of media, inoculation and incubation

The culture medium had the following composition (per litre deionized water): KH_2PO_4 1 g, $MgSO_4.7\,H_2O$ 0·5 g, glucose 20 g, ammonium sulphate 1·2 g, sodium succinate 5 g, yeast extract 5 g. Its pH was adjusted to 6·5 prior to autoclaving. Twenty ml. medium was dispensed into 100 ml. Erlenmeyer flasks, and in 50 ml. medium containing 2% agar into 8 oz. flat medicine bottles. Inocula were grown on agar slopes for 14 days, mesophiles at 25° and thermotolerants and thermophiles at 48°.

Two types of cultures were grown, 'mycelial' and 'spore'. 'Mycelial' cultures were grown by inoculating each 100 ml. Erlenmeyer flask with 1 ml. spore suspension and incubating in still culture for 14 days. Preliminary experiments had shown that under these conditions sporulation was particularly sparse, spores forming less than 0·1% of the dry weight of the culture. 'Spore' cultures were grown by inoculating each flat medicine bottle with a small inoculum transferred by sterile wire.

All cultures were incubated at the specified temperatures for 14 days and then harvested. Mycelial mats were removed from six replicate Erlenmeyer flasks, washed with deionized water, filtered on a sintered glass disc, dried overnight at 80° and used as a combined sample. Spores were removed from the surface of the agar slopes by lightly scraping them with a spatula into deionized water. Spore suspensions from a number of replicate flat medicine bottles (usually 25) were pooled, filtered through three layers of cheesecloth and centrifuged. The spore pellet was dried overnight at 80°.

Extraction and saponification of the fungal lipid

Dried spores and mycelia were each quickly powdered in a small grinder and the lipid extracted for 8 hr in a Soxhlet apparatus with benzene and ethanol (594 + 297 v/v) (Shaw, 1965). The amount of lipid in each sample was calculated by subtracting the weights of material before and after extraction. The lipid was saponified by refluxing with 0·6 M-ethanolic KOH for 1 hr.

Fatty acid analysis

The mixture of fatty acids was methylated with BF_3/methanol reagent and analysed by gas–liquid chromatography. A $9'' \times \frac{1}{4}''$ glass column of diethyleneglycolsuccinate (5%) on chromosorb G (100 to 120 mesh) was used in a Pye Model 64 gas chromatograph. The operating temperature was 175° and the nitrogen carrier gas flow 40 ml./min. The fatty acid methyl esters were tentatively identified by comparison of retention times with those of authentic standards. Standard fatty acid methyl esters were obtained from Fluka A.G., Bucks, Switzerland and included methyl esters of C 12 to C 20 saturated acids, as well as the methyl esters of palmitoleic, oleic, linoleic and α-linolenic acid. Fatty acid esters were identified completely by combined mass spectrometry and gas–liquid chromatography. The percentages of fatty acid methyl esters were calculated from the peak areas.

RESULTS

Lipid content of spores and mycelium

In all the species examined the lipid content of the spores from 'spore' cultures was lower than that of mycelium from 'mycelial' cultures. Also the lipid contents of spores and mycelium of mesophilic fungi were lower than those of thermotolerant and thermophilic fungi. These results are summarized in Tables 1 and 2.

Table 1. *Lipid content of spores and mycelium of mesophilic fungi grown at 25°*

Spores and mycelium were harvested from agar-slope cultures and stationary liquid cultures, respectively, after 14 days growth. They were dried and weighed, and the lipid extracted in a Soxhlet apparatus. The amount of lipid present was calculated by weighing each sample before and after extraction.

Organism	Fungal phase examined (spores or mycelium)	Lipid content (% of dry weight)
M. mucedo	Spores	3·7
	Mycelium	12·0
M. ramannianus	Spores	7·6
	Mycelium	15·2
M. racemosus	Spores	4·1
	Mycelium	9·8
M. hiemalis	Spores	8·4
	Mycelium	18·1

Fatty acid composition of spores and mycelium

No unusual fatty acids were found in the lipids of spores of any of the species examined. Seven fatty acids occurred in measurable amounts: myristic, palmitic, palmitoleic, stearic, oleic, linoleic and linolenic acids; α-linolenic acid was absent. The

triply unsaturated C 18 acid was identified as γ-linolenic acid. This agrees with previous reports that only the γ-isomer is present in phycomycete fungi (Bernhard & Albrecht, 1948; Shaw, 1965, 1966*a*, *b*; White & Powell, 1966; Tyrrell, 1967 and Sumner, Morgan & Evans, 1969). These results are summarized in Tables 3 and 4.

The fatty acids of spore and mycelial lipids were very similar both qualitatively and quantitatively, but in each species the spore lipids were more saturated than related mycelial lipids. In these respects the lipids of these mucoraceous fungi are similar to those of *Pithomyces chartarum* (dematiaceous imperfect fungus) (Hartman, Hawke, Morice & Shorland, 1960; Hartman, Morice & Shorland, 1962) in which the spore lipids closely resemble the mycelial lipids though they are more saturated.

Table 2. *The effect of incubation temperature on the lipid content of spores and mycelium of thermotolerant and thermophilic fungi*

Spore and mycelial cultures were harvested after 14 days growth at either 25 or 48°. After drying and weighing, the lipid was extracted in a Soxhlet apparatus. The lipid content of each sample was calculated by weighing it before and after extraction.

Organism*	Temperature of incubation (°)	Fungal phase examined (spores or mycelium)	Lipid content (% of dry weight)
Rhizopus sp.	25	Spores	16·1
		Mycelium	25·7
	48	Spores	10·4
		Mycelium	11·6
M. miehei	25	Spores	19·4
		Mycelium	24·3
	48	Spores	11·3
		Mycelium	11·9
M. pusillus	25	Spores	19·3
		Mycelium	26·2
	48	Spores	16·1
		Mycelium	23·1

Table 3. *Fatty acid composition of spores and mycelium of mesophilic fungi grown at 25°*

The lipid of spore and mycelial cultures was saponified, esterified and analysed by gas-liquid chromatography. The percentage of each fatty acid was calculated from the peak area. The degree of unsaturation of the lipid was calculated in terms of the number of double bonds/mole from the formula:

$$\triangle/\text{mole} = 1{\cdot}0 \times (\%\ \textit{monoenes})/100 + 2{\cdot}0 \times (\%\ \text{dienes})/100 + 3{\cdot}0 \times (\%\ \text{trienes})/100.$$

Organism	Fungal phase (spores or mycelium)	Fatty acids 14:0*	16:0	16:1	18:0	18:1	18:2	18:3	Degree of unsaturation
M. mucedo	Spores	2·5	21·3	3·5	12·6	27·2	21·0	12·0	1·08
	Mycelium	0·8	12·0	3·1	9·3	26·1	25·3	20·2	1·40
M. ramannianus	Spores	4·1	18·7	3·4	5·5	31·2	14·1	20·9	1·25
	Mycelium	1·6	18·5	2·6	4·4	28·0	13·5	30·9	1·50
M. racemosus	Spores	4·9	21·8	3·8	9·1	31·4	14·9	14·1	1·07
	Mycelium	2·7	16·9	2·9	4·6	36·5	16·6	19·4	1·28
M. hiemalis	Spores	2·0	15·0	2·1	17·1	28·0	17·9	18·0	1·20
	Mycelium	2·4	14·8	3·1	9·7	32·6	18·8	19·2	1·29

* The number of carbon atoms: the number of double bonds per molecule.

The spore lipids were influenced by incubation temperature in the same way as mycelial lipids, being more unsaturated when the fungus had been grown at a lower temperature. The sorts and proportions of fatty acids in spore lipids of mesophiles, thermotolerants, and thermophiles were essentially the same as those in the mycelial lipids; the lipids of mesophiles contained greater proportions of the polyunsaturated acids (linoleic and linolenic acid) and lower proportions of oleic acid, compared with lipids of thermotolerants and thermophiles.

Table 4. *Effect of temperature on the fatty acid composition of spores and mycelium of thermotolerant and thermophilic fungi*

The lipid extracted from spore and mycelial cultures grown at 25 or 48° was saponified, esterified and analysed by gas-liquid chromatography. The percentage of each fatty acid was calculated from the peak area. The degree of unsaturation of the lipid was calculated in terms of the number of double bonds/mole:

$$\triangle/\text{mole} = 1{\cdot}0 \times (\%\ \text{monoenes})/100 + 2{\cdot}0 \times (\%\ \text{dienes})/100 + 3{\cdot}0 \times (\%\ \text{trienes})/100.$$

Organism	Temperature of incubation (°)	Fungal phase (spores or mycelium)	Fatty acids 14:0*	16:0	16:1	18:0	18:1	18:2	18:3	Degree of unsaturation
Rhizopus sp.	25	Spores	6·0	27·3	2·9	12·1	28·8	10·6	12·1	0·89
		Mycelium	1·4	20·6	2·3	5·2	30·0	28·6	11·9	1·22
	48	Spores	5·8	22·6	2·8	13·8	40·1	11·0	4·0	0·77
		Mycelium	Trace	23·3	3·0	18·6	34·9	21·8	5·1	0·94
M. miehei	25	Spores	5·6	24·4	3·1	11·3	32·6	13·1	9·3	0·88
		Mycelium	1·6	22·8	3·0	4·7	47·8	15·8	4·1	0·95
	48	Spores	4·0	26·3	4·8	9·9	44·0	5·9	4·4	0·72
		Mycelium	1·8	27·7	3·0	6·3	48·0	10·3	2·0	0·76
M. pusillus	25	Spores	1·0	25·4	3·0	4·8	42·2	19·0	4·5	0·97
		Mycelium	1·2	23·9	3·0	5·6	39·7	20·2	6·3	1·02
	48	Spores	1·3	28·6	2·9	7·1	39·4	17·2	3·4	0·87
		Mycelium	1·5	26·3	3·1	4·3	42·1	19·2	3·6	0·93

* See footnote to Table 3.

DISCUSSION

The fatty acid compositions of the spores and vegetative mycelium of each organism tested were similar. However, the fact that in each species the spores contained lipid in lower concentration but of a more highly saturated nature than in the mycelium appears to indicate a modification of the fatty acid synthesizing and fatty acid desaturating systems of sporogenous mycelium. Both enzyme systems have cofactor requirements for acetylcoenzyme A (acetyl CoA), acyl carrier protein (ACP) and the reduced forms of nicotinamide adenine dinucleotide ($NADH_2$) or nicotinamide adenine dinucleotide phosphate ($NADPH_2$). However, the desaturating enzymes (desaturases) specifically require oxygen, while the synthesizing enzymes (saturases) require carbon dioxide (Mudd & Stumpf, 1961; James, 1963; Stumpf & James, 1963; Nagai & Bloch, 1966).

A correlation between the need for molecular oxygen in the desaturation reaction and the low dissolved oxygen tensions at high temperatures has been used to explain the increased synthesis of highly saturated lipids in response to increases in temperature (Bloomfield & Bloch 1960; Meyer & Bloch, 1963; Harris & James, 1969). In the present context this explanation seems perfectly acceptable when considering the lipid

composition of cultures grown at a high and a low temperature; indeed, preliminary experiments have shown that cultures of *Mucor pusillus* grown at 48° become oxygen deficient, while those at 25° do not. However, it does not explain why spore lipids are more saturated than mycelial lipids, even when 'spore' and 'mycelial' cultures are grown at the same temperature.

A number of studies on the biochemical aspects of fungal morphogenesis have indicated that respiratory changes are associated with spore production; increased spore production in *Mucor hiemalis* and *Phycomyces blakesleeanus* has been correlated with a reduction in the respiration rate of sporogenous mycelium (Hawker & Hepden, 1962), while in mitochondria of sporogenous mycelium of *Neurospora crassa* there is activation of the enzymes of the glyoxylate cycle, and corresponding decrease in activity of enzymes of the Krebs cycle (Turian, 1960; Weiss & Turian, 1966). Also increased activity of proteinase and nuclease enzymes has been demonstrated in spore-producing mycelium of *Penicillium griseofulvum* (Morton, Dickerson & England, 1960). Accumulation of only small concentrations of lipid, but of a highly saturated nature, may therefore be caused by a reduction in the activities of synthetase and desaturase enzymes resulting from a check in the respiration rate of sporogenous mycelium. A depression of respiratory rate would effectively reduce the level of a number of respiratory metabolites, e.g. acetyl CoA, $NADH_2$, $NADPH_2$, carbon dioxide, which are also substrates for fatty acid biosynthesis.

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The Binding of Crystal Violet by Isolated Bacterial Cell-wall Material

By G. WISTREICH* AND J. W. BARTHOLOMEW
Microbiology Section, Department of Biological Sciences, University of Southern California, Los Angeles, California 90007, U.S.A.

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SUMMARY

Isolated bacterial wall material from four Gram-positive and four Gram-negative organisms bound crystal violet as well as or better than intact bacteria. Dye binding ability varied between species. There was no correlation between the dye binding ability of isolated wall material and the Gram characteristics of the intact bacteria.

INTRODUCTION

Statements in the literature concerning the affinity of bacterial cell-wall material for basic dyes are indefinite. Knaysi (1951) wrote '...the cell wall has a low affinity for dyes and is not usually coloured in common staining procedures...'. Bisset & Hale (1953) reported 'In preparations stained by the routine bacteriological methods the external wall is unstained and invisible...'. Lamanna & Mallette (1954) reported that the walls of Gram-positive organisms were stainable with crystal violet but not with safranin, while those of Gram-negative organisms were not stainable with either dye. Hale & Bisset (1956), in a study of several bacterial genera, observed that the walls of Gram-negative organisms were more difficult to stain than those of Gram-positives. All of the aforementioned studies concerning the staining ability of cell-wall material were performed using intact bacteria. As far as we are aware, only one report of dye uptake by partially isolated wall material exists, namely that of Scherrer (1963). Results of his investigations showed high dye uptake for a Mickle disrupted preparation of *Bacillus megaterium* which consisted of 95% disrupted and 5% intact organisms. No comparative dye uptake studies have appeared using isolated wall material. This paper is a report on such a study, using wall material from several species of Gram-positive and Gram-negative organisms.

METHODS

Growth of organisms. Eight species of bacteria were employed, and all were obtained from the stock culture collection of the Microbiology Section, Department of Biological Sciences, University of Southern California. These included four Gram-positive organisms, *Bacillus subtilis*, *Clostridium sporogenes*, *Micrococcus roseus*, and *Sarcina lutea*; and four Gram-negative organisms, *Aerobacter aerogenes*, *Escherichia coli*, *Pseudomonas fluorescens*, and *Serratia marcescens*. All, except *Clostridium sporo-*

* Present address: Department of Biological Sciences, East Los Angeles College, Los Angeles, California 90022, U.S.A.

genes, were grown in Trypticase Soy Broth (Difco) in Erlenmeyer flasks in a reciprocating shaker water bath at 30° for 24 hr. *Clostridium sporogenes* was grown in Thioglycollate Broth (Difco) in Erlenmeyer flasks in stationary culture at 30°. *Bacillus subtilis* was grown with butyric acid to prevent sporulation (Hardwick, Guirard & Foster, 1951).

Cell-wall preparations. Intact bacteria were disrupted using the synthetic zeolite procedure described by Wistreich, Lechtman, Bartholomew & Bils (1968). Following the disruption of bacteria, the wall fragments were isolated as follows, with all centrifugations conducted at 5°. The supernatant was saved following centrifugation at 2000 *g* for 10 min., and subjected to 12,000 *g* for 30 min. This pellet was washed in M-NaCl, and then in distilled water until the supernatant was free of Cl^-. The pellet then was exposed to 100 μg./ml. of crystalline trypsin at pH 7·6 for 2 hr followed by washing in distilled water until the wall material gave no absorption peak at 260 mμ (Salton & Horne, 1951*b*; Salton, 1953). Suspensions of this material then were centrifuged at 3000 *g* for 10 min. and the pellet discarded. The supernatant was then centrifuged at 12,000 *g* for 30 min. The pellet obtained, on suspension in distilled water, was examined in the electron microscope and only those preparations were used which could be seen to consist entirely of cell-wall fragments (Salton & Horne, 1951*b*; Salton, 1953; Wistreich *et al.* 1968). The isolated wall preparations were then lyophilized and stored at 0° until needed. Dry weights were determined by drying duplicate 2·0 ml. volumes of the wall suspensions at 105° to constant weight. The procedures used were described in detail by Wistreich (1969).

Intact bacteria. Bacteria were killed by heating at 65° for 30 min., and then washed twice in sterile cold distilled water. The temperature of 65° was chosen to avoid the disruptive effects of heat reported by Salton & Horne (1951*a*, *b*). Electron microscopy confirmed that the bacteria were not disrupted by this treatment. Dry weights were determined as for the wall preparations.

Determination of dye uptake. The colorimetric method described by Finkelstein & Bartholomew (1953) was used except that the final speed of centrifugation needed to remove fragmented walls was 12,000 *g* for 30 min., and all centrifugations were done at 5°. Valid comparisons of dye binding by different material requires that comparisons be made only when the material is saturated with dye (Bartholomew & Finkelstein, 1954; Finkelstein & Bartholomew, 1953). In the present experiments saturation was achieved by keeping the dye concentrations constant in a test series, and decreasing the concentration of the wall material. As the dye/wall material weight ratios increased, the amount of dye originally present eventually reached a point when it was in excess to that needed to achieve dye saturation of the wall material present. When the data obtained was plotted, using as the ordinate 'milligrams dye taken up per mg. of wall material', and using as the abscissa the 'dye/wall material ratio (by weight) present at the start', a curve was obtained which reached its maximum height at the point of dye saturation of the bacterial material (Finkelstein & Bartholomew, 1953). The weight of dye bound per mg. of the dye saturated bacterial material could then be determined using the values indicated by the level portion of the curve.

Although Wistreich (1969) found very small residual quantities of zeolite in wall material prepared by the above method, he also reported that the zeolite present did not bind measurable amounts of crystal violet. The wall weight values reported here were corrected for the weight of any zeolite found to be present. This was done by

solubilizing a known weight of the wall material preparation in conc. HCl, at 95°, for 4 hr. The insoluble zeolite residue could then be centrifuged out and its weight subtracted from the original value.

RESULTS

Several points are demonstrated by the data presented in Table 1. Isolated bacterial wall material bound crystal violet as well as or better than intact bacteria, and there was no correlation between its ability to bind dye and the Gram characteristic of analogous intact bacteria. The ability of bacterial wall material to bind crystal violet varied considerably between bacterial species.

Table 1. *Crystal violet bound by whole organisms and isolated wall material, when saturated by the dye*

Gram reaction of intact organisms	Microorganism	mg. Crystal violet/mg. whole organisms	mg. Crystal violet/mg. isolated wall material
Gram-negative	*Escherichia coli*	0·15	0·22
	Pseudomonas fluorescens	0·16	0·22
	Aerobacter aerogenes	(*a*)	0·28
	Serratia marcescens	0·15	0·44
Gram-positive	*Clostridium sporogenes*	(*a*)	0·20
	Micrococcus roseus	0·19	0·21
	Sarcina lutea	0·30	0·28
	Bacillus subtilis	0·36	0·48 (*b*)

(*a*) = Determinations not made.
(*b*) = Dye saturation was approached, but not achieved. Final dye uptake would be greater.

DISCUSSION

In view of the previous reports in the literature, the considerable affinity for basic dye of isolated bacterial wall material reported here was surprising. Especially pertinent would be the relationship of this dye affinity to the reported role of wall staining in the Gram differentiation of intact bacteria. Our data are in contradiction to the contention of Lamanna & Mallette (1954) that Gram differentiation is the result of an affinity of crystal violet for the walls of Gram-positive organisms, and the lack of such affinity for walls of Gram-negative organisms. Certainly, the failure of dye uptake of wall material to correlate with the Gram characteristic of intact bacteria would indicate that dye affinity could not contribute in any essential manner to the process of Gram differentiation. Such a conclusion is supported by the finding of Bartholomew & Finkelstein (1958) that the dye-iodine precipitate present interior to the cell wall, by itself, is sufficient to allow Gram differentiation.

The dye concentration used in a normal Gram staining procedure is 100-fold greater than that needed to saturate the cell material present, and such high dye concentrations are known to improve the differential results obtained (Bartholomew, 1962). On the addition of iodine, most of the dye-iodine precipitate formed would therefore come from the free dye present in the cell, rather than from the small amount of dye originally bound by cell material (Smyth & Gershenfeld, 1960). Such 'charging' of the cell internal spaces with large amounts of the dye-iodine precipitate is the true function of

the dye and iodine concentrations used in Gram stain procedures (Bartholomew, Cromwell & Gan, 1965). On the basis of the above, one could predict the failure of all attempts to correlate dye affinity for any cell material, with the true mechanism of Gram differentiation.

It should not be inferred, however, that the cell wall is unimportant in Gram differentiation. It has been demonstrated repeatedly that it has an extremely important role (Bartholomew & Cromwell, 1965; Benians, 1912; Burke & Barnes, 1929; Scherrer, 1963). For Gram-positive organisms it has been proposed that exposure to a decolorizer, such as 95% ethanol, causes a physico-chemical change in the wall material which retards passage of dye and iodine molecules through the wall; a change which does not occur with the wall material of Gram-negative organisms (Salton, 1963; Wensick & Boevé, 1957). Also, Bartholomew *et al.* (1965) found that isolated cell-wall fragments could reflect the decolorization characteristics of analogous intact bacteria. These observations, along with the data presented in the present paper, strongly point to the physico-chemical nature of the wall material, and not to its dye binding ability, as the major contributing factor to the mechanism of Gram differentiation.

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Potassium Transport in Non-growing Mycelium of *Neocosmospora vasinfecta*

By K. BUDD
Department of Biology, Queen's University, Kingston, Ontario, Canada

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SUMMARY

Mycelium of potassium-limited *Neocosmospora vasinfecta* had a lowered potassium content and an increased sodium content compared with mycelium grown in presence of excess potassium and no sodium. Potassium uptake involves the exchange for Na^+ and probably also for H^+.

Total potassium uptake was decreased to half by a fivefold equivalent excess of rubidium, but was essentially unaffected by a 100-fold equivalent excess of sodium. Calcium and magnesium had intermediate effects. Potassium uptake was inhibited by sodium azide (mM) and by dinitrophenol (0·3 mM). These depressed the equilibrium potassium level but had a much smaller effect on the time required to reach equilibrium. Loss of potassium by K^+-loaded mycelium to sodium azide or dinitrophenol solutions or to water was small. Much greater losses occurred to solutions of rubidium but not of sodium.

It is suggested that the equilibrium potassium level is determined only in part by exchange-diffusion at the mycelium surface, and that net potassium uptake is a metabolic process.

INTRODUCTION

Studies of the transport of inorganic ions by mycelial fungi are scarce. The only fungus which has been systematically studied from this point of view is the yeast, *Saccharomyces cerevisiae* (Rothstein, 1965). Although some of the major features of ion transport in this fungus are now clear, it seems impossible to decide whether yeast is representative of the fungi as a group in this respect.

In the absence of growth, net movements of ions between the cells and the environmental fluid can be observed, under suitable conditions, with yeasts and other microorganisms. The use of non-growing organisms is an advantage, since it simplifies interpretation of the relationship of metabolism to the transport processes, and allows the latter to be studied in relatively simple experimental solutions. In preliminary experiments, mycelium of *Neocosmospora vasinfecta* grown in a medium of high (106 m-equiv./l.) potassium content (Budd, 1969*a*) was harvested in mid-logarithmic phase and transferred to solutions of potassium salts lacking a nitrogen source. In such solutions, growth as defined by net protein increase was not measurable. Net uptake of potassium by this mycelium was small and variable. Replacement of most of the K^+ of the growth medium by Na^+ produced a mycelium of relatively low K^+ content when harvested in late logarithmic phase. Some of the characteristics of potassium transport by this low-potassium mycelium are reported in this communication. A preliminary report of this work has already been published (Budd, 1969*b*).

METHODS

Neocosmospora vasinfecta ATCC 11686 (American Type Culture Collection, Rockville, Md., U.S.A.) was grown in liquid shaken cultures from standardized mycelial inocula for 46 to 50 hr at 25 ± 0·5°. The culture medium was a low-K^+ variant of the medium Gl previously described by Budd (1969*a*) and contained (per l. glass-distilled water): glucose, 30·0 g.; $NaNO_3$, 8·5 g.; $MgSO_4.7H_2O$, 2·5 g.; KH_2PO_4, 0·10 g.; $NaH_2PO_4.H_2O$, 0·90 g.; trace elements according to Horowitz & Beadle (1943); pH before autoclaving, 4·5. Analysis showed the K^+ and Na^+ contents of this medium to be 0·7 and 106·8 m-equiv./l. respectively, in contrast to 106·0 m-equiv./l., and essentially zero for medium Gl. Growth on the low-K^+ medium was logarithmic from the time of inoculation to the 56th hr of incubation. The mycelium was separated by filtration, washed by suspension in distilled water, and samples of 30 to 50 mg. dry wt were prepared as described by Budd & Harley (1962).

Incubation was at room temperature (22·5 to 25·5°) with aeration (Budd, 1969*a*), terminated by rapid filtration on Whatman No. 54 paper circles. After about 10 sec. rinsing with glass-distilled water on the filter the mycelium was extracted, usually with 1·0 N-HNO_3, for 24 hr in a centrifuge tube. Mycelium and extractant were then separated by centrifugation, the mycelium extracted with more 1·0 N-HNO_3 and the combined extracts made to known volume with glass-distilled water. In some experiments this procedure was replaced by wet combustion of the sample with 1 ml. 70% (w/v) HNO_3, followed by dilution to known volume with glass-distilled water. Extraction of sodium and potassium by these two procedures agreed to within ± 2%.

Sodium and potassium contents of suitably diluted portions of the mycelial extracts were determined by flame emission spectrophotometry, with a Unicam SP 90 A atomic absorption spectrophotometer in the emission mode. Standard solutions and blanks were adjusted to the same content of nitric acid as the unknown solutions. Mutual interference between sodium and potassium in flame emission analysis was negligible at their relative concentrations in the extracts. Rubidium did interfere with this method of potassium estimation. A correction factor was established by first determining the rubidium concentration of the extract as described below, and then determining the expected error in potassium flame-emission from experimentally prepared curves which related this emission to Rb^+ and K^+ concentrations in mixed solution.

Rubidium uptake was determined by using ^{86}Rb as a tracer. The isotope was obtained from the Radiochemical Centre, Amersham, Buckinghamshire, England as an aqueous solution of RbCl; this was diluted immediately before use with carrier RbCl to approximately 2·5 μCi/m-equiv. Rb^+. The amount of isotope in mycelial extracts was determined by liquid scintillation spectrometry with the Packard '3002' Tri-Carb system and the second solution of Bruno & Christian (1961) as liquid fluor. The specific activity of the RbCl supplied to the mycelium was determined in the same way at the time of assaying the extracts.

Chloride in mycelial extracts was determined by coulometric titration with a Buchler-Cotlove automatic chloride titrator. All pH measurements were made with a Heathkit model EUW-301 recording pH meter and a Sargent combination electrode.

Ouabain octahydrate was purchased from Sigma Chemical Co., St. Louis, Mo.,

U.S.A. and rubidium chloride from Alfa Inorganics, Inc., Beverly, Mass., U.S.A. All other chemicals were of analytical grade.

RESULTS

Table 1 shows the levels of sodium and potassium in mycelium grown in low-K^+ medium and in normal mycelium from G1 medium. Growth on the low-K^+ medium resulted in a significant decrease in potassium content, and an increase in that of sodium. The specific growth rates in the two media were identical for at least 56 hr (to a yield of about 2·5 g. dry matter/l.). It is clear that the levels of K^+ and Na^+ could be manipulated within the limits of these experiments without fundamentally changing the metabolic characteristics of the mycelium.

Table 1. *Sodium and potassium contents of normal and low-K^+ mycelium of Neocosmospora vasinfecta*

	Sodium			Potassium		
Mycelium	Mean*	σ	No.	Mean	σ	No.
Normal	0·98	0·35	9	62·08	8·39	12
Low-K^+	26·26	9·06	16	21·70	6·52	16

* Values are μ-equiv./100 mg. dry weight. σ = Standard deviation. No. = number of experiments. Growth conditions as in text.

On transfer to dilute solutions of potassium salts, the low-K^+ mycelium took up potassium and lost sodium, reaching new equilibrium levels of both ions after 2 to 3 hr. Figure 1 shows the results of an experiment in which KCl (0·1 mM) was the incubation medium. The pH value was monitored continuously; the changes recorded are characteristic and differed only slightly for more concentrated KCl solutions. Changes in pH value of this order had essentially no effect on the net K^+ uptake (Fig. 2). As shown in Fig. 1, potassium uptake and sodium loss were roughly equivalent; after 3 hr 5·1 μ-equiv. K^+ were absorbed for every 4·2 μ-equiv. Na^+ lost. However, results to be presented showed that the K^+:Na^+ transport ratio had no characteristic value; it was strongly affected by experimental conditions, and was not usually close to unity. It also appears unlikely that deviations of the K^+:Na^+ ratio from unity were closely related to the amounts of chloride absorbed, because chloride uptake was consistently small during the first hour, the period when the greatest adjustment in K^+ and Na^+ levels took place (Fig. 1).

Potassium uptake was essentially complete after 2 hr for KCl concentrations between 0·1 μM and 10 mM. The relationships of K^+ uptake and Na^+ loss to KCl concentration for a 2 hr incubation period are shown (Fig. 3). The total potassium uptake varied somewhat less than twofold over this 10,000-fold range of K^+ concentration, while Na^+ loss was unaffected.

Glucose (0·1 %) in the medium had no effect on the uptake of K^+ or loss of Na^+ during 2 hr.

Effects of Inhibitors. Sodium azide and 2,4-dinitrophenol (DNP) strongly decreased the K^+ level at 2 hr but they had a smaller effect on the time required to reach equilibrium, as shown for sodium azide at 1 mM in Fig. 4. The effects of DNP concentrations

are shown in Table 2. The total uptake of K^+ could be decreased by 70 to 80% with the proper concentration of inhibitor. On the other hand, Na^+ loss accompanying K^+ uptake was much less sensitive to both inhibitors. Consequently, the K^+:Na^+ transport ratio fluctuated, e.g. from 2·3 to 1·0 in Table 2.

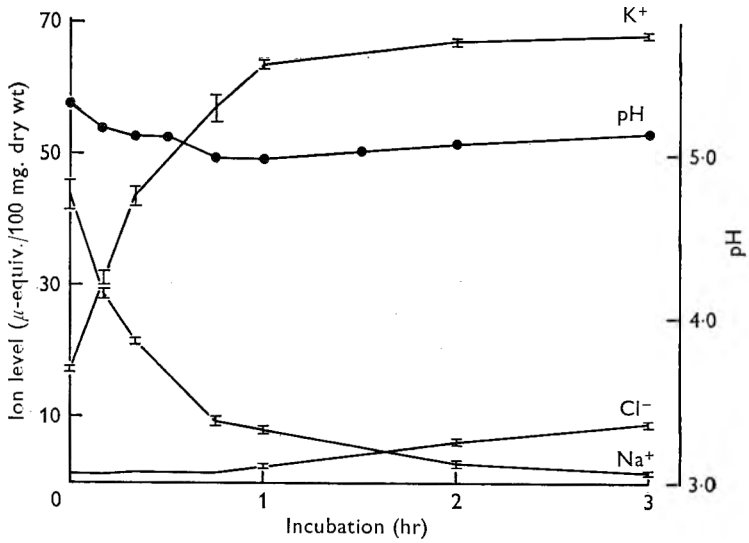

Fig. 1. Changes in *N. vasinfecta* mycelial ion levels and in extracellular pH values, during incubation of low-K^+ mycelium in 0·1 mM KCl at 25·5° (The pH values are those measured a sample size of 38 mg. dry wt incubated in 1 l. KCl.) Bars show range of duplicates.

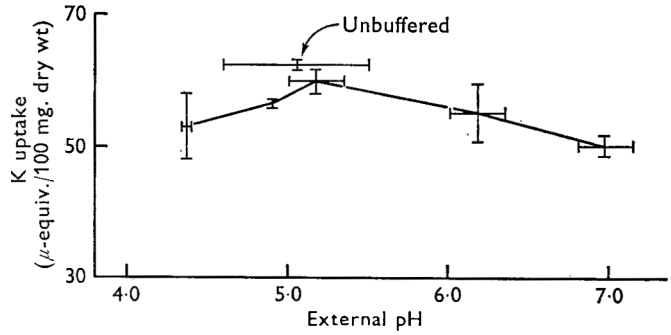

Fig. 2. Effect of pH value on potassium uptake from mM-K^+ by *N. vasinfecta*. Uptake for 2 hr at 23·5°. Buffer system below pH 5·0, KH phthalate + HCl; above pH 5·0, KH_2PO_4 + KOH. Vertical bars show range of duplicates; horizontal bars connect initial and final pH values.

Ouabain (0·1 mM) had no effect on the movement of either K^+ or Na^+ when presented simultaneously with the KCl. When the KCl was presented following pre-incubation 1 hr with ouabain, a 15% decrease in K^+ transport and no significant effect on Na^+ transport were observed over 2 hr.

Effect of other cations. Sodium and magnesium were the major cations in the growth medium used in these experiments. Of these, Na^+ had no significant effect on the 2 hr

Table 2. *Effect of 2,4-dinitrophenol on K^+ uptake and Na^+ loss by low-K^+ mycelium of Neocosmospora vasinfecta*

DNP (M)	K^+ uptake	Na^+ loss	K^+ uptake/ Na^+ loss
None (control)	48·7*	21·0*	2·3
30 μM	35·8	20·0	1·8
100 μM	16·9	15·4	1·1
300 μM	8·8	8·5	1·0

* Values (averages of duplicates) in μ-equiv./100 mg. dry wt. Incubation for 10 min. in DNP (brought to pH 5·7 with NaOH) followed by 2 hr incubation in KC mM-KCl at 24°.

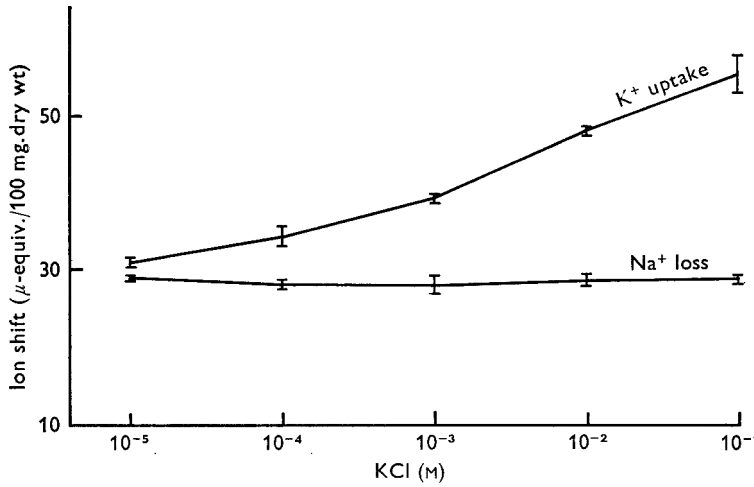

Fig. 3. Effect of KCl concentration on potassium uptake and sodium loss by *N. vasinfecta*. Uptake for 2 hr at 25°. Bars show range of duplicates.

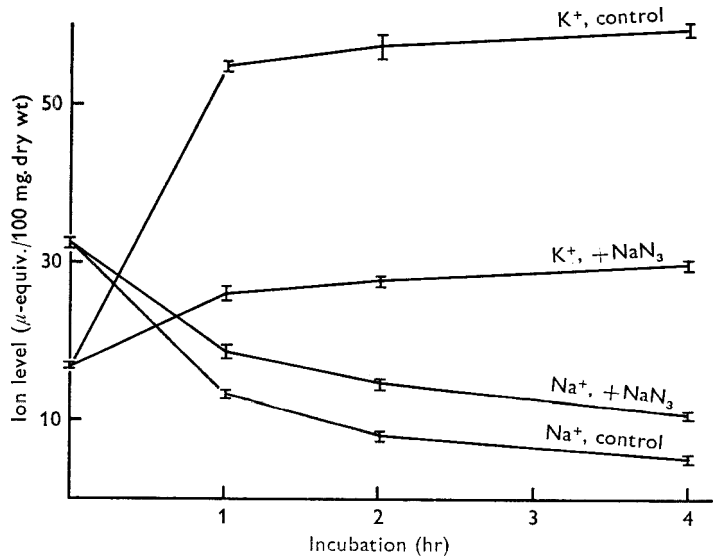

Fig. 4. Effect of mM-sodium azide on potassium uptake and sodium loss at 24·5°. Controls incubated in mM-NaCl. Bars show range of duplicates.

K^+ level at up to a 100-fold equivalent excess over K^+ in the uptake medium. Net loss of Na^+ under these conditions was, however, drastically decreased. Magnesium, when provided at 100-fold equivalent excess ($MgSO_4$ 50 mM; KCl 1 mM), did not affect the time required for K^+ equilibration but depressed the equilibrium K^+ level. This effect was hyperbolic with Mg^{2+} concentration and amounted to a 20·0% decrease in the equilibrium K^+ level at 100-fold equivalent excess of Mg^{2+}. However, there was no effect on simultaneous Na^+ loss.

Calcium acted similarly to Mg^{2+} but was more potent. At twofold equivalent excess ($CaCl_2$ 1 mM; KCl 1 mM), K^+ uptake was decreased 25% after 2 hr but Na^+ loss was unaffected.

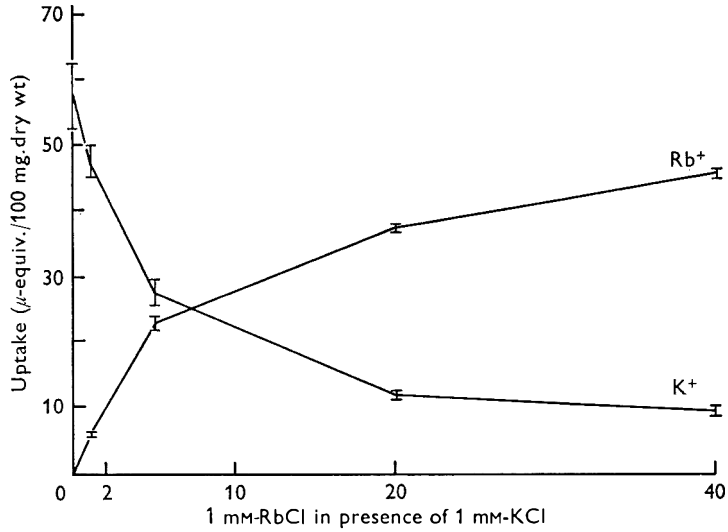

Fig. 5. Effect of various concentrations of RbCl on uptake of both Rb^+ and K^+ from mM-KCl by *N. vasinfecta* at 22·5°. Bars show range of duplicates.

The effects of various concentrations of rubidium on K^+ uptake from mM-KCl are shown in Fig. 5. As usual, the net K^+ uptake was essentially complete in 2 hr although Rb^+ continued to accumulate for at least 4 hr. Depression of the equilibrium K^+ level was hyperbolic with Rb^+ concentration, and amounted to 50% at fivefold equivalent excess of rubidium. Depression of the K^+ level was accompanied by an almost equivalent accumulation of Rb^+, although this relationship would certainly be different for a 4 hr incubation period. As in the case of Mg^{2+} the net Na^+ loss was unaffected by Rb^+ in the KCl solution.

Loss of ions from mycelium. A smaller loss of Na^+ also occurred when low-K^+ mycelium was transferred to distilled water instead of to KCl solutions; this loss was complete within 1 hr and amounted to 20 to 25% of the initial content. Simultaneously a smaller loss of K^+ sometimes took place, not exceeding 14% of the initial content.

The characteristics of K^+ loss by mycelium previously allowed to reach equilibrium in KCl solutions should indicate how the equilibrium K^+ level is established and maintained.

(*a*) *K^+ losses to water only.* As with the untreated low-K^+ mycelium, a small loss of potassium took place, complete within 1 hr, when the mycelium was transferred to

distilled water following equilibration in KCl (Table 3). The actual loss of K^+ was independent of the volume of washing water over a tenfold range. Also it is clear (lines 2 and 6, Table 3) that, following suspension of the mycelium for 1 hr in one volume of water, no further K^+ was lost on transfer to a second, identical volume of water. Lines 4 and 6 of Table 5 show that K^+ loss was not enhanced by mM-NaCl. This was also true for 10 mM-NaCl and for 20 mM-sodium phosphate (at pH 6·5).

Table 3. *Loss of potassium to water at 24° by N. vasinfecta following a period of K^+ uptake (2-hr uptake from mM-KCl at 24°)*

Wash volume (ml.)	Wash time (hr)	K^+ level*	Control (%)
—	—	69·9±3·4	100·0
1000	1	65·2±1·4	93·3
1000	2	64·7±4·1	92·6
2000	2	64·9±3·8	92·8
200	2	63·8±3·9	91·3
1000+1000	1+1	64·6±0·9	92·4

*K^+ levels (mean of triplicates) expressed as μ-equiv./100 mg. dry wt, $\pm\sigma$. K^+ level of samples *before* incubation in KCl, 36·4 μ-equiv./100 mg. dry wt.

Table 4. *Potassium loss as influenced by 2,4-dinitrophenol in N. vasinfecta*

Uptake conditions		Washing conditions		
[KCl]	Additions	Solution	Incubation time (hr)	K^+ level* μ-equiv./100 mg. dry wt.
—	—	—	—	27·5
mM	—	—	—	67·4
	—	Water	1	62·2
			3	57·8
	—	0·3 mM-DNP	1	50·3
			3	48·9
	0·3 mM-DNP	—	—	34·1

* K^+ levels averages of duplicates. K^+ uptake period, 2 hr at 22·5°. (DNP brought to pH 5·7 with NaOH.)

(*b*) *K^+ losses to inhibitor solutions.* A comparison of lines 1, 2 and 7 of Table 4 shows that DNP at a concentration already shown to inhibit K^+ uptake by over 70% (Fig. 4; Table 2), decreased net K^+ uptake to 16·5% of the control. The resulting steady K^+ level was just over half that in control samples. When presented to mycelium immediately following equilibration in KCl, however, the DNP was far less effective in decreasing the steady K^+ level (cf. Table 4, lines 3 to 6). After 3 hr, the K^+ level in DNP-treated control samples was approximately 85% of that in water-treated control samples. Similar results were obtained with sodium azide (Table 5). Depending on whether pre-equilibration took place in KCl 0·1 M or mM subsequent transfer to sodium azide depressed the K^+ level to 85 to 91 % of NaCl-treated controls (Table 4, lines 2 to 5). In parallel experiments where sodium azide (mM) was presented simultaneously with KCl, the K^+ level was depressed to less than 50% of that of the controls, irrespective of incubation period (Fig. 4). Therefore, like DNP, azide was less effective in inducing K^+ loss than in preventing K^+ uptake.

(*c*) *K^+ losses to RbCl solutions.* Potassium loss to RbCl 10 mM was compared with the effect of the same RbCl level on K^+ uptake from KCl mM. All samples not post-incubated in RbCl were held in distilled water for the same time. Treatment with RbCl was more effective than any of the preceding treatments in inducing K^+ losses from the mycelium (Table 6). Nevertheless, the K^+ level was not decreased to that of mycelium which had absorbed K^+ in the presence of RbCl, even after twice as long an exposure to Rb^+ (line 7 and line 5, Table 5). The loss of potassium was insignificant after the second hour, despite continuing uptake of Rb^+. After 4 hr, the $Rb^+:K^+$ ratio in the post-incubation medium in this experiment exceeded 200, yet the K^+ level was not decreased to that found for a $Rb^+:K^+$ ratio of 10 in the uptake medium. Therefore, the bulk of mycelial K^+ was not removed by Rb^+ ions.

Table 5. *Potassium loss as influenced by sodium azide in N. vasinfecta*

Uptake soln.	Washing soln. (mM)	K^+ level	Na^+ level
—	—	18·1	21·1
0·1 M-KCl	NaCl	58·6	10·2
	NaN_3	53·3	11·3
mM-KCl	NaCl	59·5	6·1
	NaN_3	50·9	8·0
	KCl	61·2	0·6

Ion levels (averages of duplicates) in μ-equiv./100 mg. dry wt. K^+ uptake, 2 hr at 23·5°. Washing period, 1 hr at 23 5°.

Table 6. *Effect of RbCl on the equilibrium K^+ level in N. vasinfecta*

Uptake medium	Washing medium (hr)	K^+ level*	Rb^+ level*
		μ-equiv./100 mg. dry wt	
—	—	10·08	—
mM-KCl	Water, 2	51·98	—
	Water, 4	50·40	—
mM-KCl +	Water, 2	20·14	35·44
10mM-RbCl	Water, 4	20·63	38·75
mM-KCl	10mM-RbCl, 2	36·97	19·38
	10mM-RbCl, 4	35·13	32·45

* Ion levels (averages of duplicates) K^+ uptake for 2 hr. Temperature, 23°.

DISCUSSION

The absorption of K^+ and of other ions by a variety of organisms leads to an equilibrium condition at which no further net uptake occurs. Equilibrium is reached within minutes or hours in bacteria (Schultz, Epstein & Solomon, 1963; Galdiero, 1966), while for roots of higher plants one or more days may be required (Jackson & Edwards, 1966). In the present experiments with *Neocosmospora vasinfecta* approximately 2 hr sufficed, for a wide range of external K^+ concentrations. In general, and notably for monovalent ions, the equilibrium appears to be dynamic, with efflux balancing influx (Rothstein, 1959). Although no data on simultaneous K^+ fluxes are as yet available for *N. vasinfecta*, the present work provides some information about efflux from K^+-loaded mycelium. Experiments involving washing such mycelium in water or inhibitor

solutions (Tables 3 to 5) indicate that simple diffusive efflux (leakage) is unlikely to play a major role. On the other hand, the effect of Rb^+ on K^+ loss (Table 6) is most easily explained as exchange diffusion, in which K^+ leaves in exchange for Rb^+ entering the mycelium. The specificity of K^+ loss towards Rb^+ and Na^+ resembles that of K^+ uptake, suggesting that the same transport system may be involved in both processes. Equilibria mediated by a K^+ exchange system are known in other micro-organisms (Epstein & Schultz, 1966; Slayman & Tatum, 1965).

Table 6 also shows that a relatively large proportion of mycelial K^+ appears to be unavailable for exchange with Rb^+ when the latter is presented after K^+ uptake is complete. Two explanations suggest themselves, both of which involve a secondary sequestration of K^+ following its entry into the cell. (1) A fraction of cellular K^+ may become bound in a non-exchangeable form after its discharge from the potassium transport system. (2) The secondarily sequestered fraction of cell K^+ may remain exchangeable but be associated with a cytoplasmic system which discriminates more strongly against Rb^+ than does the potassium transport system itself. A decision between these alternatives cannot be made without recourse to isotopic potassium. Nevertheless, the potassium level in the mycelium does not appear to be regulated solely by the properties of the transport system itself. The penetration of Rb^+ into *Neurospora crassa* is apparently followed by its binding into a non-exchangeable form (Lester & Hechter, 1958).

Whereas the equilibrium K^+ level appears to be associated with $K^- \leftrightharpoons K^+$ exchange, the approach to equilibrium involves exchange of K^+ with other cations. Loss of Na^+ is usually insufficient to balance K^+ uptake, and little chloride accompanies K^+ into the cells, at least during the first hour. It is reasonable to suppose that the excess K^+ absorbed is balanced by excretion of H^+, as in *Escherichia coli* (Schultz *et al.* 1963) and in yeast (Conway & O'Malley, 1946). Where measured, the actual pH decrease during K^+ uptake exceeds any expected pH decrease due to such an exchange, but the pH changes taking place in distilled water only were never observed.

The mechanism mediating $K^+ \leftrightharpoons Na^+$ exchange is unknown, but the operation of a classical sodium pump is not supported by either the wide fluctuations in the K^+:Na^+ transport ratio or the insensitivity to ouabain.

Lack of information on the trans-surface electrical potential difference prevents a final decision whether K^+ uptake in *Neocosmospora vasinfecta* is active or not (Ussing, 1949). Values of this potential difference have been reported for *Neurospora crassa* which will account for a 10,000-fold accumulation of K^+ in cells as compared with external medium, without invoking active transport (Slayman, 1965*a*). However, the effects of sodium azide and 2,4-dinitrophenol on K^+ uptake (Table 2; Fig. 4) strongly indicate the participation of energy metabolism in this process. These inhibitors do not act merely by rendering the cellular membranes leaky (Tables 4, 5; Slayman & Tatum, 1964). Two other modes of action are possible: (*a*) that potassium influx depends, at least partly, on energy transfer; or (*b*) that the trans-surface electrical potential difference is made less negative. If the K^+ transport system is electrogenic, as Slayman (1965*b*) has concluded for *N. crassa*, then it may be impossible to distinguish between these alternatives. For the present, it is sufficient to conclude that K^+ uptake is a metabolic process, although it may not qualify as active transport.

A detailed comparison of the K^+ transport in *Neocosmospora vasinfecta* with that of other fungi is not possible on the basis of the present studies. The use of low-K^+

mycelium in these experiments is analogous to the production of sodium-yeast (Conway & Moore, 1954). In the importance of ion-exchange processes to net K^+ uptake and maintenance of the equilibrium K^+ level, and in the sensitivity of K^+ uptake to alterations of cellular energy metabolism *N. vasinfecta* shows a general similarity to other fungi (Rothstein, 1959; Slayman & Tatum, 1964, 1965). Discrimination between K^+ and Na^+ by the K^+-uptake system appears to be greater in *N. vasinfecta* than in yeast (Conway & Duggan, 1958). Measurements of potassium fluxes are a clearly desirable next phase in these studies.

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Lysosomal Activity and its Control in Encysting *Hartmannella castellanii*

By A. J. GRIFFITHS AND S. MAUREEN BOWEN
Department of Microbiology, University College of South Wales and Monmouthshire, Cardiff, CF 1 3 NR

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SUMMARY

A latent, non-specific phosphatase of *Hartmannella castellanii* with an optimum pH of 4·0 has been investigated. The latency could be released in cell-free homogenates by treatment with Triton × 100, freezing and thawing, refrigeration or carbon tetrachloride. It seems likely that this enzyme is attached to a sub-cellular organelle as it was largely sedimentable when homogenates were prepared in media containing sucrose.

During encystation of the amoebae under controlled conditions there was no appreciable change in the levels of total phosphatase activity measured in frozen-thawed extracts, but there were quite large increases in free activities of fresh, unfrozen preparations. Inhibitors and promoters of encystation were also found to affect the levels of phosphatase activity in the amoebae but did not affect its sedimentability which persisted throughout the initial degradative phase of encystment.

Incubation of homogenates of Hartmannella decreased the latency of the acid phosphatase but this activation could be modified by substances which are known to affect the encystation responses of the amoebae.

It is concluded that the degradative phase of encystation is due to the activation of hydrolytic enzymes within sedimentable compartments of the amoebae and is not the result of hydrolases being released from these compartments. It seems likely that agents which are capable of promoting encystation may do so by inducing activation of lysosomal enzymes.

INTRODUCTION

When starved completely of carbon and nitrogen sources and supplied with magnesium chloride the amoebae of *Hartmannella castellanii* encyst (Griffiths & Hughes, 1968, 1969). The initiation of encystation is marked by large decreases in dry weight and protein which apparently provide the energy and precursors for the subsequent syntheses of cyst-specific components of which cellulose is the most prominent. The importance of this degradative phase suggests that encystation is a process in which it would be reasonable to expect lysosomal activity to play a significant role and there is now some evidence for the participation of autolysosomes in cyst-formation in *Acanthamoeba* sp. (Bowers & Korn, 1969) and in another amoeba, *Mayorella palestinensis* (Lasman, 1967). As encystation can now be investigated under reasonably controlled experimental conditions it would seem to present a suitable system for the study of the control of lysosomal activity in developmental processes.

METHODS

The organism used in this investigation was *Hartmannella castellanii* (NEFF strain). The amoebae were grown axenically in 4% mycological peptone (Oxoid) and encystation was induced by replacing the growth medium by 0·05M-$MgCl_2$ (Griffiths & Hughes, 1968, 1969).

Cell-free extracts were prepared as follows. Amoebae were collected by centrifuging suspensions at 500 *g* for 3 to 5 min., then the pellet was washed once with 50 mM-$MgCl_2$ at laboratory temperature. In most experiments the washed amoebae were resuspended in 0·6M-sucrose and gently disrupted in a glass hand-homogenizer fitted with a Teflon pestle (Thomas Ltd., Philadelphia) immersed in ice. The homogenate was centrifuged again at 500 *g* for 10 min. to remove unbroken amoebae and the supernatant retained as the cell-free homogenate. Further fractionation of the homogenate was accomplished by centrifuging either at 800 *g* for 10 min. followed by 20,000 *g* for 20 min., in a MSE 17 centrifuge, or at 20,000 *g* only. Pellets were resuspended in 5 to 10 volumes of 0·6M-sucrose buffered at pH 7·2 with 20 mM-tris.

Acid phosphatase was assayed at 25° with *p*-nitrophenylphosphate (Sigma Chemical Co.) as the substrate. The reaction mixture consisted of 0·2 ml. 100 mM acetate buffer (pH 4·0), 0·2 ml. 25 mM-sucrose and 0·1 ml. of the enzyme preparation (2 to 30 μg protein). The reaction was stopped after 5 to 10 min. by the addition of 4 ml. 0·5M-NaOH. The amount of *p*-nitrophenol released was measured at 400 nm. on a Unicam SP600 spectrophotometer. Total phosphatase activities were measured on extracts which had been frozen and thawed; free phosphatase was measured on fresh, unfrozen extracts.

Protein was measured by the methods of Lowry, Rosebrough, Farr & Randall (1951) and Warburg & Christian (Layne, 1957). Amoebae were counted in a Fuchs-Rosenthal haemocytometer (Griffiths & Hughes, 1969).

RESULTS

The *p*-nitrophenylphosphatase of *Hartmannella* has a pH optimum of 4·0. When assayed in freshly prepared extracts the phosphatase activity was much lower than in extracts which had been refrigerated overnight or frozen and thawed. This latency could also be released in the fresh extracts by incubating with Triton × 100 or carbon tetrachloride (Table 1). It was sufficient to freeze and thaw only once to release the maximum activity and for this reason measurements of total activity were always performed on frozen-thawed preparations.

Inclusion of sucrose in the media used to prepare cell-free homogenates of Hartmannella increased the sedimentability of acid phosphatase (Table 2). The use of sucrose concentrations up to 0·6M also brought about a decrease in the total measurable activity in association with the increase in sedimentability. Extracts prepared in 0·9M-sucrose were 95% more active than those in 0·6M-sucrose.

As expected, the sedimentable acid phosphatase also exhibited a greater degree of latency than the apparently soluble form of the enzyme. Freezing and thawing of the sedimentable fraction produced increases of 60 to 80% in the phosphatase activity compared with 10 to 20% in the soluble fraction. The sedimentability of the acid phosphatase was characteristic of amoebae at all stages of growth (Table 3). Mid-log phase amoebae which had been induced to encyst also showed a high proportion of

sedimentable phosphatase after 6 hr encystation (which corresponds to the mid-point of the hydrolytic phase of encystation (Griffiths & Hughes, 1969)). In one encystation experiment the amoebae inexplicably lysed after 5 hr incubation. It may be significant that in this culture only 28% of the recovered phosphatase activity was sedimentable.

Table 1. *Latency of acid phosphatase of H. castellanii*

Treatment	Increase in activity (%)
Triton × 100	50
Refrigeration (4°, 24 hr)	60
Freezing and thawing	80
Carbon tetrachloride (10 μl.)	40

Cell-free homogenates were prepared in 0·6M-sucrose. Triton × 100 and carbon tetrachloride were each added to the assay mixture. To relieve latency by freezing and thawing it was sufficient to freeze once (12 hr at −25°) and thaw at laboratory temperature.

Table 2. *Sedimentability of H. castellanii acid phosphatase at various sucrose concentrations*

Sucrose concentration in homogenate	Total activity (μM-*p*-nitrophenol)	Sedimented (%)	Recovery (%)
0	90	15	60
0·3M	94	38	70
0·6M	83	79	86
0·9M	162	58	70

Cell-free homogenates were prepared in media containing the indicated sucrose concentrations from the same culture of log-phase amoebae. All media were buffered at pH 7·2 with 20 mM-tris. Sedimentability was determined after centrifuging homogenates at 20,000 *g* for 20 min. and resuspending the pellets in a medium containing sucrose at a concentration corresponding to that used during cell breakage. The percentage recovery of phosphatase activity following fractionation is also indicated.

Table 3. *Sedimentability of acid phosphatase during growth and encystation*

	Per cent of total activity of homogenate			
	Hours growth			Hours encystation
	24	48	72	6
Sedimentable	79	96	128	86
Soluble	10	23	30	17
Recovery (%)	89	119	158	103

Cell-free homogenates were prepared in 0·6M-sucrose. Sedimentability was determined after centrifuging at 20,000*g* for 20 min.

Table 4 shows the activities of acid phosphatase in homogenates of Hartmannella during the degradative phase of encystation. Although the specific activity of acid phosphatase increased by 34% in the first 3 hr of encystation the phosphatase activity per cell decreased. Some of the apparent increase in specific activity may be due not to an increase in the amount of enzyme but to the not insignificant proteolysis which occurs at this stage of encystation and which proceeds at an initial rate (during the first 10 hr) of ∠ to 5%/hr. The enzyme activities were measured in frozen-thawed

extracts and therefore represent the total measurable phosphatase in the encysting amoebae. Table 3 shows the percentage of the total activity which was measurable in fresh homogenates and in fractions of amoebae after various periods of incubation in the encystation medium; this was the 'free' phosphatase activity. There were large increases in the free activities of the homogenates during the initial 5 hr of encystation which were followed by a slight decrease between 5 and 8 hr. A similar pattern was found to occur in the phosphatases of the 800 *g* pellet and the 20,000 *g* supernatant. Free phosphatase activity in the 20,000 *g* pellet remained essentially at the same level throughout the hydrolytic phase of encystation.

Table 4. *Total activities of acid phosphatase during the hydrolytic phase of encystation*

	μM-*p*-nitrophenol	
	Per mg. protein/min.	Per cell/min.
0 hr encystation	139	500
1 hr encystation	143	400
3 hr encystation	186	300
5 hr encystation	140	120

Phosphatase activities were measured on frozen-thawed cell-free homogenates prepared in 0·6M-sucrose. Samples were taken from the same culture.

Table 5. *Free acid phosphatase activity in encysting H. castellanii*

	Hours encystation		
	0	5	8
Homogenate	27	61	54
800*g* × 10 min. pellet	16	50	33
20,000*g* × 20 min. pellet	42	50	50
20,000*g* × 20 min. supernatant	62	92	77

Free activities, which were measured on fresh preparations, are expressed as a percentage of the total activities which were obtained with the same extracts after freezing and thawing.

The encystation response of Hartmannella has been found to be sensitive to a number of carbon and nitrogen sources when these are included in the replacement medium. Addition of glutamic acid to an encysting culture completely blocks encystment and brings about the eventual death of the amoebae. When glucose is included in the encystation medium however, there is a significant enhancement of cellulose synthesis and there is also a sparing effect on the dry weight lost by the encysting amoebae. (Griffiths & Hughes, 1969). In an experiment devised to determine the effect of glucose and glutamate on the behaviour of acid phosphatase the amoebae were incubated for 15 hr in the presence of these substrates. One per cent mycological peptone was used as a control as at this concentration neither growth nor encystation occur. The highest activity of phosphatase was found in amoebae which had been incubated in the normal encystation medium (Table 6). The activity in the cells incubated with glucose was only slightly higher than in those incubated in the peptone control. The highest sedimentable activities were found in those amoebae which eventually encysted.

When cell-free homogenates of Hartmannella were incubated with $MgCl_2$, glucose and glutamate, all in 0·6M-sucrose (Table 7), it was found that both glutamate and

glucose depressed the measurable free activity whereas $MgCl_2$ had an enhancing effect on the release of the phosphatase latency. Curiously, in view of their quite opposite effects on the encystation of whole amoebae, both $MgCl_2$ and glutamate induced a release of acid phosphatase into the non-sedimentable fraction of the homogenate. In these experiments there was some spontaneous activation of the enzyme as the free activity in the control at the start of the incubation period was only 30% with 20% of the activity in the soluble phase.

Table 6. *Acid phosphatase activity in amoebae following incubation with inhibitors and promoters of encystation*

		μM-*p*-nitrophenol/cell	Sediment (%)	Soluble (%)
No encystation	1% w/v Mycological peptone	425	49	44
	50 mM-glutamate + 50 mM-$MgCl_2$	122	54	39
Encystation	250 mM-glucose + 50 mM-$MgCl_2$	475	65	36
	50 mM-$MgCl_2$	595	68	27

Amoebae were incubated for 15 hr in media containing the substrates shown above. After harvesting cell-free homogenates were prepared in 0·6 M-sucrose. Sedimentability was determined after centrifuging homogenates at 20,000*g* for 20 min.

Table 7. *Activation of acid phosphatase in homogenates of H. castellanii*

	Per cent of total activity		
	Free	Sedimentable	Soluble
Control (initial)	30	70	20
Control (2 hr incubation)	74	64	27
50 mM-$MgCl_2$	87	24	94
50 mM glutamate	48	14	100
50 mM glucose	48	68	29

Amoebae were homogenized in 0·6 M-sucrose and the cell-free homogenate was incubated as shown at 30°. Free activities were measured on fresh extracts and total activities on frozen-thawed preparations.

DISCUSSION

The acid pH optimum, latency and sedimentability of the non-specific phosphatase of *Hartmannella castellanii* is strong evidence in favour of its lysosomal location. There were no spectacular increases in acid phosphatase levels in encysting Hartmannella as has been reported for the biochemically similar phenomenon of slime-mould sporulation (Gezelius, 1966). It is quite clear though, that the mere measurement of total enzyme activities in eucaryotic organisms is of limited value in studies such as these when the enzyme in question may not have free access to its substrate *in vivo*. In the case of the Hartmannella phosphatase, for example, although there was no large increase in the level of this enzyme during encystation there were significant increases in its free activity. Furthermore, these increases did not occur in all sub-cellular fractions. How far these observations are representative of the *in vivo* situation can only be determined by further investigation and is now receiving attention. But the dry weight loss and extensive protein degradation which is known to occur in encysting amoebae (Griffiths & Hughes, 1969) is, at least, circumstantial evidence in favour of such changes in the activities of the hydrolases.

The persistent sedimentability of acid phosphatase in encystation is in contrast to the behaviour of this enzyme in starved Euglena (Bertini, Brandes & Buetow, 1965) and in the development of the chick urino-genital system (Scheib-Pfleger & Wattiaux, 1962) in which hydrolytic activity is brought about by the release of the lysosomal enzymes into the soluble fraction of the cells. The highest levels of soluble acid phosphatase in Hartmannella were found in amoebae which had been prevented from encysting by the presence of glutamate or low concentrations of peptone and in the culture which had lysed. It seems likely, therefore, that the release of hydrolases from membrane-bound locales in the amoeba is inconsistent with encystation. This is understandable for certain enzymes or structures of the amoeba are required for the provision of energy and for syntheses in the differentiating cell and these must be protected from indiscriminate destruction by lysosomal enzymes released into the 'cytoplasm'.

Shibko, Pangborn & Tappel (1965) have shown that the activation of rat-kidney lysosomal enzymes takes place in two stages. Initially there is an increase of the free activities of the hydrolases without loss of sedimentability. The second stage involves an almost complete release of the enzymes into the soluble phase. In the case of the Hartmannella phosphatase only activation by magnesium involved the latter. This observation is relevant to our understanding of the role of magnesium in promoting encystation and possibly to the activation of lysosomes in other cells, for Sawant, Desai & Tappel (1694) have reported the activation of lysosomal aryl-sulphatase by magnesium ions in rat liver. In Hartmannella although the levels of acid phosphatase were higher in the amoebae which had been incubated with $MgCl_2$ alone, most of this activity was still sedimentable. In view of this finding, and the indication that high levels of soluble acid phosphatase are inconsistent with encystation, the role of magnesium in encystation may still be interpreted as an inhibitor of the leakage of macromolecules which is known to occur in non-growing Hartmannella (Griffiths & Hughes, 1968). Alternatively, the inhibition of acid phosphatase activation by glucose may also occur *in vivo* and antagonise the activating effect of magnesium chloride. There is a large accumulation of hexoses in encysting amoebae (Griffiths & Hughes, 1968) which coincides with the phase of hydrolytic activity. The inhibitory effect shown by glucose is consistent with its probable role as a precursor for cellulose synthesis during encystation and with the observation that there is a substantial reduction in dry weight loss by amoebae induced to encyst in the presence of exogenous glucose (Griffiths & Hughes, 1969).

The presence of glutamate in the encystation medium depresses the phosphatase levels of the cells and also inhibits phosphatase activation in homogenates suggesting that glutamate may inhibit encystation by acting at the level of the lysosomes.

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Transformation of *Micrococcus lysodeikticus* by Various Members of the Family Micrococcaceae

By W. E. KLOOS

Department of Genetics,
North Carolina State University, Raleigh,
North Carolina 27607, U.S.A.

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SUMMARY

Various strains originally designated as members of the genera *Micrococcus*, *Sarcina*, and *Staphylococcus* were able to transform adenine, histidine, and tryptophan auxotrophs of *Micrococcus lysodeikticus* to prototrophy at relatively high frequencies. These included certain strains of *M. flavocyaneus*, *M. flavus*, *M. luteus*, *M. lysodeikticus*, *M. sodonensis*, *Sarcina flava*, *S. lutea*, *S. subflava*, *Staphylococcus afermentans* and *St. flavocyaneus*. Many of the above strains were adenine auxotrophs and produced low frequencies of prototrophic recombinants when crossed with the adenine-dependent *M. lysodeikticus* strain ISU. Prototrophic strains of *M. conglomeratus*, *M. radiodurans*, *M. roseus*, *M. rubens*, *M. varians*, *St. aureus* and *St. epidermidis* failed to transform *M. lysodeikticus* in these studies. Prototrophic pigment mutants of *M. lysodeikticus* and *S. lutea* were able to doubly transform yellow auxotrophs of *M. lysodeikticus* to prototrophy and donor colour.

INTRODUCTION

The taxonomic status of the 'species' *Micrococcus lysodeikticus*, isolated by Fleming (1922), has been under investigation for a number of years. One significant step toward classification was made when Evans, Bradford & Niven (1955) proposed including this organism in the genus *Micrococcus* and separating it from the staphylococci on the basis of its relation to oxygen (obligate aerobe) in a standardized complex medium containing glucose. As a result of numerous physiological and biochemical studies, Kocur & Martinec (1962, 1963, 1965) and Rosypal & Kocur (1963) have suggested that strains of *M. lysodeikticus* be included together with certain strains of *M. cyaneus*, *M. flavocyaneus*, *M. flavus*, *M. luteus*, *M. sodonensis*, *Sarcina aurantiaca*, *S. citrea*, *S. flava*, *S. lutea*, *S. marginata*, *S. pelagia*, *S. subflava*, *S. variabilis*, *Staphylococcus afermentans*, and *St. flavocyaneus* into a single species, *M. luteus*. Baird-Parker (1963, 1965) also combined *M. lysodeikticus* with representatives of the above organisms into a single taxonomic group, Micrococcus subgroup 7. More recent studies by Rosypal, Rosypalova & Horejs (1966), based on DNA base composition and Adansonian analysis, have shown that most of the strains classified as *M. luteus* by Kocur & Martinec possessed high coefficients of similarity (S value) and had approximately similar GC contents in their DNA. However, they proposed subdividing these micrococci on the basis of DNA base ratios: group 1 (*M. flavocyaneus*, *M. flavus*, *M. lysodeikticus*, *M. sodonensis*, *S. citrea*, *S. flava*, *S. lutea*, *S. marginata*, *S. pelagia*, *S.*

subflava, *S. variabilis*, *St. afermentans*, *and St. flavocyaneus*) having 70·8 to 73·3% GC; group 2 (*S. aurantiaca* and several other micrococci) having 67·5 to 69·5% GC; and group 3 (*M. luteus* strain ATCC 398 and *M. cyaneus*) having 65·8 to 67·0% GC.

Preliminary studies on transformation in *Micrococcus lysodeikticus* (Kloos &

Table 1. *Transformation of nutritional markers in Micrococcus lysodeikticus by various members of the family Micrococcaceae*

Donor species*	Strain	Source†	Nutritional classification including mutant or auxotrophic genotype‡	Prototrophs/10^8 colony-forming units resulting in crosses with *M. lysodeikticus* strain ISU auxotrophs§		
				ade	*trpE* 16	*hisD* 61
Micrococcus						
M. candicans	ATCC 8456	1	−	< 1	< 1	< 1
M. citreus	ATCC 395	1	−	< 1	< 1	< 1
M. conglomeratus	ATCC 401	1	+	< 1	< 1	< 1
M. flavocyaneus	ATCC 8673	2	*ade*	26	888	283
M. flavocyaneus	CCM 622	3	*ade*	10	530	126
M. flavocyaneus	CCM 851	3	*ade*	12	572	131
			ade$^+$-1	297	1279	348
M. flavocyaneus	CCM 853	3	*ade*	16	613	172
M. flavus	ATCC 400	1	*ade*	9	238	82
M. flavus	ATCC 10240	2	+	192	470	174
M. luteus	ATCC 398	1,4	−	< 1	< 1	< 1
M. luteus	CCM 105	3	*ade*	11	1150	271
			ade$^+$-1	340	1279	230
M. luteus	CCM 308	3	+	220	1080	210
M. luteus	CCM 355	3	+	234	760	179
M. luteus	CCM 370	3	*ade*	8	852	140
M. luteus	CCM 408	3	*ade*	23	1088	96
			ade$^+$-1	414	1236	74
M. luteus	CCM 1423	3	+	696	665	263
M. luteus	CCM 1672	3	*ade*	32	1390	260
			ade$^+$-1	374	1240	188
M. lysodeikticus	ISU	5	*ade*	< 1	1176	478
			ade$^+$-1	346	1899	232
			ade$^+$-1 *trpE* 16	563	< 1	190
			ade$^+$-1 *hisD* 61	548	1260	< 1
M. lysodeikticus	PU	5	*ade*	34	2394	227
			ade$^+$-8	374	2126	307
M. lysodeikticus	UM	5	*ade*	11	1211	203
			ade$^+$-1	261	1013	180
M. lysodeikticus	WRU	5	*ade*	9	1812	198
			ade$^+$-1	381	1762	186
M. lysodeikticus	ATCC 4698	1	*ade*	9	2046	350
M. lysodeikticus	ATCC 15801	4	*ade*	16	784	102
			ade$^+$-4	338	627	118
M. lysodeikticus	CCM 1335	3	+	276	967	151
M. radiodurans	ATCC 13939	6	+	< 1	< 1	< 1
M. roseus	ATCC 412	1	+	< 1	< 1	< 1
M. roseus	ATCC 416	1	+	< 1	< 1	< 1
M. roseus	ATCC 516	1	+	< 1	< 1	< 1
M. roseus	ATCC 9815	1	+	< 1	< 1	< 1
M. roseus	B-P 94	7	+	< 1	< 1	< 1
M. roseus	B-P 97	7	−	< 1	< 1	< 1
M. rubens	ATCC 186	1	+	< 1	< 1	< 1
M. sodonensis	ATCC 11880	1	+	264	935	217
M. varians	ATCC 399	1	+	< 1	< 1	< 1

Table 1 *cont.*

Donor species*	Strain	Source†	Nutritional classification including mutant or auxotrophic genotype‡	Prototrophs/10^6 colony-forming units resulting in crosses with *M. lysodeikticus* strain ISU auxotrophs§		
				ade	*trpE* 16	*hisD* 61
Sarcina						
S. aurantiaca	ATCC 146	4	−	< 1	< 1	< 1
S. flava	ATCC 540	4	*ade*	24	972	162
S. lutea	ATCC 272	3	+	235	708	359
S. lutea	ATCC 381	4	*ade*	17	512	163
S. lutea	ATCC 382	2	(−)	500	638	190
S. lutea	ATCC 533	8	+	325	1479	161
S. subflava	ATCC 7468	4	+	240	855	320
S. ureae	ATCC 6473	4	−	< 1	< 1	< 1
S. ureae	NCDO 1548	9	−	< 1	< 1	< 1
Staphylococcus						
St. afermentans	ATCC 15307	4	+	806	2568	204
St. aureus	655	10	(+)	< 1	< 1	< 1
St. aureus	83	10	(+)	< 1	< 1	< 1
St. epidermidis	B-P 287	7	(+)	< 1	< 1	< 1
St. epidermidis	B-P 2	7	(+)	< 1	< 1	< 1
St. epidermidis	B-P 12	7	(+)	< 1	< 1	< 1
St. epidermidis	B-P 21	7	(+)	< 1	< 1	< 1
St. epidermidis	B-P 31	7	(+)	< 1	< 1	< 1
St. flavocyaneus	CCM 247	3	*ade*	18	641	221

* Strains which were originally designated *Micrococcus flavocyaneus* (CCM 622, CCM 851, CCM 853), *M. lysodeikticus* (CCM 1335), *Sarcina lutea* (ATCC 272), and *Staphylococcus flavocyaneus* (CCM 247) were received from M. Kocur as strains of *M. luteus* (Kocur & Martinec, 1962; 1963).

† 1—J. B. Evans, North Carolina State Univ., Raleigh, N.C., U.S.A.; 2—E. R. Kennedy, Catholic Univ., Washington, D.C., U.S.A.; 3—M. Kocur, Czechoslovak Collection of Microorganisms, J. E. Purkyne Univ., Brno, CSSR; 4—American Type Culture Collection, Rockville, Maryland, U.S.A.; 5—E. A. Grula, Oklahoma State Univ., Stillwater, Okla., U.S.A.; 6—A. H. Schein, Univ. of Vermont, Burlington, Vermont, U.S.A.; 7—A. C. Baird-Parker, Unilever Research Laboratory, Bedford, England; 8—T. Tornabene, Colorado State Univ., Fort Collins, Colo., U.S.A.; 9—E. I. Garvie, National Collection of Dairy Organisms, Univ. of Reading, Reading, England; 10—P. A. Pattee, Iowa State Univ., Ames, Iowa, U.S.A.

‡ *ade* = Adenine requirement (growth stimulated by adenine, adenosine, inosine, or hypoxanthine); *ade*$^+$ = adenine-independent mutant, notation followed by arabic numeral to indicate order of isolation, e.g. *ade*$^+$-1; *trpE* 16 = L-tryptophan requirement (growth stimulated by anthranilic acid, indole, or L-tryptophan); *hisD* 61 = L-histidine requirement (growth stimulated by L-histidine but not by L-histidinol); − = no detectable growth (colonies) on defined agar, phenotype unclassified; (−) = no detectable growth on defined agar, but genotype is believed to be *ade*$^+$ *trpE*$^+$ *hisD*$^+$ based on transformation data; (+) = no detectable growth on defined agar of Kloos & Schultes (1969), but prototrophic phenotype classified on defined agar for staphylococci of Kloos & Pattee (1965); + = prototroph.

§ Data represent the average of two separate DNA preparations. Two crosses were made from each preparation and each cross plated on duplicate plates. Recipient controls without DNA produced < 1 prototroph/10^6 colony-forming units.

Schultes, 1969) have shown that *Sarcina lutea* strain ATCC 272 was able to transform adenine auxotrophs of certain *M. lysodeikticus* strains to prototrophy, providing evidence of close genetic relationship. Extending transformation studies between *M. lysodeikticus* and other members of the family Micrococcaceae would offer the possibility of examining genetic exchange among these organisms and would broaden our understanding of a genetic definition of Micrococcus species, *i.e.* those micrococci

that can potentially contribute to, or share in, a common gene pool (Marmur, Falkow & Mandel, 1963). In this investigation, various Micrococcaceae have been tested for transforming activity of nutritional markers in crosses with the high transformation frequency *M. lysodeikticus* strain ISU (Kloos, 1969; Kloos & Schultes, 1969). In addition, preliminary studies on transformation of pigment markers is reported.

METHODS

Bacterial strains. Donor strains selected for this study were originally designated as members of the genera *Micrococcus*, *Sarcina* and *Staphylococcus* and were, in particular, those sensitive to either lysozyme or lysostaphin (Table 1).

Cultures received by this laboratory were checked for purity and to some extent proper identification by (1) colony morphology and pigment production on a complex agar medium (P agar) (Naylor & Burgi, 1956) incubated at 26 and 34° and (2) microscopic examination of Gram-stained cells. Each strain was also tested for prototrophy, with respect to adenine, L-tryptophan, and L-histidine, by examining colony formation on a defined agar medium (Kloos & Schultes, 1969) incubated at 26 and 34°. Strains which failed to produce detectable colonies or colonies of less than 0·1 mm. diameter by 8 days were further tested for adenine, L-tryptophan, and/or L-histidine auxotrophy on defined agar media supplemented with each metabolite (20 μg./ml.) singly or in combinations.

Table 2. *Double transformation of pigment and nutritional markers in Micrococcus lysodeikticus*

Prototrophic donor species	Strain	Pigment genotype*	Donor pigment-prototrophs/10^6 colony-forming units resulting in crosses with *M. lysodeikticus* strain ISU yellow-auxotrophs†		
			pig-y ade	*pig-y trpE16*	*pig-y hisD61*
M. lysodeikticus	ISU	pig-w	1·2	6·7	1·0
		pig-p	1·9	7·3	1·5
		pig-o	0·9	5·0	0·8
M. lysodeikticus	ATCC15801	pig-p	1·0	4·1	0·5
Sarcina lutea	ATCC272	pig-w	0·6	2·4	0·4
		pig-p	1·0	4·7	0·4
		pig-o	0·7	1·9	0·5

* pig-w = white; pig-p = pink; pig-o = orange.

† Data represent the average of two separate DNA preparations. Two crosses were made from each preparation and each cross plated on ten plates. Recipient controls without DNA or DNA isolated from yellow strains produced < 0·1 donor pigment-prototroph/10^6 colony-forming units.

Spontaneous adenine-independent mutants were isolated from certain adenine-dependent strains and used as donors. Several different pigment mutants of *Micrococcus lysodeikticus* strain ISU and *Sarcina lutea* strain ATCC272 were obtained by treatment of yellow strains with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) (300 μg./ml.) (Adelberg, Mandel & Chen, 1965) and were also used as donors (Table 2).

Recipient strains included the adenine-dependent *Micrococcus lysodeikticus* strain ISU and a tryptophan and histidine auxotroph derived from a spontaneous adenine-independent mutant (*ade*$^+$-1) of this strain by treatment with NTG (Table 1). The tryptophan and histidine auxotrophs were biochemically characterized by growth

stimulation studies and chromatographic analysis of de-repressed culture supernatants (Kloos & Pattee, 1965; Rose & Kloos, 1969).

Procedure for DNA isolation. Staphylococcus aureus and *St. epidermidis* strains were grown in tryptic soy broth (Difco) at 37° for 18 hr. All other donor strains were grown in a glucose+yeast extract broth (Rosypalova, Bohacek & Rosypal, 1966) at 30° for 18 to 24 hr. Cultures were shaken during incubation with a Burrell Wrist-Action Shaker (Burrell Corporation, Pittsburgh, Pennsylvania) at a setting of two giving 324 shakes/min. through an arc of 3°. The yield in 100 ml. of broth was about 1 to 3 g. wet packed cells. After growth, cells were harvested by centrifugation and washed twice with a NaCl-EDTA solution: 0·15M-NaCl+0·10M-ethylenediamine-tetraacetic acid (EDTA), pH 8·0. DNA was isolated by the procedure of Marmur (1961) with the modification of Saito & Miura (1963) using T1 ribonuclease (RNase) (Sankyo Company, Ltd., Tokyo, Japan) as well as bovine pancreatic RNase (Calbiochem, Los Angeles, California). The duration of lysozyme (Calbiochem) treatment varied from 1 hr to as long as 12 hr for more resistant strains. Lysostaphin (kindly supplied by Dr W. A. Zygmunt, Mead Johnson Research Centre, Evansville, Indiana) was used in place of lysozyme to lyse *St. aureus* strains. The final DNA fibres were sterilized in 75% ethanol for 2 hr and then dispersed into a $\frac{1}{10}$ dilution of sterile standard saline solution (SS: 0·1M-NaCl, pH 7·0) which was adjusted to SS as soon as dispersion was completed. DNA preparations were used within 2 days of isolation and were stored at 4°. DNA concentration was determined by the diphenylamine reaction of Dische (1955).

Procedure for transformation. An 18 hr P agar slope culture of the recipient strain was suspended in 1 ml. saline and diluted 1/100 in saline. Aliquots of 0·1 ml. (about 5×10^6 colony-forming units) from the diluted suspension were added to duplicate tubes containing 1 ml. defined broth (Kloos, 1969) supplemented with 10 μg./ml. adenine (strain ISU *ade*), 10 μg./ml. L-histidine (strain ISU *ade*$^+$-1 *hisD* 61), or 15 μg./ml. L-tryptophan (strain ISU *ade*$^+$-1 *trpE* 16). Mixtures were shaken in a 34° water bath with a Burrell Wrist-Action Shaker at a setting of four giving 324 shakes/min. through an arc of 6° for 12 hr (strain ISU *ade*$^+$-1 *hisD* 61) or 24 hr (strains ISU *ade* and ISU *ade*$^+$-1 *trpE* 16). After growth, cocci (about $1–3 \times 10^8$ colony-forming units/ml.) were centrifuged and resuspended in 1 ml. transformation buffer: 0·05M-tris (hydroxymethyl) aminomethane (tris)+0·01M-$SrCl_2$+0·5% monosodium glutamate, pH 7·0. DNA (10 μg. in 0·03 to 0·8 ml. SS) was added to each tube and the mixtures shaken in a 30° water bath at a setting of four for 1 hr. Exposure to DNA was terminated by the addition of deoxyribonuclease (5 μg./ml.) (Worthington Biochemical Corporation, Freehold, New Jersey) and 0·005M-$MgSO_4$ with incubation at 37° for 15 min. After treatment, cocci were centrifuged and resuspended in 1 ml. saline. Samples of 0·1 ml. were taken from the saline suspension, a 10^{-1} and 10^{-2} dilution in saline and spread on duplicate defined agar plates. Prototrophs were scored after incubation at 34° for 72 hr. Additional plates were prepared and incubated at 26° for 72 hr in crosses where the donor strain had a more optimum growth at 26° than at 34°. Plates failing to show significant numbers of prototrophic transformants were incubated for an additional 5 days. In crosses where pigment mutants were used as donors, pigment-prototrophs (double transformants) were scored after incubation for 6 days to allow for pronounced pigment production.

RESULTS

Nutritional characterization of donor strains. Many of the donor strains tested were able to produce relatively large colonies (0·5 to 2·0 mm. diam. in 96 hr) on the defined agar medium used in transformation experiments (Table 1). These were considered prototrophic with respect to adenine, L-histidine, and L-tryptophan as these nutrients were omitted from the medium.

Some of the strains tested proved to be adenine auxotrophs and were markedly stimulated by either adenine, adenosine, inosine, or hypoxanthine. For example, auxotrophs that showed no detectable growth or colonies less than 0·1 mm. diam. (leaky strains) on defined agar media produced colonies of 0·5 to 1·6 mm. diam. in 96 hr on defined agar media supplemented with 20 μg. of adenosine per ml. Adenine auxotrophy has been reported previously in strains of *Micrococcus lysodeikticus* (Grula, Luk & Chu, 1961; Kloos & Schultes, 1969); however, it was interesting to note that other strains having different species designations were also auxotrophic. Cultures of *M. luteus* strains CCM 105, CCM 408, CCM 1672 and *M. flavocyaneus* strain CCM 851 were received containing both adenine-independent and adenine-dependent cells in ratios (Ade$^+$/Ade) of 1/9, 1/116, 1/1890 and 1/36, respectively.

Transformation of nutritional markers. DNA isolated from certain strains of *Micrococcus flavocyaneus*, *M. flavus*, *M. luteus*, *M. lysodeikticus*, *M. sodonensis*, *Sarcina flava*, *S. lutea*, *S. subflava*, *Staphylococcus afermentans* and *St. flavocyaneus* was able to transform auxotrophs of *M. lysodeikticus* to prototrophy (Table 1). In all instances, the above strains transformed *M. lysodeikticus* strain ISU at near or equal to homologous, intra-strain frequencies. Variation between DNA preparations from the same strain generally did not exceed 60% of the mean value.

Adenine-dependent donor strains produced low but significant frequencies of prototrophic transformants when crossed with adenine-dependent *Micrococcus lysodeikticus* strain ISU. These results would suggest that adenine auxotrophy, in these strains, was the effect of mutation at sites different from the one present in the recipient strain. The very low transformation values obtained may be evidence of mutation in the same adenine locus, though consideration of mutation in different adjacent or closely linked loci, at this point, cannot be excluded. Studies are being pursued to determine the specific enzyme(s) affected in these auxotrophs.

Double transformation of pigment and nutritional markers. It would be of interest to extend genetic studies and test transformation of additional markers with those strains capable of transforming *Micrococcus lysodeikticus* to prototrophy. One class of mutants that can be identified readily and are produced in high frequencies is pigment mutants. For example, treatment of *M. lysodeikticus* strain ISU (*ade*$^+$-1) with NTG, to obtain auxotrophs used as recipients in this study, resulted in the production of about 2% pigment mutant, sectored colonies. These included various intensities of yellow, white, pink, orange, chartreuse (similar to the colour of *Sarcina lutea* strain ATCC 272), and a water-soluble yellow class which dispersed yellow pigment in the surrounding culture medium. Several of these mutants were selected and used as donors in crosses with yellow auxotrophic strains (Table 2). Pigment transformants were scored selectively as double transfromants with prototrophy. In this preliminary study, pigment mutants of *S. lutea* strain ATCC 272 were isolated and used as donors with recipients of *M. lysodeikticus*. The range of different colours produced by treat-

ment with NTG was similar to that found with *M. lysodeikticus*. As shown in Table 2, all mutants tested were able to transform yellow auxotrophs of *M. lysodeikticus* strain ISU to the donor colour. The fraction of double pigment-prototroph transformants/single prototroph transformants for each recipient auxotroph was similar and was approximately 1/360. Additional studies were conducted which showed an increase in the fraction of double transformants/single transformants as the DNA concentration was increased up to saturation levels (Kloos, 1969). Collectively, these results would suggest that the pigment markers are not linked to DNA fragments containing the adenine (*ade*), histidine (*hisD*), and/or tryptophan (*trpE*) loci.

DISCUSSION

Genetic exchange has been shown to be useful in measuring homology between various bacteria and is usually parallel to the results obtained with nucleic acid hybridization (Dubnau, Smith, Morell & Marmur, 1965; Marmur *et al.* 1963). The demonstration of genetic exchange between *Micrococcus lysodeikticus* and certain strains designated as species of the genera *Micrococcus*, *Sarcina*, and *Staphylococcus* implies close genetic relationships among these organisms.

Transformation with such a large array of strains bearing different species and generic designations seems, at first glance, to be most unusual. In particular, nutritional markers are usually transformed only among very closely related organisms (Dubnau *et al.* 1965). These results, however, are in close agreement with the suggestions of others based on other criteria, classifying these strains as members of a single species, *Micrococcus luteus*. All strains participating in genetic exchange had very similar GC ratios, S values, and occurred in Micrococcus subgroup-1a of the classification of Rosypal *et al.* (1966).

Sarcina aurantiaca and *Micrococcus luteus* strain ATCC 398, previously considered by Kocur & Martinec (1962) to be *M. luteus* strains, failed to produce prototrophic transformants when crossed with *M. lysodeikticus*. These strains had distinctly lower GC ratios and, according to the above classification, were placed in subgroups 2*c* and 3*b*, respectively. *Micrococcus luteus* strain ATCC 398 also has a different menaquinone pattern than that shown for strains in subgroup 1*a* (Jeffries *et al.* 1968). It should be noted that these strains failed to grow on the defined medium used in transformation experiments and, therefore, any interpretation of negative results should be taken with some caution. Prototrophy with respect to adenine, L-tryptophan, and L-histidine is uncertain in these strains.

The failure of *Micrococcus candicans*, *M. citreus*, *M. conglomeratus*, *M. roseus*, *M. rubens* and *M. varians* to transform *M. lysodeikticus* was not surprising, as these organisms differ significantly in certain physiological and biochemical features and/or GC ratio (Baird-Parker, 1965; Baddiley, Brock, Davison & Partridge, 1968; Bohacek, Kocur & Martinec, 1967; Jeffries *et al*, 1968; Rosypal *et al.* 1966). Transformation was not expected by strains of *Staphylococcus aureus* or *St. epidermidis* either, as members of the genus *Staphylococcus* as recommended by Evans *et al.* (1955) are facultative anaerobes and differ in numerous characteristics, including a very low GC ratio (32 to 35%) (Auletta & Kennedy, 1966; Baird-Parker, 1965; Garrity, Detrick & Kennedy, 1969; Jeffries *et al.* 1968). Two additional species designated *M. radiodurans* and *Sarcina ureae* failed to transform *M. lysodeikticus*. However, there is

strong evidence that these organisms should be omitted from the family Micrococcaceae (Baird-Parker, 1965; Herndon & Bott, 1969; Bohacek, Kocur & Martinec, 1967).

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Cytochemical Observations on the Localization of Sulphydryl Groups in Budding Yeast Cells and in the Phialides of *Penicillium notatum* Westling during Conidiation

By D. PITT

Department of Botany, University of Exeter, Exeter, Devon

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SUMMARY

Fluorescence and chromogenic cytochemistry confirmed the presence of –SH groups in the walls of *Candida albicans* and *C. utilis*, predominantly at budding sites. They were absent from pseudomycelia of *C. albicans* and the walls of the mycelium and the hyphal tips of *Penicillium notatum* but present in the walls of the tips of the phialides of *P. notatum*. Autoradiography after treatment with tritiated SH-blocking agents confirmed these findings.

INTRODUCTION

Certain yeast species have sulphydryl compounds as structural components of the walls with higher concentrations at sites of bud formation (Nickerson & Falcone, 1958). A conversion of disulphide (–SS–) to sulphydryl (–SH) groups in the protein of the wall may allow re-orientation of the wall fibrils and so permit plastic deformation and distension of the wall for bud formation. The walls of mycelial fungi contain relatively few –SH groups (Robson & Stockley, 1962).

Robson & Stockley (1962) fulfilled the prediction of Nickerson & Falcone (1958) that suitable cytochemical techniques might reveal –SH groups at budding sites in yeasts when they demonstrated such groups at budding sites in *Eremothecium ashbyi* and *Candida albicans* by using tritiated phenyl mercuric chloride.

The walls of the phialide tips in *Penicillium notatum* cover a zone which undergoes rapid division during sporulation. Such a zone might be analogous to the sites of bud formation in yeasts and spore formation might need high concentrations of –SH groups at such sites. A number of recent developments in histochemistry have provided highly sensitive and specific chromogenic reagents for protein bound –SH groups and these have been used in the current work to re-examine the budding process in yeasts, to seek confirmation of results by autoradiographic techniques and to examine the position in *P. notatum*.

METHODS

Organisms. Penicillium notatum Westling, *Candida albicans* (Robin) Berkhout and *C. utilis* (Henneb.) Lodder & Kreger-van Rij, designated E9, E25 and E135 respectively in the culture collection of the Exeter Botany Department, were used. *Penicillium notatum* was grown on coverslips for 3 days at 25° on discs of malt extract agar and the

yeasts were inoculated from stationary phase liquid cultures into 2% malt extract liquid medium and grown for 18 hr at 25° in shaken vessels.

Preparation of reagents. (*a*) *N*-(4-hydroxy-1-naphthyl) *iso*-maleimide (HNI) was prepared according to the method of Tsou, Barrnett & Seligman (1955). (*b*) *p-N,N*-dimethylaminophenylmercuric acetate was made by the method of Lillie (1965). (*c*) Tritiated phenyl mercuric chloride was prepared in very low yield by the method of Robson & Stockley (1962); subsequently the following procedure was devised: 0·25 ml. HCl (sp.gr. 1·18), 0·3 ml. H_2O and 25 mg. (10 mc.) ring-tritiated aniline (Radiochemical Centre, Amersham) were stirred at room temperature for 10 min. and then cooled to 0°. 0·5 ml. (0·17 g.) $NaNO_2$ was then added followed by vigorous stirring for ½ hr at 0°. 0·42 g. $HgCl_2$ was dissolved in 0·2 ml. HCl (sp.gr. 1·18), 1 ml. H_2O was added and this mixture was added dropwise to the stirred diazotate at 0° over 5 min. The mixture was stirred for a further ½ hr at 0°, the addition compound was filtered off in the cold and washed with iced water. The wet compound was then suspended in 5 ml. acetone at 20° and small amounts of an active copper powder were added over ½ hr with stirring until effervescence ceased. Stirring was continued for 20 min. and 5 ml. H_2O was added. The suspension was filtered and the precipitate washed and extracted with boiling xylene and recrystallized twice from this solvent. Yield 40 mg.

Microscopy. A Leitz 'Ortholux' microscope equipped with a transmitted light condenser and an incident light attachment was illuminated with a tungsten lamp or a 250 w high-pressure mercury vapour lamp situated in either the incident or transmitted light positions for white light and fluorescence microscopy. Unfixed material was sometimes examined but generally fixed materials were used after being coated with 0·5% collodion before staining.

Chromogenic reactions for sulphydryl groups. (*a*) The dihydroxy-dinaphthyl-disulphide (DDD) procedure of Barrnett & Seligman (1952) was done on material of *Penicillium notatum*, *Candida albicans* and *C. utilis* which had been freeze-dried and subsequently fixed for 2 to 3 hr in 1% acetic acid in absolute alcohol. Controls blocked with iodoacetate, *N*-ethyl maleimide (NEM) or phenyl mercuric chloride and others incubated with the coupling agents but no DDD were prepared. Also disulphides in the material were reduced with thioglycollate before applying the full DDD reaction with appropriate controls. (*b*) The *N*-(4-hydroxy-1-naphthyl) *iso*-maleimide method with coupling with Fast blue B salt (G. T. Gurr) or Fast blue RR salt (G. T. Gurr) according to Pearse (1969) was applied to Carnoy fixed material and material freeze-dried and fixed in 1% acetic acid in absolute alcohol for 2 to 3 hr and stored in absolute alcohol. Material of *P. notatum* and the two yeasts was stained for 24 hr and then examined by visible light and by transmitted light fluorescence microscopy using Leitz UG 1 + BG 38 filters and suppressor filters. In controls HNI was omitted from the incubation medium, the coupling stage was omitted from the full reaction, and iodoacetate or NEM was used to block –SH groups prior to incubation in HNI. (*c*) The performic acid + Alcian blue method of Adams & Sloper (1956), using performic acid prepared according to Pearse (1969), was applied to material fixed for 12 hr in Baker's formol + calcium fixative. Since this method stains both –SS– and –SH groups other material was reduced with thioglycollate and the original and newly formed –SH groups were blocked by iodoacetate. The differences between this material, that stained by the normal method, and that stained normally after iodoacetate blocking

indicated the abundance of –SS–groups in the original material. (*d*) Material was freeze-substituted or freeze-dried and fixed in 1% acetic acid in absolute alcohol for 2 to 3 hr and subjected to the Mercury orange staining procedure of Bennett & Watts (1958) for 1 to 3 days at 20°. A saturated solution of 1-(4-chloromercuriphenylazo)-2-naphthol, known as Mercury orange (Koch-Light Laboratories Ltd.), in dimethylformamide was used. Before it was stained control material was treated with a saturated solution of phenyl mercuric chloride in *n*-butanol for 48 to 72 hr or with NEM. The mercaptide azo coupling reaction for sulphydryl groups of Lillie & Glenner (Lillie, 1965) was also used, with incubation in 0·7% *p-N,N*-dimethylaminophenylmercuric acetate in 99% *iso*propanol at 25° for 24 to 48 hr. (*e*) Fresh and freeze-dried material was subjected to the NEM + salicyloyl hydrazide + zinc method of Stoward (1963). Preparations were brought to water, incubated in NEM for 4 hr at 37°, treated with salicyloyl hydrazide (Sigma Chemical Co.) for 20 to 120 min., rinsed in 0·05% sodium pentacyano-amine ferroate to extract excess hydrazide, and then examined by incident and transmitted light fluorescence microscopy using UG 1 + BG 38 filters and suppressor filters. 5% potassium alum or 0·1% Solochrome black in 5% potassium alum were used as counterstains (Stoward, 1967).

Autoradiographic methods for sulphydryl groups. (*a*) Preparations of *Penicillium notatum* and yeast smears were freeze-dried fixed in 1% acetic acid in absolute alcohol for 2 to 3 hr, taken into dioxan, incubated in tritiated phenyl mercuric chloride (0·001 M) for 72 hr, washed in two changes of acetone (15 min. each), coated with stripping film (Kodak AR 10), and exposed for 1 month at 4° before development in Kodak D 19b developer. The autoradiographs were dehydrated, cleared in xylene and mounted in 'Euparal'. (*b*) Iodoacetic acid-2-T (Radiochemical Centre, Amersham; specific activity 0·68 mc./mg.) was titrated to pH 8·0 with sodium hydroxide and made 0·1 M with respect to sodium iodoacetate-2-T. Material grown on slides was freeze-dried, fixed, coated with collodion, brought to water and incubated in 0·1 M-tritiated iodoacetate for 20 hr at 37°. It was washed three times in distilled water, coated with stripping film, exposed for 2 to 3 weeks, developed and permanently mounted.

Sulphydryl blocking and disulphide reduction reactions for protein end-groups. (*a*) Iodoacetate block. Collodion-coated preparations were treated for 20 hr at 37° with 0·1 M-sodium iodoacetate at pH 8·0. (*b*) Maleimide block. Material was treated for 4 hr at 37° with 0·1 M-*N*-ethyl maleimide in 0·1 M-phosphate buffer pH 7·4, and washed in 1% acetic acid and water. (*c*) Mercaptide block. Collodion-coated material was incubated at 20° for at least 72 hr in 0·001 M-phenyl mercuric chloride in *n*-propanol. (*d*) Thioglycollate reduction. Collodion-coated material was incubated for 4 hr at 37° in freshly prepared 0·5 M thioglycollic acid titrated to pH 8·0 with 0·1 N-NaOH, washed in water, rinsed in 1% acetic acid and washed again in water.

RESULTS

Chromophoric sulphydryl staining

In Penicillium notatum. The DDD method incorporating a number of diazotates strongly stained the tips of the phialides and the spores. The walls of the mycelium did not stain, but the cytoplasm near the hyphal tips did. Bis-coupling with Fast blue B salt stained the cytoplasm of both the mycelium and phialides pink and areas of

higher sulphydryl concentrations such as the nuclei, spores and walls at the phialide tips blue. When the monocoupling diazotate Fast black K salt was used the walls of the phialide tips and the spores stained intense black (Pl. 1, fig. 1). Staining was prevented by blocking with NEM and iodoacetate (Pl. 1, fig. 2) but only partially prevented with phenyl mercuric chloride. Control material incubated without DDD stained pale brown near the phialide tips when Fast blue salt B was used, but not with Fast black K salt or Fast red RC salt. Preparations reduced with thioglycollate stained similarly to unreduced ones, indicating that staining due to –SS–groups was negligible by this method.

The HNI procedure stained the cytoplasm a very pale purple colour by visible light, but fluorescence microscopy in which a range of diazotates was used revealed intense red staining in the nuclei, spores, cytoplasm of the hyphal tip and the walls at the tips of the phialides (Pl. 1, fig. 3); walls of the mycelium did not stain. Prior incubation of material in NEM or iodoacetate abolished the red fluorescence and replaced it by a dull general autofluorescence.

The performic acid + Alcian blue method stained nuclei and spores and especially hyphal tips and the walls near the tips of the phialides (Pl. 1, fig. 4). Preparations reduced with thioglycollate and blocked with iodoacetate before staining showed negligible staining (Pl. 1, fig, 5). A low level of staining in materials that had been blocked with iodoacetate beforehand, suggested some staining was due to –SS–groups.

The Mercury orange method of Bennett & Watts (1958) did not stain material even after several days' incubation. The *p-N,N*-dimethylaminophenylmercuric acetate procedure stained walls red at the phialide tips, but not elsewhere in the mycelium, when coupled with freshly prepared diazosafranin (Pl. 1, fig. 6).

The NEM + salicyloyl hydrazide + zinc method gave only a pale green stain with a general distribution and was not improved by counterstaining with alum or Solochrome black in alum.

In yeasts. Although the DDD, HNI and Lillie & Glenner's mercaptide methods all stained nuclei in *Candida albicans* and *C. utilis* by visible light methods, none of these methods was sensitive enough to detect sulphydryl groups in the wall. The Alcian blue method stained both nuclei and cell walls, and blocking and reduction reactions had effects similar to those observed in *Penicillium notatum.* Nuclei and cell walls of the yeast phases of *C. albicans* and *C. utilis* stained by the HNI method fluoresced a brilliant red, but there was only very slight fluorescence in the walls of the pseudomycelium of *C. albicans* (Pl. 1, fig. 7). Observations were not made on the mycelial state of *C. utilis* because such growth was not supported by the media routinely used.

The NEM + salicyloyl hydrazide + zinc procedure gave a pale green fluorescence which was almost indistinguishable from the autofluorescence of the materials.

Autoradiographic localization of sulphydryl groups

Preliminary experiments with 0·001 M-phenyl mercuric chloride to block cellular –SH groups before the DDD reaction showed that blocking was incomplete with *Penicillium notatum* and the yeasts even after 72 hr incubation in the blocking agent. Because it was impracticable to incubate longer without losing material from the slides 72 hr incubation was adopted for all experiments using tritiated phenyl mercuric chloride.

Autoradiographs of *Penicillium notatum* treated with tritiated phenyl mercuric

chloride showed grain concentrations around the spores and developing phialides but not in the walls of the mycelium. Preparations treated with tritiated iodoacetate showed concentrations of grains at the walls of the phialide tips but not in the spores (Pl. 1, fig. 8), and none in the walls of the mycelium.

Autoradiographs of yeast-like cells of *Candida albicans* and *C. utilis* treated with tritiated phenyl mercuric chloride showed grains in the walls especially at sites of bud formation and bud scars (Pl. 1, fig. 9). Similar results were obtained after treatment with tritiated iodoacetate. The concentration of grains was much less near the walls of pseudomycelium of *C. albicans*.

DISCUSSION

The role of protein-bound sulphydryl groups in maintaining the plastic condition of the cell wall in budding yeasts is now well established (Nickerson & Falcone, 1958; Nickerson, Falcone & Kessler, 1961), although McClary & Bowers (1965) challenged this view and considered that the wall neither distends nor disrupts during budding.

Robson & Stockley (1962) provided convincing autoradiographic evidence of –SH groups at budding sites in Candida and Eremothecium. The present work confirms these autoradiographic findings and supplies additional cytochemical evidence of the presence of –SH compounds in the walls of yeast cells of *Candida albicans* and *C. utilis* and their absence from the pseudomycelium of the former.

It is not known if –SH groups serve a similar function in maintaining plasticity in hyphal tips of mycelial fungi. The present work supports the findings of Zalokar (1959) in showing a higher concentration of cytoplasmic –SH groups near the hyphal tips than elsewhere in the mycelium. However, the observations of Middlebrook & Preston (1952) with Phycomyces suggest that there is no need to postulate a mechanism for maintaining sulphur in the reduced state as –SH groups in order to render the walls plastic because the orientation of the wall fibrils satisfies the requirements of extension growth and branching. The present work failed to show –SH groups in any part of the wall of *Penicillium notatum* besides the tips of the phialides. Such a localization in areas that proliferate cells rapidly during sporulation in a manner analogous to budding in yeasts supports the hypothesis that sporulation in *P. notatum* may have physiological processes in common with budding in yeasts. However, this localization does not necessarily implicate such compounds in sporulation. Biochemical analysis and electron microscope cytochemistry of the walls of developing phialides of *P. notatum* are necessary to provide further evidence.

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EXPLANATION OF PLATE

Cytochemical localization of −SH groups in *Penicillium notatum*, *Candida albicans* and *Candida utilis*.

Fig. 1. Sulphydryl groups in the walls of the phialide tips of *P. notatum* stained by the DDD procedure, coupled with Fast black K salt. × 1350.

Fig. 2. *P. notatum* stained as in fig. 1, but with prior blocking in iodoacetate. × 1350.

Fig. 3. Sulphydryl staining in *P. notatum* by the HNI reaction and observed by fluorescence microscopy. Note strong fluorescence in the spores and in the walls of the phialide tips (red in the original). × 1350.

Fig. 4. Alcian blue staining of −SS− and −SH groups in *P. notatum*. Strongest reaction in the spores and the walls of the phialide tips. × 1350.

Fig. 5. As fig. 4 but with thioglycollate reduction and iodoacetate blocking before staining; negligible staining. × 1350.

Fig. 6. Sulphydryl staining of *P. notatum* by Lillie & Glenner's mercaptide method. Staining in the spore walls and the walls of the phialide tips. × 1350.

Fig. 7. Cell wall staining of sulphydryl groups in *C. albicans* by the HNI fluorescence method showing increased staining at some sites in the wall. Note very pale cytoplasmic staining in the pseudomycelium with negligible wall staining. Coupled with Fast blue RR salt. × 1500.

Fig. 8. Arrow 1 indicates autoradiographic localization of −SH groups in the phialide tip of *P. notatum* with tritiated phenyl mercuric chloride as the blocking agent. × 1350.

Fig. 9. Autoradiographic localization of −SH groups in *C. utilis* with tritiated iodoacetate as the blocking agent. Note highest concentrations of grains at sites of budding and bud scars. × 2000.


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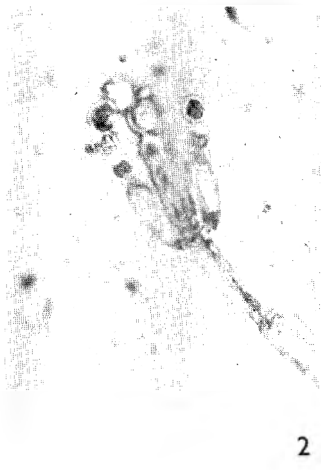
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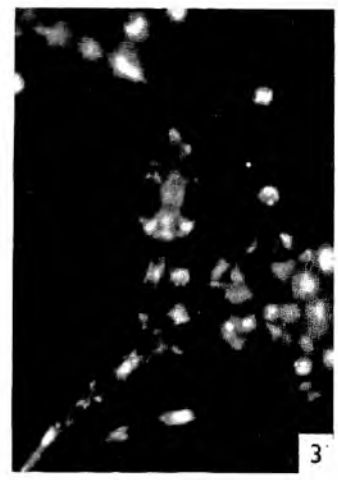
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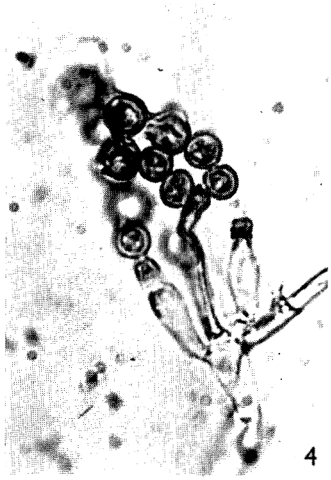
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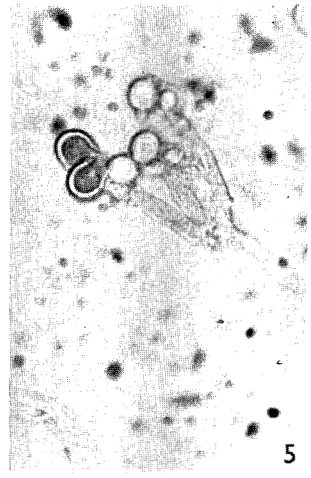
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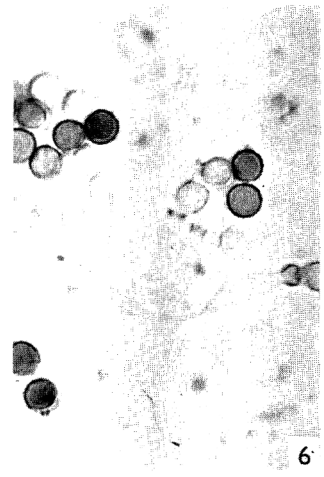
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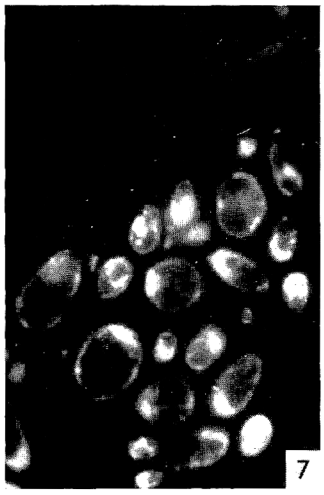
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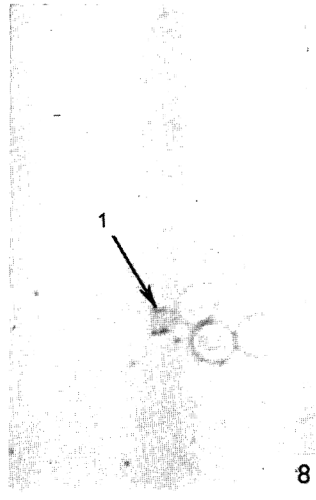

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D. PITT

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Role of Divalent Cations in the Action of Polymyxin B and EDTA on *Pseudomonas aeruginosa*

By M. R. W. BROWN AND J. MELLING
Pharmaceutical Microbiology Group, School of Pharmacy, Bath University of Technology, Bath, Somerset, BA2 7AY

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SUMMARY

Pseudomonas aeruginosa grown under conditions of Mg-depletion in batch culture in simple salts medium lost sensitivity to polymyxin B, causing lysis, release of 260 nm. absorbing materials and loss of viability. The patterns of lysis and leakage produced by polymyxin were similar with regard to the effect of growth in different Mg concentrations and the inhibitory effect of high polymyxin concentrations. The rates of lysis and leakage for any one suspension were similar. There was decreased polymyxin uptake by insensitive bacteria. Addition of Mg or one of several cations restored sensitivity both to polymyxin and to EDTA to varying degrees, but only after several cell divisions had occurred. A close similarity was observed between the effects of Mg-depletion on sensitivity to EDTA and to polymyxin; a relationship between their mechanisms of action is suggested. It is proposed that cations are essential for the synthesis of sensitive components of the envelope and may themselves be involved in the structure of the component.

INTRODUCTION

Growth of *Pseudomonas aeruginosa* in Mg-depleted cultures resulted in loss of sensitivity to the antibacterial action of ethylenediaminetetra-acetic acid (EDTA) (Brown & Melling, 1969). This phenomenon has now been further investigated and the effect of polymyxin B sulphate on *P. aeruginosa* grown in media with various Mg concentrations examined. Polymyxin was chosen since it was reported to be active against this organism (Haas & Sevag, 1953), and Newton (1954) showed its action to be antagonized by divalent cations. Polymyxin was also of interest since, unlike EDTA, it has been reported not to act by a chelation mechanism (Newton, 1953*a*). Other divalent cations were examined for ability to substitute for Mg in its role concerned with the resistance of *P. aeruginosa*, particularly in view of the finding of Eagon, Simmons & Carson (1965) who identified Mg, Ca and Zn in cell walls of *P. aeruginosa*.

METHODS

Organism. Pseudomonas aeruginosa NCTC 6750 was used throughout this work.

Chemicals. All chemicals used were of Analytical Reagent grade (AnalarR).

Cleaning procedures. All glassware was treated with chromic+sulphuric acid mixture, washed with tap water and finally with glass-distilled water.

Culture methods. The organism was maintained in a liquid medium consisting of: 0·001 M-D(+)glucose, 0·01 M-$(NH_4)_2HPO_4$, 0·01 M-$(NH_4)_2SO_4$, 0·0005 M-NaCl, 0·0005 M-KCl dissolved in glass-distilled water. In the following experiments bacteria

were grown in media identical to the above, but containing different concentrations of the divalent cations. Samples (500 ml.) of bacterial suspensions were grown in 2 l. flasks in a Mickle shaker bath (The Mickle Laboratory Engineering Co., Gomshall, Surrey) at 37·5°. Cultures with the lower concentrations of Mg gradually entered a stationary phase due to Mg-depletion. Growth eventually ceased owing to depletion of glucose at an extinction of about 0·180 measured at 470 nm. with a Unicam SP600 spectrophotometer. This extinction corresponded to a colony count of about $3{\cdot}5 \times 10^8$ bacteria/ml. When no increase in E_{470} had been observed over a period of 1 hr, samples were taken and treated with polymyxin or EDTA.

Treatment of bacteria with polymyxin or EDTA. Samples of culture (93 ml.) at 37·5° were added to 7 ml. of EDTA or polymyxin solutions of various concentrations contained in 250 ml. flasks and maintained at the same temperature. The mixture remained constant at pH 7·2.

Measurement of lysis and colony counts. These were made as described previously (Brown & Melling, 1969).

Measurement of 260 nm.-absorbing substances released from bacteria. Estimates of 260 nm.-absorbing substances released from bacteria were made by the technique of Brown, Farwell & Rosenbluth (1969). This involved removal of bacteria by membrane filtration and measurement of the extinction at 260 nm. of the filtrate with a Unicam SP500 spectrophotometer.

RESULTS

Effect of Mg-depletion on lysis by polymyxin

The E_{470} of cultures grown in a range of Mg concentrations was measured at intervals after treatment with various polymyxin concentrations. Figure 1 shows the typical effects of polymyxin treatment on bacteria grown in 1 μg./ml. (excess) and 0·05 μg. Mg/ml. (depleted), respectively. Little change in E_{470} of the culture containing 0·05 μg. Mg/ml. occurred compared to the culture containing 1 μg./ml. The changes in E_{470} observed in the latter culture indicated that lysis of bacteria occurred after treatment at the lower polymyxin concentrations, and that the rate of lysis increased as the polymyxin concentration was increased up to 32 units/ml. Further increases in polymyxin concentration resulted in decreased lysis; treatment with 256 units/ml. caused a rapid initial increase in E_{470}.

The activity of any one concentration of polymyxin in causing lysis was taken to be the decrease in E_{470} after 180 min. treatment with polymyxin compared to the E_{470} at zero time. Figure 2 shows the activity of several polymyxin concentrations in causing lysis of bacteria grown in different Mg concentrations. The rate of lysis reached a peak at a particular polymyxin concentration. There appears to be some correlation between the polymyxin concentration at which the peak occurred and the Mg concentration in the growth medium, the peak being reached at a lower polymyxin concentration as the Mg concentration increased. The culture containing 4 μg. Mg/ml. was an exception, possibly due to antagonism of polymyxin by Mg (Newton, 1954).

Figure 3 shows the effect of growth in different Mg concentration on the maximum rate of lysis resulting from polymyxin treatment and it can be seen that the greatest change occurred at Mg concentration below 0·5 μg./ml. An almost identical pattern of sensitivity to lysis with Mg-depletion has been shown with EDTA (Brown & Melling 1969).

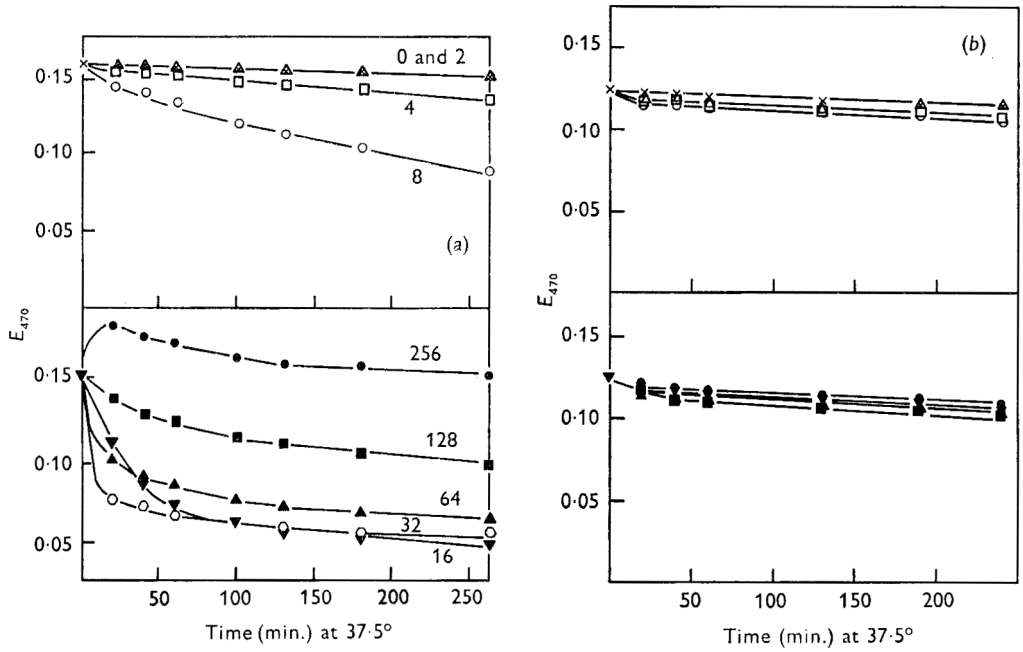

Fig. 1. Effect of polymyxin concentration on lysis of *Pseudomonas aeruginosa* grown in medium with: (*a*) 1 μg. Mg/ml.; bacteria not Mg-depleted. (*b*) 0·05 μg. Mg/ml.; bacteria Mg-depleted. Polymyxin concentrations (units/ml.) were: ×, zero, △, 2; □, 4; ○, 8; ▼, 16; ◇, 32; ▲, 64; ■, 128; ●, 256.

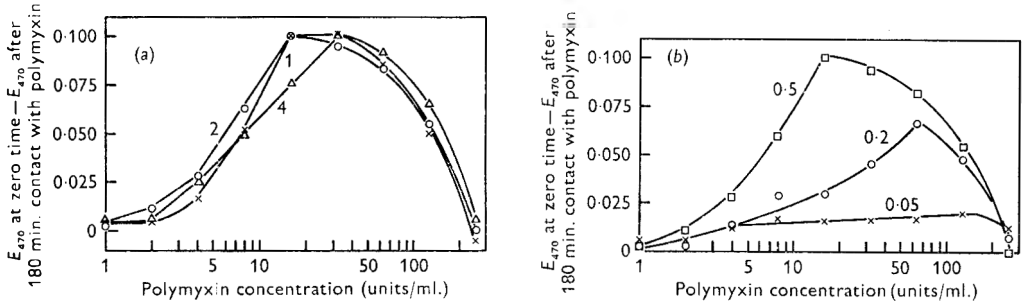

Fig. 2. Effect of polymyxin concentration on rate of lysis of *Pseudomonas aeruginosa* grown in media containing different Mg concentrations. Mg concentrations (μg./ml.) were: (*a*) ×, 1; ○, 2; △, 4; (*b*) ×, 0·05; ○, 0·2; □, 0·5.

Effect of Mg-depletion on release of 260 nm.-absorbing substances by polymyxin

Release of material absorbing at 260 nm. from *Pseudomonas aeruginosa* treated with polymyxin followed a similar pattern to that observed for lysis by this antibiotic. Typical results are shown in Fig. 4 for bacteria grown in 1 μg. and 0·05 μg. Mg/ml., respectively. Clearly little loss of 250 nm.-absorbing material occurred from bacteria grown in the lower magnesium concentration. Figure 5 shows the activity of several polymyxin concentrations in causing leakage from bacteria grown in different Mg concentrations. As the polymyxin concentration increased the rate of leakage also

increased to a maximum and then was decreased at high concentration. Examination of the effect of growth in different Mg concentrations on leakage (Fig. 6) showed that the maximum rate of leakage increased as the Mg concentration in the growth medium increased from 0·05 to 0·5 μg./ml.

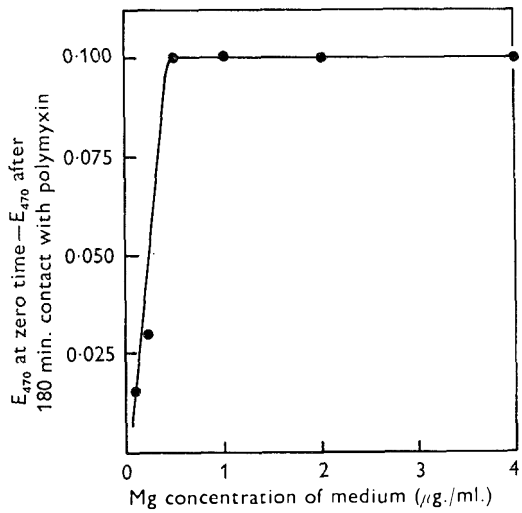

Fig. 3. Maximum lysis (E_{470}) by polymyxin (units/ml.) of *Pseudomonas aeruginosa* grown in media containing graded Mg concentrations.

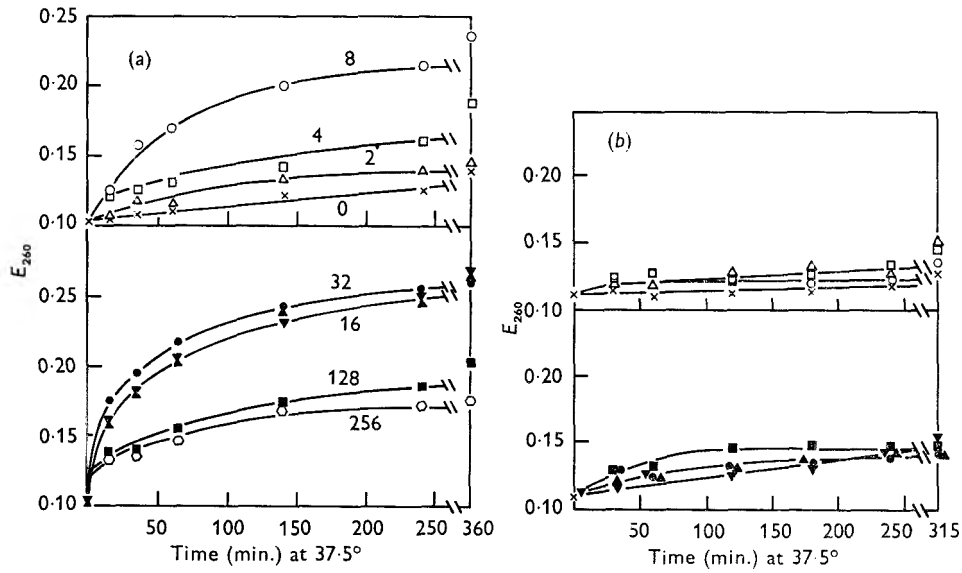

Fig. 4. Effect of polymyxin concentration on leakage (E_{260}) from *Pseudomonas aeruginosa* grown in medium with: (*a*) 1 μg. Mg/ml.; bacteria not Mg-depleted. Polymyxin concentrations (units/ml.) were: ×, zero; △, 2; □, 4; ○, 8; ▼, 16; ●, 32; ▲, 64; ■, 128; ○, 256. (*b*) 0·05 μg. Mg/ml.; bacteria Mg-depleted. Polymyxin concentrations were: ×, zero; ○, 2; □, 4; △, 16; ▼, 32; ● 64; ▲, 128; ■, 256.

Effect of Mg-depletion on release of 260 nm.-absorbing substances by EDTA

In a previous paper (Brown & Melling, 1969) dealing with the effect of EDTA on Mg-depleted *Pseudomonas aeruginosa*, the effect of EDTA on release of 260 nm.-absorbing material was not reported. This was due to an anomaly in the measurement of E_{260} in the presence of EDTA, which resulted in higher values than expected from the sum of the components. This anomaly was resolved for present purposes by making a comparison between the observed E_{260} values of solutions containing graded amounts

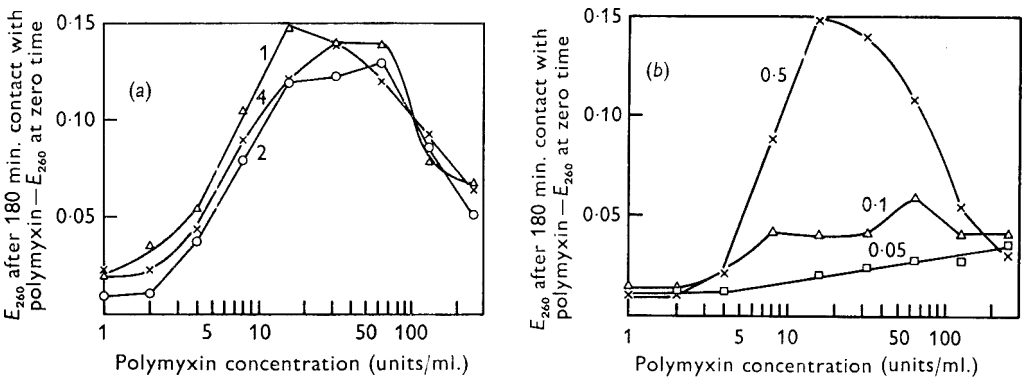

Fig. 5. Activity of various polymyxin concentrations in causing leakage (E_{260}) from *Pseudomonas aeruginosa* grown in media containing different Mg concentrations. Mg concentrations (μg./ml.) were: (*a*) △, 1; ○, 2; ×, 4; (*b*) □, 0·05; △, 0·1; ×, 0·5.

of culture filtrate + EDTA, and the expected E_{260} assuming the extinctions of EDTA and filtrate to be additive (Melling, 1968). For example, the E_{260} of a solution containing 1000 μg. EDTA/ml. was 0·007, and the E_{260} of culture filtrate was 0·138. Thus, the expected E_{260} of the mixture was 0·145; but the observed value was 0·163. However, the E_{260} of EDTA and 260 nm.-absorbing material released from bacteria washed free from the growth medium was additive. It seems probable that EDTA forms a complex with some metabolic product of *P. aeruginosa* and that this complex absorbs more strongly at 260 nm. than do its components. An extinction greater than additive was not observed with cell exudate from washed bacteria; this suggests that the complex was formed with an extracellular product of metabolism rather than the characteristic leakage products.

Release of 260 nm.-absorbing substances from *Pseudomonas aeruginosa* grown in different Mg concentrations and treated with EDTA was estimated. Typical results are shown in Fig. 7. The zero-time readings were obtained by adding the particular EDTA concentration to a sample of the culture filtrate. Thus, increases in E_{260} above the zero reading were solely a result of cell leakage since the non-additive increase in absorption did not occur with leakage products. Inspection of Fig. 7 shows that bacteria grown in the lower Mg concentration lost little 260 nm.-absorbing material as compared with those grown in the higher concentration.

The activity of any EDTA concentration in causing leakage was measured as the increase in E_{260} of the culture filtrate after 180 min. treatment with EDTA. Figure 8 shows the activity of EDTA in causing leakage from *Pseudomonas aeruginosa* grown

in different Mg concentrations. The pattern of leakage was very similar to that for lysis caused by EDTA (Brown & Melling, 1969). The EDTA concentration required to produce maximum lysis was greater the higher the Mg concentration; the maximum rate of lysis increased as the Mg concentration in the growth medium increased.

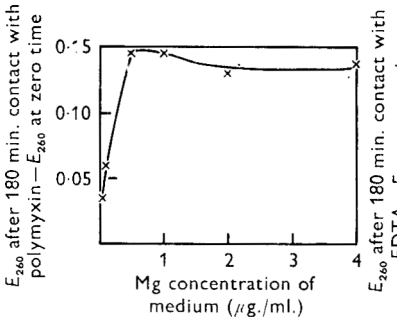

Fig. 6

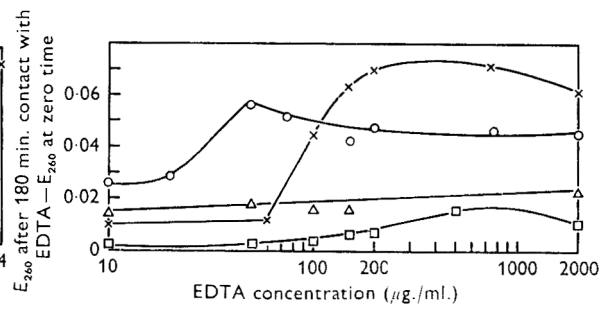

Fig. 8

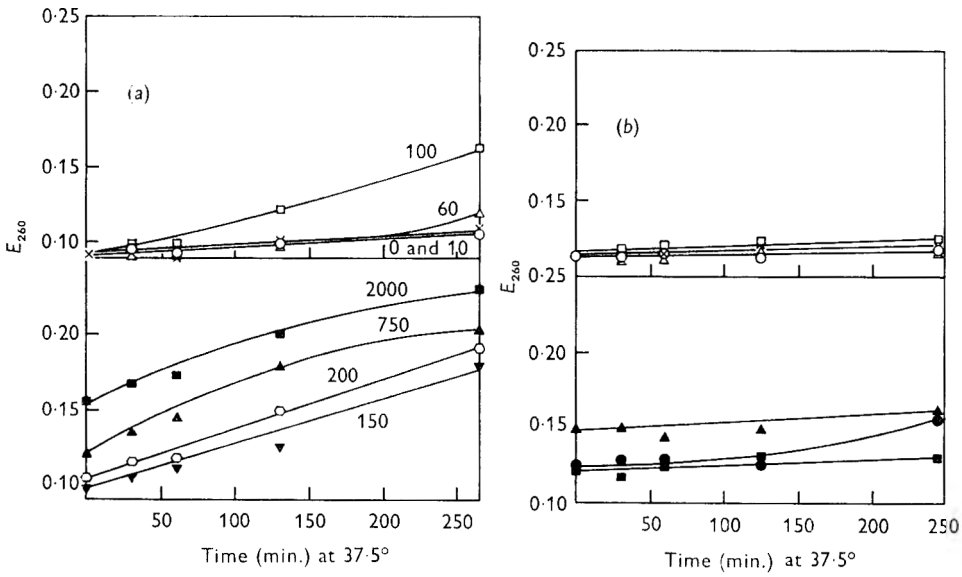

Fig. 7

Fig. 6. Maximum leakage (E_{260}) caused by polymyxin from *Pseudomonas aeruginosa* grown in media containing graded Mg concentrations.

Fig. 7. Effect of EDTA concentration on leakage (E_{260}) from *Pseudomonas aeruginosa* grown in medium with: (*a*) 4 μg. Mg/ml.; bacteria not Mg-depleted. EDTA concentrations (μg./ml.) were: ×, zero; ○, 10; △, 60; □, 100; ▼, 150; ○, 200; ▲, 750; ■, 2000. (*b*) 0·05 μg. Mg/ml.; bacteria Mg-depleted. EDTA concentrations (μg./ml.): ○, zero; △, 50; ×, 100; □, 150; ■, 200; ●, 500; ▲, 750.

Fig. 8. Activity of various EDTA concentrations in causing leakage (E_{260}) from *Pseudomonas aeruginosa* grown in media containing graded Mg concentrations. Mg concentrations (μg./ml.): □, 0·05; △, 0·1; ○, 0·5; ×, 4.

Effect of polymyxin on colony count of Mg-depleted bacteria

Two polymyxin concentrations were tested for their effect upon the viability of *Pseudomonas aeruginosa* grown in 1 and 0·05 μg. Mg/ml. Dilutions for counting were made in lecithin broth. These two Mg concentrations were selected since they resulted in the production of sensitive and resistant bacteria, respectively, when lysis and leakage were the parameters being measured. The results are shown in Fig. 9*a*. Unlike the effects on lysis and leakage 256 units/ml. polymyxin caused a greater loss of viability compared to 16 units/ml. Figure 9*b* shows the effect of growth in different Mg concentrations on the decrease in colony count after 180 min. resulting from treatment with 16 units polymyxin/ml. Again, bacteria grown in the lower Mg concentrations showed the greater resistance to polymyxin.

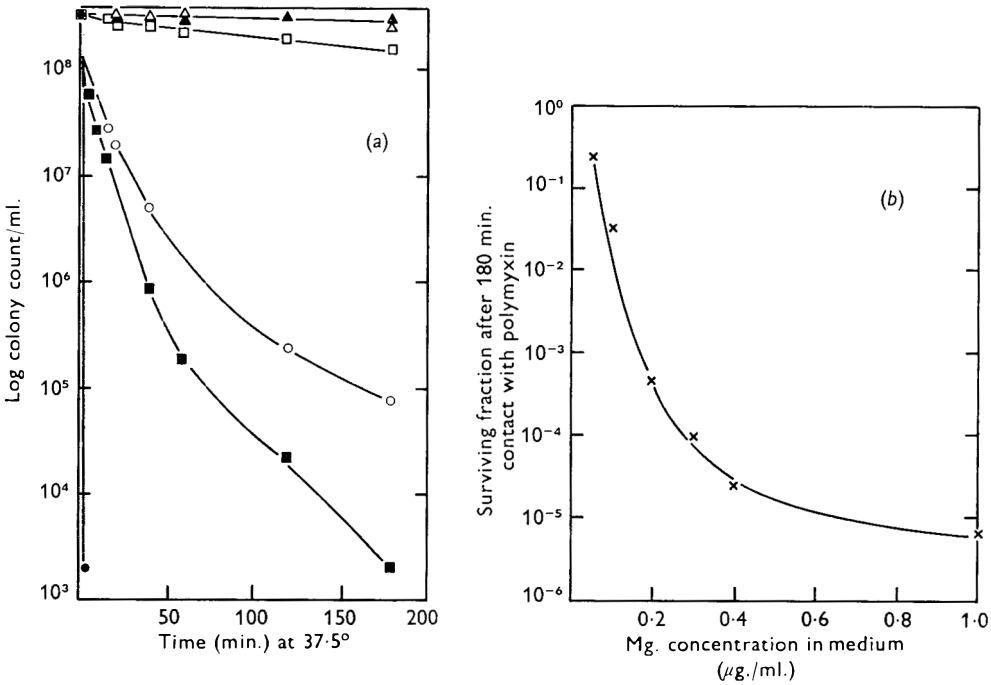

Fig. 9. Effect of treatment with polymyxin on the viability of *Pseudomonas aeruginosa* grown in media containing different Mg concentrations: (*a*) Mg concentrations (μg./ml.) were: open symbols, 0·05 (Mg-depleted); closed symbols, 1 (Mg-plentiful). Polymyxin concentrations (units/ml) were: △, ▲, zero; □, ■. 16; ○, ●, 256. (*b*) Polymyxin concentration was 16 units/ml.

Effect of adding Mg and other divalent cations to Mg-depleted cultures

Bacteria were grown in a low Mg concentration (0·05 μg./ml.) and the concentration increased to 1 μg./ml. at various times before growth ceased because of glucose depletion. Samples of the ensuing stationary phase culture were taken and treated with 16 units polymyxin/ml. or 375 μg. EDTA/ml. Since release of 260 nm.-absorbing material and loss of viability caused by the two agents were reflected by lysis of bacteria, the change in E_{470} of cultures was the parameter chosen to examine this. The

data in Table 1 indicate that the longer the bacteria had grown in a high Mg concentration the more susceptible they became to the two agents. The addition of Mg to cultures whose growth rate was decreased because of Mg-depletion caused an immediate increase in growth rate; subsequent measurements showed no immediate change, but rather a very gradual one, in sensitivity to polymyxin or EDTA.

Addition of 1 μg. Mg/ml. after cell division had ceased because of glucose depletion did not render the bacteria sensitive to EDTA or polymyxin even when 6 hr had elapsed before treatment with these agents began. Thus cell division in a high Mg concentration was required to render the organism sensitive to these agents.

Table 1. *Effect on sensitivity to lysis by polymyxin or EDTA of adding 1 μg. Mg/ml. to cultures of Pseudomonas aeruginosa containing 0·05 μg. Mg/ml., at intervals before the cultures entered stationary phase*

Time (hr) before stationary phase	E_{470} at time zero − E_{470} after 180 min. treatment with: Polymyxin (16 units/ml.)	EDTA (375 μg./ml.)
0	0·010	0·009
0·75	0·019	0·016
2·0	0·050	0·020
4·5	0·095	0·057
5·5	0·114	0·070
25·0	0·103	0·092
Control: no extra Mg	0·018	0·011

Table 2. *Sensitivity to lysis of cultures of Pseudomonas aeruginosa grown in the presence of different divalent cations and treated with polymyxin or EDTA*

Cation	Concentration (μg./ml.)	Decrease in E_{470} after 180 min. treatment with: Polymyxin (16 units/ml.)	EDTA (375 μg./ml.)
Mg	0·05	0·011	0·005
Mg	1·0	0·103	0·092
Be	0·37	0·011	0·009
Ca	1·65	0·073	0·043
Sr	3·60	0·064	0·005
Ba	5·65	0·045	0·005
Zn	2·69	0·040	0·024

Pseudomonas aeruginosa was grown with 0·05 μg. Mg/ml. and with other divalent cations in amounts which, on a molar basis, were equivalent to 1 μg. Mg./ml. Cultures were treated with polymyxin or EDTA as described above and the decrease in E_{470} after 180 min. contact with these agents determined. The data in Table 2 show that other divalent cations were able to replace Mg in varying degrees in its role concerned with sensitivity. The effects of the different cations varied according to the antibacterial agent used.

DISCUSSION

The effects of polymyxin on *Pseudomonas aeruginosa* grown in Mg concentrations above 0·5 μg./ml. correspond closely to findings of other workers. Newton (1953*b*) observed that increasing the polymyxin concentration resulted in increased leakage from *P. aeruginosa* up to a maximum and any further increase in the polymyxin concentration gave decreased leakage. The initial increase in E_{470} observed on treatment of *P. aeruginosa* grown in 1 μg. Mg/ml. with high polymyxin concentrations (Fig. 1) is also paralleled by the finding of Hugo & Longworth (1964). These authors reported that the E_{500} of suspensions of *Escherichia coli* and *Staphylococcus aureus* increased after treatment with chlorhexidine and attributed the increase to a change in the light-scattering properties of the bacteria as a result of uptake of chlorhexidine. Not only did the activity of polymyxin in causing lysis, leakage and loss of viability of *P. aeruginosa* diminish as the Mg concentration in the growth medium was decreased to 0·05 μg./ml., but also no initial increase in E_{470} occurred. Presumably there was little or no uptake of polymyxin by the pseudomonad. The patterns of lysis and leakage produced by polymyxin were very similar, both with regard to the effect of growth in different Mg concentrations and the inhibitory effect of high polymyxin concentrations. Also, the rates of lysis and leakage of a particular suspension were very similar, suggesting that these phenomena resulted from the action of polymyxin on a single site. There is evidence that polymyxin acts on the cytoplasmic membrane (Newton, 1955) and the observations of Warren, Gray & Yurchenco (1957) that pretreatment of some Gram-negative bacteria with polymyxin rendered them sensitive to lysozyme, suggested that polymyxin, like EDTA, may also affect some relatively superficial component of the cell wall.

The decrease in colony count of suspensions of *Pseudomonas aeruginosa* treated with polymyxin (Fig. 9*a*) was greater than the change in E_{470} or E_{260}. Therefore, the presence of another site of action of polymyxin seems possible particularly as Nakajima & Kawamata (1966*a*, *b*, *c*) indicated that colistin affected the RNA of *Escherichia coli*. There is a similar possibility for EDTA in view of the observation of Neu, Ashman & Price (1967) that EDTA affected RNA of *E. coli*.

Not only were lysis and leakage, caused by polymyxin (and EDTA), diminished by growing *Pseudomonas aeruginosa* in low Mg concentrations, but so too was the decrease in colony count. Loss of viability may possibly result from the effect of polymyxin on RNA; if this were so either the cell envelope altered when bacteria were grown in low Mg concentrations and no longer permitted these agents to enter the cell, or some alteration in the RNA occurred. The first possibility seems more probable for two reasons. Firstly, there was a definite indication that uptake of polymyxin by the bacteria was decreased as a result of growth in a low Mg concentration; there was a lack of initial increase in E_{470} with high polymyxin concentrations. Secondly, changes in RNA seem less likely in view of the importance of its structure and biological function. Another possibility is that lethal membrane damage preceded lysis and leakage and occurred at a faster rate.

The increase in resistance of *Pseudomonas aeruginosa* which resulted from growth in low Mg concentrations was not associated with an alteration in growth rate in every case. Increasing the Mg concentration to values up to 0·5 μg./ml. had a marked effect in increasing sensitivity to polymyxin (Fig. 2), but such a variation altered the

growth rate only below about 0·1 μg. Mg./ml. (Brown & Melling, 1969). It appears therefore that Mg is used preferentially for RNA synthesis; only when this requirement is satisfied is Mg available for other needs.

Newton (1954) proposed that antagonism of the action of polymyxin by Mg and other divalent cations resulted from competition between polymyxin and the cations for some negatively-charged site. He did not consider that a direct interaction of Mg and polymyxin was likely in view of the finding (Newton, 1953*a*) that 400 atoms of Mg were required to annul the antibacterial effect of one molecule of polymyxin. However, the striking similarity of the changes in resistance to polymyxin and to EDTA, produced in *Pseudomonas aeruginosa* by growth in low Mg concentrations suggest either a common site of action or at least a relationship between their sites of action. One explanation might be that Mg (or other cation) is required for synthesis of some sensitive component of the cell envelope and may itself be involved in the structure of this component. Thus, polymyxin might disrupt or be taken up by some negatively-charged site in the wall, while EDTA might chelate with the cation itself; in the absence of cations, sites for both inhibitors would be lacking. A polymyxin-sensitive wall site might be phospholipid which has been identified in the wall of *P. aeruginosa* and is associated with cations (Gordon & MacLeod, 1966; Clarke, Gray & Reaveley, 1967; Bobo & Eagon, 1968). This hypothesis is consistent with the work of Few (1955) who showed that polymyxin E reacted with several lipids.

Cox & Eagon (1968) reported that treatment of cell walls of *Pseudomonas aeruginosa* with EDTA caused solubilization of carbohydrates, divalent cations, some straight-chain saturated fatty acids, a quantity of unidentified fatty acids and some phosphorus (but no phospholipid). These authors suggested that divalent cations may form cross-linkages via phosphate groups contained in the lipoprotein and lipopolysaccharide components of the cell wall. They suggested that both intra- and inter-molecular cross-linking may occur throughout those components. In the absence of divalent cations such cross-linking could not occur and perhaps, as in the case of *Aerobacter aerogenes*, deprivation of Mg results in a variation in the organic constituents of the cell wall (Ellwood & Tempest, 1967). In such a case cross-linking by divalent cations might no longer be necessary for stability. Nevertheless it is especially interesting that these Mg-limited *A. aerogenes* had an increase in the cell wall content of lipopolysaccharide.

Although Mg has been shown to be adsorbed by the surface of bacteria (Tempest & Strange, 1966) it has been shown in the present work that this is not sufficient to render *Pseudomonas aeruginosa* sensitive to EDTA or polymyxin. Cell division must occur in a high Mg concentration before bacteria grown in a low Mg concentration become sensitive to these agents (Table 1). Since the mean generation time of *P. aeruginosa* under the particular growth conditions was about 90 min., at least three divisions were needed before the sensitivity approached that of bacteria grown in a high Mg concentration throughout. It therefore seems reasonable to suggest that either incorporation of Mg into the cell wall or synthesis and incorporation of some component dependent upon Mg must occur to produce sensitive organisms.

Cations other than Mg varied in their effect on the sensitivity of *Pseudomonas aeruginosa* to EDTA or polymyxin (Table 2). The greatest increase in sensitivity to EDTA resulted from addition of Ca. The effect of Zn was less, and Be, Sr and Ba had no effect. With polymyxin, however, the order of effectiveness was Ca, Sr, Ba, Zn;

beryllium had no effect. The order of chelation of these metals by EDTA, over the range pH 6·5 to 8·5 has been given as Zn > Ca > Mg > Sr > Ba (Welcher, 1958). Thus, two types of specificity appear to operate. First, there is the specificity of the organism for particular cations which would account for the fact that no other cation was as effective as Mg in producing sensitivity to EDTA and polymyxin. Secondly, there is the specificity of the inhibitor. The interaction of these two types of specificity may account for the fact that although Zn is most strongly chelated by EDTA, Ca and Mg were more effective in producing sensitive pseudomonads. The finding of Eagon *et al.* (1965) that magnesium (0·2 %), calcium (0·15 %) and zinc (0·09 %) were constituents of the cell wall of *P. aeruginosa* may give a measure of the specificity of the cell wall for particular cations under the growth conditions used.

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An Analysis of the Distribution of Volumes amongst Spores of the Cellular Slime Mould *Dictyostelium discoideum*

By M. J. SACKIN AND J. M. ASHWORTH
M.R.C. Microbial Systematics Unit and Department of Biochemistry, School of Biology, Leicester University, Leicester, LE1 7 RH

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SUMMARY

The mean volume of a diploid spore population is twice that of a haploid spore population; it is assumed that the mean volume of an aneuploid spore population is a similar reflexion of its ploidy. The distribution of volumes amongst a population of spores of *Dictyostelium discoideum* was analysed in terms of frequency of occurrence of diploid, aneuploid and haploid cells; in a wild-type strain (NC-4) these cell types were present in the ratio 8:3:89. It is suggested that 'metastable' strains are mutants in which this ratio has been altered.

The life cycle of the cellular slime mould *Dictyostelium discoideum* is divided into two mutually exclusive phases. In the first, feeding, phase the organism exists as solitary amoeboid cells and in the second, differentiation, phase these hitherto solitary amoeboid cells associate and, without any change in number, produce a fruiting body consisting of spore and stalk cells. The spores germinate in a suitable environment to give amoeboid cells, each spore giving one myxamoeba. The stalk cells are non-viable. Recent studies of mutants derived from a haploid strain of *D. discoideum* have shown that genetic exchange can occur at a low frequency during this life cycle (Loomis & Ashworth, 1968; Sinha & Ashworth, 1969; Loomis, 1969). Segregation occurs from heterozygous diploids in a manner which suggests haploidization *via* stepwise and random chromosome loss, rather than by meiotic division. Direct chromosome counts during the period of haploidization support this suggestion (Sinha & Ashworth, 1969). It appears, therefore, that *D. discoideum* has a parasexual cycle of the type described by Pontecorvo (1956) for *Aspergillus* and shown in Fig. 4. The genotype of the spores must therefore be identical with the genotype of the myxamoebae from which they are derived. There should thus exist, in any population of spores, diploid, haploid and aneuploid genotypes. Sussman & Sussman (1962) suggested that the ploidy of a spore could be correlated with its size. They reported that the mean major diameter of a diploid spore was about one and a half times the mean major diameter of a haploid spore.

We report here that the mean volume of a diploid spore population is twice that of a haploid spore population and that the distribution of volumes amongst a spore population can therefore be analysed in terms of the frequency of occurrence of haploids and diploids. Such an analysis also enables the frequency of occurrence of aneuploids to be estimated. The nature of the distribution of ploidy types in the population can be calculated if it be assumed that very few non-viable aneuploid

myxamoebae produce spores and that when aneuploids are produced from diploids each chromosome has an equal and constant chance of being lost at each division. This analysis predicts the occurrence, and describes the nature, of strains isolated by Sussman & Sussman (1962) and called by them 'metastable'.

METHODS

Organisms. Dictyostelium discoideum NC-4 is a haploid strain isolated by Raper (1935). *Dictyostelium discoideum* H-1 is a diploid, yellow pigment producing, strain isolated by Sussman & Sussman (1962) from a mixed culture of a mutant strain which produces a brown pigment and another mutant strain which produces a white pigment. Both strains were the gift of Dr M. Sussman (Brandeis University, Waltham, Mass., U.S.A.).

Volume determination. Myxamoebae were grown in association with *Aerobacter aerogenes* on agar plates and allowed to form fruiting bodies as described by Sussman (1966). Spores were collected from agar plates 36 h after the disappearance of the bacterial lawn. They were collected in a drop of 0·9 % sterile NaCl in a platinum loop and dispersed in 60 ml. of sterile 0·9 % NaCl. Volume measurements were made by using a Coulter Counter Model B with a 30 μm. aperture. This aperture size is suitable for the analysis of particles whose mean diameters fall in the range 2 to 20 μm. The mean major diameters of *D. discoideum* spores are in the range 7 to 10 μm.; similar results were obtained when a 100 μm. aperture was used. Measurements were made with the following settings on the instrument: 1/amplification = 1/aperture current = 4, upper threshold setting = 20 and locked with the lower threshold setting. Measurements were taken at the odd numbered settings of the lower threshold dial. Samples (0·5 ml.) of the spore suspensions at $\leqslant 3 \times 10^4$ spores/ml. were counted at each setting, this corresponds to a counting rate of $< 10^4$ spores/ml. Harvey & Marr (1966) showed that at counting rates greater than this the unmodified Coulter Counter gives seriously inaccurate volume distributions. The spore suspensions were stirred continuously during the measurements which are expressed in terms of the lower threshold setting of the instrument. By using spherical puff ball spores of mean diameter 3·6 μm. as a standard it was found that 10 such units correspond to a particle of volume 42·5 μm^3.

RESULTS

The distribution of volumes amongst a population of spores of the diploid strain H-1 ($n = 14$) of *Dictyostelium discoideum* is shown in Fig. 1. In this, and subsequent, distributions the particles with a volume < 4 are disregarded. This part of the distribution is caused by dust, particles from the sporangium etc., and not spores. Spores from strain H-1 were plated out clonally with *Aerobacter aerogenes*; one white and one brown clone were picked from amongst the predominantly yellow (H-1) clones. The spores from these strains were transferred to a fresh growth plate, allowed to germinate, the myxamoebae allowed to grow and the resulting spores isolated and analysed. The results of an analysis of the white spores are shown in Fig. 1. The size distribution of the spores prepared from the brown clone was like that of the white spore distribution, with a mean of 11·0. Similar strains have been isolated from H-1 by Sussman & Sussman (1963) who have shown, cytologically, that they are haploid ($n = 7$). The mean

volume of the diploid spore population (22) is exactly twice that of the two haploid spore populations derived therefrom (both 11).

Spores from strain H-1 were also subcultured without cloning. There was a progressive change in the volume distributions after each mass sub-culture (Fig. 2). In our hands strain H-1, although much more stable than other diploids (Sinha & Ashworth, 1969), could only be kept by repeated isolation of yellow spore-forming clones. Eventually, after frequent mass sub-culturing, the stock was 'lost' and became predominantly haploid. The term 'stable' as applied to strain H-1 is thus a relative term.

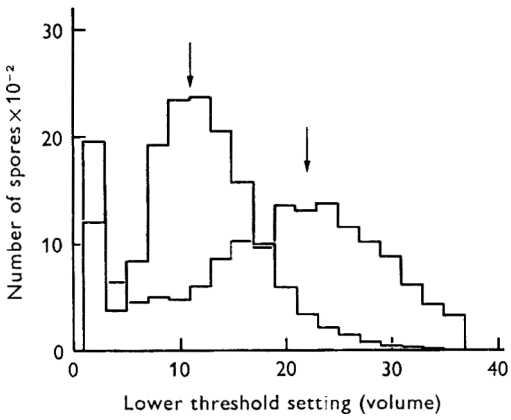

Fig. 1

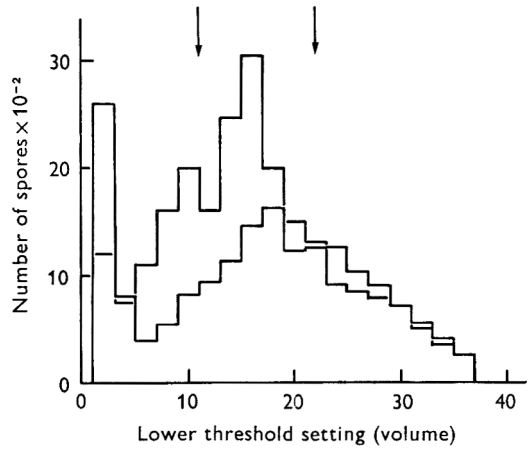

Fig. 2

Fig. 1. Distribution of volumes amongst spore populations derived from the diploid stock H-1 (mean 22) and from a haploid stock derived from H-1 (mean 11). The arrows mark the means.

Fig. 2. Distribution of volumes amongst spore populations derived from a clone of the diploid stock H-1 after 3 and 6 non-clonal sub-cultures. The arrows mark the mean volumes of the haploid and diploid populations.

The distribution of volumes amongst a population of spores from the haploid strain NC-4 is shown in Fig. 3*a*: the distribution is similar in shape to that of the haploid stocks derived from the diploid stock H-1. The mean volume of NC-4 (12·5) was, however, slightly but significantly larger than the mean volumes found for the white and brown haploid stocks isolated from H-1 (11·0), in agreement with the observations of Sussman & Sussman (1962). This volume distribution (Fig. 3*a*) is a normal distribution curve skewed to large volumes. We have shown that the volume of a spore can be correlated with its chromosome number and thus this distribution can be analysed in terms of contributions from haploid and diploid spores. At low volumes (below 12) the contribution of the haploid spores to the population will predominate. By using the techniques described by Harris (1968) the mean and standard deviation of the haploid population were calculated and the appropriate normal distribution fitted to the experimental curve. Subtraction of this distribution from the experimental distribution (Fig. 3*b*) will leave the contribution of the non-haploid spores to the total. At large volumes (above 25) the contribution of the diploid spores will predominate. By using the same techniques, a normal distribution

curve can be fitted to, and subtracted from, the experimental curve. A considerable amount of the distribution remained unaccounted for after these procedures.

Sinha & Ashworth (1969) showed by using genetic techniques that diploid stocks of *Dictyostelium discoideum* haploidized *via* transient aneuploids; the parasexual cycle they suggested is shown in Fig. 4. If the exact relationship between spore volume and chromosome number which we have shown to exist for haploid and diploid spores also holds for aneuploid spores then that portion of the total volume distribution of strain NC-4 which is unaccounted for after removal of the haploid and diploid contributions must be ascribed to aneuploid spores. The ratio of haploids:aneuploids:diploids calculated from the distribution shown in Fig. 3 is 89:3:8.

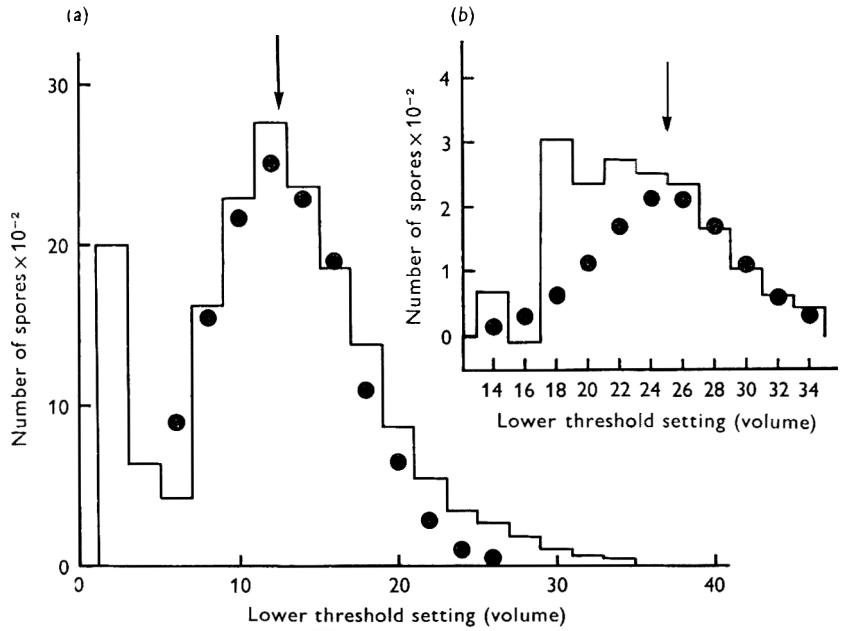

Fig. 3. (*a*) Distribution of volumes amongst a spore population derived from *D. discoideum* NC-4. ●, normal distribution with $\bar{x}$ = 12·5 and S.D. = 4·5 fitted to the observation distribution. (*b*) Volume distribution remaining after subtraction of the calculated normal distribution from the data of Fig. 3 (*a*). ●, Normal distribution with $\bar{x}$ = 25 and S.D. = 4·5 fitted to these volumes. In both cases the arrows mark the mean volumes.

Analysis of the aneuploid population

Aneuploid spores may be described as A_8, A_9, A_{10}, A_{11}, A_{12} or A_{13} classes according to the number of chromosomes they contain. Each class may be further subdivided into several different chromosome configurations. There will thus be seven different chromosome configurations for the A_8 class. An aneuploid spore is derived from an aneuploid myxamoeba which is, in turn, derived either from an aneuploid myxamoeba containing more chromosomes or a diploid myxamoeba (Fig. 4). The mechanism whereby chromosomes are lost at division is unknown but it would seem plausible that the chance of any particular chromosome being lost from a diploid or aneuploid at any one division is constant. If a diploid (or aneuploid) loses two identical chromosomes then it will presumably be non-viable even though it may still have more than

seven chromosomes. Some of these non-viable aneuploids may be capable of forming spores but the chances of their doing so seem slight; even if they did they would comprise a very small fraction of the total aneuploid population, being diluted out by the viable aneuploids. We can thus neglect the contribution made by the non-viable aneuploids (if any) to the total spore population. Two mechanisms can be suggested whereby a new aneuploid myxamoeba is produced. One possibility is that the chromosomes of an aneuploid replicate and these replicated chromosomes are then completely but unequally shared between two, or perhaps more, daughters. The

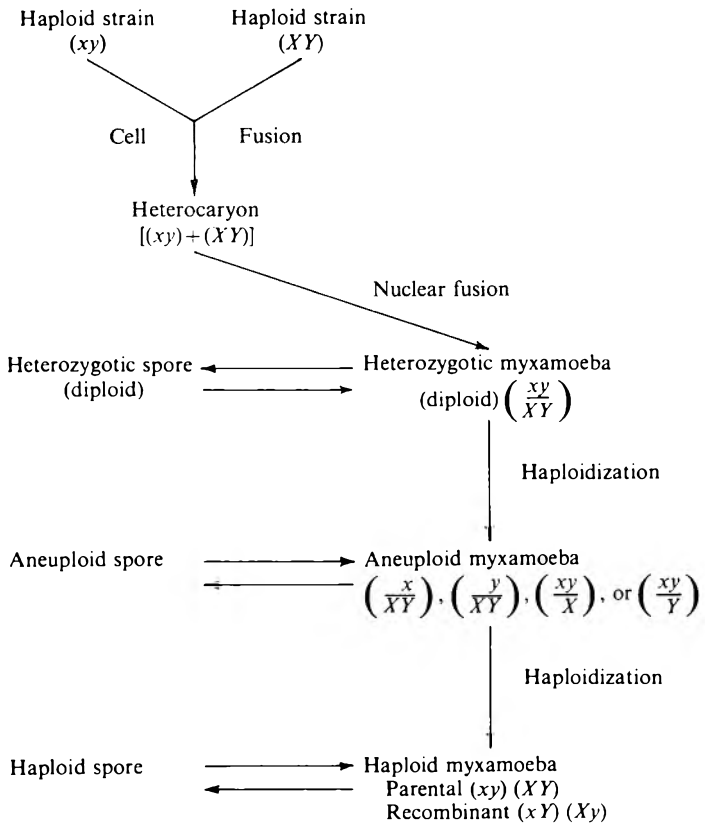

Fig. 4. The parasexual cycle of *D. discoideum*. x/X and y/Y represent two unlinked genes and the genotype of the various kinds of nuclei are enclosed in parentheses (Sinha & Ashworth, 1969).

other possibility is that no replication occurs but that within one division time an aneuploid divides to give either another aneuploid with fewer chromosomes, or a haploid and the extra chromosomes (which will be less than seven) are degraded or ejected.

The first possibility is difficult to handle mathematically. It is also unlikely in the case of *Dictyostelium discoideum* where the aneuploids are unstable (Sinha & Ashworth, 1969; Loomis, 1969). The second possibility is easier to handle mathematically and is in accord with the known instability of aneuploids. These two possibilities are not mutually exclusive. It is possible to imagine partial replication to occur before

division, and this might explain the rare tripolar mitotic figures which have been seen by us and by Sussman & Sussman (1963). However, we shall disregard partial replication here (see Discussion).

Let P be the probability that any given chromosome disappears during one division cycle. Suppose that the equilibrium frequencies of A_k cells are P_k ($k = 8, 9, \ldots, 14$). During one division cycle any k-aneuploid will have been derived from an aneuploid of class A_j, say, with a higher or equal ploidy number. Thus j may equal $k, k+1, \ldots,$ or 14. For all pairs of k and j ($8 \leqslant k \leqslant j \leqslant 14$) we shall derive an expression for the proportion of A_k cells which derive from A_j cells during one division cycle. This proportion will be a multiple of P_j and will also depend on P. Thus P_k will be a linear combination of $P_k, P_{k+1}, \ldots, P_{14}$. (Since we are dealing with equilibrium conditions P_k does not vary with time, so that a proportion P_k of A_k cells are formed during one division time.) We shall then have a set of simultaneous linear equations in P_k ($k = 8, 9, \ldots, 14$) which can be solved.

We shall use notation of the type (1 2 2 1 2 2 2) for a typical chromosome configuration; here there is a single representative of the first and fourth chromosomes and two representatives of the others.

The probability that a diploid cell (2 2 2 2 2 2 2) changes into an A_{13} cell of configuration (1 2 2 2 2 2 2) $= 2PQ^{13}$, writing $Q = 1-P$, since one of the two representatives of the first chromosome is lost, and the other 13 chromosomes are not lost. There are

$$7 \text{ (i.e. } \binom{7}{1}\text{) } A_{13} \text{ configurations.}$$

Hence

$$\text{prob } (A_{14} \text{ changes to } A_{13}) = 2 \times \binom{7}{1} PQ^{13}.$$

Similarly,

$$\text{prob } (A_{14} \text{ changes to } A_{12}) = 2^2 \times \binom{7}{2} P^2Q^{12},$$

where A_{12} is used to denote a *viable* 12-aneuploid and similarly for the other A's.

In general

$$\text{prob } (A_{14} \text{ changes to } A_k) = 2^{14-k} \binom{7}{14-k} P^{14-k}Q^k \quad (k = 8, 9, \ldots, 14).$$

We may generalize further to give:

$$\text{prob } (A_j \text{ changes to } A_k) = 2^{j-k} \binom{j-7}{j-k} P^{j-k}Q^k.$$

Thus the proportion of total cells which change from A_j to A_k during one division cycle is

$$2^{j-k}P_j \binom{j-7}{j-k} P^{j-k}Q^k.$$

Since all k-aneuploids derive from j-aneuploids ($j = k, k+1, \ldots, 14$), we have

$$P_k = \sum_{j=k}^{14} 2^{j-k}P_j \binom{j-7}{j-k} P^{j-k}Q^k \qquad \text{(I)}$$

for $k = 8, 9, \ldots, 13$.

Solving equation (I) for various values of P it appears that the relative frequencies P_k of aneuploids and diploids are an extremely sensitive function of P (Fig. 5). The experimentally determined value for the ratio diploid:aneuploid is approximately 2·4 and is given by $P_{14}/(P_8{+}P_9{+}\ldots{+}P_{13})$ in our notation. From Fig. 6 it is seen that this ratio

is given by $P \simeq 0{\cdot}35$. Solving equation (I) with this value of P we obtain the following values for the relative equilibrium frequencies of the various classes of aneuploid, assuming that the frequency of diploids remains constant: $P_8/P_{14} = 0{\cdot}0453$; $P_9/P_{14} = 0{\cdot}0926$; $P_{10}/P_{14} = 0{\cdot}1245$; $P_{11}/P_{14} = 0{\cdot}1090$; $P_{12}/P_{14} = 0{\cdot}0593$; $P_{13}/P_{14} = 0{\cdot}0182$.

The mean value v_k of the spore volume distribution corresponding to the aneuploid class A_k is given by

$$v_k = \frac{25k}{14}$$

on the assumption that v_k is proportional to the ploidy number k. It is not possible to calculate the standard deviation corresponding to this mean, but we take this to be equal to the standard deviations of the haploid and diploid populations (both 4·5) and

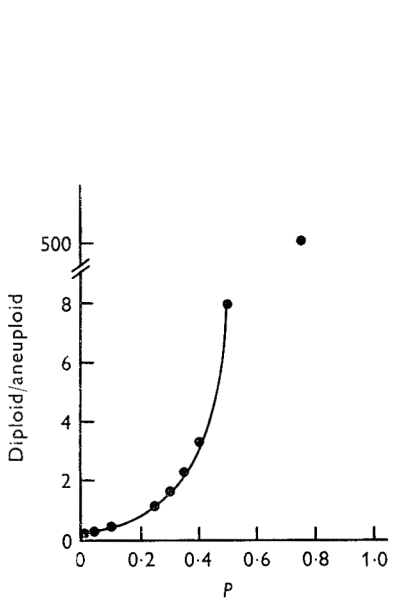

Fig. 5

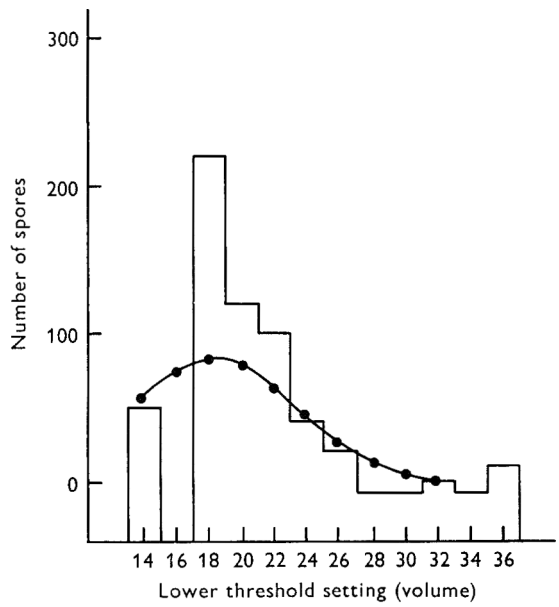

Fig. 6

Fig. 5. Dependence of the diploid:aneuploid ratio on the value of P, the probability that any one chromosome will be lost at any one division.

Fig. 6. Observed (histogram) distribution of spore volumes of *D. discoideum* NC-4 remaining after subtraction of the haploid and diploid contributions and calculated distribution of volumes (●) assuming that these spores are aneuploids with $P = 0{\cdot}35$ and S.D.S = 4·5.

assume that it is normally distributed. Using these assumptions and the values of the relative frequencies of the aneuploid classes we obtain a calculated volume distribution for the aneuploids (Fig. 6). Since the final distribution is relatively unaffected by the exact values taken for the standard deviation and the exact form of each aneuploid class volume distribution this uncertainty has little affect on our calculations.

A change in P not only alters the diploid:aneuploid ratio; it also alters the relative frequency of the various aneuploid classes. Thus at $P = 0{\cdot}35$ the predominant aneuploid class is A_{10}. As P tends to zero the predominant aneuploid classes are A_{12} and A_{13}; at $P = 0{\cdot}5$ it is A_9, and for values of P close to 1·0 it is A_8. It seems reasonable that

this should be so since, if P is large, chromosomes are lost so quickly that very few aneuploids exist and those that do exist have only one or two extra chromosomes. Conversely, if P is small, chromosomes are lost so slowly that aneuploids are quite frequent and many of them have a large number of extra chromosomes.

DISCUSSION

Sussman & Sussman (1963) reported that diploid spores of *Dictyostelium discoideum* often have an unusual and irregular shape; this was confirmed by Sinha & Ashworth (1969). It is thus not surprising that it is the mean volume of a spore population which is proportional to the ploidy and not the mean major diameter. It is not unusual to find amongst other fungi (Clutterbuck, 1969) and higher organisms (Noggle, 1946) relationships of this type.

The analysis of volume distributions of the type shown in Fig. 2 in terms of contributions from haploid, diploid and aneuploid spores gives, at best, an approximate answer, because the total number of aneuploid spores present is so small. Any procedure which entails measuring accurately the relatively small difference between two large numbers is bound to be approximate. However, the Coulter Counter can handle, accurately, large numbers of spores in a reasonable time, and, as can be seen in Figs. 3 and 6, the agreement between the calculated and experimental volume distributions is acceptable. This agreement can be taken to justify the assumptions made in calculating this distribution, namely, (*a*) aneuploids arise either from diploids or from aneuploids containing a larger number of chromosomes; (*b*) aneuploids are unstable and the probability (P) that a chromosome will be lost at any one division is a constant; (*c*) that it can be assumed that there is no chromosome replication before division of an aneuploid.

There is independent, genetic, evidence which justifies some of these assumptions (Loomis & Ashworth, 1968; Loomis, 1969; Sinha & Ashworth, 1969). Assumption (*c*) is probably only valid to a first approximation, but it would be difficult to distinguish between partial replication and no replication at all. The occurrence of partial replication can, in part, be allowed for by an alteration in the constant P. If it were possible to determine P experimentally then it would be possible to assess the extent of chromosome replication in an aneuploid before its division. Since partial replication will result in an alteration of P and since we have chosen the best value of P to fit our data empirically it is probable that we have allowed, in part, for such replication as occurs.

The frequency of diploids deduced in this way (8 %) is considerably higher than the frequency of heterozygous diploids as found by Loomis & Ashworth (1968) after mixing myxamoebae from two genetically different strains (0·04 to 2 %); this suggests that homozygous diploids are formed more readily than heterozygous diploids.

Sussman & Sussman (1962) described the isolation of mutant strains of *Dictyostelium discoideum* which had a spore size distribution similar in shape to that of the diploid or haploid spores, but with a mean major diameter between these two extremes; such strains they described as 'metastable'. Cytological investigation showed that these metastable strains contained both diploid and haploid cells, and one strain differed from another in the ratio of haploid : diploid metaphase figures observed. They did not report seeing any aneuploid figures, but these are easily overlooked and

disregarded, especially since the bacterial debris inside the myxamoebae often stains like the chromosomes.

The size distribution of the spores from metastable strains cannot be explained by assuming these strains to be a mixture solely of haploid and diploid cells. The addition of two normal distributions of the type shown in Fig. 1 cannot result in another normal distribution with a mean of about 16 and a standard deviation of 4·5. Some of the metastable strains described by Sussman & Sussman (1962) did, however, have distributions of this type.

We suggest that metastable strains of *Dictyostelium discoideum* are mutant strains in which the mutational event has affected the value of the constant P which determines the probability that a chromosome will be lost at any one division. It would seem likely that such a mutational event would also affect the haploid:diploid ratio. The aneuploid:diploid ratio is affected by the value of P (Fig. 5), and the predominant aneuploid class in the aneuploid population is also affected by the value of P. Thus, by taking appropriate values of P and the haploid:diploid ratio it is possible to construct spore-volume distribution curves which closely resemble those described by Sussman & Sussman (1962). Distribution curves similar to those of the metastable strains can be obtained by the mass sub-culture of the initially homogenous diploid strain H-1. Here the proportion of diploid:haploid:aneuploid is changing while haploidization proceeds, and distributions of the type shown in Fig. 2 are not equilibrium distributions. They show, however, that our explanation for the spore volume distributions of metastable strains in terms of a change in P and consequent change in diploid:haploid:aneuploid ratio is adequate to account for the experimental observations.

Sussman & Sussman (1962) reported that many metastable strains arose from cells ('I-cells') which initiated the aggregation phase of the life cycle of *Dictyostelium discoideum*; they thus represent the first type of cell differentiation shown by this organism. However, metastable strains are also most easily isolated from those cells which do not readily aggregate (Sussman, 1964), a fact difficult to reconcile with their being involved in initiating aggregation. These and other properties of the I-cells become comprehensible in terms of our theory, which would describe such cells as being of a particular aneuploid configuration. The evidence, and implications, for such an assertion will be presented in more detail elsewhere (Ashworth & Sackin, 1969).

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Inducible Sucrase Activity in *Bacillus subtilis* Distinct from Levan-sucrase

By LOUISE S. PRESTIDGE AND J. SPIZIZEN
Department of Microbiology, Scripps Clinic and Research Foundation, La Jolla, California 92037, U.S.A.

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SUMMARY

An inducible sucrose hydrolysing enzyme has been found in *Bacillus subtilis*. The enzyme is intracellular, unstable in the absence of EDTA and its formation is repressed by glucose. These properties contrast with those of the levan-sucrase enzyme previously investigated in this organism.

INTRODUCTION

The occurrence of a sucrase without associated levan or dextran synthesizing activity has not been reported in *Bacillus subtilis* although separate enzymes for these two functions were suggested some time ago in Aerobacter strains (Hestrin, Avineri-Shapiro & Ascher, 1943) and in *B. megaterium* (Forsyth & Webley, 1950).

In this report evidence is presented for an inducible invertase-like activity in *Bacillus subtilis* which differs from the levan-sucrase (E.C. 2.4.1.10) extensively studied by Delobbe & Dedonder (1966), Dedonder (1960), Joyeux & Seys-Borrel (1966) and Delobbe (1966). The lability, EDTA stabilizing effect, intracellular location and glucose repressibility of this sucrose hydrolysing activity differ from those reported for levan-sucrase.

METHODS

Bacterial strains. Bacillus subtilis 168 *try*-2, (designated '168' in the text) was from the collection of this laboratory while BS 5 and MC 2, kindly provided by Dr Dedonder, were mutants obtained from the 168 strain carried in his laboratory. These three strains were, respectively, low level inducible, high level inducible, and constitutive with regard to levan-sucrase production (personal communication from Dr Dedonder). They behave identically with respect to the newly detected sucrose hydrolyzing activity reported in this note.

Growth conditions and experimental techniques. Cultures were grown in either basic salts composition A (Dedonder, 1960) or B (Anagnostopoulos & Spizizen, 1961), containing 0·5 % carbon source, 20 μg./ml. L-tryptophan and 0·05 % casein hydrolysate. Inducers were added to exponential growth-phase cultures and samples removed at intervals into iced tubes containing chloramphenicol sufficient to give a final concentration of 25–50 μg./ml. and EDTA, when used, at a final concentration of 0·01–0·015 M. Suspensions were broken before assay either by lysozyme treatment or by sonic oscillation. Where necessary to eliminate compounds reacting in the assay, dialysis was carried out before determination of sucrose hydrolysis or levan synthesis activity.

Assays. Sucrose hydrolysis activity was measured by glucose formation after incubation with 0·3–0·4M-sucrose. Glucose was then determined by a modification of the method used by Messer & Dahlqvist (1966) as follows. After incubation with sucrose a total volume of 0·5 ml. diluted sample was equilibrated at 37° and 2·0 ml. assay reagent added. (The assay reagent contained 10 μg./ml. glucose oxidase, 1 μg./ml. peroxidase and 50 μg./ml. *o*-dianisidine in 0·25M-PO_4, pH 6). After a suitable incubation period, the reaction was terminated and purple colour developed by the addition of 1·0 ml., 50 % H_2SO_4. Glucose concentrations of the samples were obtained by comparison of optical density readings at 530 mμ with those of glucose standards run under the same conditions.

Levan production was estimated from turbidity readings of incubated samples in which levans of mol. wt 5×10^4 or larger were precipitated by the addition of 2 volumes of 95 % EtOH.

Chemicals. Lysozyme grade I, glucose oxidase (*Aspergillus niger*) purified type II, peroxidase (horse-radish) type I, and *o*-diansidine were products of Sigma Chemical Company. Chloramphenicol was a gift of Parke Davis and Company. Other analytical grade reagents used were products of Fisher, Mallinkrodt, or Matheson Coleman and Bell.

RESULTS

Preliminary experiments of levan-sucrase induction in *Bacillus subtilis* strain BS5 done in the presence of 0·5 % glucose in a medium of salts composition 'A' were in qualitative agreement with data presented by Dedonder (1960) for the induction of levan-sucrase in a medium utilizing glycerol as a carbon source. Both sucrose hydrolysis and levan synthesis activity were optimally induced at the highest inducer concentration tested (5×10^{-2}M), while comparatively negligible activities were found at inducer concentrations of 1×10^{-4}M or lower.

However, when lactate was substituted for glycerol or glucose as carbon source in the same salts 'A' medium, it was found that both *Bacillus subtilis* 168 and BS5 were capable of synthesizing significant levels of sucrose hydrolysis activity at inducer concentrations below 1×10^{-4}M.

Levan synthesis activity could not be demonstrated in either strain by turbidity assay of samples induced at 1×10^{-4}M-sucrose although appreciable activities were found in Strain BS5 at inducer concentrations of $0{\cdot}5 \times 10^{-2}$M or higher.

Induction of sucrose hydrolysis activity at low sucrose concentration was dramatically increased in both BS5 and 168 in lactate medium when salts 'B' was substituted for salts 'A'. However, regardless of the composition of the medium, the activity induced at low concentrations of sucrose proved to be quite unstable (unlike levan-sucrase), and various compounds were added in an attempt to stabilize the enzyme. Of the additions tested (β-mercaptoethanol, cysteine, vitamin mixtures, trace metals, yeast extract, EDTA), only EDTA was found to have an appreciable effect on sucrose hydrolysis activity. Addition of 0·01–0·015M-EDTA increased activity and stabilized extracts for a short time during storage at 4°.

Studies of the EDTA-activated sucrase (which will henceforth be referred to as 'sucrase B') were then repeated in *B. subtilis* 168 and BS5 under experimental conditions found optimal for induction and assay (i.e. the medium contained salts B and lactate as carbon source, low sucrose inducer concentrations were employed and

EDTA was added to broken-cell samples). The results contrasted strikingly with those previously obtained for levan-sucrose induction. A higher initial rate of enzyme induction was obtained with decreasing inducer concentration. The lowest concentration of sucrose tested ($1{\cdot}46 \times 10^{-5}$ M) acted as a more effective inducer than either of the two higher sucrose concentrations ($1{\cdot}17 \times 10^{-4}$ M or $2{\cdot}92 \times 10^{-4}$ M) when early time samples prior to 30 min. were compared.

The role of carbon source on induction suggested a catabolite repression effect. Addition of a low concentration of glucose (40 μg./ml.) was found sufficient to cause a lag period in initiation of sucrase B synthesis in BS5 and 168 during growth on lactate. Addition of glucose to cultures in which induction was already in progress

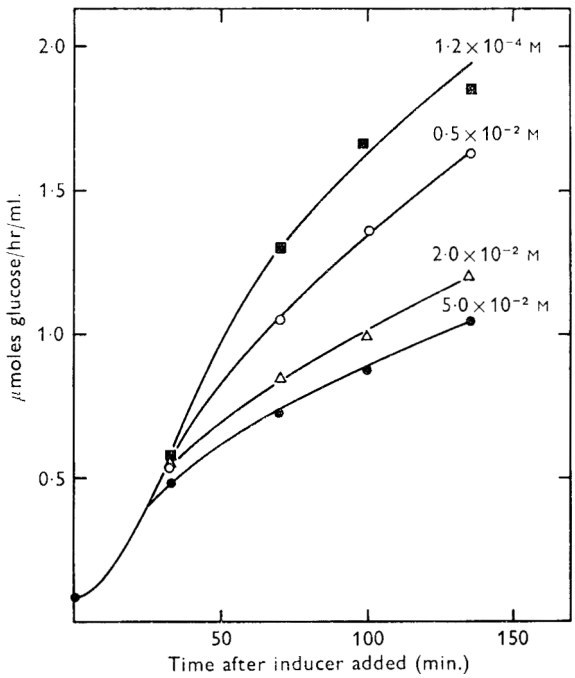

Fig. 1. A culture of *Bacillus subtilis* strain BS5 in exponential growth phase at 30° on salts A, 0·1 % glycerol, 0·1 % casein hydrolysate and 20 μg. L-tryptophan/ml. was divided among several flasks to which various concentrations of sucrose were added at 0 time. At intervals samples were removed and the organisms collected by centrifugation. Pellets were washed and resuspended in 0·02 M-sodium phosphate buffer (pH 6)+0·01 M-EDTA. (Resuspension volumes were adjusted to make all samples to the same concentration based on Klett Summerson readings made at the time of sampling.) After addition of 75 μg./lysozyme/ml., 15 μg. RNAse/ml. and 12 μg. DNAse/ml., a 30 min. incubation was carried out at 37° in order to lyse the organisms prior to subsequent determination of sucrose hydrolysis activity. No levan synthesis activity was detectable by turbidity assay.

caused abrupt and complete cessation of further enzyme production for a period of time was related to the amount of glucose added. This contrasts with levan-sucrase which is non-glucose repressible.

Sucrase B also differs from the predominantly extracellular levan-sucrase in that it appears to be completely intracellular. When sucrase B is induced by low concentration of inducer, and the cells and medium separated by centrifugation are assayed indepen-

dently, all of the activity is found with the resuspended cells. Lysis by lysozyme or sonic treatment is required for full expression of the intracellular enzyme.

Finally, sucrase B can be most clearly distinguished by its different pattern of induction under conditions comparable to those used by Dedonder to study total levan-sucrase in the culture (i.e. salts A with glycerol carbon source). When BS5 organisms were separated from the supernatant medium and maintained in the presence of EDTA their sucrose hydrolysis activity pattern was the reverse of that ascribed to levan-sucrase. Thus, at the lowest sucrose concentration ($1{\cdot}2 \times 10^{-4}$M) maximum sucrose hydrolysis activity was produced without associated levan-synthesis activity. Figure 1 illustrates these results.

DISCUSSION

Two distinct sucrose hydrolysing activities can be induced in the same culture; one (sucrase B) being intracellular and produced with greatest activity at a lower inducer concentration ($1{\cdot}17 \times 10^{-4}$M or less), and the other (levan-sucrase) found by Dedonder predominantly in the supernatant medium and present with maximal activity at high inducer concentrations (5×10^{-2}M or higher).

The two enzymes are presumably subject to different genetic controls. The MC2 mutant strain of *Bacillus subtilis* 168 (classified as levan-sucrase 'constitutive' by Dedonder) produced only a low level of levan-sucrase under conditions where levan-sucrase is fully induced in BS5. The induction pattern of sucrase B is, however, identical in both MC2 and BS5.

These observations, together with observed differences in the properties of levan-sucrase and sucrase B with respect to the effects of EDTA and glucose repression provide strong evidence for the existence of two separate inducible sucrose hydrolysing enzymes in *Bacillus subtilis*.

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