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THE JOURNAL OF GENERAL MICROBIOLOGY

THE PREPARATION OF PAPERS

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

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

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

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

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

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Proprietary Substances and Materials. At first mention, the correct designation and the manufacturer's address should be given in the text.

Chemical Nomenclature. Follow the 1967 revision of *Policy of the Journal and Instructions to Authors*, The Biochemical Society, 20 Park Crescent, London, W.1.

Nomenclature of Enzymes. The system published in *Report of the Commission of Enzymes of the International Union of Biochemistry*, Oxford: Pergamon Press, 50*s.*, is used.

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.

Nomenclature and Description of Micro-organisms. The authors' preference in naming micro-organisms is at present accepted provided that the designation is unambiguous and conforms with international rules of nomenclature; if desired, synonyms may be added in brackets when a name is first mentioned. Names of bacteria must conform with the Bacteriological Code of the International Committee on Bacteriological Nomenclature and the opinions issued by this International Committee (*Bacteriological Code* (1958), edited by the Editorial Board of the International Committee on Bacteriological Nomenclature and published by the Iowa State College Press, Ames, Iowa, U.S.A.). Names of algae and fungi must conform with the International Rules of Botanical Nomenclature which are considered and revised at each International Botanical Congress (published by the International Bureau of Plant Taxonomy and Nomenclature, 106 Lange Nieuwstraat, Utrecht, Netherlands (1952)). Names of protozoa must conform with the International Code of Zoological Nomenclature adopted by the XVth International Congress of Zoology (published for the International Commission on Zoological Nomenclature by the International Trust for Zoological Nomenclature, London (1961)). The 1913 rules will be found in C. M. Wenyon, *Protozoology*, (1926), vol. 2 (London: Baillière Tindall and Cox). One or two small changes have been made to these rules at later International Congresses.

Useful commentaries on the taxonomy of micro-organisms will be found in the articles of the Twelfth Symposium of the Society for General Microbiology, *Microbial Classification*, edited by G. C. Ainsworth and P. H. A. Sneath (Cambridge University Press).

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

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

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

S. T. Cowan & K. J. Steel, *Manual for the Identification of Medical Bacteria*, (1965). Cambridge University Press.

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

List of Common British Plant Diseases, 4th ed. (1944), compiled by the Plant Pathology Committee of the British Mycological Society. Cambridge University Press.

Medical Research Council: Memorandum No. 23. 3rd ed. (1967). *Nomenclature of Fungi Pathogenic to Man and Animals*. London: H.M.S.O.

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The Influence of Metal Ion Concentrations and pH Value on the Growth of a Nitrosomonas Strain Isolated from Activated Sludge

By J. E. LOVELESS AND H. A. PAINTER
Water Pollution Research Laboratory, Stevenage, Hertfordshire

(*Accepted for publication* 31 *October* 1967)

SUMMARY

A strain of *Nitrosomonas europaea* was isolated from activated-sludge effluent by a dilution method which readily demonstrated the presence of contaminating heterotrophs and yielded a high proportion of tubes containing pure cultures of ammonia-oxidizing bacteria. Copper, sodium, calcium and magnesium stimulated growth of pure cultures, and effects of deficiencies of these metals were demonstrated. Ethylenediaminetetra-acetic acid improved growth in the basal medium, and abolished the toxic effect of added copper; it was, however, inhibitory at low calcium concentrations. The effect of pH value on the growth of *N. europaea* appeared to be dependent on the metal ion content of the medium although the optimum pH value was always between 7·5 and 8. The growth rate constant in pure culture was similar to that previously observed for nitrification in Thames water, but double that previously observed for nitrification of sewage by activated sludge. The Michaelis constants for ammonia and oxygen were similar to those found for nitrification in activated sludge.

INTRODUCTION

In his review of the metabolism of chemolithotropic bacteria, Lees (1960) indicated that the recalcitrant nature of this group of organisms was the reason for the relatively slow progress being made in elucidating the biochemical problems they present. Nitrosomonas, which uses carbon dioxide to oxidize ammonia to nitrite, is typical of this group. The most useful recent advances in growing Nitrosomonas in pure culture were made by Engel & Alexander (1958) when they introduced a precipitate-free medium, and by Skinner & Walker (1961) who used this medium in a continuous-culture apparatus. Skinner & Walker reported that, when the population of Nitrosomonas exceeded about 3×10^8 organisms/ml., the growth rate decreased; this was attributed to inadequate aeration. Gundersen (1957) was unable to maintain strains of Nitrosomonas for more than a year.

In the present work, it was originally intended to develop a culture system which would provide large-scale batch cultures for biochemical investigations, and also background information for the continuous culture of large numbers of organisms for survival and inoculation studies in model sewage-purification systems. The kinetics of nitrification in these systems have been interpreted by the application of electronic computation to hypothetical changes in the proportions of Nitrosomonas in the mixed populations of micro-organisms which are the agents of purification (Downing,

Painter & Knowles, 1964; Knowles, Downing & Barrett, 1965). However, it became quite clear from early experiments that none of the media hitherto used was entirely satisfactory. It seems likely that essential trace elements have commonly arisen fortuitously as contaminants of water, chemicals and glassware. It would also appear that under certain conditions growth rates can be dramatically decreased by traces of toxic ions arising from similar sources. The main theme of the present work thus became an attempt to define more closely some of the conditions necessary for growth. Much still remains to be done along these lines before consistently good growth can be obtained.

The isolation of the organism has also remained a tedious and haphazard process. Van Niel (1954), in reviewing autotrophic bacteria, suggested that the isolation of Nitrosomonas was in itself an accomplishment worthy of publication. Further progress towards a satisfactory technique for the isolation in pure culture was made by Lewis & Pramer (1958), when they combined the precipitate-free medium of Engel & Alexander (1958) with a dilution technique. It is one of the objectives of this paper to indicate further improvements in this isolation technique and specify more closely the conditions necessary for success.

METHODS

Apparatus. A satisfactory pH-controlled and sterilizable batch culture unit was designed after referring to the work of Skinner & Walker (1961), Engel & Alexander (1958) and Callow & Pirt (1956). The output stage of a pH meter was connected in series with a relay unit (Transitrol, Ether Ltd., Stevenage) which operated an electromagnetic valve controlling the entry of alkali to the culture vessel. The calomel electrode was immersed in saturated potassium chloride in a separate bottle, and electrical contact with the medium in the culture vessel was maintained by a bridge filled with 2% agar containing potassium chloride, and ending in a sintered glass plug immersed in the culture medium. The glass electrode was sterilized by soaking overnight in 0·1 M-hydrochloric acid or soaking for 1 hr in a 1% (w/v) solution of β-propiolactone (Taplin, 1962) before being inserted aseptically into the freshly sterilized medium cooled to below 50°. Instability in the pH reading was traced to static charges on various parts of the apparatus but was overcome by adequate earthing; in extreme situations even the cases of the pH meters, the potassium chloride reservoir and the medium had to be earthed.

The culture vessel was a 10 l. aspirator, and aeration was by means of a porous porcelain candle connected to an airline and fitted in a silicone-rubber bung in the lower opening; this bung also held a sampling tube fitted with a shielding hood and the agar bridge from the calomel electrode. The neck of the aspirator was closed with a cotton-wool plug wrapped in cellophan to prevent wetting by the spray thrown up during aeration. Glass tubes inserted in the plug served to introduce the inoculum and the sterilized glass electrode. The alkali used for neutralizing the nitrous acid produced during growth was usually supplied as approximately 3 N-ammonium carbonate, but in some earlier experiments ammonium hydroxide was tried, with and without the addition of sodium carbonate.

Aeration of cultures. From the known rate of flow of air to the cultures and from their measured respiration rate, it was calculated that sufficient air was supplied at all times. This was occasionally confirmed by determining the concentration of dissolved oxygen by means of the dropping-mercury or the Mackereth electrode (Painter &

Jones, 1963; Briggs & Viney, 1964). At no time was the concentration below 2 mg. O_2/l., and since this value is well above the Michaelis constant (see Results) it was concluded that oxygen was not limiting growth.

Some cultures were grown in screw-capped bottles, in which no direct determinations of the concentration of dissolved oxygen were made. Initially the medium was saturated with respect to oxygen. During incubation samples were withdrawn, no further liquid was added and the bottles were shaken, thereby introducing further oxygen. The values for doubling times observed in these cultures were about the same as those obtained in the large vessels, suggesting that oxygen supply was satisfactory.

Media. The media used were developed from the suspension-free medium described by Skinner & Walker (1961). Sodium was found to stimulate growth. It was also found that the substantial pH change which occurred on autoclaving some media was due to loss of ammonia. This hazard was largely eliminated by decreasing the concentration of ammonia-nitrogen and substituting ammonium carbonate for ammonium sulphate. The final medium contained 36 mg. Na (as $Na_2SO_4.10H_2O$), 15 mg. Ca (as $CaCl_2$), 10 mg. Mg (as $MgSO_4.7H_2O$), 32 mg. P (as KH_2PO_4), and 0·2 mg. Fe (chelated) per litre, dissolved in de-ionized distilled water. The chelated iron was added by diluting 1 ml. of a solution of 0·48% $FeCl_3.6H_2O$, 0·312% NaCl and 0·643% 'Chel 138' (Geigy Chemical Co., New York; ethylene diamine di(*o*-hydroxyphenyl) acetic acid) to 1 l. of medium (Engel & Alexander, 1958). For the isolation, 100 mg. yeast extract/l. was added so that heterotrophs became evident after 2–3 days of incubation. In the pH-controlled batch and enrichment cultures, the pH value was maintained at the predetermined value with ammonium carbonate, which also provided nitrogen and supplemented the supply of carbon dioxide from the atmosphere. In medium prepared for small volume experiments in bottles and flasks, nitrogen was added as ammonium bicarbonate at a concentration of 20 mg. N/l. and final adjustments of pH value were made with dilute sodium hydroxide. This medium was augmented for making isolations with 100 mg. Difco yeast extract/l., the pH value being adjusted to 7·9; it was then dispensed in test-tubes in convenient volumes and sterilized in a domestic pressure cooker for 10 min. at 121°. In experiments done in 1 oz. screw-capped bottles, improved pH control was achieved by adjusting pH values up to 7·9 with sodium bicarbonate; for any further increase in alkalinity sodium hydroxide was used. The bottles were filled to the brim at the beginning of experiments and the caps were screwed on tightly to minimize the loss of volatile components from the liquid phase. All additions, including the inocula, were made to the largest practicable volumes of the medium before dispensing into smaller lots. The size of the inocula was usually 1–2·5 ml./l. medium so that the concentration of nitrite carried over was below 0·1 mg. N/l.

Source and isolation of organism. Preliminary experiments on the effects of Na_2SO_4 on growth were made with a pure culture of Nitrosomonas given to us by Dr N. Walker (Rothamsted Experimental Station, Harpenden, Hertfordshire) and originally isolated by Dr H. L. Jensen (Lyngby, Denmark), but this culture was lost at an early stage and most of our work was therefore done with a strain isolated from the effluent from an activated-sludge plant. This isolation was made from an enriched culture inoculated with 10 ml. of effluent in 9 l. of the mineral salt medium and allowed to grow in the pH-controlled apparatus to a population density equivalent to 500–1000 mg.

nitrite-N/l. While still in the exponential growth phase, dilutions of this culture were made in isolation medium to a concentration of about 2 bacteria/ml., based on a rough count using a haemocytometer (Skinner & Walker, 1961); volumes (0·5 ml.) of this suspension were distributed aseptically into test-tubes of sterile isolation medium and incubated at 27° for 11–14 days before sampling for nitrite.

Preparation of glassware. Flasks and bottles were prepared by washing in 'Haemo-Sol', a buffered alkaline detergent (manufactured by Meineke and Co.), rinsing several times in tap water and twice in distilled water. They were finally filled with de-ionized water and autoclaved for 1 hr at 121°.

Nitrite was determined by the modified Griess-Ilosvay diazotization method due to Montgomery & Dymock (1961).

Estimation of growth. Throughout the present work the concentration of nitrite produced has been used as the main criterion of bacterial growth, because the greater sensitivity, simplicity and accuracy of the method of determination of this compound make it a better choice than the estimation of cell carbon, mass or numbers. Nitrite production is a true measure of growth of Nitrosomonas, at least up to 500 mg. N/l., as shown by Buswell, Shiota, Lawrence & Van Meter (1954), and by Engel & Alexander (1958). As mentioned earlier ('Aeration of cultures'), the concentration of nitrite in screw-capped bottle cultures increased logarithmically with time but the growth rate was not always determined; instead, in some experiments the results are expressed as concentration of nitrite after a stated period of incubation. It was valid to compare these concentrations in a single experiment since the inoculum was the same for all vessels; but the comparison could not be made between experiments since the activity of the inocula varied from experiment to experiment.

RESULTS

Isolation of pure strains

A high success rate was achieved by the method described. Seven out of nine experiments intended to isolate or re-isolate the organism were successful, the number of pure isolates obtained in 14 days varying between 5% and 70% of the tubes inoculated in each experiment, whereas previously, when using the unmodified medium and unenriched seeds, isolates were not obtained even after 21 days. Confirmation of purity was obtained by finding no growth in nutrient broth and no evidence of contaminating organisms on microscopical examination.

The pure strain chosen for growth experiments was very motile; the bacteria were somewhat elliptical in shape and measured about 1–1·25 μ in diameter. The bacteria were well-dispersed and motile in the early stages of a culture; later a substantial flocculent deposit usually became apparent and within flocs micro-colonies of a few dozen organisms could often be seen. The characters of the organism isolated fit the description for *Nitrosomonas europaea* in *Bergey's Manual* (1957).

Reproducibility of growth of the stock strain in pure culture

In the first pH-controlled batch cultures, exponential growth at a constant, though low, rate (doubling time 48 hr) was achieved up to nitrite concentrations of 2500 mg. N/l. (equivalent to 96 mg. organic C/l.) (curve A, Fig. 1), which is much higher than any other reported. Lower maximum concentrations of nitrite were obtained in the

next group of experiments, and it appeared, surprisingly, that when more care was taken in preparing glassware and in selecting reagents, it became more difficult to achieve high yields.

To test the possibility that there had been a mutation in the stock strain, its growth curve was compared with simultaneous growth curves of cultures in the same medium inoculated with 1 ml./l. of water from an unpolluted river or of clear supernatant fluid from an aqueous suspension of soil. In all these cultures, both the final nitrite production and the doubling time were nearly the same, indicating that the falling-off in growth was due to the influence of the medium and not to genetic change in the stock strain.

To test the reproducibility of results within one experiment, six batch cultures were grown under identical conditions; the results of plotting the logarithm of nitrite concentration against time are shown in Fig. 1 (curves B, C). Consistent results were obtained, particularly for five of the vessels, doubling times in the initial stages varying from 19·2 to 21 hr. The decline in the rate of growth in the later stages was typical of the results usually obtained in the present work.

Reproducibility in experiments in screw-capped bottles was not so good as in the large batch cultures. However, it was possible to set up many more parallel cultures in each experiment to enhance the reliability of the mean obtained. Some of the inconsistency was found to be due to a 'glassware factor' and many experiments were made with a variety of vessels, types of glass and washing procedures in order to determine the best way of diminishing this source of error. Although these experiments were complicated to some extent by pH changes, caused by loss of NH_3 and CO_2 from vessels where the closures were not effective, it became clear that variations between replicates could be substantially decreased by following the strict washing procedure already described.

Source of water for making media

Originally media were prepared with distilled water from a high-rate still lined with tinned Admiralty metal (containing tin, copper and antimony). Analysis of this water after cultural difficulties had become serious revealed variable concentrations of copper within the range 0·05–0·08 mg./l. and zinc within the range 0·05–0·1 mg./l.; analysis was also made for antimony but none was detected. A measure of the toxicity of this 'crude' distilled water is shown in Fig. 2, in which growth on medium prepared from it is compared with that on media prepared from (1) water twice-distilled in an all-glass apparatus, (2) demineralized distilled water (using 'Bio-deminrolit', The Permutit Co. Ltd.), and (3) 'crude' distilled water containing 5 mg./l. ethylenediaminetetraacetic acid disodium salt (EDTA). The toxic nature of the 'crude' distilled water is clearly demonstrated and the improved growth in the presence of EDTA suggests that metals are responsible.

Effect of pH value on growth

In experiments to determine the effect of pH value on growth rate, daily measurements of the concentration of nitrite were made in samples from each of six cultures controlled at different pH values; in Fig. 3 the results of two such experiments have been expressed by plotting the mean doubling time against pH value. In the first of these experiments, 'crude' distilled water was used and the curve obtained had a fairly

sharp trough at about pH 7·8; this contrasted with the relatively flat curve obtained with demineralized water over the pH range 7–8·2. These results suggest that the effect of pH value on growth rate is different at different concentrations of heavy metal ions, and *vice versa*. Working with small cultures in screw-capped bottles, the effect of pH value was determined at different concentrations of added copper. Although an interrelationship between the concentration of the element and pH value was demonstrated, the effect was not as great as would be expected from the results in Fig. 3. Several metal ions were tested individually over a range of concentrations for evidence of biological activity at pH 7·3 and 7·9. In Table 1 are summarized the results of

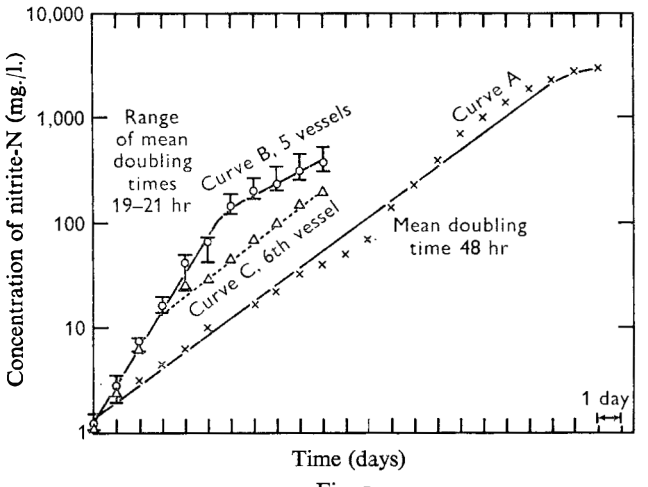

Fig. 1

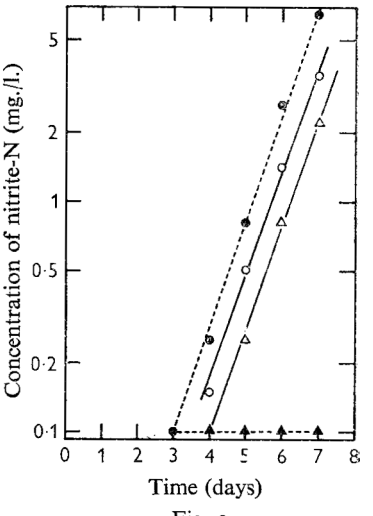

Fig. 2

Fig. 1. Exponential growth curves of *Nitrosomonas europaea* in pH-controlled batch culture. ○——○, Mean of 5 replicates; △-----△, 6th replicate; ×——×, exponential growth to a high concentration of nitrite (single vessel). Vertical lines indicate range.

Fig. 2. Effects of various treatments of distilled water on growth rate of Nitrosomonas in small bottles. ●-----●, Twice-distilled water (in glass); ○——○, demineralized water; △——△, 'crude' distilled water with EDTA (5 mg./l.); ▲----▲, 'crude' distilled water. Each point is an average of 6 determinations.

Table 1. *Summary of the effect of various metals on growth of Nitrosomonas*

Element	Range tested (mg./l.)	Concentration at which effect observed (mg./l.)	
		Stimulation	Inhibition
Cu	0·005–0·56	0·005–0·03	0·05–0·56
Cu*	0·005–0·48	None	None
Ca	0·5–20	None	None
Ca*	0·5–20	0·5–10	None
Mg	12·5–100	12·5–50	50–100
Zn	0·005–0·5	None	0·08–0·5
Co	0·005–0·5	None	0·08–0·5
Al	0·005–1·0	None	None
Sr	0·005–0·05	None	None
Pb	0·005–0·05	None	None
B	0·005–0·05	None	None

* In presence of 5 mg./l. EDTA.

these screening tests and of other more detailed tests on those metals which showed biological activity. The effect of five concentrations of calcium (0·5–20 mg./l.) at five pH values (pH 6·9–8·5) was determined in the absence and presence of 5 mg. EDTA 1 l. The calculated value of calcium carried over with the inoculum was about 0·05 mg. l. There was little difference in the concentrations of nitrite reached in the presence of the five different concentrations of added calcium at any pH value; the mean values are given in Table 2. In the absence of EDTA there was little difference between the values obtained with or without added calcium. However, in the presence of 5 mg. EDTA 1 l. growth was depressed in the absence of added calcium while at pH 7·3 and above in the presence of added calcium the nitrite values reached were substantially higher than in the absence of EDTA.

Table 2. *Effect of calcium, in presence and absence of EDTA, and of magnesium on growth of Nitrosomonas in small bottles*

Nitrate formation was taken as proportional to growth yield

	Average concentration of nitrite after 6 days incubation (mg. N/l.)					
	Concentration of calcium added (mg./l.)				Concentration of magnesium added (mg./l.)	
	No EDTA added		5 mg. EDTA/l. added			
pH of medium	0 (15)	0·5–20 (60)	0 (15)	0·5–20 (60)	0 (15)	12·5–100 (60)
6·9	3·0	3·5	2·0	1·2	0·1	2·0
7·3	4·5	5·0	1·0	4·0	0·1	2·2
7·7	5·0	4·0	2·0	5·0	2·0	4·0
8·3	5·5	6·0	3·5	9·0	5·0	6·0
8·5	6·0	6·5	3·7	9·0	6·5	6·0

Figures in parentheses are numbers of determinations.

Table 3. *Effect of copper in the presence and absence of EDTA on growth of Nitrosomonas in small bottles*

Concentration of copper added (mg./l.)	Concentration of nitrite after 11 days incubation (mg. N/l.) (average of 15 determinations)	
	No EDTA	5 mg. EDTA/l.
0	0·25	5·3
0·005	1·0	4·5
0·02	2·3	6·1
0·04	2·4	6·4
0·06	2·8	6·4
0·48	0·25	6·0

The effect of adding magnesium within the range 12·5–100 mg./l. (background concentration about 0·02 mg./l.) was tested at five different pH values; apart from a slight decrease in growth rate at high values magnesium appeared to have very little effect. However, as shown in Table 2, the effect of deficiency of magnesium was far more marked at the low pH values than at high.

Copper was also tested for its effect on growth, both in the absence and presence of 5 mg. EDTA/l. Table 3 contains the mean values for nitrite production at five different pH values. Increasing stimulation by copper was found up to an added amount

of 0·02 mg./l. with a levelling off up to about 0·06 mg./l.; at an added concentration of 0·48 mg./l. the stimulating effect of copper had disappeared. The stimulation by copper was reproducible, since of ten experiments nine showed enhanced growth in its presence (0·03 mg./l.) over controls with no added copper. The results of these experiments when compared with those of similar experiments in which EDTA had been added are interesting. Not only did EDTA increase the tolerance to high concentrations of copper, but it also improved the growth at all concentrations of copper tested (Table 3) and was advantageous at most pH values (Fig. 4). This was in contrast to the growth-depressing effect observed at threshold concentrations of calcium (Table 2).

Zinc and cobalt were the only other metals tested which showed any biological activity. Toxicity was fairly marked with both these metals at 0·5 mg./l. in the presence of 0·03 mg. Cu/l.

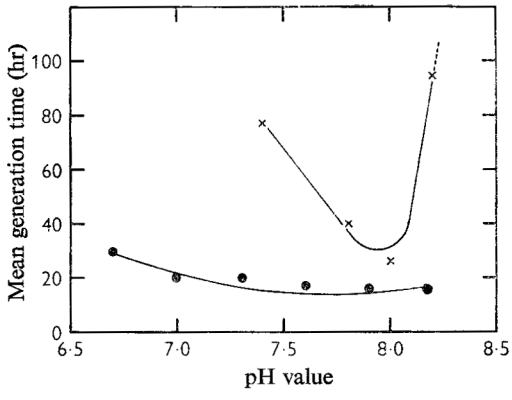

Fig. 3

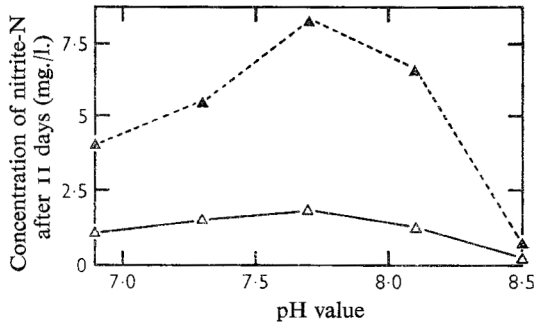

Fig. 4

Fig. 3. Dependence of mean generation time of Nitrosomonas on pH value of medium. Generation time measured by changes in nitrite concentration in pH-controlled batch cultures. ×——×, Distilled water; ●——●, demineralized water.

Fig. 4. Effect of EDTA at various pH values on growth of Nitrosomonas in small bottles. ▲- - - - -▲, With EDTA (5 mg./l.); △——△, no EDTA. Each point is the average of three determinations.

Effect of sodium

When studying Nitrosomonas, it has been standard practice to use ammonium sulphate as a source of ammonia, and to neutralize the nitrous acid produced with sodium bicarbonate; this often resulted in the accumulation of high concentrations of sodium and sulphate ions. For example, in the cultures of Skinner & Walker (1961) sodium appears to have reached a concentration of about 0·4% and sulphate ions about 0·5%.

In Fig. 5 the effect of various concentrations of sodium sulphate on the doubling time of the Jensen strain in small bottles is shown. A marked stimulation was obtained at sodium 0·06–0·15%, with inhibition at 0·7%. In other experiments stimulatory effects were observed with similar concentrations of sodium added, either as sulphate or chloride (Fig. 5), indicating that sodium rather than sulphate or chloride was involved. Marked stimulation of this and the activated-sludge strain by sodium also occurred, as evidenced by the doubling times calculated from data collected from pH-controlled batch experiments (Table 4).

Growth-rate constant

Values obtained in the present work for the growth-rate constant and the mean generation time of Nitrosomonas are presented in Table 4 together with values found by other workers. It can be seen that the Jensen strain grew at higher rates than did the isolate from activated sludge with which the shortest doubling-time obtained was 16 hr. The stimulatory effects on growth of sodium, magnesium and EDTA are also illustrated.

Table 4. *Summary of growth-rate constants of Nitrosomonas (batch culture)*

Investigation	Medium	pH value	Temperature (°)	Growth-rate constant (days^{-1})	Mean generation time (hr)
Present work					
Jensen strain	Complete* (distilled water)	8·0	25	1·38	12
	Complete (distilled water)	7·6	25	0·88	19
	Complete (distilled water)	8·0	25	0·69	24
	Na omitted	8·0	25	0·39	46
Isolate from effluent of activated-sludge process	Complete* (distilled water)	7·6	25	0·88	19
	Complete (distilled water)	8·0	25	0·69	24
	Na omitted	8·0	25	0·39	46
	Complete* (deionized water)	7·9	25	1·03	16
	5 mg./l. Mg	8·0	25	0·42	40
	10 or 50 mg./l. Mg	8·0	25	0·69	24
	Complete* (deionized water)	7·8	25	0·79	21
	Na omitted	7·8	25	0·57	29
	Complete* (deionized water)	8·0	25	0·28	58
	EDTA (5 mg./l.)	8·0	25	0·83	20
Engel (1930)	Suspended solids present	~ 8·0	30	1·19	14
Bömeke (1946)	Suspended solids present	~ 8·0	30	1·03	16
Lees (1952)	Suspended solids present	~ 8·0	30	0·46	36
Buswell *et al.* (1954)	BOD dilution water	8–8·5	30, 25, 20	1·74, 1·39 0·94	9·5, 12, 17·5
Engel & Alexander (1958)	Soluble	8·0	25	1·5	11
Skinner & Walker (1961) (Jensen strain)	Soluble	7–7·4	28–32	2·1, 1·51†	8, 11–16†

Values linked with braces refer to simultaneous determinations.
* See text. † Continuous culture.

There was a substantial, unexplained, variation from experiment to experiment in the mean generation time of the activated-sludge isolate grown on the complete medium (16–58 hr), although, as has already been mentioned, good replication was obtained in simultaneous determinations.

The effect of temperature on growth is illustrated in Fig. 6, in which are plotted the average results of six parallel cultures at each of six different temperatures. Best growth and shortest lag time occurred at 30°. Comparison with data (Fig. 6) for a pure culture of an isolate from a percolating filter (Buswell *et al.* 1954), and with data for production of nitrite in activated sludge and in Thames water (Knowles *et al.* 1965), shows that the growth constant increased by roughly threefold from 10° to 20° and that above about 25° the curves for both pure cultures began to deviate from the straight line while those for impure cultures did not.

Yield constant

In a few of the pH-controlled batch cultures the concentration of bacteria was determined by centrifuging, drying and weighing the deposit from a convenient volume. The 'yield constant' or 'economic coefficient' was calculated by dividing the concentration of bacteria by the concentration of nitrite nitrogen formed. The results showed a fairly wide variation between about 0·1 and 0·03. It may be significant that the higher values were from the least concentrated cultures and therefore those most sensitive to error because of the presence of mineral precipitates. These values were, however, of the same order as those reported by other workers (see Downing *et al.* 1964).

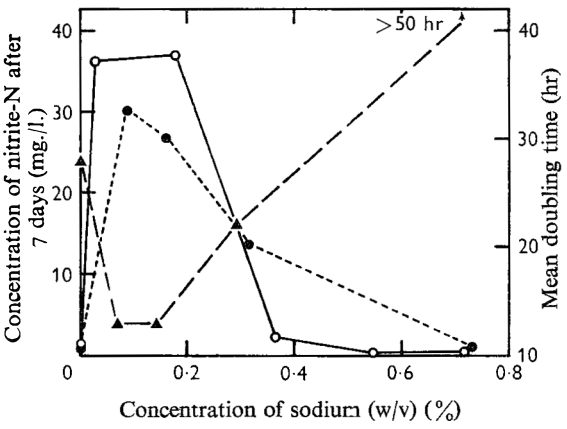

Fig. 5

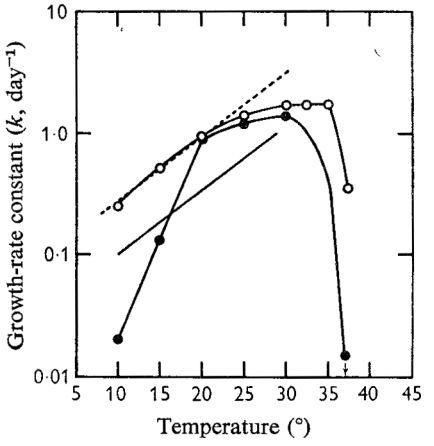

Fig. 6

Fig. 5. Effect of sodium salts on growth of Nitrosomonas (Jensen strain). Experiment 1 (in small bottles): O——O, effect of sodium, as chloride, on nitrite production; ●-----●, effect of sodium, as sulphate, on nitrite production. Each point is the average of 4 determinations. Experiment 2 (in pH controlled large batch cultures): ▲——▲, effect of sodium as sulphate on mean doubling time.

Fig. 6. Effect of temperature on growth rate of Nitrosomonas in pure culture compared with that in Thames water and activated sludge. ------, Thames water (Knowles, Downing & Barrett, 1965). ●——●, Pure culture (from activated sludge—present work). O——O, Pure culture (Buswell *et al.* 1954) ——, Activated sludge (Knowles *et al.* 1965).

Michaelis constants

We were unable to do much biochemical work on suspensions of the organisms because of substantial loss of activity, usually over 90%, on harvesting and resuspension. Various combinations of centrifugal force, time and variations in suspending solutions were tried; none resulted in a marked improvement in the activity of the recovered material. In general, centrifugation for longer times at lower values of g and with suspending solutions similar to the growth medium gave higher recoveries. However, one batch of the Jensen strain retained more activity than usual after harvesting and this was used to determine the Michaelis constants for ammonia-N and for oxygen by the polarographic method of Painter & Jones (1963). The value for ammonia was found to be about 1 mg. N/l. and for dissolved oxygen about 0·3 mg./l.

at 20°. These values agree well with those found for nitrification in the activated-sludge process (*Department of Scientific and Industrial Research*, 1963) and fairly well with the findings of Knowles *et al.* (1965) for Thames water.

DISCUSSION

With the technique described, little difficulty was encountered in the isolation of pure cultures of Nitrosomonas, which were active in the oxidation of ammonia. Contamination by heterotrophs was decreased by including only a little organic matter (in the form of chelated iron) in the medium and following the exponential accumulation of nitrite to a fairly high value before attempting dilutions for isolation. The addition of yeast extract to the isolation medium did not appear to impair seriously the growth of Nitrosomonas and caused heterotrophs to be readily apparent after 2–3 days of incubation. Repeated efforts to grow colonies on plates prepared with Difco Noble agar, as recommended by Engel & Alexander (1958), were unsuccessful.

A potential deficiency in media usually employed was implicit in the work of Nicholas *et al.* (1962), which indicated that copper was an essential trace metal involved in an oxidase system of Nitrosomonas. This has been further confirmed by the present work, which shows that media prepared with analytical grade chemicals and high-quality water can be deficient in copper. There appears to be little doubt, in contrast to the statement of Engel & Alexander (1958), that sodium is also a necessary trace metal since it was not difficult to demonstrate sodium deficiency in media prepared without added sodium. Added magnesium was found to stimulate growth, contrary to the findings of Skinner & Walker (1961) under comparable conditions. Recently, Khare, Chauhan & Tandon (1966) have implicated zirconium, cerium, indium, thallium and beryllium in the nutrition of Nitrosomonas, but examination of the data presented shows that although the addition of these elements increased the final concentration of nitrite, the rate of production of nitrite was little affected.

The increase in growth when EDTA was added to the medium without added copper could be explained if the basal medium contained adequate copper, provided that some unidentified toxic metal which competitively interfered with copper nutrition was inactivated by chelation. (Postgate & Hunter, 1962, found as much as 0·012 mg. Cu/l. in de-ionized water and Dixon & Webb, 1958, listed metals between which competition exists for sites on enzymes.) On the other hand, the decreased growth resulting when EDTA was added without added calcium can be explained by assuming that this element is essential, but was present in the medium in such concentration that on chelation the free concentration of the element remaining was so low as to limit growth. The stimulation of growth usually observed on addition of EDTA to media containing added trace elements might be due to the co-operation or partition effects described by Albert (1965).

That further deficiencies or inhibitions existed was shown by the variations (Table 4) in mean generation times obtained in the complete medium and the repeated failure to reproduce the results of the experiment in which a concentration of 2500 mg. nitrite-N/l. and 96 mg. organic C/l. was reached, which are the highest values so far recorded in the literature. These variations and failures occurred in spite of the addition of what were regarded as the optimum concentrations of sodium, magnesium, calcium and copper. Figure 1 gives typical curves showing changes in the growth rate at around

20–200 mg.N/l. Skinner & Walker (1961) recorded somewhat similar changes in growth rate but they took place at higher concentrations (1000 mg. N/l.).

The limitation of growth at 2500 mg. nitrite-N/l. was not due to lack of dissolved oxygen since the concentration of the gas was always over 2 mg./l., a value well above the Michaelis constant. It is possible, however, that inhibition was caused by the combined effect of 2500 mg. nitrite-N/l. and an approximately equal concentration of ammonia-N, added to neutralize the acidity formed. Lewis (1959) reported that 2500 mg. nitrite-N/l. was completely toxic in the logarithmic phase, although Pokallus (1963) found only 50% inhibition at this concentration.

It is relevant to note the difficulty that various workers have experienced in trying to maintain their cultures in a viable state for long periods. Gundersen (1957) mentioned that Nitrosomonas seemed to be more frail than most other bacteria and Kingma Boltjes (1935) kept his cultures on agar slants enriched with a preparation of egg albumin. This specific preparation is no longer available and there are no reports of similar preparations having stimulatory effects; he apparently lost his cultures during the war, but no reason has been given. Meiklejohn (1950) lost all her cultures after about 18 months storage under various conditions, and Gundersen (1957) lost all his after 12–25 months, again under a variety of conditions. The strain isolated by Jensen (1950) has also died out, although it must have survived in the hands of various workers for some 13 years. The activated-sludge strain used for the current work survived on various media for over 18 months and was then still as active as at the beginning. It is, however, quite certain that the strain would have been lost on several occasions if large numbers of parallel cultures had not been maintained under a variety of conditions, since during this period growth did fail several times. One reasonable interpretation of these experiences is that they are brought about by inadequate control of trace elements and more consistent results are likely to be achieved with a medium more carefully balanced with trace nutrients and buffered against toxic effects by the inclusion of adequate chelation capacity.

Many contradictions appear in the literature on the effect of pH value on respiration rate of Nitrosomonas and a variety of pH curves have been published. Typical of the extreme cases is that of Engel & Alexander (1958), which is relatively flat between pH 7 and 9, falling to about 50% at 6·2 and 9·6, and that of Meyerhof (1917), which has a sharp peak at 8·6, and 50% values at 7·9 and 9·3. In the present work similar extremes have been produced and appear to be due to the pH value strongly influencing the toxicity of copper and possibly other metals. The general picture may also be complicated by quantitative and possibly qualitative changes in the stimulatory effects of metals at different pH values, as has been shown for low concentrations of magnesium. Under the 'cleanest' conditions used in the present work pH value seemed to have little effect on growth over the pH range 7–8; in all other cases the best pH value appeared to be in the range 7·6–8·0.

The optimum temperature of the Nitrosomonas isolate was determined as 30° and this is consistent with the findings of other workers. It is interesting to note, however, that the growth rate constant was substantially higher in pure culture and in Thames water than in activated sludge (Fig. 6). This may be due to an adverse environment in the activated-sludge system, e.g. toxic effects such as have been observed with peptone (Kingma Boltjes, 1935; Buswell *et al.* 1954) or to slower-growing strains being selected in virtue of their flocculation.

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The Carotenoids of *Corynebacterium fascians* Strain 2 Y

By J. PREBBLE
Bedford College, Regent's Park, London, N.W.1

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SUMMARY

An analysis of the carotenoids of *Corynebacterium fascians*, strain 2 Y, showed the presence of 13 fractions. The main pigments identified were β-carotene, a β-carotene-like fraction (possibly β-isorenieratene), leprotene, a mono-hydroxy pigment, P 450, a second xanthophyll, P 452 and a glucoside of hydroxy-chlorobactene. The minor constituents have been identified as phytoene, phytofluene, β-zeacarotene, neurosporene, ζ-carotene, lycopene and either γ-carotene or chlorobactene. P 452 forms a purple derivative with an absorption maximum at 560 mμ in the presence of calcium salts which can be converted to the original P 452 form by treatment with traces of acid or alkali. The total carotenoid content of the organisms is estimated at between 0·5 and 0·6 mg. carotenoid/g. dry weight of organism.

INTRODUCTION

The corynebacteria include a number of highly pigmented organisms. Analyses of the pigments in *Corynebacterium michiganense* (Saperstein & Starr, 1954; Saperstein, Starr & Filfus, 1954), *C. poinsettiae* (Starr & Saperstein, 1953), *C. erythrogenes* (Hodgkiss, Liston, Goodwin & Jamikorn, 1954) and a *Corynebacterium* sp. (Hodgkiss *et al.* 1954) have shown the pigmentation to be due to carotenoids which, from *C. michiganense*, have been isolated in a complex with protein (Saperstein & Starr, 1955). A function for carotenoid pigments of bacteria in inhibiting lethal photo-oxidations was shown initially in photosynthetic organisms (Stanier & Cohen-Bazire, 1957) and later in non-photosynthetic bacteria (see, for example, Mathews & Sistrom, 1959, 1960; Mathews & Krinsky, 1965; Roth, 1967) including *Corynebacterium poinsettiae* (Kunisawa & Stanier, 1958). Although protection against photo-oxidations is the only known function of carotenoids in non-photosynthetic bacteria, the carotenoids present in the organisms so far examined appear to differ widely from species to species; however, the number of species examined in any one group is very limited. The present paper describes the carotenoid pigments of a further species, *Corynebacterium fascians*. A preliminary report of the pigments found in *C. fascians* has already been published (Prebble, 1962), but a closer investigation of this organism has shown the carotenoids present to differ slightly from those originally described. In particular, the pigments cryptoxanthin and zeaxanthin were incorrect identifications for two related but apparently hitherto undescribed carotenoids designated P 450 and P 452. Also, the pigments originally believed to be forms of β-carotene are probably three unrelated pigments.

METHODS

Strain 2Y (isolated by Mohanty, 1951) of *Corynebacterium fascians* (Tilford) Dowson was grown on a medium containing (%, w/v) 3 mannitol, 0·2 NH_4Cl, 0·2 asparagine, 0·1 glycine, 0·1 valine, 0·0001 thiamine, 0·5 KH_2PO_4, 0·5 Na_2HPO_4, 0·02 $MgSO_4$, 0·01 NaCl, 0·0001 $FeCl_3$, 2 agar; incubated at 26° for 4 days. The organisms were scraped from the agar and repeatedly extracted with methanol until no further appreciably coloured extract was obtained.

For direct separation on alumina columns, 20 % (v/v) water was added to the methanol before exhaustive extraction with light petroleum (b.p. 40–60°) followed by ether to remove the more polar pigments. Additions of acetone were made to break emulsions. The petroleum extract was washed with water, dried over Na_2SO_4, and evaporated to small volume under nitrogen. Hypophasic and epiphasic fractions were obtained by partition of the petroleum solutions with 90 % (v/v) methanol in water.

Saponification was done overnight at room temperature (18°) in the dark after the addition of half a volume of 33 % (w/v) KOH in methanol to the crude methanol extract.

Fractionation of extracts was achieved mainly on alumina columns of various Brockmann activities; 1 and 2 were obtained from Hopkin & Williams, Chadwell Heath, Essex, while the lower activities were prepared according to Brockmann & Schodder (1941). Pigment preparations were placed on alumina columns in petroleum and the pigments were eluted with increasing concentrations of ether, acetone and ethanol in light petroleum. Calcium carbonate and zinc carbonate columns were used for the separation of the more polar pigments.

Isolated carotenoids were chromatographed with standards on thin layers of alumina by using mixtures of acetone (5–20 %, v/v) in light petroleum for development.

The major carotenes were treated with iodine under illumination to form *cis*-isomers and isomers of dehydro-β-carotene (isocarotene) (Polgar & Zechmeister, 1942; Zechmeister & Wallcave, 1953).

A quantitative estimation of the sugar present in fraction P 462 was made by the method of Park & Johnson (1949); as standard, a sample of glucose treated in the same manner as the carotenoid was used. Concentrated HCl (0·2 ml.) was added to 1 ml. methanolic carotenoid solution and the hydrolysis done at 60° for 20 min., 1 ml. water was then added and the solution extracted with light petroleum to remove carotenoid components. The aqueous preparation was adjusted to between pH 10·5 and 11·0 and the reducing sugar estimated.

Identification of the sugar was by means of thin-layer chromatography. After hydrolysis and removal of carotenoid, the preparation was de-ionized in an electrolytic de-ionizer, evaporated to small volume under reduced pressure and chromatographed on Kieselguhr G-sodium acetate plates according to the method of Stahl & Kaltenbach (1961). Under these conditions glucose gave two spots, because of some degradation.

Samples of standard carotenoids were obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks. (synthetic β-carotene), Professor Weedon's laboratory (leprotene, hydroxy-chlorobactene and 4-hydroxy-β-carotene) and Dr B. Gilchrist (3-hydroxy-β-carotene). Preparations of carotenoids were made from maize seedling leaves (β-carotene), tomatoes (lycopene) and rose hips from *Rosa canina* (rubixanthin).

RESULTS

Extraction of carotenoid from bacteria grown on the mannitol–valine–glycine–asparagine medium gave a yellow solution with a maximum absorption in methanol at 458 mμ. Assuming an $E^{1\%}_{1\,cm}$ value of 2500, these cultures contained between 0·5 and 0·6 mg. carotenoid/g. dry wt. bacteria. Preliminary experiments with a simplified mannitol (1 %) asparagine–salts medium showed that this concentration of carotenoid was sensitive to environmental conditions. Static liquid cultures having a poor oxygen

Table 1. *Chromatography of the total pigment extract of Corynebacterium fascians strain 2Y on an alumina column (Brockmann activity 2)*

Band	Approximate absorption maxima (mμ)			Elution solvent	Phase partition between light petroleum and 90 % (v/v) methanol in water	Final carotenoid identification
1. Colourless with bluish-green fluorescence	275 333	285 350	300 369	4 % (v/v) ether in light petroleum	Epiphasic	Phytoene, phytofluene
2. Orange	(428)	450	477	8 % (v/v) ether in light petroleum	Epiphasic	β-carotene (all-*trans*)
3. Orange	427	450	476	15 % (v/v) ether in light petroleum	Epiphasic	'Carotene 450' (? β-iso-renieratene)
4. Orange	(430)	452	480	20 % (v/v) ether in light petroleum	Epiphasic	Leprotene, β-zeacarotene
5. Deep orange	.	448	.	5 % (v/v) acetone in light petroleum	Epiphasic	Neurosporene, γ-carotene or chlorobactene, ζ-carotene, lycopene (β-zeacarotene)
6. Orange-yellow	.	450	(472)	10 % (v/v) acetone in light petroleum	Mainly hypophasic	P 450
7. Orange-yellow (with occasional grey-blue band)	.	453	.	5 % ethanol in light petroleum	Hypophasic	P 452 (P 560)
8. Orange-red	.	462	485	Firmly bound, only partially eluted with HCl-methanol mixtures	Hypophasic	P 462, hydroxy-chlorobactene glucoside

supply synthesized substantially less pigment than comparable surface cultures on agar medium. The addition of certain substances to an agar medium promoted pigment synthesis; for example valine at 0·1 % (w/v) produced up to a 50 % increase in pigment content while increases in mannitol from 1 % to 3 % had a similar effect.

Chromatography of a pigment extract on an alumina column (Brockmann activity 2) gave the eight fractions listed in Table 1. Separation of the pigment extract into epiphasic and hypophasic fractions, followed by chromatography on alumina and zinc carbonate, respectively, showed that fractions 1–5 were epiphasic, 7 and 8 hypophasic, while 6 was mostly, though not entirely, hypophasic. Saponification, before partition and chromatography, produced no apparent change in the subsequent behaviour of the pigments, from which it was concluded that probably none of the pigments was present in an esterified form in this organism.

Epiphasic pigments

Fractions 2–4 with spectra almost identical to that of β-carotene (see Table 2), ran very close together on the column and were difficult to separate completely from one another. Comparison of the spectral and thin-layer chromatographic properties of each fraction with samples of β-carotene indicated that fraction 2 was all *trans*-β-carotene. Fractions 3 and 4 are unlikely to be *cis*-isomers produced as artifacts during the extraction procedure since they were present at high levels relative to fraction 2 (see Zechmeister, 1962). However, *cis*-isomers of β-carotene have been found in nature (Suzuki & Tsukida, 1959); attempts were therefore made to convert fractions 2–4 to common products using catalytic amounts of iodine under illumination. Fraction 2 gave rise to a mixture of isomers, none of which were identical with fractions 3 and 4. Fraction 4 would not isomerize appreciably under the conditions used successfully with β-carotene. Fraction 3 reacted to form a range of products giving at least three spots on thin-layer chromatograms; this range did not coincide with the isomers obtained from β-carotene and did not include a spot corresponding to all-*trans*-β-carotene. Thus it is doubtful whether this fraction can be classed as a *cis*-isomer of β-carotene and it is therefore designated carotene 450.

Fraction 4 differed from fractions 2 and 3 in its failure to react readily with catalytic amounts of iodine and in its low solubility in light petroleum. Grundmann & Takeda (1937) isolated from *Mycobacterium phlei* a pigment closely resembling β-carotene. The structure of this pigment, leprotene, has been shown to differ from β-carotene mainly in having aromatic end rings (Jensen & Weedon, 1964). Fraction 4 was shown to be identical with leprotene in its chromatographic and spectral properties and to resist isomerization under the conditions used here.

Fraction 1 running in front of β-carotene was resolved into two fractions by further chromatography on alumina. The more strongly adsorbed fraction gave a greenish fluorescence in ultraviolet radiation and had an absorption spectrum corresponding to phytofluene, while the more rapid fraction had spectral properties of phytoene.

During purification of the β-carotene and leprotene fractions, small quantities of a pale yellow pigment were isolated. This pigment had spectral and chromatographic properties which suggest its identity with β-zeacarotene.

Fraction 5 was a mixture of several carotenoids all present in low concentration. The mixture was analysed on weakened alumina columns (Brockmann activity 4). The first two closely associated constituents were separated by developing columns with increasing concentrations of ether in light petroleum (1–5 %, v/v) and were identified by their spectra as neurosporene and either γ-carotene or chlorobactene. The two latter pigments have identical spectra and similar chromatographic properties. Unfortunately the available amounts of these pigments did not permit a final identification. The third polyene, closely associated with pigment P 450, was eluted with 20 % ether and identified as ζ-carotene. A lycopene-like fraction, more strongly adsorbed, was separated and behaved similarly to lycopene on thin-layer chromatography.

Hypophasic pigments

Three readily separable hypophasic fractions, all major constituents, were obtained. The first of these was eluted from alumina (Brockmann activity 2) with 20 % (v/v) acetone in light petroleum. Its adsorption on columns and its partition characteristics

suggested that it was a monohydroxy pigment, while the spectral characteristics were not dissimilar to those of cryptoxanthin although the purified pigment had a main maximum at 477 mμ in light petroleum (Table 2). Repeated rechromatography of this pigment resulted in a spectrum which differs from that of cryptoxanthin in two respects: the main maximum is at a wavelength less than 448 mμ and there is a broad shoulder at about 472 mμ but no second maximum (Fig. 1). Thin-layer chromatography with samples of 3-hydroxy-β-carotene and 4-hydroxy-β-carotene showed that the corynebacterial pigment ran slightly in front of the two standards and well behind echinenone, the order of increasing adsorption being echinenone, the test pigment, 4-hydroxy-β-carotene, 3-hydroxy-β-carotene. The corynebacterial pigment was further differentiated from 4-hydroxy-β-carotene in its inability to react with HCl/chloroform. In view of the failure to determine the identity of this pigment it has been necessary to designate it as pigment P 450.

Table 2. *The absorption maxima of the main carotenoids in Corynebacterium fascians strain 2Y in various solvents*

Carotenoid	Light petroleum (b.p. 40–60°)	Ethanol	Carbon disulphide	Chloroform	Benzene
β-Carotene	450 477	—	—	—	465 493
Carotene 450 (? β-isorenieratene)	429 450 477	—	—	—	441 465 493
Leprotene	450 477	—	—	—	464 492
P 450	447	450	478	461	460
P 452	449	452	482	464 (488)	462
P 560	Insol.	566–571	580	573–574	556
P 462 (hydroxy-chlorobactene glucoside)	465 493 (Almost insoluble)	462 491	494 524	472 501	474 502

The last pigment to be eluted with 5 % ethanol in light petroleum from an alumina column also has a maximum at about 450 mμ and is designated pigment P 452. On re-chromatography this carotenoid was found to have an absorption spectrum with a single peak at 449 mμ and a very slight shoulder at about 480 mμ in light petroleum (see Table 2). This spectrum does not correspond to either zeaxanthin or isozeaxanthin but resembles those of conjugated ketones (Fig. 2). However, reduction of the pigment with sodium borohydride gave a complex mixture of material absorbing at wavelengths about 400 mμ.

The most significant property of pigment P 452 is its ability to form a purple derivative, designated P 560, in the presence of calcium salts. A fresh pigment preparation placed on a calcium carbonate column in light petroleum became progressively bluer when adsorbed on the column and was eluted with 10 % acetone in light petroleum to give a purple solution having a spectrum with a broad maximum at about 560 mμ (Fig. 2). The exact position of this absorption maximum varied considerably from preparation to preparation, probably due to isomerization, since a range of spots could be demonstrated by chromatography. The occasional occurrence of the purple form on alumina columns was presumably due to the presence of trace amounts of calcium hydroxide in the alumina. Pigment P 560 may also be formed by adsorbing pigment P 452 on solid calcium hydroxide followed by treatment with a mixture of equal parts of pyridine and acetone; a purple solution is slowly obtained

on standing. Pigment P 560 is almost completely insoluble in light petroleum but readily soluble in acetone and ethanol. It is eluted from an alumina column with 10% (v/v) acetone in light petroleum. The P 452 form may be regenerated from P 560 by gently shaking a petroleum solution with 0·1 N-HCl in water or by treatment with methanol containing 1% (w/v) NaOH.

The most strongly polar pigment present in *Corynebacterium fascians*, P 462, was not eluted from an alumina column by normal solvent systems, and was best prepared by chromatography on zinc carbonate or calcium carbonate after partition between 90% (v/v) methanol and light petroleum. It was eluted from these columns with 5% (v/v) ethanol in light petroleum. The pigment was almost insoluble in petroleum and showed a strong affinity for sodium sulphate. The spectrum of a purified preparation shows a main maximum at 462 mμ with a second peak at 491 mμ in ethanol and closely resembles that of the mono-hydroxy carotenoid, rubixanthin (Fig. 3,

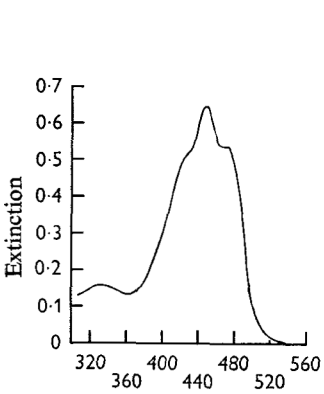

Fig. 1

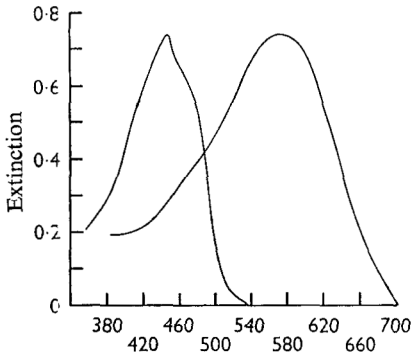

Fig. 2

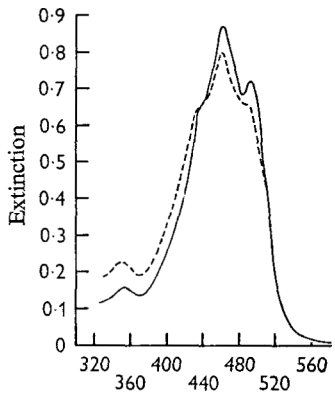

Fig. 3

Fig. 1. *Corynebacterium fascians* strain 2Y. The absorption spectrum of pigment P 450 in light petroleum.

Fig. 2. *Corynebacterium fascians* strain 2Y. The absorption spectra of pigment P 452 in light petroleum (left-hand curve) and P 560 in ethanol (right-hand curve). P 560 was derived from P 452 by adsorption on calcium hydroxide and elution with acetone and pyridine.

Fig. 3. *Corynebacterium fascians* strain 2Y. The absorption spectra of pigment P 462 (hydroxy-chlorobactene glucoside) in ethanol (——) and the pigment derived by acid hydrolysis of P 462 (hydroxy-chlorobactene) in ethanol (– – –).

Table 2). Saponification procedures had no effect on the nature of the pigment, but treatment with acid methanol (20%, v/v, conc. HCl in methanol) for 10 min. at 61° resulted in changes in the pigment's properties; the strongly hypophasic pigment became epiphasic. After this acid treatment the absorption spectrum remained essentially the same (Fig. 3) but the pigment now chromagraphed as a mono-hydroxy carotenoid. The mono-hydroxy carotenoids, rubixanthin and hydroxy-chlorobactene, have almost indistinguishable spectra with a main maximum at 461 mμ. Both have been reported from bacteria, although Jensen, Hegge & Jackman (1964) suggest that rubixanthin could be an incorrect identification for hydroxy-chlorobactene in many of the species from which it is reported. These two pigments may be separated on alumina layers using 15% (v/v) acetone in light petroleum, the hydroxy-chloro-

bactene being more strongly adsorbed than rubixanthin. The mono-hydroxy pigment from *C. fascians* was shown to be identical with, and inseparable from, hydroxy-chlorobactene on chromatography.

The result obtained on acid treatment suggests either extensive dehydration or, more likely, the hydrolysis of a strongly polar derivative of hydroxy-chlorobactene. The hypophasic layer was found to give a positive Molisch test. Estimation of the amount of reducing sugar produced from an arbitrary amount of pigment P 462 gave a value equivalent to 6·9 μg, against an expected value of 6·8 μg, assuming an $E^{1\%}_{1\,cm} = 2500$ for hydroxy-chlorobactene and the presence in each carotenoid glycoside molecule of a single residue of reducing hexose. The sugar was, in fact, identified as

Table 3. *The carotenoids of Corynebacterium fascians strain 2Y*

Estimates of the amount of each carotenoid present in the organism. It is assumed that during analysis the rate of oxidation of each pigment is the same. $E^{1\%}_{1\,cm}$ values are taken from Davies (1965) or, where not known, a value of 2500 has been used. In calculating the amount of carotenoid a value of 0·55 mg./g. dry wt. organism was used.

Carotenoid	$E^{1\%}_{1\,cm}$	λ_{max}	% of total pigment	μg. carotenoid/g. dry wt. organism
Phytoene	1250	285 petroleum	4·8	26
Phytofluene	1350	348 petroleum	1·9	10·4
β-Carotene	2505	451 petroleum	8·2	45
Carotene 450 (? β-isorenieratene)	2500	450 petroleum	5·2	38
Leprotene (isorenieratene)	2250	465 benzene	24·2	133
β-Zeacarotene	2570	428 hexane	0·7	3·8
Neurosporene	2990	440 petroleum	0·3	1·6
γ-Carotene	3100	462 petroleum	0·6	3·3
ζ-Carotene	2270	400 petroleum	1·0	5·5
Lycopene	3450	472 hexane	0·4	2·2
P 450	2500	450 petroleum	16·6	91
P 452	2500	450 petroleum	20·6	113
P 462, hydroxy-chlorobactene glucoside	2500	462 ethanol	15·7	86

glucose. It was found that the sugar derived from the carotenoid produced two spots on chromatography which were identical with those produced from standard glucose given the same acid treatment. It is therefore concluded that P 462 is a glucoside of hydroxy-chlorobactene.

Attempts have been made to hydrolyse P 462 using enzymes. Neither maltase nor emulsin hydrolysed the carotenoid glucoside in ethanol–water mixtures where it was known that the enzyme would attack a standard substrate (cf. Smith, 1963*a*; Hertzberg & Jensen, 1967).

DISCUSSION

Evidence for the existence of 13 different carotenoid pigments in strain 2Y of *Corynebacterium fascians* has been presented. Estimates, based on extinction coefficients, of the relative and absolute quantities of the individual pigments are shown in Table 3. The principal constituents in this mixture are β-carotene; a β-carotene-like pigment, carotene 450; leprotene (isorenieratene); two apparently new xanthophylls, P 450 and P 452; and hydroxy-chlorobactene glucoside. The remaining pigments, present in low concentration, are probably all intermediates in the synthesis of the

principal constituents. An earlier communication of the carotenoids of this organism failed to distinguish leprotene from β-carotene and identified pigments P 450 and P 452 with cryptoxanthin and zeaxanthin respectively. At this stage it was not realized that the purple pigment P 560 was derived from P 452. This led to a carotenoid composition that had much in common with that of higher plants, now obviously an erroneous view.

Some of the pigments described require further comment. The carotene closely related to β-carotene, running slightly behind β-carotene on columns and thin layers, is probably a new pigment rather than a naturally occurring *cis*-isomer of β-carotene, for the following reasons. Iodine treatment of the pigment produced a group of substances similar to, but slightly less strongly adsorbed on alumina than, those formed from β-carotene, but no pigment corresponding to all-*trans*-β-carotene. In addition, the main absorption band of carotene-450 is at a wavelength not less than that of β-carotene and the spectrum of the new carotene shows no *cis*-peak. It therefore seems appropriate to suggest that this may be a pigment intermediate in structure between leprotene and β-carotene. Such a pigment, β-isorenieratene, has been isolated, together with both β-carotene and leprotene, from Phaeobium by Jensen (1965*a*). Carotene-450 has chromatographic and spectral properties identical with those described for β-isorenieratene (cf. Tables 1, 2).

Of the three apparently new xanthophylls, P 450 seems to be almost but not quite identical with 4-hydroxy-β-carotene and hence would appear to be a mono-hydroxy pigment. The more strongly adsorbed pigment P 452 is assumed to be a carotenoid on the basis of its spectrum, chromatographic properties and solubility in hydrocarbon solvents. However, it is significant in its ability to form a purple derivative in the presence of calcium salts. To my knowledge, no other naturally occurring pigment with this property has been described. Two carotenoids from invertebrates are known to form purple derivatives; actinioerythrin is believed to form a purple derivative after careful treatment with NaOH (Heilbron, Jackson & Jones, 1935), while astaxanthin will form purple complexes with proteins (Cheesman & Prebble, 1966; Cheesman, Lee & Zagalsky, 1967) and a purple derivative after treatment with potassium butoxide, which is believed to be a potassium salt. Neither of these pigments has been shown to react with calcium. It should be noted that there is no evidence at present that P 560 contains any calcium although the properties suggest that it may be a calcium salt, in which case a pigment possessing two adjacent hydroxyl groups seems likely.

Naturally occurring carotenoid glycosides were unknown until Smith (1963*a*, *b*) described a neurosporyl glucoside from Mycoplasma. Subsequently two further glucosides have been thoroughly characterized from *Mycobacterium phlei* strain VERA by Hertzberg & Jensen (1967). The hydroxy-chlorobactene glucoside described here is probably only one of many naturally occurring glucosides yet to be identified since strongly adsorbed pigments are not infrequently described in micro-organisms; for example, Hodgkiss *et al.* (1954) described a pigment, corynexanthin, from Corynebacterium species which resembles P 462 in its chromatographic properties but has its main absorption maximum at 437 mμ in ethanol. Smith suggested that the function of the neurosporyl glucoside of Mycoplasma was that of a permeability factor mediating glucose transfer across the membrane; a similar function here seems unlikely since the sole carbohydrate present in the medium is mannitol.

None of the corynebacteria investigated earlier were reported to contain either

leprotene or the xanthophylls described here. Strains of *Corynebacterium michiganense* synthesize cryptoxanthin, lycopene, spirilloxanthin, β-carotene and canthaxanthin (Saperstein *et al.* 1954), while *C. poinsettiae* synthesizes cryptoxanthin, lycoxanthin, lycopene and spirilloxanthin (Starr & Saperstein, 1953), although the presence of spirilloxanthin has since been denied (Jensen, 1965*b*). *C. erythrogenes* contains neoxanthin, sarcinaxanthin and corynexanthin (Hodgkiss *et al.* 1954). Indeed a greater affinity with the leprotene-synthesizing Mycobacterium might be claimed, particularly since the mycobacterial xanthophylls B and C of Goodwin & Jamikorn (1956) could be closely related to the xanthophylls of *C. fascians*. The problematical position of *C. fascians* and closely related Mycobacterium species was discussed by Gordon (1966), who suggested that there is no clear delineation of the two genera. It is therefore not surprising to find that the carotenoid composition of the strain 2Y of *C. fascians* has similarities with that of some Mycobacterium strains.

I wish to thank Professor B. C. L. Weedon (Queen Mary College, London) for supplying samples of leprotene, 4-hydroxy-β-carotene and hydroxy-chlorobactene, and Dr B. Gilchrist (Bedford College, London), for a sample of 3-hydroxy-β-carotene.

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Gene Transfer in Strains of *Pasteurella pseudotuberculosis*

By W. D. LAWTON
Biological Sciences Laboratory, Fort Detrick, Frederick, Maryland, U.S.A.
B. C. MORRIS AND T. W. BURROWS
Microbiological Research Establishment, Porton Down, Salisbury, Wilts.

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SUMMARY

Pasteurella pseudotuberculosis strain 321V accepted the F'*lac* episome from *Escherichia coli* strain 23.10S and behaved as a gene donor in crosses with several different auxotrophs of *P. pseudotuberculosis*. Some selected donor markers were transferred at frequencies of 10^{-4}–10^{-5} per donor cell while others appeared not to be transferred. Up to 40% of recombinants were Lac+. Selected recombinants showed differing unselected marker frequencies with differing selected markers; those obtained by using double marker selection showed increased unselected marker frequencies. Some alternative explanations for the origin of recombinants (syntrophic growth, mixed clones, multiple recipient reversions) were not supported by experiment.

INTRODUCTION

Previous attempts to discover a gene transfer system in *Pasteurella pestis* involving conjugation, transduction or transformation were unsuccessful (Burrows, 1962). The report by Martin & Jacob (1962) that an avirulent strain of *P. pestis* could accept the F'*lac* episome from *Escherichia coli* and observations at the Microbiological Research Establishment that strains of *P. pseudotuberculosis* could accept R-factors (J. Boyle, unpublished) stimulated efforts to obtain episome-mediated gene transfer in Pasteurella. Although our ultimate interest is in the genetics of *P. pestis* we are approaching this subject through the very closely related but more easily handled organism *P. pseudotuberculosis* and have studied the transfer of markers from an F'*lac* infected donor strain to different recipient strains of this latter species. To avoid tedious repetition of non-committal terms such as 'presumed donor', 'presumed recombinant' in this first report, we use the terminology currently employed by others in their descriptions of the firmly established fertility system of *E. coli* and assume its applicability to the system here described for Pasteurella. In this paper we present evidence from which we deduce the occurrence of gene transfer and show that other explanations for the development of 'recombinant' strains are less acceptable.

METHODS

Strains. Escherichia coli K12 strain 23.10S Met-(F'*lac*) was kindly supplied by Dr R. C. Clowes. *Pasteurella pseudotuberculosis* strain 321V was obtained from Professor E. Thal, Stockholm, in 1958 and held since at the Microbiological Research Establishment under the number MRE259. Auxotrophic derivatives of strain MRE259

were derived by presumed single-step mutations following treatment with nitrous acid (Kaudewitz, 1959). Derivatives resistant to *P.pseudotuberculosis* phage IV were isolated as colonies arising on lawns of sensitive bacteria lysed by phage. Streptomycin-resistant strains were isolated from sensitive parent bacteria by plating on complete medium supplemented with streptomycin to 100 μg./ml. Strains were stored *in vacuo* at 2° as dried gelatin pellets (Stamp, 1947). Abbreviations designating genotypes and phenotypes of strains follow the recommendations of Demerec, Adelberg, Clark & Hartman (1966) with the additional symbols.

p4s = locus determining sensitivity to phage IV (phenotype symbols P4S and P4R for sensitivity and resistance respectively).

pth = Locus determining the presumed single-step mutation leading to a double requirement for any purine+thiamine (phenotype symbols Pth+ and Pth− for independence and dependence on these factors respectively).

The relevant genotypes of the strains used are shown in the tables.

Media. Tryptic digest of meat broth (TMB) or agar (TMA) and blood agar base (BAB) no. 2 (Oxo Limited) were used as complete media. Minimal agar medium (MA) was prepared as follows. Solution A (g./l. distilled water): Agar (Oxoid no. 3), 30; sodium citrate $2H_2O$, 1; autoclaved 115° for 15 min.; solution B (g./l. distilled water) K_2HPO_4, 21; KH_2PO_4, 9; $(NH_4)_2SO_4$, 2; sodium citrate $2H_2O$, 1; $MgSO_4.7H_2O$, 0·1; adjusted to pH 7·2 and autoclaved similarly. The final medium contained equal volumes of solutions A and B+glucose (sterilized by filtration) to give 0·2 % (w/v).

Minimal salts solution (MS) was solution B+an equal volume of distilled water. Selective media were MA variously supplemented, as required, with growth factors to give the concentrations (mM): arginine, 0·5; cysteine, 0·4; glycine, 1; histidine, 0·2; isoleucine, 0·5; methionine, 0·2; phenylalanine, 0·5; serine, 1; threonine, 1; tryptophan, 0·1; tyrosine, 0·5; purine, 0·05; thiamine, 0·001.

Lactose indicator medium was BAB medium+lactose 1 % (w/v), bromothymol blue 0·0025 % (w/v) and triphenyltetrazolium chloride 0·005 % (w/v). On this medium Lac+ colonies were yellow and Lac− colonies reddish purple.

Crossing procedure. The strain MRE2027 was used as donor throughout. It had the relevant genotype *cys-5 pth-2 p4s str-11* (F′ *lac*). Donor bacteria were grown in 10 ml. TMB medium+lactose (0·1 %, w/v) at 28° with rotation. After 18 hr incubation 5 ml. of the culture was added to 5 ml. of fresh medium and incubated, with rotation, for a further 2 hr. The exponentially growing bacteria were centrifuged and resuspended in 10 ml. of MS. Five ml. of this suspension (containing about 1×10^9 organisms/ml.) were exposed, with gentle agitation in a Petri dish, to a 15 W. ultraviolet lamp (Hanovia Limited) at a distance of 36 cm. for 30 sec. and then diluted 10-fold in MS to provide the donor bacteria suspension.

The various recipient strains were grown on TMA at 28° for 20 hr. They were then suspended in TMB to give concentrations of about 5×10^9 organisms/ml. to provide the recipient bacteria suspensions.

One-tenth ml. of recipient suspension was spread over the appropriate selective media plates followed by the same volume of donor suspension spread over the recipient lawns. Control plates spread with recipient suspension alone and with donor suspension alone were included in every experiment. All plates were incubated at 28° and scored for recombinants after 4–6 days.

Purification and analysis of recombinants. Colonies appearing on the different selective media were streaked on plates of the same media on which they had arisen and single colonies from these streaks were restreaked as before. Single colonies, one from each of the final growths, were inoculated to marked positions on plates of the same medium to provide master plates from which colonies could be replicated to variously supplemented media (by using a 76-needle replicator) to score for unselected markers. The purified recombinants always were replicated to donor selective medium to ensure their freedom from contaminating donor bacteria whose presence would have made unselected marker analysis suspect. In later experiments it was found adequate to replicate recombinant colonies 3 times on selective agar (without streaking and picking single colonies) to ensure their freedom from contamination by parent bacteria.

Recombinants were scored for phage sensitivity by replicating to BAB overlayered with 3 ml. soft agar containing 1×10^8 p.f.u. of phage IV per ml. and for streptomycin resistance by replicating to BAB + streptomycin, 100 μg./ml.

RESULTS

Isolation of donor strain MRE2027

From the *Pasteurella pseudotuberculosis* strain MRE259 we derived the Cys− strain MRE312 and from this the SmR strain MRE2077. This was grown in broth culture with *Escherichia coli* 23.10S and plated on minimal agar containing cysteine, lactose (in place of glucose) and streptomycin. Colonies arising on this medium were indistinguishable from strain MRE2077 except for being Lac+. Representative colonies transferred *lac* to other strains of *P. pseudotuberculosis*, to *P. pestis* and to *E. coli*, and were presumed therefore to have acquired the transmissible element F′*lac*. One such representative, MRE2007, was used as parent strain for the production of a series of auxotrophs, one of which had the double requirement for purine + thiamine and appeared to be the most efficient gene donor of a number tested in crosses with other, Lac−, auxotrophs. This Pth− derivative was chosen for further study and numbered MRE2027. Donor suspensions prepared for use in crosses usually contained about 2% Lac− bacteria.

Gene transfer

The crossing technique described under Methods was developed after considerable experience. Crosses made under conditions effective for *Escherichia coli*, i.e. mating in broth at 37° followed by dilution and plating on selective media, were sterile. It seemed that the growth conditions and treatment of the donor suspension were particularly critical. Ultraviolet irradiation of donor bacteria decreased viability to about 50% but increased the yield of recombinants 5- to 10-fold (i.e. a 10- to 20-fold increase per viable donor bacterium) above that of un-irradiated cells. When following ultra-violet irradiation the donor bacteria were incubated for 1 hr in TMB at 28° before crossing (Hayes, 1953) a further increase (about 1·5-fold) in yield sometimes occurred. This additional treatment, however, seemed to be an unwarranted modification of the technique for routine use.

Minimal enrichment of the selective media on which crosses were performed was necessary for fertility. Thus, when donor and recipient mixtures were incubated in TMB or in MS for 5 hr at 28°, the bacteria deposited, washed, resuspended in MS and plated on selective medium, no recombinants were obtained. The same washed

suspensions plated on selective medium enriched with 0·1 ml. TMB per plate yielded recombinants. Enrichment with 0·1 ml. TMB appeared to be optimal for maximum fertility with minimum background growth and was conveniently made by using recipient cells suspended in TMB.

Table 1. *Fertility of an F′ lac donor and infertility of its Lac− segregant in crosses with different recipient strains of Pasteurella pseudotuberculosis*

Donor: MRE 2027: *cys-5 pth-2 str-11* (F′ *lac*).

Recipient strain			Numbers of colonies on selective media*				
			Crosses		Controls		
No.	genotype	Selected phenotype	F′ *lac* donor	Lac− donor	Recipient alone	F′ *lac* donor alone	Lac− donor alone
MRE 2024	*leu-1 gly-2 ser-1*	Leu +	335	0	0	0	0
MRE 2056	*tyr-1 his-13*	Tyr +	200	5	5	0	0
		His +	20	0	0	0	0
MRE 2118	*met-5 arg-8*	Met +	0	0	0	0	0
		Arg +	112	4	8	0	0
MRE 2117	*met-5 leu-3*	Met +	0	0	0	0	0
		Leu +	320	0	1	0	0

* The different selective media were MA plus all growth factors of the particular recipient strain except that for which selection for independence was being made.

Table 2. *Occurrence of unselected donor markers in recombinants from crosses between strains of Pasteurella pseudotuberculosis*

Donor: MRE 2027: *cys-5 pth-2-str-11* (F′*lac*)

Recipient					Unselected donor markers* (%)			
Strain no.	Relevant genotype	Expt no.	Selected phenotype	Number analysed	*lac*	*p4s*	*met*	*Others*
MRE 2056	*tyr-1 his-13*	1	Tyr+	98	25	.	.	*his* 0
		2	His+	27	4	.	.	*tyr* 0
MRE 2117	*met-5 leu-3*	3	Leu+	69	20	.	0	.
MRE 2205	*met-5 arg-8 p4 -2*	4	Arg+	49	18	37	0	.
MRE 2258	*met-5 arg-8 ile-3 p4r-2*	5	Arg+	67	24	36	0	*ile* 5
			Ile +	68	22	6	0	*arg* 16
		6	Arg+	225	17	25	0	*ile* 2
			Ile +	304	18	4	0	*arg* 3
MRE 2263	*met-5 arg-8 aro-2 p4r-2*	7	Arg+	376	14	36	1	*aro* 3
			Aro+	300	25	9	18	*arg* 14
MRE 2256	*met-5 arg-8 his-8 p4r-2*	8	Arg+	138	28	37	0	*his* 81
		9	Arg+	304	22	35	0	*his* 85
MRE 2291	*met-5 arg-8 his-8 trp-3 p4r-2*	10	Arg+	225	20	32	0	*his* 85 *trp* 4
			Trp+	225	29	47	1	*his* 43 *arg* 36
			Arg+ Trp+	225	40	76	2	*his* 95

* No recombinants showed the donor phenotype SmR.

Table 1 summarizes the results of crosses involving four differently marked recipient strains. The recovery of many colonies per plate in the crosses in contrast to the small numbers on control plates indicated that the donor markers *arg*, *leu*, *his* and *tyr* were transferred to the different recipients at frequencies ranging from about 10^{-4} to 10^{-5} per donor bacterium. There was no indication of fertility when the donor was crossed with auxotrophs having requirements for methionine, nicotinamide, thiamin or uracil, nor when the donor strain was replaced by one of its Lac− spontaneous segregants.

From the Met− Arg− strain MRE2118 (Table 1) additionally marked strains were derived to test double marker selection and to permit unselected marker analysis of recombinants. As shown in Table 2 the markers *arg*, *ile*, *aro* and *trp* were transferred both as selected and as unselected markers and *his*, *p4s* and *met* as unselected markers. The donor marker *str* was not observed in recombinants in these experiments. Recombinants were obtained with double selection for the markers *arg* and *trp*; these showed increased unselected marker frequencies for *his* and for *p4s* when compared with recombinants obtained with single selection for *arg* or for *trp*.

Alternatives to gene transfer

Mechanisms other than gene transfer seemed unlikely to have been responsible for the development of colonies on selective media and for these colonies to have possessed unselected donor properties. Nevertheless, some other possibilities were examined. To explain the development of larger numbers of colonies from mixed parent platings than from parent control platings it could be argued that syntrophism, between the parental strains, permitted larger populations of recipient cells and consequently larger numbers of reversions. If this explanation held one would expect that following the plating of an Arg− recipient strain on *arg* selective media to which was added increasing concentrations of arginine, increasing numbers of revertants would arise to equal, or exceed, the numbers of recombinants in crosses with this recipient. The recipient strain MRE2205 (Table 2) tested in this way showed a maximum of 14 colonies from reversions of the *arg-8* locus (Table 3) in contrast to more than 100 recombinants/plate in crosses. A visual comparison of the background growth from mixed parent platings with those on arginine supplemented medium in these experiments indicated that the former did not exceed $1{\cdot}2 \times 10^9$ bacteria per plate. A total of 76 Arg+ revertants derived from these experiments were tested for unselected markers as if they had been Arg+ recombinants. None scored P4S or Lac+ in contrast to recombinants selected for Arg+, of which about 30% were P4S and about 20% were Lac+. The donor strain plated on minimal medium with increasing specific growth factor additions showed no double reversions. With this strain full supplementation with purine + thiamine allowed the detection of rare Cys+ revertants but no reversions to Pth+ were seen on plates fully supplemented with cysteine.

As a further test to exclude syntrophism a membrane filter was interposed between donor and recipient bacteria on selective media. These experiments resembled those of Dushman (1963) who showed that syntrophism could account for the apparent fertility of auxotrophic strains of *Serratia marcescens*. Our results (Table 4), in contrast to those reported with Serratia, showed that the number of colonies arising when parent bacteria were separated was less than 5% of that obtained when free contact of parent bacteria was permitted. Colonies arising on areas covered by membranes mostly

were restricted to the margins of the membranes where separation of parental bacteria may not have been complete. Further (Table 4), when parent bacteria were applied to selective media plates in separate, thin (0·3 mm.) agar layers, virtually no colonies developed whether or not the agar layers were separated by a membrane. However, when parent suspensions were mixed before adding to melted agar so that both parents were contained within the same agar layer, many colonies developed.

In an additional test of syntrophism confluent lawns of donor and of recipient cells (MRE2256) grown on separate BAB plates were replicated with the same 76-point replicator to another BAB plate which was incubated at 28° for 18 hr. This master plate, known to carry mixed clones, was replicated to the differently supplemented

Table 3. *Populations and numbers of spontaneous Arg+ revertants of Pasteurella pseudotuberculosis strain MRE2205 plated on Arg+ selective medium increasingly supplemented with arginine*

Strain MRE2205, Met− Arg− P4R, was grown and suspended in TMB as for use as a recipient in crosses and 0·1 ml. volumes (*c.* 5×10^8 organisms) spread on MA plus methionine plates containing increasing additions of arginine. One-tenth ml. of MS was then spread on the plates (to simulate application of donor cells) and the plates incubated for 5 days at 28°. Macroscopically obvious colonies were counted, removed from the agar, the remaining background population suspended in MS and measured turbidimetrically.

		Arginine addition (μM final concentrations)								
		0	4	8	16	32	64	125	250	500
Expt 1	Revertants per plate*	0·5	1·5	1	3·5	9	12	5·5	?	?
	Population per plate* ($\times 10^{-9}$)	0·93	1·23	1·59	1·44	1·83	9·9	9·0	17·7	20·7
Expt 2	Revertants per plate†	0	0·2	1	3	2·4	13·4	10·6	?	?
	Population per plate* ($\times 10^{-9}$)	0·6	0·63	0·87	0·84	1·25	2·7	8·1	18·7	19·3

? = Revertants not recognizable against the dense background growth.
* Means of duplicates.
† Means of five plates.

minimal media used in the analyses of recombinants. Two parallel series of plates were replicated with the donor alone and with the recipient alone and all plates incubated at 28° for 5 days. All separate donor and recipient replicates scored correctly. Mixed replicates grew both on minimal medium selective for the donor and on that selective for the recipient and all scored P4R and SmR on complete medium. On lactose indicator medium they produced brownish colonies. No growths were visible on the differently supplemented minimal media which would have been selective for syntrophic growths, reversions or for recombinants. The same results were obtained with the recipient strains MRE2258 and MRE2263. From the above experiments we obtained no support for the possibility that syntrophism followed by reversions could account for the fertility we had inferred. Further, the experiments excluded the possibility that recombinants falsely showed donor markers through contamination with donor cells since, unlike mixed clones, no recombinants grew on donor specific medium, none were SmR and several were P4S.

Table 4. *Reduction of the fertility of Pasteurella pseudotuberculosis crosses by interposing a membrane between parent bacteria or by separating them in different agar layers*

Suspensions of parent bacteria were prepared as for use in crosses. Donor, or recipient, bacteria were first applied to selective agar plates either by spreading 0·1 ml. of suspension or by adding this to 2 ml. of selective agar at 45° and overlayering the mixture. A semicircular membrane filter (standard grade, Oxo Limited, London) was then applied to cover one half of the agar surface, followed by the addition of recipient, or donor, bacteria as previously. The plates were incubated at 28° for 5 days and the colonies counted. The donor strain MRE 2027 was used throughout.

			Recipient strains				
			MRE 2267	MRE 2258		MRE 2291	
			Selection for				
Parent bacteria applied to plates as	Sequence of applications to plates	Separated (+) or not separated (−) by membrane	Arg+	Arg+	Ile+	Arg+	Trp+
			Numbers of colonies*				
Suspension (0·1 ml.) spread	†1. Recipient 2. Membrane 3. Donor	+ −	0 1000	18 1000	0 430	4 570	1 22
Suspension (0·1 ml.) added to 2 ml. agar and over-layered	Donor alone	.	0	0	0	0	0
	Recipient alone	.	0	6	0	1	1
	1. Donor 2. Membrane 3. Recipient	+ −	1 2	2 4	0 0	0 2	0 2
	1. Recipient 2. Membrane 3. Donor	+ −	0 10	0 6	0 0	0 12	0 1
Mixed suspension (0·2 ml.) added to 2 ml. agar and over-layered	Donor and recipient simultaneously	.	400	233	26	128	1

* Sums of colonies on duplicated half-plate areas. On membrane-covered half-plate areas colonies growing under and on the membranes are summed; they were stained deep red, to permit counting, by allowing 1 ml. triphenyltetrazolium chloride solution (0·25 %, w/v) to be absorbed by the membrane, followed by 20 min. incubation at 28°.

† Similar results were obtained with the reverse sequence. Membranes located above or below parental mixtures in normal crosses did not interfere with fertility.

Preliminary observations on the kinetics of marker transfer

Male specific phage $\mu 2$ (Dettori, Maccacaro & Piccinin, 1961) formed plaques on lawns of the donor strain but not on those of recipient strains, suggesting that it could be used selectively to eliminate the donor parent from mixtures with recipients. When parental mixtures were incubated in TMB for periods of 10 min. to 7 hr and then treated with phage before plating on selective media no recombinants were obtained. This result suggested either that gene transfer had not occurred in broth during the period (as earlier concluded) or that recombinants were sensitive to phage and were eliminated. Because of the failure to obtain gene transfer in broth, attempts were made to time the entry of the marker *arg* in crosses conducted on membranes (Matney & Achenbach, 1962). Donor and recipient (MRE 2205) suspensions prepared as for use in crosses were applied to membranes placed on selective medium plates and incubated at 28°. At intervals thereafter membranes were removed, the attached

organisms suspended in broth, the suspensions violently agitated using a vibrating mixer and divided into two. One half of the vibrated suspension was treated with phage for 20 min at 37°, the other untreated and both replated on selective medium. Recombinants appeared on untreated suspensions with all samples; they first appeared in phage treated samples after 16 hr had elapsed from the time of applying the mixtures to membranes. After 24 hr treated and untreated samples yielded similar numbers of recombinants. It would seem therefore that many recombinants could survive phage treatment and that *arg* transfer and expression required a minimum of 16 hr contact of parent strains under the conditions of the experiment.

DISCUSSION

We conclude from the experiments reported here that gene transfer occurred between strains of *Pasteurella pseudotuberculosis*. Some alternative explanations for the development of colonies on selective media, which we regard as recombinants, were not substantiated by experiment. It seemed most unlikely that syntrophism of parental mixtures leading to multiple reversions had given a false impression of fertility. First, cross plates did not show the heavy background growth indicative of syntrophism; secondly, permitting increased recipient populations by providing the growth factor for which selection was made, did not result in numbers of revertants approaching the numbers of recombinants obtained in crosses; thirdly, replacement of the donor by an F-Lac− derivative, which would be expected to cross-feed the recipient equally well, resulted in infertility; fourthly, separation of parents by a membrane greatly reduced fertility; and lastly, a large number of recombinants showed unselected donor markers.

Multiple reversions in the recipient strain to give the false impression that donor markers had been acquired seemed equally improbable. Spontaneous revertants subjected to the purification and replication treatment applied to recombinants showed no alteration in other properties for which they were tested. The relative ease with which recombinants appeared with double marker selection for *arg trp* and the fact that 95% of these were His+ is not explicable on a spontaneous mutation basis. The spontaneous mutation frequencies of the *arg-8* and *trp-3* loci were both about 1×10^{-8} and that for *his-8* considerably lower (none have yet been observed). The probability of obtaining triple reversions spontaneously would thus be less than 1×10^{-24}. Similarly recombinants could not have been revertants of the donor strain which carried the non-revertable *pth-1* locus, nor mixed clones of donor and recipient since no recombinants showed the donor marker *str-11*.

The apparent need for firm contact between parent bacteria for the production of recombinants and the inheritance of several donor markers indicate that gene transfer in *Pasteurella pseudotuberculosis* occurs by a process analogous to that mediated by F′*lac* in *Escherichia coli* and involving cell conjugation (Jacob & Adelberg, 1959). While there is at present no reason to doubt that the mechanisms of gene transfer would be similar in principle in the two genera we would expect to find differences in detail and to meet a number of puzzling situations at this early stage of studies with *P. pseudotuberculosis*. Thus, we failed to obtain recombinants in crosses with Met−, Nic−, Ura− or Thi− recipients. Possibly this means that the loci involved are terminal markers (as *str-11* appears to be) and rarely transferred by our donor or excluded by

lethal zygosis, or they may be closely linked to contraselected donor markers, or *P. pseudotuberculosis* may have more than one linkage group. Again, we have not yet succeeded in obtaining transfer in broth cultures. Possibly this indicates that unions between mating pairs are more fragile than those of *E. coli* or do not form except under conditions where movement of bacteria is restricted, as when held on agar surfaces or membranes. It is puzzling that gene transfer takes some 16 hr under our conditions; does this mean that it is a slow process or do parent bacteria require this time to become competent to mate? Probably with further experience and improvements in technique these and other peculiarities of our system will be resolved. Meanwhile, however, departure from the behaviour shown by fertile systems in *E. coli* cannot be held as evidence against the occurrence of gene transfer in *P. pseudotuberculosis*.

A variable number of recombinants accepted *lac* in addition to the selected marker. Lac+ recombinants varied in the stability of this property and spontaneously produced Lac− sectors, or clones, with different frequencies. The Lac− segregants however showed no loss of their other selected and unselected donor markers which thus were stably inherited (Morris & Burrows, in preparation). We infer that Lac+ recombinants carry an integrated fragment of the donor chromosome and an autonomous F′*lac* plasmid.

The base composition of DNA extracted from our donor strain of *Pasteurella pseudotuberculosis* (determined by W.D.L. using the method of Marmur & Doty, 1962) was 45·6% G+C (T_m 88·0), and that from *Escherichia coli* strain 23.10S, determined in the same experiment, was 50% G+C (T_m 89.8). This 4·4% difference is noteworthy since donor chromosome mobilization by the F factor had not been observed in bacteria that differed in base composition from *E. coli* by more than 1% G+C (S. Falkow, personal communication). However, we know from gel-diffusion analyses (unpublished) that Pasteurella and Escherichia show at least three common antigens, they have some common phage sensitivities (Stocker, 1955; Smith & Burrows, 1962; Hertman, 1964) and show close taxonomic relationships in classifications based on Adansonian principles (Sneath & Cowan, 1958). The availability of a fertility system in *P. pseudotuberculosis* should eventually permit the construction of a chromosome map for this species and allow comparison with those of *E. coli* (Taylor & Thoman, 1964) and *Salmonella typhimurium* (Sanderson & Demerec, 1965).

Two other interesting problems would now seem to be amenable to investigation. Genetic studies on bacterial virulence have been initiated in strains of *Shigella flexneri* (Falkow, Schneider, Baron & Formal, 1963) and *Salmonella typhimurium* (Krishnapillai & Baron, 1964). The accumulated knowledge of factors determining virulence in Pasteurella, (Burrows, 1963) coupled with the ability to promote gene transfer in this genus should provide an excellent system for fruitful studies of the important property of virulence. Secondly, genetic studies should permit an assessment of the validity of claims for the inter-conversion of *Pasteurella pestis* and *P. pseudotuberculosis*, made by several investigators but never using genetically marked strains, nor in an entirely convincing manner (see discussion by Brubaker, Surgalla & Beesley, 1965). In recent experiments we have shown that crosses between *P. pseudotuberculosis* and *P. pestis* are fertile; there would therefore appear to be no impediment to a future decision on the interconvertibility of the two species.

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The Genetics and Biochemistry of Mutants of *Aspergillus nidulans* Resistant to Chlorinated Nitrobenzenes

By R. J. THRELFALL
Department of Botany, Imperial College, London, S.W.7

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SUMMARY

2,3,5,6-Tetrachloronitrobenzene (TCNB) was a more effective fungistat than pentachloronitrobenzene (PCNB) for *Aspergillus nidulans*. Four mutants selected for resistance to TCNB or PCNB were resistant to both these compounds and to other halogenated nitrobenzenes, diphenyl, methylene blue and brilliant cresyl green. Resistance was conferred by either of two recessive genes in linkage group III. Three of the mutants were allelic. In crosses the frequency of resistant ascospores was less than 50%. Diploids heterozygous for resistance exposed to PCNB produced fast-growing resistant haploid or homozygous diploid segregants. PCNB decreased growth more than DNA synthesis in sensitive strains but these were unaffected in resistant strains. Five times as much TCNB was extracted from the mycelium of sensitive strains than from that of resistant strains. Resistance is probably caused by an inability to take up the chemicals rather than to an ability to metabolize them.

INTRODUCTION

When a chemical compound is used to control or prevent the growth of a micro-organism strains resistant to the compound may subsequently appear. How the compound acts on the organism and how this action is avoided or overcome by the resistant strain has considerable biochemical and genetical interest. Pentachloronitrobenzene (PCNB) and 2,3,5,6-tetrachloronitrobenzene (TCNB) are widely used for the control of diseases caused by *Rhizoctonia solani* and *Botrytis cinerea* (Brown & Montgomery, 1948). Strains of the imperfect fungi *B. allii* (Priest & Wood, 1961) and *Fusarium caeruleum* (McKee, 1951) resistant to chlorinated nitrobenzenes have been described, but not analysed genetically. In *Hypomyces solani* f. *cucurbitae* any one of three loci can mutate to give resistance to PCNB and TCNB (Georgopoulos, 1963). In *Aspergillus nidulans*, strains resistant to acriflavine (Roper & Käfer, 1957), fluoroacetate (Apirion, 1962) and *p*-fluorophenylalanine, teoquil, iodoacetate, actidione and malachite green (Warr & Roper, 1965) have been described and analysed genetically. No reports have appeared on strains of *A. nidulans* resistant to fungicides used in agriculture. This is perhaps not surprising, because the fungus is not a pathogen. The ease with which genetic analysis can be done with *A. nidulans* was the reason for its use in this study of the nature of its resistance to PCNB and TCNB.

METHODS

The general and genetic techniques used in this work were those of Pontecorvo *et al.* (1953). Incubation was at 37°.

Organisms. Mutant stocks of *Aspergillus nidulans* were obtained from Dr B. W. Bainbridge (Department of Microbiology, Queen Elizabeth College, Campden Hill Road, London, W.8), the Department of Genetics University of Glasgow, or the Fungal Genetics Stock Center, Department of Biological Sciences, Dartmouth College, Hanover, New Hampshire, U.S.A. The mutants used in this work were *y*, yellow conidia; *w*2, white conidia; *ad*20, *arg*2, *bi*1, *lys*5, *meth*2, *nic*8, *phen*2, *pro*1, *pyro*4, *ribo*2, *s*3 growth requirements, respectively, for adenine, arginine, biotin, lysine, methionine, nicotinic acid, phenylalanine, proline, pyridoxine, riboflavin and sulphite; *gal*1, unable to grow with galactose as sole carbon source; *Acr*1, resistance to acriflavine and *su*1 *ad*20, suppressor of *ad*20. Details of these mutant alleles are given in Pontecorvo *et al.* (1953), Roper & Käfer (1957), Käfer (1958) and Roberts (1963). These stocks were maintained on complete medium. Where necessary these stocks and others obtained during the work were purified by the isolation of a single conidium with a de Fonbrune micromanipulator.

Media. Minimal medium (MM): $NaNO_3$, 6·0 g.; $MgSO_47H_2O$, 0·52 g.; KCl, 0·52 g.; KH_2PO_4, 1·52 g.; glucose, 10·0 g.; trace $FeSO_4$ and $ZnSO_4$; distilled water to 1 l.; adjusted to pH 6·5 with 5% (w/v) NaOH. Complete medium (CM): yeast extract (Difco), 1·0 g.; peptone (Difco), 1·0 g.; Casamino acids (Difco, certified), 1·0 g.; glucose, 10·0 g.; vitamin solution, 1 ml. (biotin, 0·01 g.; thiamine HCl, 0·01 g.; riboflavin, 0·01 g.; *p*-aminobenzoic acid, 0·01 g.; nicotinic acid, 0·01 g.; pyridoxin HCl, 0·01 g.; water to 100 ml.); distilled water to 1 l., pH 6·0. For the growth of adenine auxotrophs media were supplemented with 0·15 g. adenine/l. CM was deficient in arginine, methionine, phenylalanine and riboflavine so that these were added to the media when required by mutant stocks. Media were solidified by the addition of 15 g. Oxoid Ionagar no. 2/l. Cultures grown in liquid media were set up in 250 ml. Oxoid flasks containing 50 ml. medium. The components of liquid MM were autoclaved separately, mixed, the pH adjusted with 5% (w/v) NaOH and dispensed into sterile flasks. Liquid CM was prepared in bulk, distributed and sterilized in the flasks.

Chemicals. The five fungicides used were pentachloronitrobenzene (PCNB), 2,3,5,6-tetrachloronitrobenzene (TCNB) and 2,5-dibromonitrobenzene (DBNB) recrystallized from ethanol and 1,3-difluoro-7,6-dinitrobenzene (DFDNB) recrystallized from acetic acid. Other chemicals were of Analar grade where available. Stains were obtained from G. T. Gurr (136/177, New Kings Road, London, S.W.6). Purified calf thymus DNA, glucose oxidase and peroxidase were purchased from Seravac Laboratories, Maidenhead, Berkshire.

Analysis of cultures grown in liquid media. The fungal mycelium was collected by filtration through Whatman no. 571 filter paper and when necessary a sample of the filtrate was stored at −20° for analysis later. The mycelium was washed with water, twice with 25 ml. industrial methylated spirit (74 o.p.), then with 25 ml. acetone, squeezed between blotting paper, removed from the paper, dried on a glazed tile at 55–60° for at least 4 hr and then weighed. DNA was measured as follows. Not more than 100 mg. dry mycelium was extracted twice with 2·5 ml. and then with 5·0 ml. ethanol+chloroform mixture (3+1, v/v) for periods of 20 min. at 75°. It was then extracted with 2·5 ml. 6% (v/v) perchloric acid for 20 min. at 70°. To 2·0 ml. of the perchloric acid extract was added 4·0 ml. diphenylamine reagent (Burton, 1956), incubated overnight at 37° and the extinction read at 600 mμ. A sample of calf thymus

DNA was hydrolysed and standards prepared at the same time. Culture filtrates were analysed for phosphate by the method of Fiske & SubbaRow (1925), for glucose with a glucose oxidase reagent in 0·5 M-tris at pH 7·0 (Dahlqvist, 1961) and for amino acids by the method of Moore & Stein (1948) with leucine as standard.

Analysis of 2,3,5,6-tetrachloronitrobenzene. A modification of the method of Auerbach (1950) was used. One-half ml. of a 25 % (v/v) solution of tetra-ethylammonium hydroxide (British Drugs Houses Ltd., Poole, Dorset) was diluted with 12·5 ml. absolute ethanol. To 0·1 ml. of this was added 4·9 ml. acetone extract containing 0–60 μg. TCNB and the extinction read at 550 mμ between 4 and 7 min. after mixing.

Cytological technique. Strips of thin sterile Cellophane (2 × 1 in.) were placed on the surface of CM agar in Petri dishes and inoculated with a suspension of conidia. After incubation the strips were removed, fixed in Helly's fluid for 10 min., washed in 70 % (v/v) ethanol in water, hydrolysed with N-hydrochloric acid at 60° for 10 min. and stained in diluted Giesma for 3 hr (Giesma R 66 1·5 ml.; M/15 phosphate buffer, pH 6·9, 30 ml.). The material was differentiated by washing the strips for about 75 sec. in distilled water containing a trace of acetic acid. The strips were mounted for examination in dilute stain (Giesma R 66, 7 drops; M/15 phosphate buffer, pH 6·9, 20 ml.).

RESULTS

Effect of PCNB and TCNB on growth and morphology of wild type

A series of Petri dishes (8·5 cm. diameter) of CM agar containing known amounts of PCNB (0–0·5 mg./ml.) were prepared by adding PCNB dissolved in acetone to the warm medium before the plates were poured. The plates were inoculated with about 200 conidia/plate. After incubation for 3 days the colonies were counted. On plates containing more than 0·05 mg. PCNB/ml. the colonies did not exceed 2 mm. in diameter. There was no significant difference ($P = 0{\cdot}01$) between the control and PCNB plates. A similar experiment was made with 0·01–10·0 mg. PCNB deposited in the lids of a series of dishes from acetone solutions. Again the viability of conidia was unchanged.

The effect of PCNB on growth was examined by inoculating a series of plates at the centre with a 2 mm. disc of mycelium cut with a cork borer from the periphery of a colony growing on CM agar. The average diameter of the colonies after 6 days is shown in Fig. 1. At 0·005 mg. PCNB/ml. growth was decreased, while at 0·05 mg./ml. the colonies were only about one-sixteenth the area of control colonies. Increasing the concentration above 0·05 mg./ml. did not lead to greater inhibition. At concentrations above 0·01 mg./ml. conidiation was progressively decreased and was almost absent at 0·05 mg./ml.

TCNB also decreased growth and conidiation; above 0·05 mg./ml. there was no conidiation and the colonies were bright pink. At 0·1 mg./ml. inhibition was complete but the organisms were not dead because growth started again on transfer of the inoculum to medium without TCNB. Thus PCNB and TCNB were fungistatic rather than fungicidal.

Isolation of PCNB- and TCNB-resistant strains

At any time during exposure to PCNB fan-shaped fast-growing sectors developed. Ten sectors taken at random, each from different colonies of the wild type growing on CM agar and exposed to the vapour from 1·0 mg. PCNB in the lid of the dish,

were examined in detail. They were found to differ from each other in several ways. Three of the sectors grew poorly on CM agar, produced an excess of aerial hyphae with few conidiophores and the edge of the colony was indented. Looked at through the bottom of the dish these strains were dark brown. When grown on CM agar containing 0·2 mg. PCNB/ml. these strains were normal in every way. Three other sectors had normal morphology in the absence of PCNB but produced excessive aerial hyphae in its presence. Another three sectors appeared to be normal whether PCNB was present or not; one of these sectors had a growth rate similar to that of the wild-type parent but the other two grew slowly. The tenth sector lost its resistance to PCNB on subculture while the others retained their resistance through several transfers by using conidia or mycelium. None of the resistant strains derived from the wild-type parent had any nutritional requirement.

Table 1. *Strains of Aspergillus nidulans resistant to pentachloronitrobenzene (PCNB) and tetrachloronitrobenzene (TCNB)*

Genotype	Gene for resistance	Isolated in presence of
pro 1 *y*; *Acr* 1	*pcnb* 1	PCNB
ad 20; *pyro* 4	*pcnb* 2	PCNB
pro 1 *y*; *Acr* 1	*pcnb* 3	PCNB
bi 1	*tcnb* 1	TCNB
pro 1 *y*; *Acr* 1; *pcnb* 3	*pcnb* 3 A	Dibromonitrobenzene

Plates of CM agar containing 0·2 mg. PCNB/ml. were inoculated with mutant auxotrophic strains of *Aspergillus nidulans*. After incubation one resistant sector was picked. Repeated attempts to isolate a resistant strain in the presence of TCNB were unsuccessful. This may have been because so little growth was made that the probability of a mutation to resistance taking place among so few nuclei was slight. Therefore plates of CM agar were inoculated and 10 mg. TCNB added to the lid after 2 days. After further incubation a few tolerant sectors developed but their frequency was much less than that of sectors developing in the presence of PCNB. One sector resistant to TCNB and three resistant to PCNB have been studied. The genotypes of the parental strains are shown in Table 1, where resistance has been tentatively ascribed to four genes. On CM agar in the presence of dibromononitrobenzene the original *pro* 1 *y*; *Acr* 1; *pcnb* 3 strain produced a fast-growing sector which still carried *pcnb* 3. The strain grown from this sector was called *pcnb* 3 A; a second mutation may have occurred, either at the *pcnb* 3 locus or elsewhere, but this was not investigated.

Response of resistant strains to other inhibitors

The effect of several chemicals on the growth of the isolates of Table 1 and of prototrophic-resistant recombinants obtained from crosses (see Table 2) was measured by the average of two diameters at right angles of colonies incubated for 6 days on CM agar containing the chemical. The source of inoculum, whether taken from cultures grown with or without PCNB (or TCNB) had no effect on the final size of the colony. Figure 1 shows that the strain having the greatest resistance to PCNB was that isolated in the presence of TCNB. In the absence of PCNB strains *pcnb* 1 and *pcnb* 2 grew much less than the other strains; *pcnb* 2 was stimulated by PCNB. At high

concentrations of PCNB growth of *pcnb* 1 was decreased more than that of *pcnb* 2 or *pcnb* 3. These properties were shown on MM agar but the differences between strains was less marked. Figure 2 shows that the strain selected in the presence of TCNB was no more resistant to it than were those selected in the presence of PCNB. At more than 0·05 mg. TCNB/ml. growth of all resistant strains was suppressed and all became pink in colour. Strains carrying *pcnb* 1 or *pcnb* 2 were found to be resistant to dichloronitrobenzene and dibromonitrobenzene at concentrations up to 0·1 mg./ml. All strains were completely inhibited by difluorodinitrobenzene at 0·01 mg./ml. and by dinitrophenol at 0·05 mg./ml. Priest & Wood (1961) found that strains of *Botrytis*

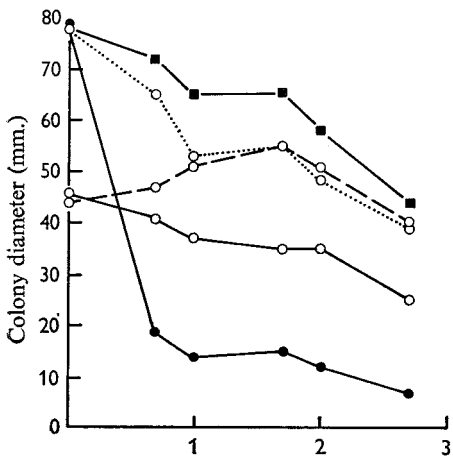

Fig. 1

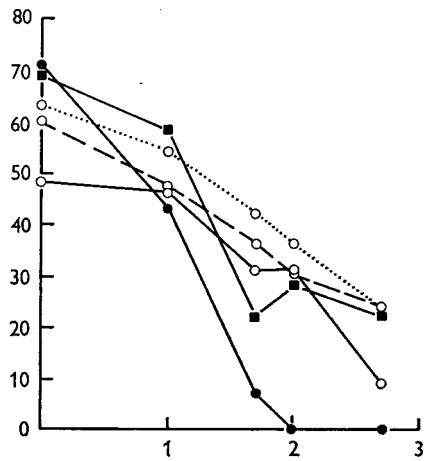

Fig. 2

Fig. 1. *Aspergillus nidulans*: diameters of colonies of *wild type* (sensitive) and *pcnb* 1, *pcnb* 2, *pcnb* 3 and *tcnb* 1; *bi* 1 (resistant) after incubation for 6 days at 37° on complete medium containing 0–500 µg. pentachloronitrobenzene (PCNB)/ml. (means of four replicates). ●, *Wild type*; —○—, *pcnb* 1; – –○– –, *pcnb* 2; ····○····, *pcnb* 3; —■—, *tcnb* 1; *bi* 1.

Fig. 2. *Aspergillus nidulans*: diameters of colonies of *wild type* (sensitive) and *pcnb* 1, *pcnb* 2, *pcnb* 3 and *tcnb* 1; *bi* 1 (resistant) after incubation at 37° for 6 days on complete medium containing 0–500 µg. 2,3,5,6-tetrachloronitrobenzene (TCNB)/ml. (means of four replicates). ●, *Wild type*; —○—, *pcnb* 1; – –○– –, *pcnb* 2; ····○····, *pcnb* 3; —■—, *tcnb* 1; *bi* 1.

allii resistant to chloronitrobenzenes were also resistant to 2,6-dichloronitroaniline (DCNA). *Aspergillus nidulans* was insensitive to DCNA; no difference in sensitivity of sensitive and chloronitrobenzene-resistant strains was shown at 0·25 mg. DCNA/ml. At this concentration the medium was bright yellow, but surrounding each colony there was a bleached border, suggesting that the compound may have been destroyed. Georgopoulos & Vomvoyianni (1965) noted that mutants of *Hypomyces solani* f. *cucurbitae* resistant to PCNB and TCNB were also resistant to diphenyl. With *A. nidulans* all the chloronitrobenzene-resistant mutants were able to grow on media containing 0·05 mg. diphenyl/ml. Under these conditions the wild type sensitive strain could not grow. Conidiation of strains *pcnb* 1 and *pcnb* 3 was unchanged but that of *pcnb* 2 and *tcnb* 1 was diminished. All the resistant strains were able to grow and conidiate normally on CM agar containing 0·05 mg. methylene blue or brilliant cresyl/ml., whereas the wild-type strain grew poorly and did not produce conidia.

Genetics of chloronitrobenzene resistance

A prototrophic diploid of *Aspergillus nidulans* was selected from a stable heterokaryon between *pro*1 *y*; *Acr*1; *pcnb*1 and *su*1 *ad*20 *y ad*20; *Acr*1; *phen*2; *pyro*4; *lys*5; *s*3; *nic*8; *ribo*2. Loopfuls of diploid conidia were stabbed into plates of CM agar at five equidistant points and 1·0 mg. PCNB added to the lid. After incubation for 3 days, fast-growing sectors appeared from the stunted colonies. The sectors were either dull yellow (diploid) or bright yellow (haploid) and about equally frequent. One sector was picked from each colony and analysed genetically. All the haploids were

Table 2. *Aspergillus nidulans: crosses between chloronitrobenzene-resistant strains*

Cross no.	Parental genotypes	Selection	No. of ascospores analysed	No. resistant
1	+ *ad*20 + *pcnb*2 + + *pyro*4 / *y* + *Acr*1 *pcnb*1 *arg*2 *gal*1 +	*ad*20$^+$ *arg*2$^+$ *pyro*4$^+$	900 (897*y*; 3*y*$^+$)	900
2	*y pro*1 *pcnb*1 + *nic*8 + / *y pro*1 *pcnb*3 *pyro*4 + *s*3	*pyro*4$^+$ *nic*8$^+$ *s*3$^+$	821	821
3	*y* + *pcnb*3 *pyro*4 *s*3 / + *ad*20 *pcnb*2 + +	*ad*20$^+$ *pyro*4$^+$	251 (212*y*; 39*y*$^+$)	251
4	+ *bi*1 + *pcnb*2 *arg*2 + / *y* + *meth*2 *tcnb*1 + *phen*2	None	98	97

Table 3. *Aspergillus nidulans: crosses of chloronitrobenzene strains to bi 1 arg 2*

Cross no.	Parental genotypes	Meiotic products: *pcnb arg*2	Meiotic products: + +	Meiotic products: *pcnb* +	Meiotic products: + *arg*2	Recombination (%)
5	*pro*1 *y* + *Acr*1 *pcnb*1 + / + + *bi*1 + + *arg*2	11	40	130	223	12·6
6	*ad*20 + *pcnb*2 + *pyro*4 / + *bi*1 + *arg*2 +	12	24	49	97	19·7
7	*pro*1 *y* + *Acr*1 *pcnb*3 + *pyro*4 *s*3 / + + *bi*1 + + *arg*2 + +	12	17	43	71	20·3
8	*pro*1 *y* + *Acr*1 *pcnb*3A + / + + *bi*1 + + *arg*2	39	8	88	212	13·6

phen$^+$ but showed assortment of the other heterozygous markers. Thus *pcnb*1 is located in linkage group III; *pcnb*3 and *tcnb*1 were also located on this chromosome by the same method. Attempts to measure the growth rate of diploids heterozygous for resistance in the presence of PCNB failed because fast-growing sectors appeared sooner or later. However the sensitivity of heterozygous diploids indicated that resistance behaved as a recessive character. Table 2 shows that in crosses between resistant mutants *pcnb*1, *pcnb*2 and *pcnb*3 there were no sensitive recombinants among the several hundred analysed; this suggests that the three genes are either closely linked or alleles. In a cross between *pcnb*2 and *tcnb*1 (cross no. 4) one sensitive recombinant *y* was found in 98 analysed, so these genes may be distinct. Table 3

shows that the *pcnb* locus is linked to *arg* 2 but the frequency of resistant ascospores is less than half so that the marker is unsuitable for use in meiotic analysis. A three-point cross was made between *bi* 1;*tcnb* 1 and *y*;*meth* 2 *gal* 1;*nic* 8;*ribo* 2. Of 108 ascospores analysed 79 (15+64) were non-crossovers. There were 11 (6+5) crossovers between *meth* 2 and *tcnb* 1, 17 (3+14) between *tcnb* 1 and *arg* 2, and 1 (1+0) with crossing-over in both regions. (Figures in parentheses refer to number of resistant and sensitive ascospores respectively.) The results indicate that *tcnb* 1 is situated between *meth* 2 and *gal* 1. A diploid heterozygous for *pcnb* 2 and *arg* 2 in coupling was exposed to PCNB and of 18 resistant diploid recombinants all required arginine, suggesting that they are homozygous for *arg* 2 and indicating that the gene for resistance is between *arg* 2 and the centromere. Slow-growing resistant strains occasionally gave faster-growing sectors which retained the resistance of the parent. The possibility that this was due to mutations taking place elsewhere in the resistance cistron or elsewhere in the genome has not been explored.

The mechanism of chloronitrobenzene action and the nature of resistance

The chloronitrobenzenes are almost insoluble in water (PCNB 1·5 μmole/l.) but may accumulate in the biophase and act as toxicants in a non-specific way. Any change which decreased uptake might confer some degree of tolerance on the fungus. That there were differences of this sort was shown by the following experiment. A sensitive (wild type) and resistant (*pcnb* 1) strain of *Aspergillus nidulans* were grown in liquid CM for 3 days and then 10 mg. TCNB added to each flask. After incubation for a further 3 days the mycelium was collected, washed, dried, weighed and extracted for 24 hr with acetone at 4°. The TCNB in the extracts was then estimated. Table 4 shows that more than five times as much TCNB was extracted from the sensitive mycelium as from the resistant mycelium.

Cytological examination of hyphae produced by conidia incubated overnight in an atmosphere of PCNB showed striking differences between sensitive and resistant strains. Sensitive strains developed short much-branched hyphae with many cross-walls. The cells had thick walls and were crowded with nuclei. Under the same conditions conidia of resistant mutants gave long thin hyphae with few cross-walls and few nuclei per cell, and were indistinguishable from hyphae of either strain grown in the absence of PCNB. These effects were studied quantitatively on cultures grown in liquid MM containing 0·1 mg. PCNB/ml. Table 5 shows that PCNB depressed growth more than DNA synthesis in the sensitive strain, so that the DNA content was increased. There was no significant effect of PCNB on the resistant strain. Even in the absence of the fungistat the sensitive strain utilized glucose at a rate lower than did the resistant strain. There were no significant differences between the inorganic phosphate content of culture filtrates. When this experiment was repeated with 0·1 mg. TCNB/ml. the results shown in Table 6 were obtained. No values for the sensitive strain with TCNB are given because no growth was made. These experiments show that PCNB did not inhibit mitosis as suggested by Horsfall (1956) although TCNB may do so.

PCNB and TCNB may release nascent chlorine which could act as an oxidizing agent and/or produce an antimetabolite by causing chlorination of a metabolite (Rich, 1960). Chloramine T is known to act in this way but there was no difference between sensitive and resistant strains in their response to this chemical. Rich (1960)

also suggested that chloronitrobenzenes might be competitive inhibitors of inositol synthesis. Addition of 0·05–0·50 mg. inositol/ml. had no effect on the growth of sensitive strains in the presence of PCNB.

Table 4. *Aspergillus nidulans: tetrachloronitrobenzene content of resistant and sensitive strains*

Strain (genotype)	Dry wt. mycelium (mg.)	μg. TCNB/mg. mycelium
Sensitive (wild type)	98·9	35·4
Resistant (*pcnb* 1)	268·5	6·5

Each value is the mean of five replicate cultures.

Table 5. *Aspergillus nidulans: effect of 0·1 mg. PCNB/ml. on mycelium dry weight, DNA content and glucose uptake of sensitive and resistant strains*

Strain	Treatment	Mycelium dry wt. (mg.)	Total DNA (μg.)	DNA (μg./100 mg. dry mycelium)	Glucose (μg./ml. culture filtrate)
Sensitive (wild type)	PCNB	69	247	362	6800
	Control	151	324	216	2740
Resistant (*pcnb* 3)	PCNB	145	336	232	17
	Control	162	324	230	123

Each figure is the mean of five replicate cultures.

Table 6. *Aspergillus nidulans: Effect of 0·1 mg. TCNB/ml. on mycelium dry weight DNA content and glucose uptake of sensitive and resistant strains*

Strain	Treatment	Mycelium dry wt. (mg.)	Total DNA (μg.)	DNA (μg./100 mg. dry mycelium)	Glucose (μg./ml. culture filtrate)
Sensitive (wild type)	Control	119	701	477	3090
Resistant (*pcnb* 3)	TCNB	18	232	1329	10400
	Control	136	698	515	520

Each figure is the mean of five cultures.

If PCNB or TCNB act as uncouplers of oxidative phosphorylation respiration would be stimulated, and the loss of energy by failure of ATP synthesis might be enough to retard growth. No change in the respiration rate of sensitive or resistant strains was detected over a period of 2 hr after the addition of 0·125 mg. PCNB or TCNB/ml.

PCNB and TCNB might increase cell permeability so that loss of low molecular weight substances limited growth, but no differences in the amino acid content of culture filtrates from sensitive or resistant cultures was detected 1 or 6 days after the addition of PCNB to cultures to give a concentration of 0·4 mg./ml.

DISCUSSION

Resistant mutants of *Aspergillus nidulans*, which appear to arise more frequently in the presence of PCNB than with TCNB, are mutant for a gene in linkage group III. One gene may be responsible or two closely linked genes. Those resistant mutants selected by PCNB are allelic but have different growth rates and sensitivities to other chemicals. The production of homozygous resistant diploid segregants by sensitive heterozygous diploids to PCNB should provide a method for mapping genes on this arm of the chromosome by mitotic recombination. The cause of toxicity has not been discovered; inhibition of inositol metabolism or evolution of nascent chlorine appear unlikely to explain the fungistatic action of PCNB or TCNB.

Lehninger (1949) showed that when methylene blue or brilliant cresyl green substitutes for the cytochrome system in electron transport there is an uncoupling of ATP synthesis. The resistance to the dyes shown by PCNB- and TCNB-resistant mutants may indicate that these compounds also act in this fashion. However, they may act quite differently inside the cell but share the same path for uptake or entry. If this path is blocked in a mutant strain then resistance will be shown to all those compounds that use it. This line of argument can account for resistance to other halogenated nitrobenzenes and to diphenyl.

The high content of TCNB extracted from a sensitive strain of *Aspergillus nidulans* as compared with a resistant strain may reflect either the inability of the resistant strain to accumulate TCNB or its ability to inactivate it. The fate of halogenated nitrobenzenes *in vivo* has been studied in the rat and rabbit but not in fungi. The labile nitro group of PCNB and TCNB is replaced by a cysteinyl group with the formation of a mercapturic acid (Bray, Hybs, James & Thorpe, 1953); cysteine required for this synthesis is derived from glutathione by a specific enzyme glutathiokinase (Al-Kassab, Boyland & Williams, 1962; Booth, Boyland & Sims, 1961). Georgopoulos & Vomvoyianni (1965) looked for this enzyme in PCNB-resistant mutants of *Hypomyces solani* f. *cucurbitae* without success. They pointed out that if it had been present it could not have accounted for resistance to diphenyl. Resistance would therefore seem to result from a decreased ability to accumulate the toxic chemical rather than from an ability to metabolize it to some harmless product. *A. nidulans* is more sensitive to TCNB than PCNB; *Botrytis cinerea* behaves in this way (Reavill, 1954), whereas the reverse is true for *Rhizoctania solani* (Brown & Montgomery, 1948). Both compounds are virtually inactive against Pythium and Phytophthora (Reavill, 1954). These variations in sensitivity may indicate differences in the nature or organization of the hyphal wall.

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Chemical Composition of Cell-wall Polysaccharide of Rough Mutants of *Salmonella typhimurium*

By T. HOLME
Department of Bacteriology, Karolinska Institutet, Stockholm
A. A. LINDBERG
Department of Bacteriology, National Bacteriological Laboratory, Stockholm
P. J. GAREGG AND T. ONN
Department of Organic Chemistry, University of Stockholm

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SUMMARY

Quantitative analyses of monosaccharide constituents of the cell-wall polysaccharide of the smooth form and rough mutants of *Salmonella typhimurium* were made by gas-liquid chromatography of glycitol acetates produced from acid hydrolysates of phenol + water extracts of cell-wall preparations. The presence, in varying amounts, of sugars characteristic of the S-specific repeating unit was detected in all rough mutants investigated. Definite conclusions about the core structure of the different rough mutants could not be drawn by using only the basis of the monosaccharide composition of the cell-wall polysaccharide. Ribose was found in the cell-wall polysaccharide of the smooth form in small amounts.

INTRODUCTION

The essential structural features of the cell-wall polysaccharide in Salmonella have been elucidated by immunochemical and chemical methods (Kauffmann, Lüderitz, Stierlin & Westphal, 1960; Kauffmann, Krüger, Lüderitz & Westphal, 1961; Beckmann, Subbaiah & Stocker, 1964; Osborn *et al.* 1964; Lüderitz *et al.* 1965; Nikaido, Naide & Mäkelä, 1966). The polysaccharide molecule is composed of a core to which long side chains containing repeating units are attached (Fig. 1). The repeating units contain the specific structures of the O antigen determinants. The chemical composition of some of these determinants has been completely clarified. (For a review see Lüderitz, Staub & Westphal, 1966.) Rare monosaccharides which have not been found in the core structure are often present in the repeating unit. The core region is believed to be similar in all salmonellas. A side chain containing glucose, galactose and *N*-acetylglucosamine is linked to an internal backbone composed of Lipid A, 2-keto-3-deoxyoctulosonic, ethanolamine, phosphate and heptose. The biosynthesis of this side chain appears to involve a sequential transfer of the monosaccharides in the form of nucleotide sugars to the backbone by transferase systems (Osborn, 1966). Studies of the core have been made possible through the use of rough mutants defective in different biosynthetic steps. The designation of these mutants is based on both the chemical composition of the cell-wall polysaccharide—

Ra, Rb, Rc, Rd, Re according to Lüderitz, Staub & Westphal (1966) and the serological specificity, RI–RII (Beckmann, Lüderitz & Westphal, 1964; Lüderitz, Beckmann & Westphal, 1964).

Studies on the monosaccharide composition, by using thin-layer chromatography, of the cell-wall polysaccharide of rough mutants of *Salmonella typhimurium*, selected by the use of phages, revealed the unexpected presence of mannose (Holme, 1965). The application of gas-liquid chromatography for the separation of monosaccharides as described by Sawardeker, Sloneker & Jeanes (1965) offered a more sensitive method for such studies. The aim of the investigations is to study the structure and function of the receptors for different bacteriophages. The present report deals with the chemical composition of the cell-wall polysaccharide of smooth and rough strains of *S. typhimurium* and *S. minnesota*.

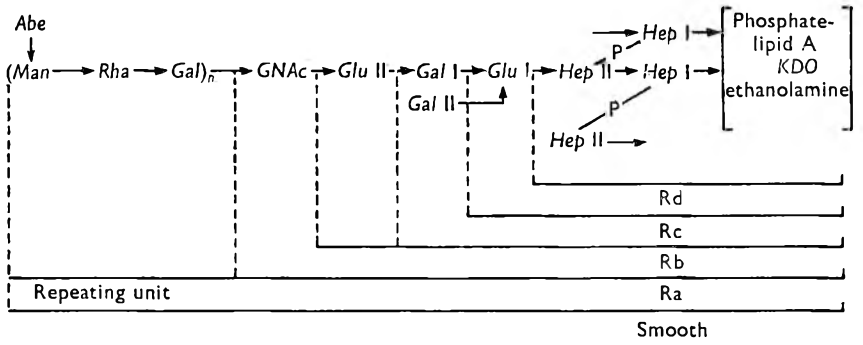

Fig. 1. Main features of the proposed structure of the cell-wall lipopolysaccharide of *Salmonella typhimurium*. The abbreviations used are: *KDO*, 2-keto-3-deoxy-D-manno-octulosonic acid; *Hep*, L-glycero-D-manno-heptose; *Glu*, D-glucose; *Gal*, D-galactose; *GNAc*, *N*-acetyl-D-glucosamine; *Rha*, L-rhamnose; *Man*, D-mannose; *Abe*, abequose (3,6-dideoxy-D-*xylo*-hexose).

METHODS

Bacterial strains. Salmonella typhimurium 395MS was the parent strain of the isolated rough mutants. The mutant strains derived from *S. typhimurium* LT2—TV160, TV161, TV163, TV166, TV208, TV225, TV226 and TV227—were obtained from Dr B. A. D. Stocker (Lister Institute of Preventive Medicine, London). The mutants TV119 and TV148 were obtained from Dr M. Raynaud (Institut Pasteur, Paris). *Salmonella minnesota* S99 and the mutants R60 and R345 derived from this strain were supplied by Dr O. Lüderitz (Max Planck-Institute für Immunbiologie, Freiburg).

Phage stocks. The Salmonella phages Felix 0–1 (Felix & Callow, 1943) and 4 (Lilleengen, 1948) were propagated by the soft agar layer method (Adams, 1959).

Isolation of mutants. For the isolation of phage-resistant mutants, the bacteria were plated with an appropriate dilution of the phage stocks using the soft top-layer agar technique (Adams, 1959). The initial bacterial number on each plate was approximately 10^8. The input multiplicity of phage was 10. After incubation at 37° for 18 hr five colonies were picked from each plate and transferred to nutrient agar plates. Daily transfer was done until no free phage could be detected by spot test against the parent strain. No adsorption of the phages FO-1 or 4 to the isolated mutants could be demonstrated.

Another series of mutants was isolated by picking rough-looking colonies from broth cultures of the strain *Salmonella typhimurium* 395MS. The mutant 395MR0 was isolated from a 1-week-old nutrient broth culture. The mutants 395MR1–R4 were prepared as follows. An 18 hr culture of the parent strain was subjected to ultraviolet (u.v.) irradiation so as to obtain approximately 0·1 % survival. Ten 0·05 ml. portions from the u.v.-treated suspension were each inoculated into 10 ml. nutrient broth. From these cultures rough mutants were isolated after incubation at 37° for 2 weeks. Mutants with different antigenic patterns in the gel precipitin test were chosen for further study. The mutants used are listed in Table 1.

Virulence and protection tests. The methods used have been described previously (Holme & Edebo, 1961, Edebo & Holme, 1965). In the virulence tests 20 g. mice were used.

Preparation of cell walls. Bacteria were grown in a medium of the following composition (g.): glucose, 5; tryptone (Difco), 10; yeast extract (Difco), 5; K_2HPO_4, 7; KH_2PO_4, 3; NaCl, 0·5; in 1000 ml. distilled water.

Table 1. *Rough mutants of Salmonella typhimurium 395MS*

Method of isolation	Mutant no.
Broth culture	395MR0
U.v., broth culture	395MR1 395MR2 395MR3 395MR4
Phage FO-1	395MR5 395MR6 395MR10
Phage 4	395MR7 395MR8 395MR9

Cultivation was in 5 l. indented Erlenmeyer flasks; the culture volume in each flask was 2 l. The flasks were placed on a rotary shaker and incubated for 18 hr at 37°. The yield varied between 1·5 and 2·5 g. dry wt bacteria/l. culture. Cell walls were prepared by disintegrating the centrifuged organisms in the frozen state in the X-press (Edebo, 1960). Initial separation of walls from cell debris was effected by centrifugation at 10,000*g* for 20 min. in an IEC-PR2 refrigerated centrifuge at +4°. The cell walls collected as a loosely packed layer on the pellet surface; they were washed off by careful rinsing. The wall preparation was then washed twice in 0·1 M-phosphate buffer (pH 7), once in M-sodium chloride and once in distilled water. The washing procedure was done in the cold. The cell walls were then suspended in distilled water. No intact bacteria could be observed when the preparations were examined by phase microscopy.

Extraction of lipopolysaccharide. Lipopolysaccharide was extracted from the cell walls by the phenol+water procedure of Westphal, Lüderitz & Bister (1952). A scanning spectrophotometer (Beckmann Dk2) was used for the detection of ultraviolet absorbing substances in the preparations.

Estimation of hexosamines. Hexosamine was estimated according to Gatt & Berman (1966) on polysaccharides obtained by hydrolysis in acetic acid of lipopolysaccharides according to Sutherland, Lüderitz & Westphal (1965).

Gas-chromatographic analysis of the carbohydrate composition of cell-wall lipopolysaccharide hydrolysates. The analysis was based on the method described by Sawardeker *et al.* (1965). The dialysed cell-wall extracts contained between 0·6 and 4·6 mg. polysaccharide/ml. by the anthrone method and expressed as glucose (Whistler & Wolfrom, 1962). For the analysis a volume containing approximately 2 mg. was taken. The extracts were hydrolysed in 0·5 N-sulphuric acid at 100° overnight.

The acid was removed by Dowex 3 (free base) and the solution concentrated under reduced pressure to about 1 ml. The mixtures of aldoses were reduced to the corresponding alditols with sodium borohydride overnight. Excess borohydride was destroyed by Dowex 50 W-X8 and boric acid removed as methyl borate by several distillations with methanol under reduced pressure. The syrups were dried by distillation under reduced pressure with benzene, and then fully acetylated with acetic anhydride + pyridine (1:1 v/v) at 100° for 10–15 min. Excess of acetic anhydride was removed by

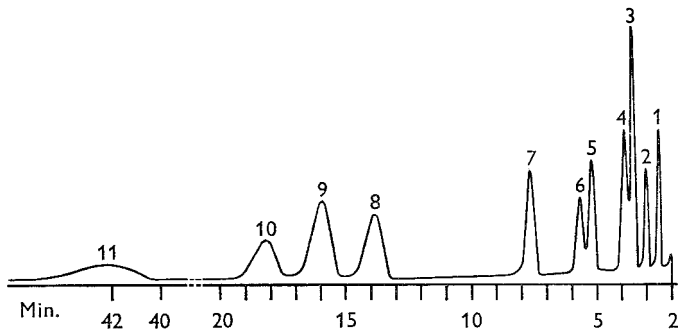

Fig. 2. Chromatogram of alditol acetates of reference sugars. Column: 3 % ECNSS-M on Gas-Chrom Q. Injector temperature 270°, column temperature 170°, detector temperature 240°. Flow: 30 ml. N_2/min. 1, Tyvelose (3,6-dideoxy-D-*arabino*-hexose); 2, abequose (3,6-dideoxy-D-*xylo*-hexose); 3, rhamnose; 4, fucose; 5, ribose; 6, arabinose; 7, xylose; 8, mannose; 9, galactose; 10, glucose; 11, L-glycero-D-manno-heptose.

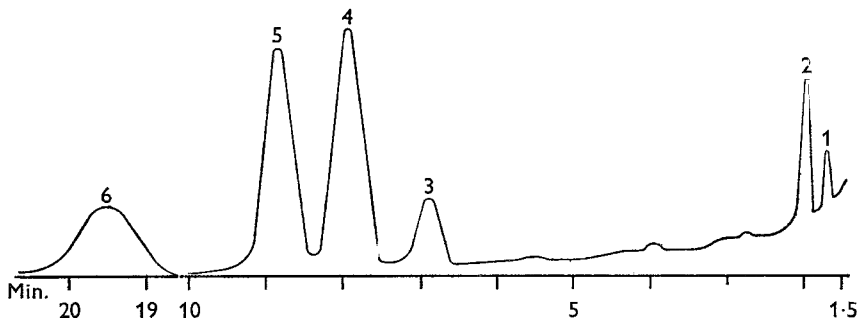

Fig. 3. Chromatogram of alditol acetates of sugars from *Salmonella typhimurium* TV225 cell-wall polysaccharide. Column: 3 % ECNSS-M on Gas-Chrom Q. Injector temperature 270°, column temperature 180°, detector temperature 240°. Flow: 32 ml. N_2/min. 1, Abequose; 2, rhamnose; 3, mannose; 4, galactose; 5, glucose; 6, heptose.

adding water and the resulting solution concentrated to dryness. The resulting mixture of fully acetylated alditol acetates was dissolved in chloroform and analysed by gas–liquid chromatography. The solutions (1 μl.) were injected on to an ECNSS (polyester-silicone) glass column fitted in a Perkin-Elmer Model 801 gas chromatograph. The injection temperature was 265–270°, column temperature 170–175°, detector temperature 235–240° and gas flow (nitrogen) 30–32 ml./min. The retention times of the various acetates were identical to those of authentic polyacetates of each of the various glycitols. The glycitols were further identified by adding reference materials

to the mixtures. Complete separation of the glycitol acetates was obtained (Figs. 2, 3). The response of the flame-ionization detector to all the glycitol acetates was found to be linear and was determined for each one. The relative peak areas were determined for the mixtures from the various lipopolysaccharides and molecular ratios for the constituent sugars calculated from these, after suitable correction for the different response of the detector to the various glycitol acetates. The method was tested by subjecting known mixtures of the various sugars to the above procedure. The ratios found differed from those calculated by not more than ± 3 mole %. Losses of the most volatile of the glycitol acetates, that derived from abequose, during the working-up of the acetylation mixture, were found to be negligible.

RESULTS

Growth characteristics of the mutants

All the mutants developed rough colonies after incubation for 48 hr on nutrient agar. On galactose Endo agar all strains except R9 showed positive fermentation reaction. The mutant R9 developed smooth colonies on this medium. This mutant was also unable to grow on minimal medium with galactose as the sole carbon source. All the mutants grew on minimal media with glucose as the sole carbon source.

Biological characteristics

The parent strain was highly virulent for mice. All mice in a group of 10 died within 7 days after inoculation of approximately 50 viable bacteria/mouse. For virulence tests of the rough mutant strains, doses of $2–8 \times 10^5$ viable bacteria were inoculated into groups of 10 mice. All strains of *Salmonella typhimurium* except R4 listed in Table 1 were tested. Strain R4 was lost after the chemical analyses had been made, and no virulence tests could be done. No deaths were recorded.

Protection tests were made using suspension of heat-killed cells of the smooth form and the mutants R0, R5, R6, R7, R8, R9 and R10 as immunizing agents. PD50 of the smooth form was 6×10^{-5} mg. (dry weight). No protection was obtained in groups of mice treated with 0·3 mg. of cells of the rough mutants.

Chemical composition of cell-wall polysaccharides

The data from the gas chromatographic analysis of the polysaccharide hydrolysates and the separate determination of glucosamine are given in Table 2. The quantities of the individual sugars are given in mole % of the detected sugars to facilitate the comparison of ratios between polysaccharides from different strains.

Various peaks obtained from contaminating materials were always observed, particularly in the region of the peaks for pentitol and deoxyhexitol acetates. The alditols in this region were therefore always particularly carefully identified by adding reference glycitols, thereby obtaining 'mixed' chromatograms. The assignments were further confirmed by employing a mass spectrometer connected to the gas chromatograph. The mass spectra of abequitol, rhamnitol, pentitols and hexitols were clearly different. The peaks of the glycitol acetates obtained from the hydrolysates gave mass spectra identical to those of the corresponding reference substances.

In the region of the hexitol peaks, including that given by mannitol acetate, inter-

fering non-carbohydrate material was absent. Between the peaks of glucitol and heptitol an unidentified peak was regularly found. The presence of mannose in some of the hydrolysates was confirmed by paper and thin-layer chromatography. The detection of rhamnose and abequose was facilitated by the use of gas chromatography, the sensitivity of which is much greater than that of paper chromatography.

Table 2. *Chemical composition of cell-wall polysaccharides from Salmonella typhimurium 395MS and rough mutants, rough mutants of S. typhimurium LT2 (TV strains) and S. minnesota S99 and rough mutants R60 and R345*

Figures for all monosaccharides except glucosamine represent mole % of detected sugars. Glucosamine is given in % of polysaccharide by the anthrone method. ND = not detected.

Mutant	Hep	Glu	Gal	Man	Rha	Abe	GN % of polysacch.	Proposed chemical designation
395MS	< 1	30	22	21	18	9	3·6	XIV
395MR0	32	31	33	3	1	< 1	16·7	Ra
395MR1	40	21	31	4	3	1	1·4	Rb
395MR2	38	22	37	2	1	< 1	2·6	Ra
395MR3	46	22	25	4	2	1	5·0	Ra
395MR4	30	30	31	5	3	1	5·0	Ra
395MR5	23	19	26	17	11	4	1·8	Rb
395MR6	27	19	28	12	8	6	1·0	Rb
395MR7	43	27	19	7	3	1	2·1	Rb
395MR8	50	22	20	5	2	< 1	0·8	Rb
395MR9	41	46	5	4	2	2	0·7	Rc
395MR10	67	3	9	14	5	2	1·3	Rd
TV160	32	22	32	7	5	2	1·1	Rb
TV161	34	19	32	9	4	2	0·6	Rb
TV163	27	26	28	9	7	3	3·3	Ra
TV208	7	9	34	24	17	9	5·2	Ra
TV225	30	26	31	7	4	2	2·7	Ra
S99	23	23	54	ND	ND	ND	—	II
R60	26	36	38	ND	ND	ND	—	Ra
R345	30	20	50	ND	ND	ND	—	Rb

Hep = heptose, Glu = glucose, Gal = galactose, Man = mannose, Rha = rhamnose, Abe = abequose, GN = glucosamine.

The parent strain, 395MS, showed an extremely low value of heptose, which indicates that the core region is only a minor component of the S polysaccharide. The same tendency was apparent for strain TV208. One mutant, R10, with the proposed chemotype Rd, displayed a heptose value of 67. The remaining mutants showed values between 23 and 50 mole %. Glucose was significantly lower in the mutants R10 and TV208 than in the other strains. Galactose was low in strains R9 and R10. The galactose-non-fermentor R9 is of chemotype Rc.

The glucosamine values for the proposed Ra mutants fell between 2·6 and 16·7. Mutants belonging to chemotypes Rb, Rc and Rd had glucosamine values between 0·6 and 2·1. The quantities of glucosamine in the *Salmonella minnesota* strains were not determined, since the specific structure contains galactosamine, and the method of analysis did not distinguish between these hexosamines. Ribose was found in small amounts, about 1 %, in the cell-wall polysaccharide of the smooth form of *S. typhimurium* but not in the smooth form of *S. minnesota*. It was also present in small amounts in some of the mutants representing all chemotypes. The ribose values

were not incorporated in Table 1 because of the uncertainty of the origin of this sugar, when it was present in very small amounts.

The S-specific sugars mannose, rhamnose and abequose were found in appreciable amounts in all the rough mutants. The strains TV208, R5 and R6 contained these sugars in especially high quantities. Mannose was always present in greater amounts than rhamnose and abequose.

DISCUSSION

Various methods for the analysis of the monosaccharide composition of lipopolysaccharides have been used. Analysis by paper chromatography is complicated by the number of components present. This makes the use of several solvent systems necessary. The use of enzymic methods on the other hand makes the combination of these various analyses into relative ratios for the monosaccharides somewhat uncertain.

The method used in the present work is essentially that described by Sawardeker *et al.* (1965) in which hydrolysates containing the monosaccharide mixture are converted into the corresponding glycitol acetates. This mixture is readily separated and analysed by gas chromatography. The method is rapid, relatively simple and has the advantage of giving a simultaneous determination of the components. In the procedure used here the glucosamine is, however, removed from the mixture.

The presence of mannose, rhamnose and abequose in all the rough mutants of *Salmonella typhimurium* raised the question whether the strains were unstable and frequently reverted to the wild type. The conclusion that this was not the case was drawn from the following facts. (1) The mutants from *S. typhimurium* 395MS have been kept in the laboratory for 5 years without any signs of reversion, only rough colonies being observed when the strains were plated on nutrient agar. (2) The antigenic pattern in gel precipitin tests has been constant during this time (unpublished). (3) Vaccines prepared from the mutant strains did not confer protection in mice against challenge with viable *S. typhimurium* 395MS. (4) The mutants behaved as typical rough strains in serological tests and phage typing (Lindberg & Holme, 1968). (5) All mutants were avirulent. The dose used in these tests was approximately 5×10^5 viable bacteria. Since the smooth bacteria are highly virulent ($LD50 < 50$), revertants would have been detected in a proportion of less than 1 in 10^4 bacteria. Such a low proportion of smooth bacteria could not have any influence on the results of the chemical and immunological investigations.

Several explanations can be given for the presence of mannose, rhamnose and abequose in the rough mutants. The enzymic block which can lead to roughness may result in defective synthesis of one or more of the component monosaccharides, or it may result in a defective transfer of the sugars to the lipopolysaccharide core. A partial synthesis of the S-specific polysaccharide would result if the enzymic block was not complete or if secondary synthetic pathways could be used in the synthesis. Gemski & Stocker (1967) attributed the capacity of some rough mutants to maintain the propagation of the smooth specific phage P22 and the evocation of P22 transductants from such strains to incomplete deficiencies (leaky mutations). Kalckar, Laursen & Rapin (1966) attributed the presence of small amounts of galactose in an UDP-galactose-4-epimerase negative mutant of *Escherichia coli* K12 to the possibility that galactose was synthetised by an alternative pathway. The small epimerase

activity detected was considered insufficient to explain the amount of galactose detected in the lipopolysaccharide.

The parent smooth strain and some of the rough mutants of *Salmonella typhimurium* displayed small amounts of ribose. The presence of 1–4% ribose in the lipopolysaccharide of RII-mutants of different Salmonella was reported by Lüderitz *et al.* (1966). Lipopolysaccharides from Salmonella TI strains, which lack the S-specific side chains, contain large amounts of ribose and galactose. Genetic analysis showed that TI strains have active genes (*rft 1* gene) for producing TI specificity which are lacking or inactive in the smooth strain (Sarvas, 1968). Recombination experiments between TI strains and well-defined rough mutants indicated that the *rft 1* gene was only expressed when the complete core was present. Ribose should then be found only in mutants of chemotype Ra and in smooth strains if the *rft 1* gene is not completely repressed. The determination of this sugar is, however, complicated by the possibility of cytoplasmic contamination of the lipopolysaccharide preparations. The lipopolysaccharide extracted from cell walls of the smooth strain *S. typhimurium* 395MS did not show any absorption at 260 mμ which indicates that the ribose (approximately 1%) detected in this extract was not derived from contaminating ribonucleic acid. No ribose was found in the extracts of *S. minnesota* R60, which is of chemotype Ra. This finding needs further investigation, since it was reported by Lüderitz *et al.* (1966) that this mutant incorporates small amounts of ribose in its lipopolysaccharide.

Ribitol was found by Lilly (1962) in extracts from isolated cell walls from *Escherichia coli* 26-26. Since the gas chromatographic method was based on the separation of glycitols, no differentiation could be made between ribose and ribitol. By using paper chromatography it could be shown that the hydrolysates of cell walls from *Salmonella typhimurium* 395MS contained small amounts of ribose as compared to galactose, mannose and rhamnose.

It was not possible to designate the mutants to the groups Ra, Rb, etc., using only the basis of the chemical composition of the cell-wall polysaccharide (Table 2). The presence of haptenic polysaccharide containing S-specific determinants in supernatant fluids from phenol+water extracts from whole bacteria (L1-fraction of Beckmann, Subbaiah & Stocker, 1964), which was easily detected by the gel precipitin reaction (unpublished), could not be used as a criterion for the Rb mutants, since two of the known Ra mutants, TV163 and 225, both displayed this antigen. The proposed chemical grouping of Table 2 was therefore based on phage patterns and immunological investigations (see Lindberg & Holme, 1968), in addition to cultural and chemical data. A difference in the glucosamine content between the Ra mutants, on the one hand, and Rb, Rc and Rd mutants, on the other, was recorded, but the amounts detected did not make a sharp distinction possible. The presence of large amounts of glucosamine in Lipid A complicates an accurate determination of this amino sugar in the polysaccharide. After the removal of the lipid by acid hydrolysis, small amounts of contaminating glucosamine might remain in the preparation. Part of the glucosamine in some of the mutants was, however, probably associated with the presence of the S-specific sugars.

The fact that the rough mutants selected for resistance against the phages FO-1 or 4 were of chemotype Rb, Rc and Rd, supports the assumption that the receptor sites for these phages are associated with the Ra structure (Lindberg, 1967). Mutants

selected for rough colony type appear to be predominantly of the chemotype Ra.

The mutant R9, which was a galactose-non-fermenter, showed characteristics of being deficient in the enzyme UDP-gal-4-epimerase. The presence of S-specific sugars in this mutant raised the question whether the culture medium used contained galactose. When analysed by the gas chromatographic method described above no traces of galactose, mannose, ribose, rhamnose or abequose could be detected in the tryptone or the yeast extract. Strain R10, which was the only mutant showing a low glucose content of its cell-wall polysaccharide, was believed to be deficient in the glucosyl-I-transferase system (Osborn *et al.* 1964), since it showed normal growth characteristics on a glucose-salts medium.

A further class of mutants of Salmonella was described by Naide *et al.* (1965). These mutants were termed semi-rough and displayed characteristics intermediate between the smooth and the rough forms with respect to cultural and serological behaviour. The lipopolysaccharide of these strains all contained the S-specific sugars but in smaller amounts than found in the wild-type strain. The results of phage typing and serological investigations presented by Lindberg & Holme (1968) disclose the possibility that the mutants used in the present investigation belong to the semi-rough group.

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Immunochemical Studies on Cell-wall Polysaccharide of Rough Mutants of *Salmonella typhimurium*

By A. A. LINDBERG
Department of Bacteriology, National Bacteriological Laboratory, Stockholm
AND T. HOLME
Department of Bacteriology, Karolinska Institutet, Stockholm

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SUMMARY

Immunological studies were made in order to characterize the lipopolysaccharides of rough mutants of *Salmonella typhimurium* and *S. minnesota*. Groups of mutants, specified by their phage pattern, also showed group homogeneity with respect to their immunological specificity, especially when using the passive haemagglutination inhibition technique. Cross-reactions between mutants belonging to different phage groups could generally be explained on the basis of the carbohydrate composition of the lipopolysaccharide.

INTRODUCTION

Lipopolysaccharides isolated from rough strains of different Salmonella species have been serologically differentiated by means of haemagglutination and haemagglutination inhibition techniques (Beckmann, Lüderitz & Westphal, 1964; Lüderitz, Beckmann & Westphal, 1964; Lüderitz *et al.* 1966*a*, *b*). In contrast to the numerous O-specificities encountered in the wild-type O-antigens of different Salmonella strains only a few specificities are found in R-antigens regardless of the serotype from which the mutant has been derived. Rough mutants of Salmonella strains have also been defined by their sensitivity to rough-specific phages (Subbaiah & Stocker, 1964; Wilkinson & Stocker, 1964; Lüderitz *et al.* 1966*a*; Brandis, 1966). Wilkinson & Stocker used a collection of eight R-specific phages, of which four were obtained from Brandis, in an attempt to separate rough mutants of *Salmonella typhimurium* strain LT2. More than eight different phage patterns could be established. Results obtained by Lüderitz *et al.* (1966*a*) indicated a certain relationship between the phage pattern, the sugar composition of the lipopolysaccharide and the reactivity with R-antisera in groups of rough mutants from *S. minnesota* and *S. ruiru*. The present paper reports on the further characterization of the *S. typhimurium* rough mutants, investigated chemically in a preceding paper (Holme, Lindberg, Garegg & Onn, 1968), with the aid of serological techniques and the sensitivity to different phages.

METHODS

Strains. The *Salmonella typhimurium* and *S. minnesota* strains were the same as used in the preceding paper by Holme *et al.* (1968).

Lipopolysaccharides. The strains were grown and the lipopolysaccharides extracted

from the bacteria by the phenol+water procedure (Westphal, Lüderitz & Bister, 1952). The lipopolysaccharides were purified by three cycles of washing and ultracentrifugation at 105,000*g* for 4 hr in a Christ Omega preparative ultracentrifuge. The pellet was lyophilized and the lipopolysaccharide resuspended in saline before use.

Antisera. Antisera were obtained by injecting rabbits intravenously three times a week for 9 weeks with heat-killed suspensions of the bacteria. Serum specific for the O-antigenic factor 4 of group B was obtained from the Salmonella Centre, National Bacteriological Laboratory, Stockholm. Serum against *Salmonella typhimurium* TV 119, obtained by immunization of a horse, was obtained from Dr M. Raynaud (Institut Pasteur, Paris). All sera were inactivated at 56° for 30 min., absorbed with packed washed sheep red cells at 37° for 2 hr and stored at −20° until use.

Passive haemagglutination. Sheep red cells were collected aseptically in sterile Alsever solution and washed three times in phosphate buffered saline before use. The lipopolysaccharides were treated with sodium hydroxide to enhance the affinity to red cells. The procedure was done according to Beckmann *et al.* (1964).

For sensitization 10 ml. saline containing 250 μg. alkali-treated lipopolysaccharide were added to 1·25 ml. of a 4% (v/v) suspension of washed sheep red cells. After incubation at 37° for 30 min. the sensitized red cells were washed 3 times with saline and finally diluted to 10 ml. with phosphate-buffered saline. Serial twofold dilutions of serum were prepared with phosphate-buffered saline (pH 7). To each tube containing 0·2 ml. serum dilution an equal volume of sensitized red cells was added. The tubes were kept at room temperature and read after 3 and 18 hr. The haemagglutinating titre was determined as the last tube showing macroscopically visible haemagglutination. Serum with unsensitized sheep red cells, and sensitized red cells in saline only, were used as controls in each experiment. The reactions were identical or deviated in only one dilution step on repeated tests.

Haemagglutination inhibition. A twofold dilution series of the lipopolysaccharide used as inhibitor was added to appropriately diluted sera. The serum dilution used was 4–8 haemagglutinating units (serum diluted 4–8 times less than the highest dilution causing haemagglutination). Serial twofold dilutions of the lipopolysaccharide with concentrations ranging from 256 to 1 μg./ml. were prepared in saline; 0·2 ml. of each lipopolysaccharide dilution was added to an equal volume of the appropriate serum dilution. The mixtures were incubated at 37° in a water bath for 30 min. Thereafter 0·2 ml. of sensitized sheep red cells was added to each mixture. Haemagglutination was read after incubation for 30 min. at 37° and 3 hr at room temperature. The lowest concentration of lipopolysaccharide giving a total inhibition of the haemagglutination was determined. The haemagglutination titre without inhibition was determined in each experiment. Homologous inhibition with the lipopolysaccharides used for sensitization was also done, as well as the controls described in the preceding section.

Phage classification. The strains were characterized by their pattern of sensitivity to a collection of phages. The phages used were classified as smooth-specific (P 22), rough specific (P 221, 6 SR, Br 2, Ffm, Br 60 and C 21) and phages active on smooth and rough strains (P 22h and FO-1). The phages were obtained from Professor H. Brandis (Hygiene-Institut der Universität Göttingen) and Professor B. A. D. Stocker (Dept. of Medical Microbiology, Stanford, California, U.S.A.). The phages were propagated by the soft-agar layer method of Adams (1959) or in broth culture. The

lysates of the phages contained 10^8–10^{10} plaque-forming units (p.f.u.)/ml. Freshly prepared lawns of the strains on nutrient agar (Difco) were tested for sensitivity to tenfold dilution steps of the phages by the spot technique. Readings were carried out after 5 and 18 hr incubation at 37°.

RESULTS

Phage classification of the strains

Drs Wilkinson & Stocker (personal communication) used a collection of phages for grouping rough mutants of *Salmonella typhimurium*. Differences in the sensitivity

Table 1. *Phage patterns of (1) wild-type Salmonella typhimurium 395MS and derived mutants, (2) known mutants of S. typhimurium LT2 and (3) known mutants of S. minnesota S99. Phages: smooth specific (P 22), smooth and rough specific (P 22h, FO-1) and rough specific (P 221, 6 SR, Br 2, Ffm, Br 60, C 21).*

Phage patterns obtained by using the collection of nine phages in spot test on the *S. typhimurium* and *S. minnesota* strains. +, Clear or turbid confluent lysis with the phage undiluted, scattered plaques in higher dilutions. −, No lytic effect of the phages.

	Phage									
Strain	P22	P22h	FO-1	P221	6 SR	Br 2	Ffm	Br 60	C 21	Classification
S. typhimurium										
395MS	+	+	+	−	−	−	−	−	−	Smooth
395MR0	−	−	+	+	+	+	+	+	−	Rough sensitive
395MR1	−	−	−	+	−	+	+	+	−	Rough resistant-1
395MR2	−	−	+	+	+	+	+	+	−	Rough sensitive
395MR3	−	−	+	+	+	+	+	+	−	Rough sensitive
395MR4	−	−	+	+	+	+	+	+	−	Rough sensitive
395MR5	−	−	−	+	−	−	+	+	−	Rough resistant-2
395MR6	−	−	−	+	−	−	+	+	−	Rough resistant-2
395MR7	−	−	−	−	−	−	+	+	−	Rough resistant-2
395MR8	−	−	−	+	−	−	+	+	−	Rough resistant-2
395MR9	−	−	±	+	−	−	+	+	+	Galactose-epimerase-less
395MR10	−	−	−	+	−	−	+	+	+	Glucosyl-I-transferase-less
TV119	−	−	+	+	+	+	+	+	−	Rough sensitive
TV148	−	−	−	+	−	−	+	+	−	Rough resistant-2
TV160	−	−	−	+	−	+	+	+	−	Rough resistant-1
TV161	−	−	−	+	−	+	+	+	−	Rough resistant-1
TV163	−	−	+	+	+	+	+	+	−	Rough sensitive
TV166	−	−	−	+	−	+	+	+	−	Rough resistant-1
TV208	+	+	+	+	+	−	−	+	−	Not recognized
TV225	−	−	+	+	+	+	+	+	−	Rough sensitive
TV226	−	−	+	+	+	+	+	+	−	Rough sensitive
TV227	−	−	+	+	+	+	+	+	−	Rough sensitive
S. minnesota										
S99	−	−	+	−	−	−	−	−	−	Not recognized
R60	−	−	+	−	−	+	+	+	−	Rough sensitive
R345	−	−	−	−	−	+	+	+	−	Rough resistant-1

to the various phages may depend on the composition of the cell-wall lipopolysaccharide of the mutants. Thus, the sensitivity to phage P 22 was limited to the smooth strains of Salmonella having the O-antigen 12 (Zinder & Lederberg, 1952). Phage FO-1 lysed smooth strains and mutants belonging to chemotype Ra and was therefore designated as a SR-specific phage. Six phages lysed only rough mutants and were

named R-specific. The strains used in the present work were grouped on the basis of their sensitivity pattern to nine phages. The phage patterns are represented in Table 1. The designation of the patterns is in accordance with Wilkinson & Stocker (1964).

Smooth specific. The parent strain, *Salmonella typhimurium* 395MS was lysed by the smooth specific phage P 22 and the smooth and rough specific phages P 22h and FO-1. The phage pattern of strain TV208 was not recognized since the strain was lysed by phages from all three groups.

Rough sensitive. Nine of the ten strains included in this group displayed a pattern characterized by lysis with the phages FO-1, P 221, 6 SR, Br 2, Ffm and Br 60. *Salmonella minnesota* R60 was lysed only by the phages FO-1, Br 2, Ffm and Br 60, but adsorbed the phages P 221 and 6 SR. Therefore this strain was classified as rough sensitive.

Rough resistant-1. Mutants in this group were lysed by the rough specific phages P 221, Br 2, Ffm and Br 60. This group contained four strains: R1, TV160, TV161 and TV166. *Salmonella minnesota* R345 was included in this group since it adsorbed phage P 221.

Rough resistant-2. The four strains in this group, R5, R6, R8 and TV148, were separated from the rough resistant-1 group by their resistance to phage Br 2. The mutant 395MR7 was included in this phage pattern although it was resistant to phage P 221 since the receptor structure for this phage appears to be independent of the polysaccharide structure (unpublished).

UDP-Galactose-4-epimerase-less mutant pattern. Only one strain, R9, was represented in this class. This galactose non-fermenter was sensitive to phages P 221 Ffm, Br 60 and C 21.

Glucosyl-I-transferase-less mutant pattern. The mutant R10 showed the same phage pattern as strain R9. The designation glucosyl-I-transferase-less is based on the phage pattern, its ability to ferment galactose and the presence of S-specific polysaccharides in the cytoplasmic fraction.

Haemagglutination and haemagglutination inhibition reactions

The lipopolysaccharides of the *Salmonella typhimurium* and *S. minnesota* strains were tested against 11 antisera with passive haemagglutination technique (Table 2). The homologous haemagglutination titres ranged between 1/640 and 1/1280. Duplicate or triplicate determinations were made in all experiments with positive reactions. The variability did not in any case exceed one dilution step. Sera drawn from rabbits before immunization showed no reactions in dilution exceeding 1/40. Titres 1/160 or above were considered as significantly positive reactions.

The lipopolysaccharides were further investigated in haemagglutination inhibition tests against eight homologous systems, representative for the smooth form and the different groups of rough mutants. Homologous inhibition was observed with the lipopolysaccharides in concentrations ranging from less than 1 to 4 μg./ml. (Table 3).

The results of the immunological studies will be presented in a following section of this paper.

Table 2. *Passive haemagglutination of Salmonella antisera with Salmonella lipopolysaccharides*

Haemagglutination systems. Data recorded are the highest dilution of serum, before addition of the lipopolysaccharide sensitized erythrocytes, giving visible haemagglutination. Entries left blank indicate negative reaction or reaction only in the dilution 1/40.

		Reciprocal of titre of antiserum to							
	Salmonella factor	*S. typhimurium*						*S. minnesota*	
Lipopolysaccharide	0–4	TV208	TV119	TV163	TV160	R6	R9	R60	R345
S. typhimurium									
395MS	1280	160	320	80	80	.	160	.	.
395MR0	.	.	1280	640	.	.	.	80	.
395MR1	.	80	.	.	160	160	80	.	80
395MR2	.	.	1280	640	.	.	.	160	.
395MR3	.	80	1280	640	.	80	.	320	.
395MR4	160	.	1280	640	.	.	.	160	.
395MR5	160	80	80	.	160	160	160	.	.
395MR6	.	.	.	.	.	640	.	.	.
395MR7	.	.	.	.	.	320	.	.	.
395MR8	.	.	.	.	.	160	.	.	.
395MR9	320	.	.	.	80	320	640	.	.
395MR10	.	.	.	.	.	.	.	.	.
TV160	.	.	.	.	640	80	.	.	80
TV163	320	80	1280	1280	160	80	80	.	.
TV208	640	640	160	.	160	80	160	80	.
S. minnesota									
R60	.	.	1280	1280	80	80	.	640	.
R345	.	.	.	.	160	80	.	.	640

Table 3. *Haemagglutination inhibition of Salmonella antisera with Salmonella lipopolysaccharides*

Haemagglutination inhibition systems: homologous lipopolysaccharide and serum. Data recorded are the minimal concentration (μg./ml.) of lipopolysaccharide from the *S. typhimurium* and *S. minnesota* strains needed for inhibition of the homologous system. Entries left blank indicate that lipopolysaccharide concentrations higher than 256 μg./ml. were needed for the inhibition.

	S. typhimurium					*S. minnesota*	
Lipopolysaccharide	TV208	TV163	TV160	R6	R9	R60	R345
S. typhimurium							
395MS	16	.	.	.	.	.	.
395MR0	.	8	.	.	.	.	.
395MR1	.	.	8	.	.	.	.
395MR2	.	8	.	.	.	.	.
395MR3	.	4	.	.	.	.	.
395MR4	.	8	.	.	.	.	.
395MR5	.	.	.	4	.	.	.
395MR6	.	.	.	< 1	64	.	.
395MR7	.	.	.	32	.	.	.
395MR8	.	.	.	4	32	.	.
395MR9	.	.	.	.	< 1	.	.
395MR10	.	.	.	.	128	.	.
TV160	.	.	4	.	.	.	.
TV163	.	4	.	.	.	.	.
TV208	4	.	.	.	.	.	.
S. minnesota							
R60	.	8	.	.	.	< 1	.
R345	.	.	.	.	128	.	< 1

Table 4. *Salmonella typhimurium and S. minnesota smooth and rough strains: phage pattern, composition of cell-wall polysaccharide and serological cross-reactions*

Approximate molar ratios of sugars are given relative to heptose taken as 1 for all strains except *S. typhimurium* 395 MS where the figures are given relative to glucose as 1. The abbreviations used are: hep = heptose, glu = glucose, gal = galactose, GN = glucosamine, rib = ribose, man = mannose, rha = rhamnose, abe = abequose. The glucosamine values are given in % of total polysaccharide. The antisera causing haemagglutination in a dilution 1/160 are recorded as well as the homologous lipopolysaccharide-antiserum systems being inhibited by the lipopolysaccharides tested.

Strain	Phage pattern	Approximate molar ratios of sugars						GN (%)	Haemagglutination of antiserum	Haemagglutination inhibition of LPS-antiserum system
		Hep	Glu	Gal	Man	Rha	Abe			
S. typhimurium 395 MS	Smooth	< 1	1	0·7	0·7	0·6	0·3	3·6	O 4, R9, TV 119, TV 208	TV 208
S. minnesota S99	Not recognized	1	1	2·4	nd	nd	nd	nt	nt	nt
S. typhimurium TV 208	Not recognized	1	1·3	4·9	3·4	2·4	1·3	5·2	O–4, R9, TV 119, TV 160, TV 208	TV 208
S. typhimurium 395 MR 0	Rough sensitive	1	1	1	0·1	< 0·1	< 0·1	16·7	TV 119, TV 163	TV 163
395 MR 2		1	0·6	1	< 0·1	< 0·1	< 0·1	2·6	TV 119, TV 163, R 60	TV 163
395 MR 3		1	0·5	0·5	< 0·1	< 0·1	< 0·1	5·0	TV 119, TV 163, R 60	TV 163
395 MR 4		1	1	1	0·2	0·1	< 0·1	5·0	O–4, TV 119, TV 163, R 60	TV 163
TV 163		1	1	1	0·3	0·3	0·1	3·3	O–4, TV 119, TV 160, TV 163	TV 163
TV 225		1	0·9	1	0·2	0·1	< 0·1	2·7	nt	nt
S. minnesota R 60		1	1·4	1·5	nd	nd	nd	nt	TV 119, TV 163, R 60	TV 163, R 60
S. typhimurium 395 MR 1	Rough resistant-1	1	0·5	0·8	0·1	< 0·1	< 0·1	1·4	R 6, TV 160	TV 160
TV 160		1	0·7	1	0·2	0·2	< 0·1	1·1	TV 160	TV 160
TV 161		1	0·6	0·9	0·3	0·1	< 0·1	0·6	nt	nt
S. minnesota R 345		1	0·7	1·7	nd	nd	nd	nt	TV 160, R 345	R9, R 345
S. typhimurium 395 MR 5	Rough resistant-2	1	0·8	1·1	0·8	0·5	0·2	1·8	O–4, R 6, R9, TV 160	R 6
395 MR 6		1	0·7	1	0·4	0·3	0·2	1·0	R 6	R 6, R 9
395 MR 7		1	0·6	0·5	0·2	< 0·1	< 0·1	2·1	R 6	R 6
395 MR 8		1	0·4	0·4	0·1	< 0·1	< 0·1	0·8	R 6	R 6, R 9
S. typhimurium 395 MR 9	Galactose-epimerase-less	1	1·1	0·1	0·1	< 0·1	< 0·1	0·7	O–4, R 6, R9	R9
S. typhimurium 395 MR 10	Glucosyl-I-transferase-less	1	< 0·1	0·1	0·2	< 0·1	< 0·1	1·3	—	R9

nd = not detected: nt = not tested: — = negative results

Correlation between chemical composition, sensitivity to phages and immunochemical investigations

In Table 4 the results of the different methods used for characterization of the rough mutants are summarized. The phage patterns which were obtained have been used as a basis for classification because they offered the most simple overall view.

Smooth specific pattern. The cell-wall polysaccharide from the parent strain *Salmonella typhimurium* 395MS contained small amounts of ribose and the S-specific sugars. The phage pattern was smooth specific. In haemagglutination tests positive reactions were observed with sera against the O-antigen 4, the Ra mutants TV119 and TV208 as well as the Rc mutant R9. Haemagglutination inhibition showed a cross-reaction with the TV208 system. *Salmonella minnesota S99*, only lysed by the FO-1 phage, had heptose:glucose:galactose ratios of 1:1:2·4.

Salmonella typhimurium TV208. This strain was originally described as a Ra mutant (Nikaido, Nikaido, Subbaiah & Stocker, 1964). The lipopolysaccharide contained great amounts of glucose, galactose, mannose, rhamnose and abequose compared to the other Ra-mutants. In serological tests TV208 showed cross-reactions with S-specific as well as several R-specific sera. Also in phage typing the strain behaved as both a smooth and rough strain. Its sensitivity to the phage P 22 indicated the presence of antigen 12 of the S-specific repeating unit. In contrast to the smooth form the strain was sensitive to three of the rough-specific phages.

Rough sensitive pattern. Four of the mutants in this group, R0, R4, TV163 and TV225, had heptose:glucose:galactose molar ratios of 1:1:1 whereas for three of the mutants the figures varied from 1:0·5:0·5 for R3, 1:0·6:1 for R2 and 1:1·4:1·5 for *Salmonella minnesota* R60. The content of *N*-acetyl-glucosamine in the R0 mutant was much higher than in any of the other *S. typhimurium* strains. Small amounts of mannose, rhamnose and abequose were found in all the mutants with the exception of *S. minnesota* R60. In serological tests the strains were fairly regular with one notable exception. The *S. minnesota* R60 homologous system was not inhibited by any of the lipopolysaccharides from the six *S. typhimurium* mutants of this group.

Rough resistant-1 pattern. This group contained four strains, *Salmonella typhimurium* 395MR1, TV160, TV161 and *S. minnesota* R345. The polysaccharide composition differed from that of the rough sensitive group as the strains contained a significantly smaller amount of glucosamine. Again the S-specific sugars were detected in small amounts. In serological tests cross-reactions were detected with the TV160 antiserum. Antiserum against *S. minnesota* R345, the test strain for RI specificity according to Lüderitz *et al.* (1966*a*), showed weak reactions in the haemagglutination test with the R1 and TV160 lipopolysaccharides.

Rough resistant-2 pattern. The phage pattern of strains in this group differed from the rough resistant-1 group as the strains were not lysed by phage Br 2. *Salmonella typhimurium* 395MR5 and R6, selected for their resistance to phage FO-1, had heptose:glucose:galactose molar ratios of approximately 1:0·75:1 and appreciable amounts of mannose, rhamnose and abequose. In haemagglutination tests lipopolysaccharide from R5 cross-reacted with O-factor 4, R6, R9 and TV160 sera whereas R6 only showed the homologous reaction. In haemagglutination inhibition tests lipopolysaccharide from R6 inhibited the R9 system. The mutants R7 and R8, selected for their resistance to the Lilleengen phage 4, had heptose:glucose:galactose molar ratios of approxi-

mately 1:0·5:0·5 with smaller amounts of the S-specific sugars than the strains R5 and R6. Lipopolysaccharide from R7 and R8 cross-reacted with R6 antisera in haemagglutination tests. The homologous systems R6 and R9 were both inhibited by lipopolysaccharide from R8.

UDP-Galactose-4-epimerase-less pattern. The only strain in this class, R9, was characterized by its sensitivity to phage C21 in addition to the phages P 221, Ffm and Br 60. The polysaccharide of this strain contained heptose and glucose in a 1:1 ratio and small amounts of galactose and S-specific sugars. Upon addition of galactose the strain R9 developed smooth specific characteristics as judged by colony morphology, agglutination reactions and phage pattern. Sometimes this strain showed an unexpected sensitivity to phage FO-1 in galactose-free media but not to the phages 6 SR and Br 2 at the same time. In haemagglutination tests the lipopolysaccharide cross-reacted with O-factor 4 and R6 antisera.

Glucosyl-I-transferase-less mutant pattern. Salmonella typhimurium 395MR10 was a galactose-fermenter with the same phage pattern as the UDP-galactose-epimerase-less mutant R9. Its polysaccharide contained small amounts of galactose, mannose and traces of glucose, rhamnose and abequose. The R10 lipopolysaccharide did not show cross-reactions in haemagglutination tests but inhibited the R9 system in high concentrations.

DISCUSSION

Attempts to classify rough mutants of Salmonella according to both the chemical composition, serological specificity and sensitivity to phages were made on *Salmonella minnesota* and *S. ruiru* mutants by Lüderitz *et al.* (1966*a*). The sugar composition of the lipopolysaccharides of the rough mutants separated them into five groups. Haemagglutination inhibition tests revealed that many antisera cross-reacted with lipopolysaccharides extracted from rough mutants belonging to different chemotypes.

The results presented in a preceding paper (Holme *et al.* 1968) showed that the rough mutants of *Salmonella typhimurium* in addition to the sugars of the basal core also contained the S-specific sugars in varying amounts. It was not possible, on the basis of the molar ratios of the sugar components calculated for the different *S. typhimurium* and *S. minnesota* rough mutants, to separate the strains into distinct groups. In haemagglutination and haemagglutination inhibition tests, presented in this paper, the lipopolysaccharides of the different rough strains showed a variety of cross-reactions which also made a separation between the mutants difficult. The overall picture was therefore based on the phage patterns obtained when applying the collection of phages used in an investigation of the phage susceptibility of mutants of *S. typhimurium* LT2 by Drs Wilkinson & Stocker (personal communication).

With the use of this collection of nine phages the rough mutants of *Salmonella typhimurium* were separated into five groups. Two of the *S. typhimurium* mutants had phage patterns which had not been recognized earlier. The three *S. minnesota* strains also showed phage patterns not recognized earlier but were included into the groups as they adsorbed the corresponding phages. Each phage pattern was stable when the strains were retyped several times as well as when the strains were grown both in the absence and in the presence of glucose. This indicated that the mutants were stable rough forms as revertants to the smooth form would have given growth in the area lysed by the R-specific phages in spot tests.

The mutant *Salmonella typhimurium* TV208 was lysed by both the S-specific and some of the R-specific phages. This strain was originally described as a Ra mutant defective in the synthesis of TDP-rhamnose (Nikaido *et al.* 1964). These workers also suggested that strain TV208 showed a high frequency of reversion to the smooth form. The molar ratios of the sugar components of the TV208 polysaccharide revealed high amounts of the S-specific sugars as compared to the other Ra mutants. In serological investigations the strain showed both R and S specificity. The results would be compatible with the assumption that the observed S-specificity could be due to smooth revertants. In virulence tests, however, no mice were killed by strain TV208; if smooth revertants were frequent in cultures of T, V, 208 no mice would have survived. It appears that the strain TV208 is a non-virulent rough mutant with unusually high content of S-specific sugars, which confer susceptibility to the S-specific phage P 22 and serological cross-reactions with the smooth form. In the rough sensitive group the sugar composition was similar for four of the strains—R0, R4, TV163, TV225—with heptose:glucose:galactose ratios of 1:1:1, more than 2·5% of glucosamine in the polysaccharide and small amounts of mannose, rhamnose and abequose. Two of the mutants—R2 and R3—did not show the 1:1:1 ratios. The observed differences in carbohydrate composition were, however, not reflected in the serological reactions where the strains were quite homogeneous with the exception of *Salmonella minnesota* R60.

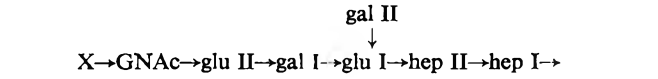

Fig. 1. Possible structure of core of Salmonella lipopolysaccharide. Hep = heptose, glu = glucose, gal = galactose, GNAc = *N*-acetyl glucosamine, X = unidentified unit, possibly involved in RII specificity.

The lipopolysaccharides of all the Ra mutants inhibited the homologous *S. typhimurium* TV163 system in haemagglutination inhibition tests whereas the homologous *S. minnesota* R60 system was not inhibited by any of the *S. typhimurium* lipopolysaccharides. This non-reciprocal inhibition could be attributed to the possible attachment of an acid-labile unit 'X' to the terminal *N*-acetylglucosamine of the core polysaccharide (Fig. 1). This unit is supposed to be involved in the production of the complete core by *rfb* mutants of the antigenic R II character, i.e. *S. minnesota* R60 (Lüderitz *et al.* 1966*a*). The *rfa* mutant *S. typhimurium* TV163 contains both the glucosamine of the core and the cytoplasmic S-specific polysaccharide. The latter has been demonstrated by gel precipitation (unpublished). This strain therefore should not possess the 'X'-unit, which is indicated by the haemagglutination inhibition tests. However, as evidenced by the uniformity in phage pattern within this group the presence of the 'X'-unit does not appear to have any influence on the susceptibility of the strains to the different phages.

The eight mutants classified as chemotype Rb were complex in phage pattern, carbohydrate composition and serological specificity. The *Salmonella typhimurium* mutants with the rough resistant-1 pattern had heptose:glucose:galactose ratios of approximately 1:0·5:1, which indicates that the core ended with the gal I-residue (Fig. 1). The *S. minnesota* R345 mutant did not show the same sugar ratios and differed also in serological specificity. The mutants 395MR5, R6, R7 and R8 showed the rough resistant-2 pattern and were rather homogeneous in haemagglutination inhibition

tests. The heptose:glucose:galactose ratios for the R7 and R8 mutants were approximately 1:0·5:0·5, which could be due to that either the gal I or gal II residue of the core is lacking. This would explain the difference between the two classes within the chemotype Rb with respect to phage pattern and serological specificity. The heptose: glucose:galactose ratios for the R5 and R6 mutants were not the same as for the R7 and R8 mutants, which could be due to a high degree of leakiness resulting in the synthesis of a few S-specific side chains. The side chains do not, however, prevent the reaction with R-specific antisera.

The UDP-galactose-4-epimerase-less mutant R9 contained small amounts of galactose and S-specific sugars. Possible explanations of this finding were discussed in the preceding paper by Holme *et al.* (1968). The assumption that rhamnose, mannose and abequose were linked in a configuration determining O-specificity was supported by the finding of cross-reactions between the anti R9 serum and lipopolysaccharides from the smooth specific strain *Salmonella typhimurium* 395MS and *S. typhimurium* TV208. The lipopolysaccharide from R9 also reacted with the O-factor 4 serum in haemagglutination tests. Furthermore this strain showed an unexpected sensitivity to the FO-1 phage on some occasions. This FO-1 sensitivity was regularly accompanied by a lysis with phage C 21 which otherwise has been reported to lyse only mutants of the Rc and Rd chemotypes (Shedlovsky & Brenner, 1963; Stocker, Wilkinson & Mäkelä, 1966). No lysis with phage P 22 or P 22h was ever observed. The fact that this strain could be sensitive to both FO-1 and C 21 indicates that the FO-1 receptostructure can be present without conferring a masking effect on the C 21 receptor structure. The same inconstancy in phage pattern has also been observed for the UDP-galactose-4-epimerase-less mutant *S. typhimurium* SL 1070. Thus for the *S. typhimurium* mutants the phage pattern was constantly rough, the only exception being the strain TV208. The presence of small amounts of the S-specific sugars was not accompanied by any adsorption of phages P 22 or P 22h.

Naide *et al.* (1965) described a class of mutants in Salmonella intermediate between the smooth and the rough form both in biological properties and lipopolysaccharide composition. They were named semi-rough (SR) mutants and displayed a smooth colony type on solid media, the O-antigen 4 was present in all mutants and their recombinants, and the O-antigen 5 was detected in some. The S-specific sugars were present in small amounts in all the strains. Some of the strains used in the present investigation have characteristics which could indicate that they were SR-mutants, namely R4, R5 and TV163. SR mutants display typical phage patterns in addition to the characteristics described above (Drs Wilkinson & Stocker, personal communication). Both the mutants R4 and TV163 showed a phage pattern which was recognized as specific for the rough sensitive group. The mutants R5 and TV163 synthesize the soluble hapten which was not found in any of the SR-mutants (Naide *et al.* 1965). The results indicate that none of the mutants could be classified as semi-rough using the definition of Naide *et al.* (1965).

In haemagglutination tests cross-reactions were observed between mutants belonging to different phage groups. The presence of the cross-reacting antigens was reflected in the monosaccharide composition of the lipopolysaccharide. With the haemagglutination inhibition technique the reactions were more specific for each phage group.

Additional information on the structure of the polysaccharide core may be obtained

by the use of the different R-specific phages in inhibition experiments with lipopolysaccharides extracted from the rough mutants. The changing susceptibility to the rough specific phages observed for the different mutants indicates that the monosaccharide components determine the specificity of the phage receptors.

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Histochemical Demonstration of Certain Hydrolytic Enzymes within Cytoplasmic Particles of *Botrytis cinerea* Fr.

By D. PITT
Department of Botany, University of Exeter

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SUMMARY

Established histochemical methods were used to locate the activities of several acid and neutral hydrolases within cytoplasmic particles of *Botrytis cinerea* Fr. The Gomori procedure revealed acid phosphatase activity and this was used as a marker to show that these particles in fresh material were inactive until subjected to treatments which affected the permeability of lipid-protein membranes. This behaviour was interpreted as indicating that these particles may be comparable with the lysosomes of animal cells. It was shown that acid phosphatase, acid deoxyribonuclease II, β-galactosidase and several esterases were localized within these particles. Attempts to demonstrate β-D-glucuronidase activity were unsuccessful; aryl sulphatase activity was weak.

INTRODUCTION

It is considered that acid phosphatase activity in fungi is localized within cytoplasmic particles (Novikoff, 1961; Pitt & Walker, 1967). It is also believed (de Duve, 1959) that the lysosomes of animal cells contain all the cytoplasmic acid phosphatase and are rich in other hydrolases. Pitt & Walker (1967) showed lysosomal properties in cytoplasmic particles of several fungi, and Zalokar (1960) showed the distribution of acid phosphatase within the mitochondrial fraction of freeze-substituted centrifuged hyphae. However, the evidence for the existence of lysosomes in fungi is incomplete. The present work was designed to examine the distribution of some acid and neutral hydrolases in *Botrytis cinerea* and to determine whether these enzymes are localized in particles having affinities with the lysosomes of cells of animals and higher plants.

METHODS

A strain of *Botrytis cinerea* (DM 32), newly isolated from rotting anemone leaves, was grown on coverslips for 3 days at 25° on discs of malt extract agar according to Ridell (1950). The discs were removed and the tissue adhering to the coverslips was used directly or after appropriate fixation.

Histochemical methods. These generally were established procedures with modifications as indicated. Fixation, unless otherwise stated, was in Baker's formol-calcium fixative for 16 hr at 0–4°.

Acid phosphatase. (*a*) The Gomori (1952) lead nitrate method was used for fresh and fixed tissues with incubation for 1–60 min. depending upon experimental procedure. Latency of enzyme activity was examined by the methods of Gahan (1965) and included pre-incubation in 4% (w/v) aqueous formaldehyde solution, in buffers and in aqueous

Triton X-100. (*b*) The standard coupling azo dye technique (Grogg & Pearse, 1952) with sodium α-naphthylphosphate and Fast garnet GBC salt (G. T. Gurr, Ltd.) and incubation times of 20–30 min. at 37° was done, with and without osmotic protection provided by 7·5% polyvinyl pyrrolidone in the medium. (*c*) The post-coupling azo dye method (Rutenberg & Seligman, 1955) was used for fixed tissues with sodium 6-benzoyl-2-napththylphosphate with incubation for 1 hr at 37° followed by coupling with Fast blue B salt (G. T. Gurr, Ltd.) at 0°. Appropriate controls were: (i) incubation without substrates; (ii) incubation in the presence of 10^{-2} M-sodium fluoride; (iii) with tissues heated in free steam for 5 min.; (iv) omission of ammonium sulphide (Gomori) or the coupling dyes.

Esterases. (*a*) *Holt's indoxyl method* (Holt, 1958) was applied to fixed tissue with O-acetyl-5-bromoindoxyl (K and K Laboratories Inc., New York) and an incubation time of 30 min. at 22°. Appropriate controls were used and the effects of known esterase inhibitors examined. Tissues were incubated for 1–4 hr in diethyl-*p*-nitrophenyl phosphate (*E*600) at 10^{-3}, 10^{-5}, 10^{-7} and 10^{-9} M in 0·05 M-acetate buffer (pH 6·0) at 22°, and in 5×10^{-3} M-iodoacetamide in tris HCl buffer (0·1 M, pH 7·0) and in 0·1 M-*N*-ethylmaleimide in similar buffer before incubation in the indoxyl medium for the usual time. (*b*) *Substituted coupling azo-dye methods.* The modified α-naphthyl-acetate method of Nachlas & Seligman (1949) was done on fresh tissues with incubation for 10 min. in the presence of Fast blue B salt at 22°. The Davis & Ornstein (1959) modification with 'hexazotized' *p*-rosaniline as the coupling dye was also used with fixed and fresh tissues, with incubation for 20 min. at 22°. The method of Gomori (1952) was used with naphthol-AS acetate as the substrate and Fast red RC salt (G. T. Gurr, Ltd.) as the coupling dye under conditions mentioned below. These azo dye methods were also performed after incubation in appropriate buffer at 22° containing *E*600 at 10^{-3}, 10^{-5} and 10^{-7} M. (*c*) *Thioacetic acid method* (Wachstein, Meisel & Falcon, 1961) was done on fresh and fixed tissues with incubation for 1–1½ hr at 22°. The effect on staining of incubation for 1 hr at 22° in 10^{-3}, 10^{-5}, 10^{-7} and 10^{-9} M concentrations of *E*600 in 0·2 M-acetate buffer (pH 5·5) was also examined. Appropriate controls included the omission of substrate and of lead nitrate.

Acid deoxyribonuclease II. Attempts were made to investigate the distribution of this enzyme in fixed material by using the method of Vorbrodt (1961). Difficulties were encountered with the method owing to the non-specific deposition of lead salts in localized areas. Investigation showed such non-specific lead staining over wide ranges of pH values, lead nitrate and buffer concentrations with incubation for more than 45 min. at 37°. A modification of the Vorbrodt incubation medium was eventually used in which 0·3 ml. lead nitrate (0·1 M) was used in 50 ml. medium. Unpolymerized calf thymus deoxyribonucleic Na salt (British Drug Houses) and acid phosphatase from wheat germ, 0·15 E.U./mg. (British Drug Houses) were used at the concentrations suggested by Vorbrodt. Incubation was for 1 hr at 37° followed by washing in water for 1 min., 1% (w/v) acetic acid for 2 min., development in ammonium sulphide solution for 2 min. Controls included: (i) omission of substrate, (ii) omission of acid phosphatase, (iii) medium consisting of buffer containing lead nitrate, (iv) complete medium containing 0·1 M-NaF to judge the contribution towards the staining reaction of the non-specific lead localization.

Double staining methods. These involved the Gomori and standard coupling methods

for acid phosphatase, the indoxyl method for esterase and the procedure for deoxyribonuclease II (DNase II), in techniques where staining was first done by one method, followed by subsequent staining by a second method for another hydrolase, to determine whether a single particle contained one or more enzyme types.

Aryl sulphatase. The distribution of this enzyme (or a non-specific esterase) was determined in fresh and fixed materials by using the method of Rutenberg, Cohen & Seligman (1952) with a substrate of potassium 6-bromo-2-naphthylsulphate and coupling with Fast blue B salt at 0–4°. A simultaneous coupling procedure was also done with naphthol-AS sulphate in 0·1 M phosphate buffer (pH 6·2) at 37°, coupled with Fast red TR salt (G. T. Gurr, Ltd.).

β-D-glucuronidase. Attempts to show the activity of this enzyme in fixed tissues (2 hr in cold neutral formalin) were unsuccessful. The substrates used were 8-hydroxyquinoline-β-D-glucuronide coupled with Fast blue RR salt (British Drug Houses) according to Pearse (1960) and 6-bromo-2-naphthyl-β-D-glucuronide coupled with Fast blue B salt (Seligman, Tsou, Rutenberg & Cohen, 1954), with incubation up to 16 hr. Unfixed tissues were similarly examined.

β-Galactosidase was localized by using a substrate of 6-bromo-2-naphthyl-β-D-galactopyranoside and incubation for 20 min. at 37° followed by post coupling to Fast blue B salt at 4° (Rutenberg *et al.* 1958).

RESULTS

Localization of acid phosphatase activity

Fresh material was incubated in the Gomori test and control media and samples taken at intervals were examined for the presence of acid phosphatase activity. Localization of the reaction occurred in discrete sites within the cytoplasm 0·2–1·0 μ in diameter and present in variable numbers (Pl. 1, fig. 1). The results are summarized in Table 1. Staining in control sections did not occur except in lead nitrate+buffer

Table 1. *Staining reaction for acid phosphatase in Botrytis cinerea following incubation in Gomori medium at 37°*

Incubation time (min.)	Cytoplasmic		Nuclei	Controls
	Particulate	Diffuse		
3	o	o	o	o
7	o	±	o	o
12	±	±	o	o
24	+	+	o	o
36	±	+	±	o
48	±	+	+	±
60	±	+	+	+

+ = positive; ± = slight reaction; o = no reaction.

controls and in sodium fluoride controls after 45 min. incubation. Similar results were obtained with tissues placed in 5 % polyvinyl alcohol for 15 min. before staining. This treatment resulted in sharper particulate localization with decreased diffuse staining at the shorter incubation times.

Fresh tissues which had been fixed for 1 hr at 4° in 4 % (w/v) aqueous formaldehyde solution, and fresh tissues pre-incubated in 0·05 M-acetate buffer (pH 5·0) at 37° for

15 min. before transfer to the test media became stained after a very brief incubation. Tissues which had been fixed in cold 4 % neutral formalin for 1 hr and then incubated in 0·25 % (w/v) aqueous Triton X-100 before prolonged incubation in the control media showed no reaction (Table 2). Prolonged incubation of sections in the test medium resulted in staining outside the particulate sites when the cytoplasm took on diffuse stain with ultimate nuclear staining.

Table 2. *Effects of pre-treatments upon localization of acid phosphatase in Botrytis cinerea using the Gomori procedure at 37°*

Treatment	Staining reaction	Incubation time (min.) 2	5	10	20	40	60
		Reaction					
Triton X-100	Particulate	o	o	o	o	o	o
	Diffuse	o	o	o	o	o	o
	Nuclear	o	o	o	o	o	o
4 % (w/v) aqueous formaldehyde	Particulate	±	+	+	+	±	±
	Diffuse	o	±	+	+	+	+
	Nuclear	o	±	+	+	+	+
0·05 M-acetate buffer pH 5·0	Particulate	±	+	+	±	±	±
	Diffuse	o	±	+	+	+	+
	Nuclear	o	±	+	+	+	+

+ = positive; ± = slight reaction; o = no reaction.

The standard coupling (Pl. 1, fig. 2) and the post-coupling azo dye methods gave a particulate localization of acid phosphatase activity like that revealed by the Gomori procedure. These particles had the same size and distribution as those shown by the lead method; controls were unstained. As with the Gomori method prolonged incubation resulted in diffuse staining of the cytoplasm; nuclear staining was not detected. The presence of polyvinyl pyrrolidone in the test medium in the standard azo dye method resulted in decreased diffuse staining and sharper particle delimitation.

Distribution of esterase activity

Excellent particulate localization of esterase activity was revealed by the indoxyl method in tissues fixed for 16 hr in cold formol + calcium fixative (Pl. 2, fig. 3). Incubation for longer periods than the optimum time of 30 min. resulted in an intensification of the staining with gradual development of diffuse staining. Short periods of fixation, e.g. 2 hr, gave heavy diffuse staining after 1 hr of incubation which tended to obscure particulate staining.

Pre-incubation of tissues in 10^{-9} and 10^{-7} M solutions of *E*600 did not affect staining. Some decrease of diffuse staining was obtained in the presence of 10^{-5} M-*E*600; particulate staining was unaffected. Diffuse staining was completely suppressed at 10^{-3} M-*E*600 with slight decrease in particulate staining. Controls for the inhibitor studies were pre-incubated in buffer alone, since Holt (1963) considered that washing of sections before incubation removed the enzyme responsible for the staining reaction. These controls showed slightly diminished staining. Iodoacetamide and *N*-ethyl-maleimide did not influence staining at the concentrations used in these experiments. Controls in all test media were unstained.

When fresh tissues were incubated for 5 min. with α-naphthylacetate in the presence of Fast blue B salt at 4°, dark blue diffuse staining of the cytoplasm was produced with darker particulate localization. The use of 'hexazotized' *p*-rosaniline as the coupling dye under similar conditions resulted in a dark-red diffuse cytoplasmic distribution of the stain so intense as to obscure any possible particulate localization. When the above methods were applied to fixed tissues, diffuse staining was decreased and particulate staining was readily visible (Pl. 2, fig. 4). Controls which excluded the substrates, dyes, or both were unstained.

Pre-incubation for 1 hr at 0° in 10^{-5} and 10^{-6} M-*E*600 in 0·1 M-phosphate buffer (pH 7·4) suppressed particulate staining and greatly decreased diffuse cytoplasmic staining.

Staining did not occur with naphthol-AS acetate as a substrate in the presence of Fast red TR salt. However, coupling with 'hexazotized' *p*-rosaniline in fresh material resulted in dark-red diffuse cytoplasmic staining; but fixed tissues gave only a slight diffuse reaction. Particulate localization was not shown by any method utilizing naphthol-AS acetate. Controls gave negative results.

Fixed tissues incubated for 3 hr at 22° in the thioacetic acid medium gave excellent particulate localization of esterase activity (Pl. 2, fig. 5). Prolonged incubation resulted in the gradual intensification of diffuse cytoplasmic staining. Similar results were obtained with unfixed tissue, but development of diffuse staining was quicker and more intense. Pre-incubation in 10^{-9}M-*E*600 had no inhibitory effect on staining. Concentrations of *E*600 of 10^{-7} and 10^{-5}M almost prevented diffuse cytoplasmic staining without affecting particulate staining. A concentration of 10^{-3}M-*E*600 eliminated diffuse staining and decreased particulate staining; but on prolonged incubation the intensity of staining at particulate reaction sites gradually increased. The particulate localization obtained for esterases by the above methods was similar in size and distribution to that obtained for acid phosphatase.

Acid deoxyribonuclease II localization

Difficulties were experienced in using this method because of patchy non-specific lead staining which occurred at the optimum incubation time of 1 hr. These difficulties were overcome to some extent by using very low lead nitrate concentrations in the test media and by using adequate controls of lead nitrate + buffer solutions and complete media containing 0·1 M-NaF. The extent of DNase activity was then assessed by comparison with the control tissues. Although patchy non-specific lead staining was seen in controls this was in contrast to the even staining which occurred at particulate reaction sites through DNase activity. All tests were done on tissues fixed for 16 hr in cold formol + calcium fixative (Pl. 2, fig. 6). Incubation for longer than 1 hr resulted in heavy diffuse staining.

Detection of other hydrolases

Aryl sulphatase. Fresh material incubated in the presence of 6-bromo-2-naphthyl-sulphate by the method of Rutenberg *et al.* (1958) for 3 hr gave a very pale blue particulate localization of the stain. The simultaneous coupling method resulted in a pale brown diffuse cytoplasmic staining with a slightly darker red-brown particulate staining after incubation for 1·5 hr at 37°; longer incubation resulted in flocculation

of the diazonium reagent. Controls from which substrate was omitted gave no reactions.

β-D-glucuronidase. Attempts to localize sites of activity of this enzyme were unsuccessful.

β-Galactosidase. Dark blue particulate staining was seen after incubation of fresh material for 20 min. at 37° or 30 min. at 25°. These particles had a similar size and distribution to those found to contain other hydrolases. A considerable degree of pink diffuse cytoplasmic staining also occurred which intensified with increasing incubation time, and after 1 hr obscured particulate reaction sites. Material previously fixed for 2 hr in cold formol + calcium showed more intense diffuse staining and fewer particulate sites of activity.

Double staining methods

Of the combinations of staining methods used the most successful were those in which fixed material previously stained for acid phosphatase activity by the Gomori or standard coupling azo dye methods was subsequently subjected to the indoxyl procedure for esterase localization. The use of the indoxyl method as the second procedure was particularly useful since the gradual double staining at room temperature could be observed with the microscope to give ultimately black particles. Such methods clearly showed that a proportion of particles present in the cytoplasm gave positive reactions for acid phosphatase and esterase. Similarly, staining of acid phosphatase-containing particles by the standard coupling azo dye method followed by staining for DNase activity resulted in a certain proportion of the particles becoming doubly stained. Similar results were obtained with a combination of DNase and indoxyl staining methods. A small number of particles previously stained for esterase activity by the indoxyl method became stained with non-specific lead deposits when incubated for 1–2 hr in lead nitrate + buffer control solutions.

DISCUSSION

The latency of the acid phosphatase activity within the cytoplasmic particles, detected by use of the Gomori lead salt procedure, suggests a similarity between such particles and the lysosomes of animal cells as defined by de Duve (1959), and the lysosome-like particles of plant cells (Gahan, 1965). Also the Triton X-100 effect supports this view since it is generally assumed that this detergent disrupts the lipid-protein membrane of lysosomes, thus liberating the enzymes into the surrounding medium. The diffuse cytoplasmic staining following prolonged incubation is possibly a result of diffusion of the lead phosphate reaction product (Danielli, 1953). On the other hand, such diffuse staining might be due to the activity of other enzymes in the cytoplasm which are capable of a slower utilization of the β-glycerophosphate substrate and which are resistant to NaF inhibition (Gahan, 1965), or to non-specific lead staining. That some contribution to such staining arises from the latter is supported by the findings of heavy lead deposition in the controls incubated for periods longer than 60 min. The occasional particulate nature of this staining was similar to that found with the lysosome-like particles except that it was not affected by NaF. However, results with the Gomori method were not invalidated by such staining since the optimum reaction time for the method never exceeded 30 min., by which time non-specific lead deposits were negligible. The excellent particulate

localization of acid phosphatase activity made visible by the azo dye methods was free from the possible criticism associated with the Gomori procedure and confirmed the existence and nature of the localized activity of the enzyme.

The demonstration of carboxylic esterase activity which was achieved by several methods, and the response to formalin fixatives and specific enzyme inhibitors, indicated a situation as complex as that found with animal tissues (Holt, 1963). Hess & Pearse (1958) suggested that the esterase activity revealed by the indoxyl method is due to the activity of cathepsin C. The indoxyl esterase in the present work, however, was resistant to 10^{-3} M-*E*600 and to iodoacetamide and *N*-ethylmaleimide. If one accepts the argument of Holt (1963) concerning inhibition of cathepsin activity it is apparent that this enzyme in *Botrytis cinerea* is neither cathepsin B, C nor D, and in these respects is similar to the indoxyl esterase investigated in mammalian tissues by Holt, the nature of which is currently unknown. The inhibition of particulate staining by *E*600 in the α-naphthylacetate *p*-rosaniline method might indicate the presence of an organophosphorus-sensitive esterase (possibly of type B similar to that found by Müller (1962), in Paramecium). But the inhibitor studies made in the present work do not allow complete characterization of the esterases so far detected.

In spite of various modifications to the Vorbrodt (1961) method for acid DNase II activity this method was not found so suitable for fungal histochemistry in the present work as it has been with animal tissues. With appropriate controls some evidence was obtained for the existence of this enzyme within the fungal cytoplasmic granules, and the double-staining methods showed that acid phosphatase, indoxyl esterase and DNase II were located within the same particles. However, only a proportion of such particles were seen to be double-stained.

The staining for aryl sulphatase activity was very weak in contrast to the speed and intensity of reaction reported by Avers (1961) in grass roots. Prolonged incubation at high temperature, found necessary in the present work, may have resulted in false localization, since according to Pearse (1960) the reaction products are lipid-soluble and liable to diffuse. In the absence of biochemical evidence for the existence of aryl sulphatase in fungi and because of the limitations of present histochemical methods the evidence for its particulate localization in *Botrytis cinerea* is weak.

The localization of some activity of β-galactosidase within cytoplasmic particles is consistent with the observations of Zalokar (1960) who obtained localization of this enzyme in the mitochondrial fraction of freeze-substituted centrifuged cells of Neurospora. The failure to detect β-D-glucuronidase activity does not necessarily mean the absence of this enzyme, but Avers (1961), using similar methods, also failed to detect this enzyme in higher-plant tissues.

The present work has shown the localization of activities of several hydrolytic enzymes within cytoplasmic particles of *Botrytis cinerea* which have some features in common with the lysosomes of animal cells. Other important lysosomal enzymes sought in *Botrytis cinerea* showed only weak activity or were not detected by methods successfully employed for their detection in animal tissues.

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EXPLANATION OF PLATES

Particulate localization of hydrolases in *Botrytis cinerea*.

PLATE 1

Fig. 1. Particles containing acid phosphatase revealed by the Gomori lead salt method. × 1827.

Fig. 2. Acid phosphatase particles, standard coupling azo dye method. × 1370.

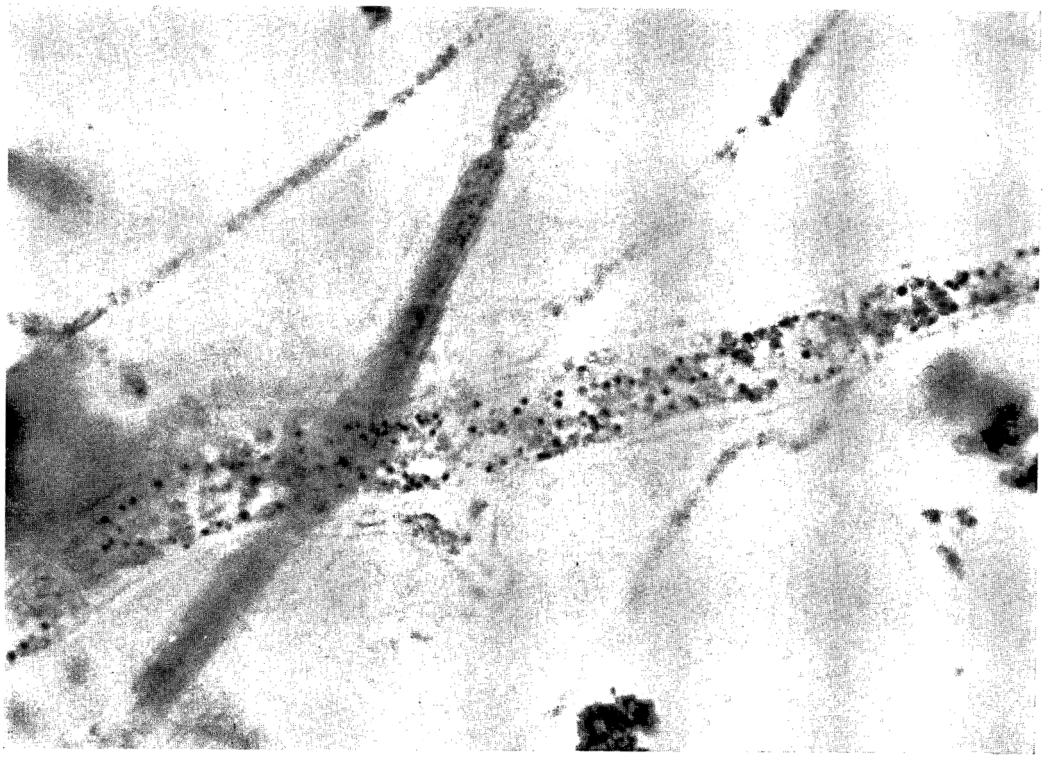

Fig. 1

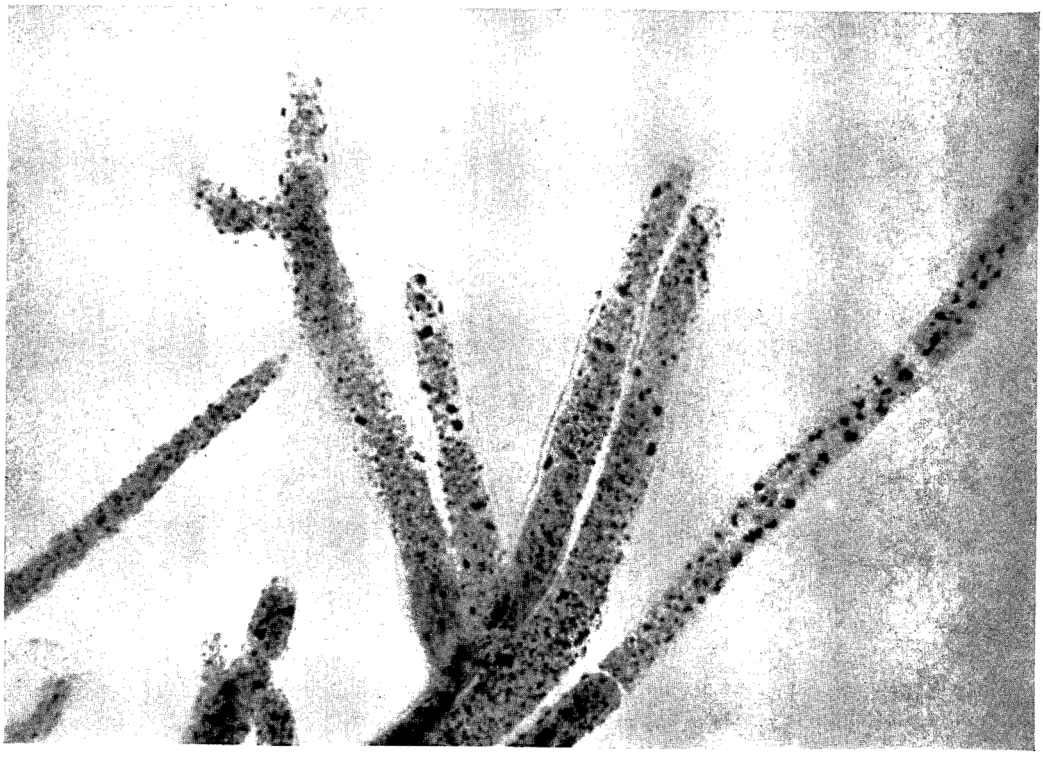

Fig. 2

(*Facing p.* 74)

Fig. 3

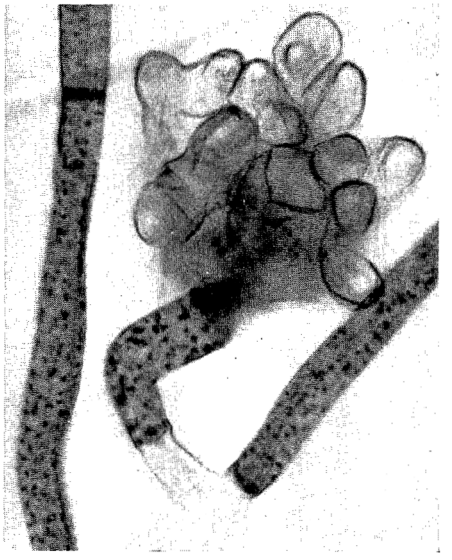

Fig. 4

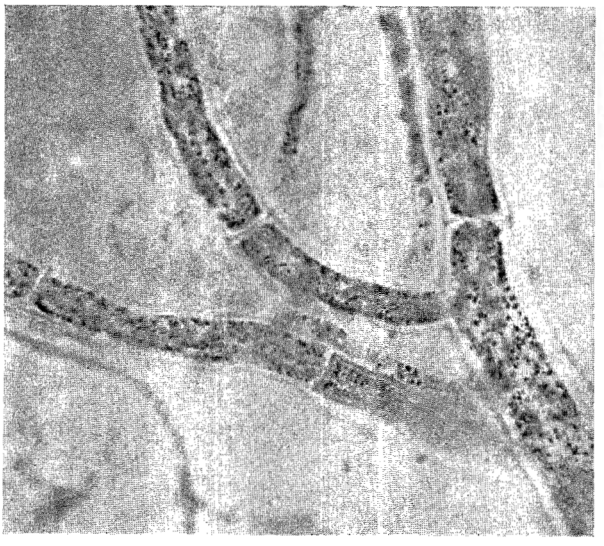

Fig. 5

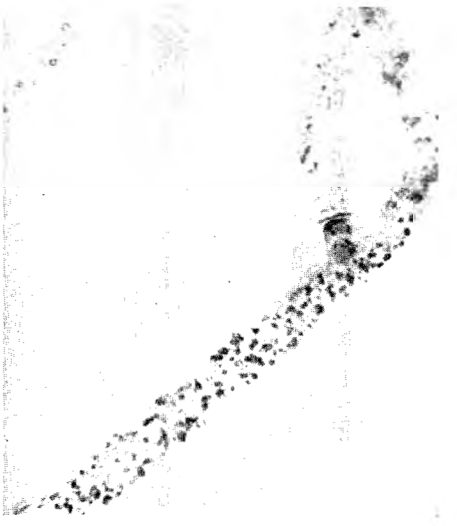

Fig. 6

PLATE 2

Fig. 3. Esterase activity made visible by the indoxyl method. × 1027.

Fig. 4. Particulate localization of esterase by the α-naphthylacetate + 'hexazotized' *p*-rosaniline method. × 1027.

Fig. 5. Esterase activity localized by the thioacetic acid procedure. × 1198.

Fig. 6. Particulate localization of DNase activity by the Vorbrodt (1961) method. × 1198.

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Serological Relationships of Corynebacteria

By I. LAZAR
'Tr. Savulescu' Institute of Biology, Bucharest, Romania

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SUMMARY

The serological relationships between 39 strains of pathogenic corynebacteria from man, animals and plants were studied by precipitin, double gel-diffusion, and immuno-electrophoretic techniques, with polysaccharides and nucleoproteins as antigens. Five distinct serological groups were established among the plant pathogenic corynebacteria: (1) *Corynebacterium flaccumfaciens*, *C. flaccumfaciens* var. *aurantiacum*, *C. poinsettiae* and *C. betae*; (2) *C. michiganense* and *C. insidiosum*. (3) *C. tritici* and *C. rathayi*; (7) *C. sepedonicum*; and (5) *C. facians*. *C. diphtheriae* (type *gravis*, *mitis*, *intermedius*, and atypical forms), *C. hoffmanii*, (*syn. C. pseudodiphtheriticum*) and the animal pathogens *C. equi*, *C. renale* and *C. kutscheri* belonged to two or three serological groups. The plant pathogen *C. fascians* appeared to occupy an intermediate position between the plant and animal pathogens, and shared many antigens in common with the plant pathogens in group 1 and the animal pathogens. Other cross-relationships were detected between the groups. An immuno-electrophoretic study was made of protein fractions from the plant pathogenic bacteria.

INTRODUCTION

Studies of the serological relationships between various species of the genus *Corynebacterium* are few; most have been concerned with a limited number of species from only a few habitats. Rosenthal & Cox (1953, 1954), in their detailed study of four plant pathogenic species and one species from soil emphasized the desirability of a serological comparison between coryneform bacteria from plant and animals. Mushin, Naylor & Lahovary (1959) studied some of the Corynebacterium species from plants, animals and man with the object of using serology as a diagnostic tool for the plant pathogens, rather than as a method for investigating their interrelationship and their relation to corynebacteria from other sources. Cummins & Harris (1956, 1958), Cummins (1962*a*, *b*) and Perkins & Cummins (1964) established relationships between several Corynebacterium species by comparing the antigens and composition of their cell walls. The work of Barber *et al.* (1963, 1965*a*, *b*, 1966) and Saragea, Barber, Meitert & Maximesco (1963) with *Corynebacterium diphtheriae* and with some corynebacteria from animals and plants indicated the importance of detailed serological studies in understanding the relations within the group of mammalian pathogens comprising the typical and atypical forms of *C. diphtheriae*, *C. hoffmanii* (syn. *C. pseudodiphtheriticum*) and *C. xerosis*, and revealed some of the serological relationships between these organisms and the animal and plant pathogenic corynebacteria.

Relationships within the corynebacteria have also been demonstrated by other

methods. Robinson (1966) investigated the esterase, catalase and peroxidase activity, and protein components of several coryneform bacteria by starch-gel electrophoresis, and established a relationship between the human, animal and plant pathogens, which enabled him to divide these organisms into groups and subdivisions. Ramamurthi (1959) made a comparative study of morphological and physiological characters of some coryneform plant pathogens and of single isolates of *Corynebacterium creatinovorus*, *C. equi* and *C. renale*. An Adansonian study of some corynebacteria and related organisms by Harrington (1966), who used a wide range of biochemical and physiological characters, showed that some of the organisms need to be reclassified. Lelliott (1966) pointed out that further morphological, physiological and serological work is needed to confirm his suggestions on the division of plant pathogenic coryneforms and their relation to other corynebacteria.

The work presented in this paper was done with a view to extending the understanding of the serological, and perhaps taxonomic, relationships between coryneform bacteria from plants, animals and man, by means of precipitin, double gel-diffusion and immuno-electrophoretic techniques.

METHODS

Strains examined. Thirty-nine strains of various Corynebacterium species were used; ten species were from plants, three from animals and three from man. These cultures were obtained from various foreign and local collections (Table 1).

Production of antisera. The technique used to obtain antisera with high antibody titres was that described by Barber *et al.* (1963, 1965*a*) and Saragea *et al.* (1963), with slight modifications. Briefly this technique consists of the hyperimmunization of rabbits with cultures in Freund incomplete adjuvant: lanolin, 20 ml.; paraffin oil, 40 ml.; bacterial suspension at $1{\cdot}6$–$2{\cdot}0 \times 10^9$ bacteria/ml., 40 ml. For plant pathogenic corynebacteria, living suspensions from 48 hr cultures on agar slopes (corn-steep, 1·5% (w/v); peptone, 0·5%; glucose, 1%; NaCl, 0·5%; $CaCo_3$, 0·05%; distilled water, 100 ml.) were used, and for animal and human corynebacteria, suspensions of 24-hr cultures on meat broth agar slopes were used. Four ml. of antigen suspension were injected into rabbits (2·8–3·0 kg.) subcutaneously into the nape region three times at 21-day intervals. Twenty-one days after the third inoculation of antigen with Freund adjuvant, five or six intravenous inoculations were given at 6 to 7-day intervals with nucleoprotein extracts, in increasing doses from 1 to 5 mg. When the test bleeding did not give a satisfactory precipitin reaction in tubes with homologous polysaccharide, immunization was continued for another two or three intravenous injections.

Production of antigens. Nucleoprotein and polysaccharide antigens were used. They were obtained by extraction of acetone-dried bacteria by the technique described by Barber *et al.* (1963, 1965*a*) and Saragea *et al.* (1963). Cultures were grown on the media described above in *Production of antisera.* To obtain nucleoprotein extracts, 5 g. of dry bacteria were suspended in 80 ml. 2% (w/v) sodium deoxycholate solution for 24 hr at 37°. After centrifugation the supernatant fluid was dialysed for 4–5 days against running tap water, followed by centrifugation of the dialysis residue and dialysis of the supernatant fluid for 2–3 days against distilled water at refrigerator temperature, further centrifugation and finally sterilization by filtration. For polysaccharide extracts, the sediment resulting after treatment with sodium deoxycholate

solution was resuspended in 50–60 ml. of 0·3 M-HCl and kept for 45 min. at 90° in a water bath with continuous stirring. After centrifugation or filtration, the supernatant fluid or filtrate was treated with trichloracetic acid (final concentration 10%. w/v). The resultant precipitate was removed by filtration and the filtrate dialysed for 3 days in running tap water and for 2 days in distilled water at refrigerator temperature.

Gel diffusion and immuno-electrophoresis. Double gel-diffusion was done by the Ouchterlony technique (1949, 1958) and immuno-electrophoresis as shown in detail in another paper (Lazar, 1967), generally by using the techniques described for

Table 1. *Corynebacterium species studied*

Species and culture no.	Source	Obtained from:
C. diphtheriae type *gravis* 4895, 5429	Man	'Dr I. Cantacuzino', Institute, Bucarest
C. diphtheriae type *mitis* 411		
C. diphtheriae P.W.8.		
C. diphtheriae type *intermedius* 1180, 3522, 3536, 4109, 4465		
C. diphtheriae, atypical form 4409 4528, 4852		
C. hoffmanii 4855		
C. equi 4031	Animals	Faculty of Vet. Med., Bucarest
C. renale 2276		
C. kutscheri 239		
C. betae 373, 375	Plants	NCPPB*
C. flaccumfaciens 559, 567		NCPPB
C. flaccumfaciens 706		Ottawa, Canada
C. f. var. *aurantiacum* 558		NCPPB
C. poinsettiae 845, 848		NCPPB
C. michiganense 1		Our own isolation
C. michiganense 515		NCPPB
C. michiganense 201		Leningrad, U.S.S.R.
C. michiganense 704		Quebec, Canada
C. insidiosum 83		NCPPB
C. insidiosum 702		Quebec, Canada
C. tritici 255, 471		NCPPB
C. rathayi 797		NCPPB
C. sepedonicum 299		NCPPB
C. sepedonicum 708		Ottawa, Canada
C. fascians 156, 469		NCPPB
C. fascians 716		Ottawa, Canada

* National Collection of Plant Pathogenic Bacteria, Harpenden, Herts, England.

proteins from human serum (Grabar & Williams, 1953; Grabar & Burtin, 1960; Heremans, 1961; Bratu, 1962). The gel was prepared by dissolving 1·2% of Noble agar (Difco) in veronal buffer (veronal, 2·0766 g.; medinal, 13·4170 g.; sodium acetate, 1·2952 g.; potassium oxalate, 0·8346 g.; distilled water 2 l.; pH 8·6). The best results were obtained when 13 × 18 cm. glass plates were covered with 40–45 ml. gel. For nucleoprotein migration a series, each of two plates, was introduced into the electrophoresis bath (which contained veronal buffer) at a working potential of about 250–300 V, adjusted to an estimated 1·5 mA/1 cm. of plate. The experiment was carried out in an electrophoresis apparatus of the QE-201 type, manufactured in Hungary. Plates that showed a suitable immuno-electrophoretic reaction were stained with 'Amidoschwarz' solution (90% (v/v) methanol, 10% glacial acetic acid, 0·3%

'Amidoschwarz' prepared in a water bath for 4–6 hr. After staining, the plates were decolourized with a 2 % acetic acid solution, for one or several days, until the background of the plate was clean. The fractions represented by precipitation arcs along the migration axis were identified by comparison with those reported for fractions in human serum by Grabar & Williams (1953), Heremans (1961), Schmid (1961) and, Haggis *et al.* (1964).

RESULTS

The results of this study are presented in Tables 2–5 and partially illustrated in Figs. 1 and 2.

Tube precipitin reactions

Tube precipitin reactions (Table 2) indicated that only *Corynebacterium fascians*, among the plant pathogens, shared more than a limited amount of nucleoprotein antigen with *C. diphtheriae*. Lesser amounts were shared with *C. diphtheriae* by *C. poinsettiae*, *C. flaccumfaciens*, *C. betae*, *C. tritici* and *C. equi*, and little or none by *C. insidiosum*, *C. michiganense*, *C. sepedonicum*, *C. rathayi*, *C. renale* and *C. kutscheri*. The species from animals shared common antigens with *C. diphtheriae*, flagellate plant pathogenic corynebacteria and *C. fascians*.

Reactions within the plant pathogenic species suggested five serological groups: (1) *C. poinsettiae*, *C. flaccumfaciens*, *C. betae*; (2) *C. insidiosum*, *C. michiganense*; (3) *C. tritici*, *C. rathayi*; (4) *C. sepedonicum*; (5) *C. fascians*. Small amounts of common antigens were found between some species of these groups (Table 2).

Gel diffusion with nucleoprotein extracts

The results of the precipitation reaction by double gel-diffusion are shown in Table 3. The reactions between the antisera of the different types of *Corynebacterium diphtheriae* and the antigens of the phytopathogenic species indicated some relationship, expressed by weak or very weak precipitation lines, between *C. diphtheriae* and the following plant pathogenic species: *C. fascians*, *C. poinsettiae*, *C. flaccumfaciens*, *C. flaccumfaciens* var. *aurantiacum*, *C. insidiosum*, *C. michiganense*, *C. tritici*, *C. rathayi* and *C. sepedonicum*.

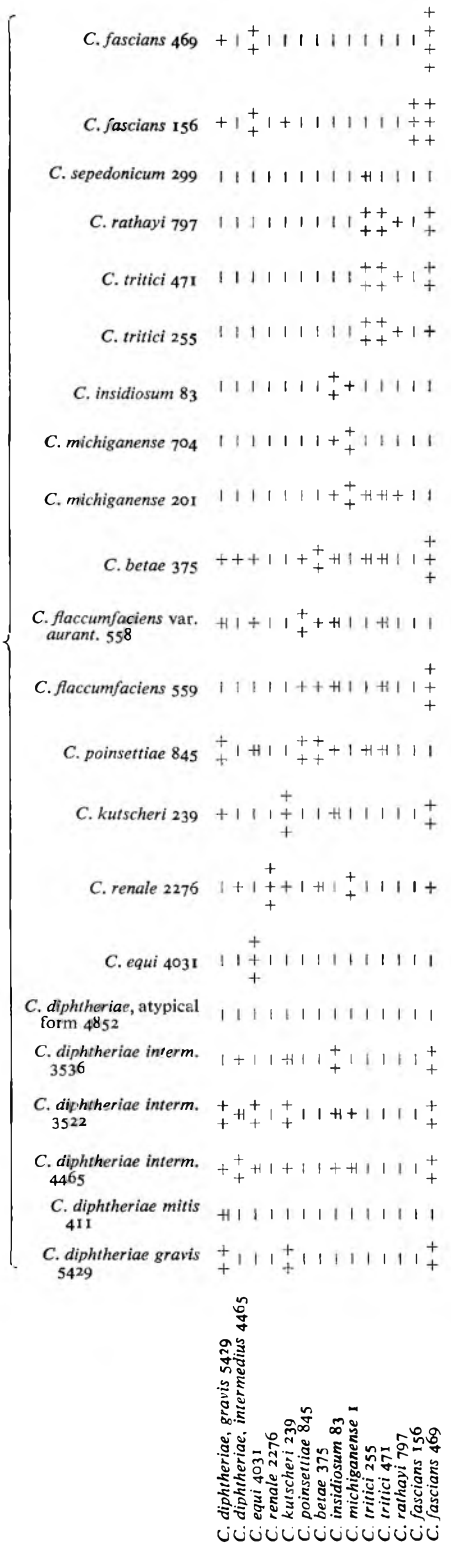
The antigens of species from animals did not react with the available diphtheriae antisera. However, positive reactions were obtained between the antigens (particularly the nucleoprotein antigens) of *Corynebacterium diphtheriae* and the antisera of the animal pathogens *C. renale* and *C. equi*.

Antigens of species from plants reacted positively with the antisera of species from animals, the more intense reactions being: *Corynebacterium betae*, *C. flaccumfaciens*, *C. poinsettiae*, *C. fascians* and *C. michiganense* with antiserum of *C. equi*; *C. insidiosum* and to a lesser degree *C. poinsettiae* and *C. flaccumfaciens* var. *aurantiacum* with antiserum of *C. kutscheri*; *C. flaccumfaciens* var. *aurantiacum*, *C. flaccumfaciens*, *C. poinsettiae* and *C. fascians* with the antiserum of *C. renale*.

The antigenic structural relationship between species from man and from plants was clearer when diphtheria antigens were tested with antisera for plant pathogens than when diphtheria antisera were used against antigens from plant pathogens. This was because in some cases higher-titre antisera were obtained for plant pathogenic species than for species from man. *Corynebacterium fascians* appeared to be more closely related serologically to the three types of *C. diphtheriae* than were the other

Table 4. *Reactions obtained by double gel diffusion with polysaccharide extracts*

The method used is the same as that described at the head of Table 3.



plant pathogens. A positive reaction, though only with the *mitis* type of *C. diphtheriae*, was obtained with the antisera of the various strains of *C. michiganense*, *C. insidiosum* and *C. betae*. The nucleoprotein antigens of various strains belonging to atypical forms of *C. diphtheriae* also gave one or two precipitation lines with the antisera of *C. flaccumfaciens* var. *aurantiacum* and *C. poinsettiae*, and one single and weak precipitation line with the antiserum of *C. michiganense* 704; a very weak or doubtful reaction was obtained with *C. michiganense* 1 and 515.

Some antisera of the plant pathogenic species formed a clear precipitation line with some antigens of different species from animals: *Corynebacterium insidiosum* with *C. equi* and *C. kutscheri*, *C. fascians* 469 with *C. kutscheri*; *C. sepedonicum* 299, and less clearly *C. michiganense* 704, with *C. renale*.

The same five groups within the plant pathogenic corynebacteria were distinguished by gel diffusion as were demonstrable by tube precipitin reactions (see above).

Gel diffusion with polysaccharide extracts

The results of double gel diffusion with polysaccharide antigens are given in Table 4. The weaker relationships between groups were less clear than with nucleoprotein extracts. Thus the antisera of *Corynebacterium flaccumfaciens* 599, *C. flaccumfaciens* var. *aurantiacum* 558, *C. poinsettiae* 848, *C. sepedonicum* 299, *C. michiganense* 705, *C. diphtheriae* type *mitis* 411, as well as a few others, gave no satisfactory reactions with homologous antigens, probably because these strains produced little polysaccharide or because what polysaccharide they did produce was serologically inactive. Generally the relationships demonstrated were the same as those with nucleoprotein extracts. Antisera of *C. diphtheriae* gave usually weak reactions with the antigens of one or more strains of *C. renale*, *C. kutscheri*, *C. poinsettiae*, *C. flaccumfaciens* var. *aurantiacum*, *C. betae* and *C. fascians*. Some of the antisera against species from animals reacted with antigens of one or more strains of *C. diphtheriae* type *intermedius*, *C. fascians*, *C. betae*, and *C. flaccumfaciens* var. *aurantiacum*. The antisera of plant pathogens reacted with some of the antigen preparations of *C. diphtheriae* type *intermedius*, *C. diphtheriae* type *gravis*, *C. renale* and *C. kutscheri*. The reactions between the polysaccharide antigens and the antisera of plant pathogenic species reinforced the serological groups of species determined by the reaction with nucleoprotein antigens (see above).

The interrelationships schematized in Fig. 2 show that plant pathogenic corynebacteria of groups 1, 2 and 5 share more polysaccharide antigens with the human and animal corynebacteria than do groups 3 and 4. *Corynebacterium fascians* (strain 469) is very rich in polysaccharide antibodies, as well as in nucleoprotein antibodies (Tables 3, 4) and shows a particularly clear relationship with the corynebacteria from man and animals and also with the other groups of plant pathogenic corynebacteria.

Immuno-electrophoresis

The results obtained by double gel diffusion were confirmed by immuno-electrophoresis. It was also possible by using immuno-electrophoretic techniques to identify and make a more exact estimation of the number of antigenic fractions.

Antisera with high antibody titres, and the application for migration of about 0·2 ml. of not less than 1·5–2·0 mg. nucleoprotein antigen/ml., were necessary to

Table 5. *Immuno-electrophoretic analysis of nucleoprotein antigens from Corynebacteria*

Fractions were separated in a QE-201 electrophoretic bath at 250–300 V. working potential and 1·5 mA. per cm. using a gel of veronal buffer, pH 8–6, and 1·2 % agar.

Nucleoprotein antigens from:

Antisera for	*C. betae* 373					*C. poinsettiae* 845					*C. poinsettiae* 848					*C. flaccumfaciens* 567					*C. flaccumfaciens* v. *aurantiacum* 558					*C. michiganense* 1					*C. insidiosum* 83					*C. tritici* 255				
	O	A	α	β	γ	O	A	α	β	γ	O	A	α	β	γ	O	A	α	β	γ	O	A	α	β	γ	O	A	α	β	γ	O	A	α	β	γ	O	A	α	β	γ
C. betae 373	−	+	+	+	−	±	+	+	−	−	−	+	+	−	−	+	+	+	−	−	±	+	+	−	−	−	−	−	−	−	−	±	−	−	−	−	−	−	−	−
C. betae 375	−	+	+	+	−	+	+	+	−	−	−	+	+	−	−	+	+	+	−	−	+	+	+	−	−	−	−	−	−	−	−	±	−	−	−	−	−	−	−	−
C. poinsettiae 845	−	−	+	±	−	+	+	+	±	−	+	+	+	+	−	±	+	+	−	−	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
C. poinsettiae 848	−	−	+	±	−	−	+	+	±	−	−	+	+	±	−	+	+	+	±	−	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
C. flaccumfaciens 567	−	+	+	−	−	−	+	+	−	−	−	+	+	−	−	+	+	+	−	−	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
C. f. var. *aurantiacum* 558	−	+	+	+	−	+	+	+	−	−	+	+	+	−	−	±	+	+	−	−	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
C. michiganense 1	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−	±	±	−	−	−	−	−	−	−
C. tritici 255	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−
C. fascians 469	−	−	+	−	−	−	+	+	−	−	−	+	+	−	−	−	−	±	−	−	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
C. renale 2276	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

Nucleoprotein antigens from:

	C. rathayi 797					*C. sepedonicum* 299					*C. fascians* 156					*C. fascians* 469					*C. diphtheriae gravis* 5429					*C. diphtheriae mitis* 411					*C. diphtheriae*, atypical form 4852					*C. renale* 2276				
	O	A	α	β	γ	O	A	α	β	γ	O	A	α	β	γ	O	A	α	β	γ	O	A	α	β	γ	O	A	α	β	γ	O	A	α	β	γ	O	A	α	β	γ
C. betae 373	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
C. betae 375	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
C. poinsettiae 845	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	±	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
C. poinsettiae 848	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	±	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
C. flaccumfaciens 567	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
C. f. var. *aurantiacum* 558	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	±	+	−	−	−	−	−	−
C. michiganense 1	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
C. tritici 255	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
C. fascians 469	−	−	−	−	−	−	−	−	−	−	−	+	±	+	−	−	−	±	+	−	−	+	+	−	−	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−
C. renale 2276	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	±	±	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−	−

O = Orosomucoids; A = albumens; α, β, γ = fractions whose position along the migration axis is similar to that of α, β and γ fraction in human serum. + = positive reaction; ± very weak or doubtful reaction; − no reaction.

detect and identify some of the antigens detectable by gel diffusion. These conditions were not realized with some of the nucleoprotein extracts, including those of *Corynebacterium sepedonicum* 299, *C. flaccumfaciens* 559, *C. insidiosum* 83, *C. rathayi* 797 *C. kutscheri*, *C. diphtheriae* (some types), etc., and some antisera, including those of *C. insidiosum*, *C. sepedonicum*, *C. fascians* (some strains), *C. kutscheri*, *C. equi*. For this reason some of the antigens and antisera tested are not included in Table 5.

The electrophoretic migration of the nucleoproteins was towards the positive pole and their deposition was in zones comparable to those of the orosomucoids, albumins, and group α and β fractions from human serum. In runs in which the nucleoprotein extracts of the different Corynebacterium species were compared with human serum, fractions with the same mobility as the α group and the albumins were detected most frequently; fractions comparable with the orosomucoids, and with group β less frequently. No fractions comparable to group γ were detected.

The species richest (5–8) in fractions identifiable with the proteins from human serum were: *Corynebacterium flaccumfaciens* var. *aurantiacum* 558, *C. poinsettiae* 845, 848, *C. flaccumfaciens* 567, 706, *C. betae* 373, 375, *C. fascians* 469, 156, and *C. michiganense* 1. A few comparable fractions (2–3) were identified in: *C. tritici* 255, *C. rathayi* 797, *C. fascians* 716, *C. diphtheriae* 5429, 411, 4465, 4528, *C. renale* 2276 and *C. kutscheri* 239. It is possible that, using more concentrated nucleoprotein antigens and antisera richer in antibodies, these strains and those of others including *C. sepedonicum* *C. insidiosum* and *C. equi*, could be shown to have more protein fractions in the nucleoprotein antigens extractable from bacterial cells with sodium deoxycholate.

In addition to confirming the groups obtained by tube precipitin and gel-diffusion techniques, immuno-electrophoresis confirmed that group I was very rich in antigenic fractions and group 3 poor. It also indicated closer relationship between *Corynebacterium flaccumfaciens* var. *aurantiacum* and *C. poinsettiae* than between *C. flaccumfaciens* var. *aurantiacum* and *C. flaccumfaciens*. *C. flaccumfaciens* var. *aurantiacum* and *C. poinsettiae* have fractions identical in number and in their electrophoretic position.

Plant pathogenic corynebacteria from groups 1, 5 and 3 appear to be related to human and animal coryneforms in albumens and fractions from group α and possibly in fractions from group β.

DISCUSSION

By means of the classical methods and criteria used for differentiating diphtheroids, Lazar (1968) has shown that most of the plant pathogenic corynebacteria resemble the pseudodiphtheria organisms from man and animals in many of their characters. *Corynebacterium fascians* presented a striking resemblance to *C. diphtheriae* and particularly to its atypical forms; this is not in agreement with the suggestion that *C. fascians* should be classified as a member of the genus *Nocardia* (Ramamurthi, 1959) or that it is a boundary form between the genera *Corynebacterium*, *Mycobacterium* and *Nocardia* (Lacey, 1955).

The present serological study of a wide range of coryneform bacteria by precipitation-double gel-diffusion, and immuno-electrophoresis techniques has confirmed and amplified previous results obtained with agglutinins, cell extracts and cell-wall, composition (Mushin *et al.* 1959; Cummins, 1962*a*, *b*; Cummins & Harris, 1956, 1958; Perkins & Cummins, 1964; Barber *et al.* 1965*b*, 1966), and has indicated the

broad relationships demonstrated in Figs. 1 and 2. It appears that plant pathogenic coryneforms can be placed in five distinct serological groups: (1) *Corynebacterium flaccumfaciens*, *C. flaccumfaciens* var. *aurantiacum*, *C. poinsettiae*, *C. betae*; (2) *C. michiganense* and *C. insidiosum*; (3) *C. tritici* and *C. rathayi*; (4) *C. sepedonicum*; (5) *C. fascians.*

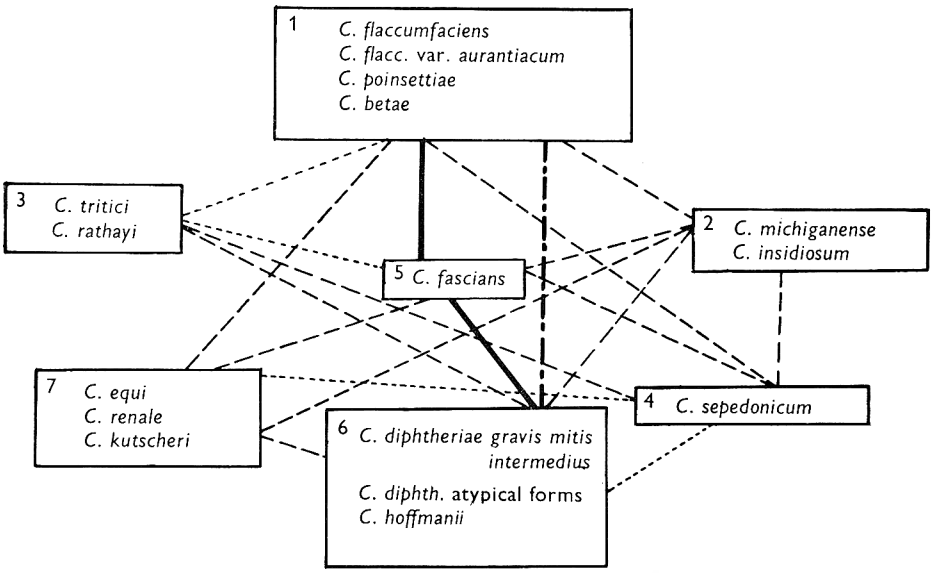


Fig. 1. Serological relationships as determined by double gel diffusion and immunoelectrophoresis techniques, using nucleoprotein extracts as antigen. ——, strong reaction; —·—, reaction; – – –, weak reaction; ....., very weak or doubtful reaction.

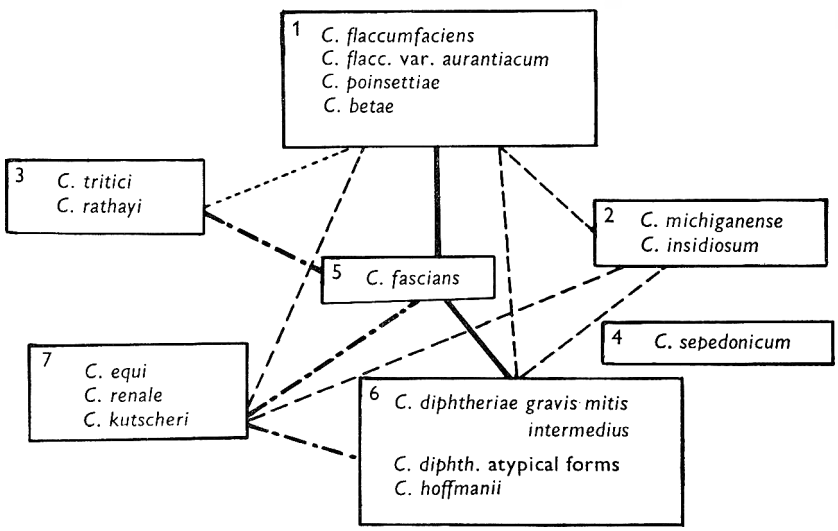


Fig. 2. Serological relationships as determined by double gel diffusion method, using polysaccharide extracts as antigen. ——, Strong reaction; —·—, reaction; – – –, weak reaction; ....., very weak or doubtful reaction.

The results have confirmed the work of Rosenthal & Cox (1953, 1954) who, by cross-absorption, showed serological identity between the *Corynebacterium michiganense* and the *C. insidiosum* AB serotypes, and the very close serological relationship of *C. poinsettiae* with *C. flaccumfaciens*. The results also confirm the finding by Katznelson & Sutton (1956) that *C. sepedonicum* shares one or more antigens with *C. michiganense* and *C. flaccumfacens*, but also show that *C. sepedonicum* shares antigens with *C. poinsettiae*, *C. fascians*, *C. betae* and *C. rathayi*. The results also confirm the division of the plant pathogenic coryneform bacteria suggested by Lelliott (1966), but show that his first group contains three serological groups, and his second group two serological groups.

Corynebacterium species from plants, animals and man are antigenically related (Figs. 1, 2). *Corynebacterium fascians* and the species in the *C. flaccumfaciens* group (group 1) are the plant pathogens most closely related to corynebacteria from man and animals. *Corynebacterium fascians* appears to be most closely related to *C. diphtheriae*, and species in group 1 to the atypical forms of *C. diphtheriae*. *Corynebacterium fascians* appears to occupy a central position, showing very close affinity with the human and animal corynebacteria, and also with the flagellated group 1. Other approaches to the problem have also indicated this intermediate or central position of *C. fascians*. Robinson (1966) showed that *C. fascians* produced a pattern of enzymes similar to that produced by some plant, some human and some animal pathogenic corynebacteria. Its peroxidase pattern was similar to that of the plant pathogens but its esterase and catalase pattern was like that of the animal pathogens. Robinson also found that *C. tritici* had an enzyme pattern which showed very little relationship with the other plant pathogens; a similar lack of relationship in nucleoprotein antigens between *C. tritici* and *C. rathayi* and the other plant pathogenic corynebacteria is shown in Fig. 1. An Adansonian taxonomic study (Harrington, 1966) has also indicated that *C. fascians* appears to be the plant pathogen most closely related to the animal strains.

Barber *et al.* (1963, 1965*a*) and Saragea *et al.* (1963) showed that *Corynebacterium diphtheriae* and the other Corynebacterium species from man are related to each other only through their nucleoprotein antigens, and that their polysaccharide antigens are specific. They also showed a serological specificity for the polysaccharides isolated from different plant pathogenic corynebacteria (Barber *et al.* 1966). The results reported here, however, show that the polysaccharide antigens of several Corynebacterium species from plants *C. fascians* 469, 156; *C. betae* 375; *C. poinsettiae* 845; *C. flaccumfaciens* var. *aurantiacum* 558) react with *C. diphtheriae* antisera and, even more clearly, that the polysaccharides of *C. diphtheriae* react with the antisera of *C. fascians* 469, *C. insidiosum* 83, *C. kutscheri* 239 and *C. equi* 4031. Other examples of common polysaccharide antigens are given in Table 4.

The serological relationship found between certain species from plants and *Corynebacterium diphtheriae* (including its atypical forms) seems to be at least as close as that ascertained by Barber *et al.* (1963, 1965*a*) and Saragea *et al.* (1963) between *C diphtheriae* on the one hand, and the atypical forms and *C. hoffmanii* and *C. xerosis* on the other. It appears justifiable to consider that, if the place of pseudodiphtheroids and atypical forms within the genus *Corynebacterium* cannot be doubted, then neither can that of the diphtheroids from plants among which *C. fascians* is included. As Gorlenko (1964) suggested, *C. fascians* may be an intermediary form in this group of

bacteria. To understand more clearly the interrelationships of corynebacteria, it will be necessary to study the relationship of members of the genus *Corynebacterium*, particularly *C. fascians* and *C. renale*, with representative species of the genera *Arthrobacter* and *Nocardia*.

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The Involvement of Cellulase and Laminaranase in the Formation of Pythium Protoplasts

By D. E. EVELEIGH, J. H. SIETSMA AND R. H. HASKINS
National Research Council of Canada, Prairie Regional Laboratory, Saskatoon, Saskatchewan, Canada

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SUMMARY

Five inducible streptomycete lytic enzyme complexes were equally capable of producing protoplasts from a *Pythium* sp. strain PRL 2142, when compared on a quantitative basis. Both cellulase and laminaranase from various microbial sources were required to produce the protoplasts under standard conditions. Protease was not required; lipase shortened the survival time of protoplasts. It was concluded from an analysis of the products of enzymic hydrolyses that *Pythium* sp. PRL 2142 cell wall contained cellulose, β-D-(1→3) glucan, and a β-D-(1→3) glucan possessing D-glucose units attached by β-(1→6)-linkages.

INTRODUCTION

Glycosidases, lipases and proteases have all been shown to be involved in the formation of fungal 'protoplasts'. However, little quantitative data is available in published form about these enzymes, especially with regard to the degradation of cell wall of oomycetous genera (Villanueva, 1966). The cell walls of these genera are known to possess both cellulose and β-D-(1→3) glucans and associated gentibiose components (Aronson, Cooper & Fuller, 1967; Bartnicki-Garcia & Lippmann, 1967; Wang & Bartnicki-Garcia, 1966). Streptomycete enzyme systems capable of producing 'protoplasts' from *Pythium* sp. PRL 2142 have been described by Sietsma, Eveleigh, Haskins & Spencer, (1967). A qualitative and quantitative analysis of these lytic enzyme systems is now presented; attention is focused on the cellulase, laminaranase, lipase and protease components. Several components of the cell wall are now identified by enzymic techniques.

METHODS

The preparation of the streptomycete lytic enzyme complexes used in the production of Pythium 'protoplasts' has been described by Sietsma *et al.* (1967). Other enzymes, used comparatively, included a *Streptomyces* sp. B 814 cellulase (E.C. 3.2.4.4, from E. T. Reese: Reese & Mandels, 1963), *Aspergillus niger* cellulase, subtilisin (E.C. 3.4.4.16; from Nutritional Biochemical Corporation, Cleveland, Ohio), *Rhizopus arrhizus* laminaranase (E.C. 3.2.1.6; Reese & Mandel, 1963), bacterial laminaranase (given by Glaxo Laboratories, England: see Manners & Patterson, 1966), trypsin (E.C. 3.4.4.4; from Armour Laboratories, Chicago, Illinois), wheat germ lipase (E.C. 3.1.1.3; from Pentex, Kankakee, Illinois), purified pcrk pancreatic lipase (given by C. G. Youngs, P.R.L., N.R.C., Saskatoon).

Substrates used. These were as follows: laminaran (β-D-(1→3)-glucan) and nigeran (mixed α-D-(1→3), α-D-(1→4)-glucan) (Koch-Light Labs., Colnbrook, England); carboxymethylcellulose (CMC-cellulose—courtesy Hercules Trading Corp., Wilminton, Delaware, U.S.A.); pustulan (β-D-(1→6)-glucan; from *Umbilicaria pustulata* —given by E. T. Reese). Purified chitan (β-(1 → 4) linked 2 acetamido-2-deoxy-D-glucan-crustacean shell) and crown gall (β-(1 → 2)-D-glucan from *Agrobacterium tumefaciens* IIBV7) were available at this laboratory. Pythium cell-wall preparations were made by boiling the mycelium (7-day culture) in water for 10 min. to inactivate autolytic enzymes, and then homogenizing it in a Waring blendor followed by ultrasonic treatment. The wall preparations were washed three times with water. Although only 50% breakage of the mycelium was achieved, its coenocytic nature allowed complete loss of cytoplasm as judged by random microscopic examination.

The glycosidases of the lytic enzyme preparations were investigated by reacting enzyme solution (0·1) with a range of polysaccharides (3 mg./ml.; 0·1 ml.) for 16 hr at room temperature. Thiomersalate 0·005% was used as a bacterial inhibitor. The reactions were terminated by heating at 100° for 10 min. The degree of hydrolysis and the lytic products were characterized by paper chromatography (solvent c—see below). The enzyme preparations were quantitatively tested for β-D-(1 → 3)-glucanase (laminaranase) and cellulase (Cx) by using laminaran and carboxymethylcellulose as substrates respectively. The assays were done with 0·5 ml. substrate (5 mg./ml.) + enzyme and 0·05 M-sodium acetate buffer (pH 5·8) to 1·0 ml. at 30°, for 15 (or occasionally 30) min. The reactions were allowed to run to a maximum of 5% substrate degradation in order to maintain a stoichometric response between the product and the amount of enzyme added (Eveleigh, 1967; Miller, Blum, Glennon & Burton, 1960). An enzyme unit is defined as that amount of enzyme which released 1 μmole glucose equivalent/min. at 30°. Reducing end-groups (as glucose equivalents) were estimated by the Nelson-Somogyi method (Neish, 1952). Initially cellulase assays were made on CMC-cellulose 0·7 degree of substitution (DS); later assays were made on the 0·4 DS CMC-cellulose which is four times more reactive than the 0·7 DS substrate. The data for the cellulase assay were presented on the basis of this latter substrate. Lipase was determined titrimetrically (Marchis-Mouren, Sarda & Desnuelle, 1959). Olive oil was used as a standard and one lipase unit defined as that amount of enzyme which released 10 μ-equiv. acid/min. under standard conditions (Marchis-Mouren *et al.* 1959). Proteolytic activity was determined by using the protein-dye complex azocoll, as substrate (*Calbiochem. tech. Bull.* 1966); a unit is defined as 0·001 extinction change/min. at 37°, pH 7·5. Proteins were determined by the Folin phenol method (Lowry, Rosenbrough, Farr & Randall, 1951) with bovine serum albumin as a standard.

Paper chromatographic analysis was done on Whatman filter paper no. 1 with the following solvent systems: (*a*) *n*-butanol + ethanol + water (3 + 1 + 1 by vol.); (*b*) ethyl acetate + pyridine + water (10 + 4 + 3 by vol.); (*c*) ethyl acetate + acetic acid (glacial) + water (9 + 2 + 2 by vol.); (*d*) ethyl acetate + formic acid + acetic acid + water (18 + 1 + 3 + 4 by vol.). Reducing compounds were detected with *p*-anisidine. Partial acid hydrolysis of the cell walls was done in 0·4 N-H_2SO_4 at 100° for periods of 20 min., 1 hr and 3 hr. A more complete hydrolysis was performed in N-H_2SO_4 at 100° for 20 hr. Hydrolysates were neutralized with $BaCO_3$, filtered and concentrated *in vacuo* at room temperature.

RESULTS

The initial survey of enzymes present in the crude lytic streptomycete system was done by chromatographic analysis of enzymic hydrolysates and results are presented in Table 1. Endo-β-D-(1 $\rightarrow$ 3)-glucanase was shown to be present in each system by the production of β-(1 $\rightarrow$ 3)-D-linked-oligosaccharide series as degradation products from laminaran. Exo-laminaranase was also shown to be present in three systems (Table 1) by their degradation of a *Claviceps* sp. PRL 1980 glucan (Perlin & Taber, 1963) to yield glucose and gentiobiose. The presence of both endo- and exo-laminaranases in the *Streptomyces* sp. 0143 complex was also demonstrated by an n.m.r. analysis of the glucose and oligosaccharide anomers released initially during enzymic degradation of laminaran (i.e. α-glucose and β-laminaribiose + β-laminaritriose—unpublished observations). Cellulase and β-glucosidase were present in each system, though lower levels of the latter enzyme were present in the *S. albidoflavus* system. The activity of two systems towards pustulan is noteworthy. The α-glucosidases present in these systems are probably constitutive, as starch has not been reported from Pythium mycelium, and only glucose and mycelium were used for the induction of the lytic enzymes.

Table 1. *Glycosidase activity of the streptomycete lytic complexes*

Substrate	*Streptomyces* sp. 0143	*S. satsumaensis* 1399	*Streptomyces* sp. 1294	*S. globisporus* B 2872	*S. albidoflavus* A 14
Cellobiose	+	++	++	++	+
Laminaran	++	++	++	++	++
Cellulose (CMC)	++	++	++	+	+
Lichenan	++	++	++	++	+
Claviceps sp. PRL 1980 glucan	+	++	+	nt	nt
Pustulan	o	+	o	+	o
Crown gall	o	o	+	o	o
Chitan	o	o	o	o	o
Maltose	++	+	o	+	o
Starch	++	++	+	o	o
Nigeran	o	o	o	o	o

++, Strong positive reaction; +, positive reaction; o, negative reaction; nt, not tested.

This initial survey was followed by a more detailed analysis of the laminaranase and cellulase activities of the preparations. Lipase and protease were also determined because Acha, Aguirre, Lopez-Belmonte, Villanueva (1966) and Tabata & Terui (1963), respectively, reported their involvement in 'protoplast' production. The results are presented in Table 2.

It was previously reported (Sietsma *et al.* 1967) that *Streptomyces* sp. 0143 and *S. globisporus* lytic enzymes were capable of producing 'protoplasts' from Pythium species, while those from *S. satsumaensis* caused extensive lysis only. A strong correlation is seen between those results and the results presented in Table 2, i.e. systems producing the greatest lysis possessed generally greater amounts of laminaranase and cellulase. The *S. satsumaensis* system also had a major lipase component. The failure to form protoplasts by this system is probably due to the action of this lipase component, as the addition of wheat germ lipase to the other positive systems causes lysis of proto-

Table 2. *Major enzymes present in the streptomycete lytic complexes*

	Protein (mg./ml.)	β(1,3)-Glucanase		β(1,4)-Glucanase		Lipase		Protease	
		Units/ml.	Specific activity	Units/ml.	Specific activity	Units/ml.	Specific activity	Units/ml.	Specific activity
Streptomyces sp. 0143	1·68	1·245	0·740	0·492	0·293	0·0155	0·0092	12·1	7·2
S. satsumaensis 1399	2·42	0·363	0·150	0·532	0·220	0·0288	0·0119	115	47·5
Streptomyces sp. 1294	3·88	0·486	0·125	0·620	0·160	0·0119	0·0031*	0	0
S. globisporus B-2872	2·53	0·335	0·132	+	–	–	–	–	–
S. albidoflavus A-14	4·12	0·963	0·234	0·052	0·012	0·0875	0·0212	206	50·0
Cellulase (Cx) (*Streptomyces* sp. B-814)	0·11	0·00026†	0·0024	0·14	1·27	0·0242	0·2200	0	0
Cellulase (Cx) (*Aspergillus niger*)	0·56	0·03	0·053	0·46	0·82	0·0256	0·0457	0	p
Laminaranase (bacterial)	0·41	0·043	0·105	0·036	0·098	0·0342	0·0845	15·9	38·8
Laminaranase (*Rhizopus arrhizus*)	0·065	0·040	0·62	0·002	0·003	0·0057	0·0867	0	0
Lipase (pork pancreatic)	1·30	–	–	–	–	0·4000	0·3077	–	–
Lipase (wheat germ), pH 9·0	0·84	–	–	–	–	0·0266	0·0269	–	–
Lipase (wheat germ), pH 7·4	0·84	–	–	–	–	0·0051	0·0061	–	–
Lipase (wheat germ), pH 6·2	0·84	–	–	–	–	0	0	–	–
Trypsin	0·447	–	–	–	–	–	–	151	338
Subtilisin	1·366	–	–	–	–	–	–	1620	1186

*3 hr assay. †2·5 hr assay. –, not tested; +, not tested quantitatively.

plasts (Sietsma *et al.* 1967). Two of the systems (*S. satsumaensis*, *S. albidoflavus*) possessed marked protease activity. These proteases were assumed to possess limited specificity from a comparison of their reaction kinetics with those of trypsin and subtilisin and other proteases (*Calbiochem. tech. Bull.* 1966).

The streptomycete lytic enzymes were compared in their ability to produce protoplasts on a quantitative basis with regard to laminaranase activity. The induction of protoplast formation was done as previously described (Sietsma *et al.* 1967) using 50 mg. mycelium (equiv. 5 mg. dry wt). The lytic enzymes were added at an equivalent of 0·621 unit laminaranase, i.e. a concentration at which the *Streptomyces* sp. 0143 preparation (0·5 ml.) showed good protoplast formation. Three streptomycete preparations produced protoplasts after 24 hr incubation at 30°, while that from *S. satsumaensis* caused extensive lysis only (Table 3). The *S. albidoflavus* preparation also produced protoplasts, but after continued incubation (36 hr). The slower action of this

Table 3. *Comparative amounts of laminaranase and cellulase used for protoplast formation*

	Laminaranase (unit)	Cellulase (unit)	Protoplast produced after
Streptomyces sp. 0143	0·621	0·246	24 hr
S. satsumaensis	0·621	0·910	Complete lysis in 24 hr
Streptomyces sp. 1294	0·621	0·784	24 hr
S. globisporus	0·621	NT	24 hr
S. albidoflavus	0·621	0·032	36 hr

latter preparation was thought to be due to a suboptimal concentration of Cx cellulase. Further trials with various amounts of enzyme (maximum 0·096 Cx units), which included successive dosing with the lytic preparation every 12 hr. did not shorten the incubation period of 36 hr. Thus the original preparation apparently did not lack sufficient Cl cellulase and 0·1 unit Cx cellulase is probably a suboptimal amount of enzyme.

The preparation of Pythium protoplasts was further investigated by using microbial laminaranases and cellulases from various sources. The activities of these preparations are reported in Table 2. Equivalent amounts of the two laminaranases (0·75 unit) and of the two cellulases (0·5 unit) were added to the standard protoplast-producing incubation system, both singly and together. Samples were examined periodically by phase-contrast microscopy. The results are presented in Table 4. A good correlation is seen between the production of protoplasts and the combined use of laminaranase and cellulase. One exception was found in that the *Streptomyces* sp. B 814 cellulase, which had a low value of laminaranse, did produce a few protoplasts after 48 hr incubation. The deleterious effect of high concentrations of lipase on protoplast stability was again noticed when bacterial laminaranase or wheat germ lipase was used.

Hydrolysis of Pythium cell walls with dilute sulphuric acid produced glucose (major), laminaribiose and cellobiose, these sugars being identified chromatographically in three solvent systems (a, b, c). The cell wall was further characterized by enzymic degradation by using the streptomycete lytic preparations. The major products released were glucose, laminaribiose and gentiobiose; cellobiose, laminaritriose,

laminaritetraose and cellotriose were minor products, with a trace of 4-O-β-D-laminaribiosyl-D-glucose. Laminaritriose, laminaritetraose, cellobiose 4-O-β-D-laminaribiosyl-D-glucose and 3-O-β-D-celliosyl-D-glucose can be separated chromatographically (solvent, d, quadruple development). Neither of the latter two compounds were produced in appreciable amounts during time-sequence enzymic cell-wall analysis, whereas the other oligosaccharides did gradually accumulate in spite of the presence of a β-glucosidase component in the lytic systems. This would indicate that the *Pythium* sp. PRL 2142 cell walls do not possess a major mixed-linkage β-D-glucan component of the lichenan type. The cell walls on hydrolysis with a purified basidiomycete exo-β(1 → 3)-D-glucanase (Reese & Mandels, 1963) yielded both glucose and gentiobiose. Thus a glucan consisting of a β-D-(1 → 3)-linked main chain with D-glucose units attached as branches by β-D-(1 → 6)-linkages, similar to that obtained from a *Claviceps* sp., PRL 1980 (Perlin & Taber, 1963) appeared to be present.

Table 4. *Production of Pythium protoplasts by synergistic action of laminaranase and cellulase*

Expt.	Enzymes	Amounts of Enzymes (units)		Protoplast formation
		Laminaranase	Cellulase	
1	*Rhizopus arrhizus* laminaranase	0·75	0·038	−
2	Bacterial laminaranase	0·75	0·628	++*
3	*Aspergillus niger* cellulase	0·033	0·5	−
4	*Streptomyces* sp. B 814 cellulase	0·0009	0·5	+
5	*R. arrhizus* laminaranase + *A. niger* cellulase	0·783	0·538	++
6	*R. arrhizus* laminaranase + *Streptomyces* sp. B 814 cellulase	0·7501	0·538	+++
7	*R. arrhizus* laminaranase + *Streptomyces* sp. B 814 cellulase + wheat germ germ lipase (0·5 mg)	0·7501	0·538	+++*
8	Bacterial laminaranase + *Streptomyces* sp. B 814 cellulase	0·7501	1·128	++*

+, Few protoplasts (48 hr); ++, numerous protoplasts (44 hr); +++, numerous protoplasts (22 hr).

* Protoplasts lysed relatively rapidly.

Endo-β-D-glucanases do not attack this type of glucan. Hence the presence of laminaribiose (major) and cellobiose as products from the action of the streptomycete lytic complexes on Pythium cell walls indicated the presence of an unsubstituted β-D-(1 → 3) glucan as the major, and cellulose as a minor, component of these walls. The large amounts of gentiobiose produced in such hydrolyses may have arisen from the β-D-(1 → 6) substituted β-D-(1 → 3) glucan, for the lytic complexes from the *Streptomyces* sp. strains 0143, 1294 and 1399 were shown to possess an exo-glucanase by their positive action on the β-D-(1 → 6) substituted Claviceps glucan (Perlin & Taber, 1963). There still remains the possibility that the gentiobiose arose from a β-D-(1 → 6) glucan. Pustulanase activity was observed in two of the lytic preparations (Table 1). An alkali insoluble glucan fraction has been reported from *Phytophthora cinnamomi* which contained either separate β-D-(1 → 3) and β-D-(1 → 6) homopolymers or a mixed linkage polymer (Bartnicki-Garia & Lippman, 1967).

DISCUSSION

Streptomycete lytic complexes that are capable of lysing the cell walls of the *Pythium* sp. PRL 2142 have been shown to possess, as major components, exo- and endo-laminaranases and a cellulase. Initially we reported that only two of the five lytic preparations were capable of producing 'protoplasts' (Sietsma *et al.* 1967). However, when these preparations were compared quantitatively with respect to laminaranase activity four of the preparations produced 'protoplasts', while the other caused complete lysis (Table 3). The amounts of enzyme cited allowed the ready formation of protoplasts under our conditions in 24 hr. Much smaller amounts of these enzymes plus cellulase will produce some protoplasts. The lowest amount of cellulase (Cx) necessary for protoplast formation appeared to be 0·1 unit from the experiments carried out with the *Streptomyces albidoflavus* complex. The amount of laminaranase generally used 0·621–0·75 unit (Tables 3, 4) was probably excessive, since the *Streptomyces* sp. B 814 cellulase (0·5 unit) which contained only 0·00093 unit of laminaranase was still capable of forming a few protoplasts in 24 hr (Table 4). Laminaranase 0·00093 unit can release 240 μg. glucose-equivalent in 24 hr and is quite sufficient, theoretically, to produce protoplasts in this system, especially if the enzyme action was localized. The *Pythium* sp. PRL 2142 possesses a laminaranase (unpublished observation), and the possibility also exists that this enzyme may be activated by the addition of the streptomycete cellulase preparation. An anomaly is seen, however, in that *Aspergillus niger* cellulase (0·5 unit) containing 0·038 unit laminaranase was not capable of producing Pythium protoplasts. Other enzymes may be essential; for instance the presence or even necessity of a Cl cellulase (Mandels & Reese, 1964) has not been established. Several other factors will also have to be determined (e.g. product inhibition) before the system can be put on a rigorous quantitative basis. At present, a comparison of the amounts of the enzymes used with those of other workers is not informative because of variations in assay procedures and organism studied. A general inference is that in this study smaller amounts of enzyme and longer incubation periods have been used than by other workers (Lloyd, Noveroske & Lockwood, 1965; Mitchell & Alexander, 1963). Bartnicki-Garcia & Lippman (1967) used the same *Streptomycete* sp. B 814 cellulase to study the cell walls of *Phytophthora cinnamomi* and to form protoplasts. The amounts of cellulase they used were higher (about 0·2 unit cellulase/mg. cell wall) than in the present work. A unit of this cellulase (see Methods) approximately equals 10·8 units of Reese & Mandels (1963). Though the major component of this enzyme preparation is a cellulase, Bartnicki-Garcia & Lippmann stated that the lytic effectiveness was dependent also on the action of an endo β-D-(1 $\rightarrow$ 3) glucanase.

Lipases have been shown to aid fungal cell-wall degradation (Satamuro, Ono & Fujomoto, 1960) and in short-term incubation experiments also aid protoplast formation (Acha *et al.* 1966). It is evident from the results presented with the *Pythium* sp. PRL 2142 that the amounts of lipase are critical in protoplast formation; all the preparations tested in this study possessed some lipase activity. Removal of the incubation medium from the protoplasts lengthened their survival time, while addition of more lipase to the incubation medium shortened it. The substrate on which the lipases act at relatively low pH during protoplast formation is unknown. No wheat-germ lipase activity was detected affer a 3 hr assay at pH 6·2 with olive oil as a substrate

(Table 2). However, the optimum pH value for lipase action can depend on the substrate and can be relatively low (Wills, 1965). One interesting observation is that protoplasts of the *Pythium* sp. PRL 2142 produced from mycelium grown in the presence of cholesterol are more stable than those obtained from mycelium grown without it. Any relationship between steroids and membrane stability is speculative at present. Proteases have been reported to act synergistically with glycosidases in the degradation of a *Sclerotium* sp. and yeast cell walls (Satamuro *et al.* 1960; Tabata & Terui, 1963). They are, however, not essential for the production of protoplasts from the *Pythium* sp., since a mixture of lytic preparations from *Rhizopus arrhizus* and a *Streptomyces* sp. B 814 which lacked protease (Table 2) were effective protoplasts producers (Table 4). It is hoped to purify the components of the *Streptomyces* lytic enzymes and re-evaluate the protoplast-forming system.

The main components of the *Pythium* sp. PRL 2142 cell walls appear to be an unsubstituted β-D-(1 $\rightarrow$ 3)-glucan, a β-D-(1 $\rightarrow$ 6) substituted β-D-(1 $\rightarrow$ 3) glucan and cellulose, as judged from the analysis of the products of enzymic hydrolysis. They appear, therefore, to be quite similar to the walls of other oomycetous genera (Aronson *et al.* 1967; Bartnicki-Garcia & Lippman, 1967; Wang & Bartnicki-Garcia, 1966). Mitchell & Sabar (1966) reported the presence of β-D-(1 $\rightarrow$ 2) glucan in the walls of *P. butleri*, but no evidence for this glucan was found in *Pythium* sp. PRL 2142. Evidence of layering of cell-wall components has been presented by enzymic techniques (Skujins, Potgieter & Alexander, 1965; Villanueva, 1966). However, no evidence of layering of the Pythium walls has been found from an analysis of 'time-sequence' enzymic hydrolysates.

Several fungi (Lloyd *et al.* 1965; Potgieter & Alexander, 1966) show marked resistance to degradation by lytic preparations from other micro-organisms. Lloyd *et al.* (1965) suggested that fungal lysis in the soil may be an autolytic process, induced by toxic metabolites from antagonistic micro-organisms. The control of *Pythium de baryanum* in the soil has been achieved by the introduction of micro-organisms capable of lysing its mycelium (Mitchell & Hurwitz, 1965). The results found with *Pythium* sp. PRL 2142 indicate that heterolytic mycolysis might be the singular process active in the control of Pythium in the soil.

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The Effect of Aerosolization upon Survival and Potassium Retention by Various Bacteria

By J. D. ANDERSON*, F. A. DARK AND S. PETO
Microbiological Research Establishment, Porton, Salisbury, Wiltshire

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SUMMARY

Previous studies with populations of *Escherichia coli* strain B, recovered from aerosols, showed that, of the biochemical changes which were shown to precede death, the most dramatic was a rapid loss of ability to maintain cellular potassium concentrations. Loss of control over potassium may cause or contribute to death in bacteria recovered from aerosols and also probably implies a loss of control over other ions and substrates. Potassium ion efflux studies with this organism have been extended here to *E. coli* var. *communis*, *E. coli* strain JEPP, *Aerobacter aerogenes* strain H, *Serratia marcescens* strain 8 UK and *Staphylococcus epidermidis* strain NCTC 7291. After aerosolization all these organisms rapidly lost ability to retain intracellular potassium, as a consequence of damage initiated in the aerosol. Evidence for a positive correlation between survival and potassium retention was found over a limited range of conditions for most of the organisms examined. The significance of these results in relation to death processes in aerosolized bacteria is discussed.

INTRODUCTION

Loss of ability to synthesize an inducible protein, and loss of control over phosphate and potassium ions appears to precede death in populations of *Escherichia coli* strain B which have been recovered from aerosols. These changes in *E. coli* strain B are thought to precede death because their relative extent is generally far greater than the corresponding loss of viability, and because of evidence for correlations between survival and certain of the changes. These changes are also thought to be partly reversible because populations which are substantially viable may be recovered from aerosols under certain conditions and yet have undergone a temporary disruption of normal biochemical processes (Anderson, 1966; Anderson & Dark, 1967). The disruption is probably not due to generalized denaturation of protein because the β-galactosidase of *E. coli* strain B, which is a convenient and representative protein to study, is unaffected by aerosolization (Anderson, 1966; Anderson & Crouch, 1967). Of the metabolic changes observed in populations recovered from aerosols of *E. coli* strain B, a rapid loss of ability to control cellular potassium concentrations was the most dramatic consequence of aerosolization. Since loss of control over K^+ could be directly or indirectly lethal and implies a general disorganization of bacterial ion and substrate transport mechanism, K^+ movements merit particular attention. Studies with *E. coli* strain B have therefore been extended here to other strains of this organism, and to other species in order to discover whether phenomena observed with *E. coli*

* Present address: The Medical School, University of Bristol, Bristol 2.

strain B are of general occurrence. Evidence for correlations was looked for between the age and relative humidity of the bacterial aerosol, the survival on recovery, and the extent of the rapid loss of potassium into the collecting fluid.

METHODS

The apparatus, materials and methods used here for the study of six organisms were similar to those already described for studies of efflux of [^{43}K] from populations of *Escherichia coli* strain B (Anderson & Dark, 1967). Therefore only a brief outline of the methods is given here.

Organisms. Cultures of *Aerobacter aerogenes* strain H, *Escherichia coli* strains B, JEPP, *E. coli* var. *communis*, *Serratia marcescens* strain 8 UK and *Staphylococcus epidermidis* NCTC 7291 were grown from freeze-dried stocks and maintained in a liquid meat broth medium.

Radiolabelled bacterial suspensions were prepared by re-incubation of concentrated suspensions of resting phase organisms in 'low potassium', [^{43}K]-labelled tryptone medium. The radio-isotope was determined with a solid crystal scintillation counter.

Determination of bacterial survival values. Total numbers of bacteria recovered from aerosols were calculated from the viable organism count and the radiotracer content of the impinger samples, and the viable and radiotracer content of unsprayed suspensions. In general, samples of unsprayed suspensions removed from the spray pot before each experiment served as a control and were given a nominal viability of 100%. Suspensions of *Staphylococcus epidermidis* in distilled water tended to clump slightly; material which had undergone violent agitation in the spray pot was therefore sampled at the end of each experiment, as a control. Plate counts and radio-tracer determinations were made under conditions whereby survival estimates generally had a 95% confidence belt of better than $\pm$ 10% of observed values.

Apparatus for the study of bacterial aerosols. Monodisperse aerosols were generated from aqueous suspensions of bacteria by using an air-blast atomizer, then mixed with a further supply of air to give the desired relative humidity and stored in a rotating drum (Goldberg, Watkins, Boerke & Chatigny, 1958). Samples containing about 3×10^6 organisms/ml. were collected into 'raised Porton impingers' (May & Harper, 1957) containing phosphate buffer + alginate (Henderson, 1952).

Determination of the rate of loss of [^{43}K] *from labelled organisms.* The extent of [^{43}K] efflux from controls and from populations recovered from aerosols was determined at various times after collection by filtration of portions of impinger samples through membrane filters. Estimates of the [^{43}K] content of bacterial samples generally had 95% fiducial limits of less than $\pm$ 5%.

RESULTS AND DISCUSSION

Table 1 shows that the procedure for labelling the bacteria was satisfactory and that in all cases only a small proportion of radiotracer could be removed from the organisms by merely washing with water. Any sudden loss of ability to retain [^{43}K] in aqueous suspension thus indicated a breakdown of normal ion control processes.

Aerosols generated from washed suspensions of the different bacteria were stored at various relative humidities. At each relative humidity value bacterial samples were

recovered from the aerosol cloud into impingers at 1·2 sec., 5 and 30 min. after generation. The [^{43}K] content of each of these populations, and of unsprayed controls, was determined at 2, 15 and 30 min. after the mean time of sampling. All the bacterial species examined suffered a rapid initial loss of radiotracer followed by a very much slower loss. The plot of log [^{43}K] content versus time after recovery from the aerosol was practically linear for the three sampling times (2, 15 and 30 min.). The rate of this slower loss could not be accurately determined because of the comparatively large, but

Table 1. *Incorporation of* [^{43}*K*] *and loss by suspensions of various bacteria*

Organism	Uptake of labelled ion (% of the amount added to growth medium)	Amount of labelled ion remaining in organisms after one wash with water (% of the amount added to growth medium)
Escherichia coli strain B	64	55
E. coli var. *communis*	47	40
E. coli strain JEPP	34	26
Aerobacter aerogenes	70	50
Serratia marcescens	68	54
Staphylococcus epidermidis	37	33

Table 2. *Effect of vigorous 'washing' processes in the spray pot upon the value of the 'initial'* [^{43}*K*] *efflux from aqueous suspensions of various bacteria*

Organism (number of determinations in parentheses)	Mean value of 'initial'* [^{43}K] efflux (%) from suspensions taken from the spray pot *before* each experiment	Mean value of 'initial'* [^{43}K] efflux (%) from suspensions taken from the spray pot *after* each experiment
Escherichia coli strain B (13)	5·8	8·3
E. coli var. *communis* (6)	6·5	6·7
E. coli strain JEPP (7)	0·5	14
Aerobacter aerogenes (10)	9·0	18
Serratia marcescens (8)	4·0	8·1
Staphylococcus epidermidis (6)	5·0	5·5

* 'Initial' [^{43}K] efflux is defined in the text.

statistically acceptable, scatter in individual radiotracer determinations. Intercepts (at zero time) of the plots of log [^{43}K] content versus time were calculated for all species both for the unsprayed 'controls' and for samples which had been recovered from aerosols. Since the [^{43}K] content of bacterial populations could not be conveniently measured at less than 2 min. after the mean sampling time, the intercept of 'initial' value for potassium loss obtained from the plot of log [^{43}K] content against time was used as a measure of the extent of the initial rapid loss of potassium which followed recovery from the aerosol. The extent of the 'initial' [^{43}K] loss was regarded as a measure of the degree of failure of mechanisms responsible for the retention of this ion. This 'initial' loss of [^{43}K] by *Escherichia coli* strain B has already been shown (Anderson & Dark, 1967) to be a net loss of this ion rather than a facilitated exchange between the radio-

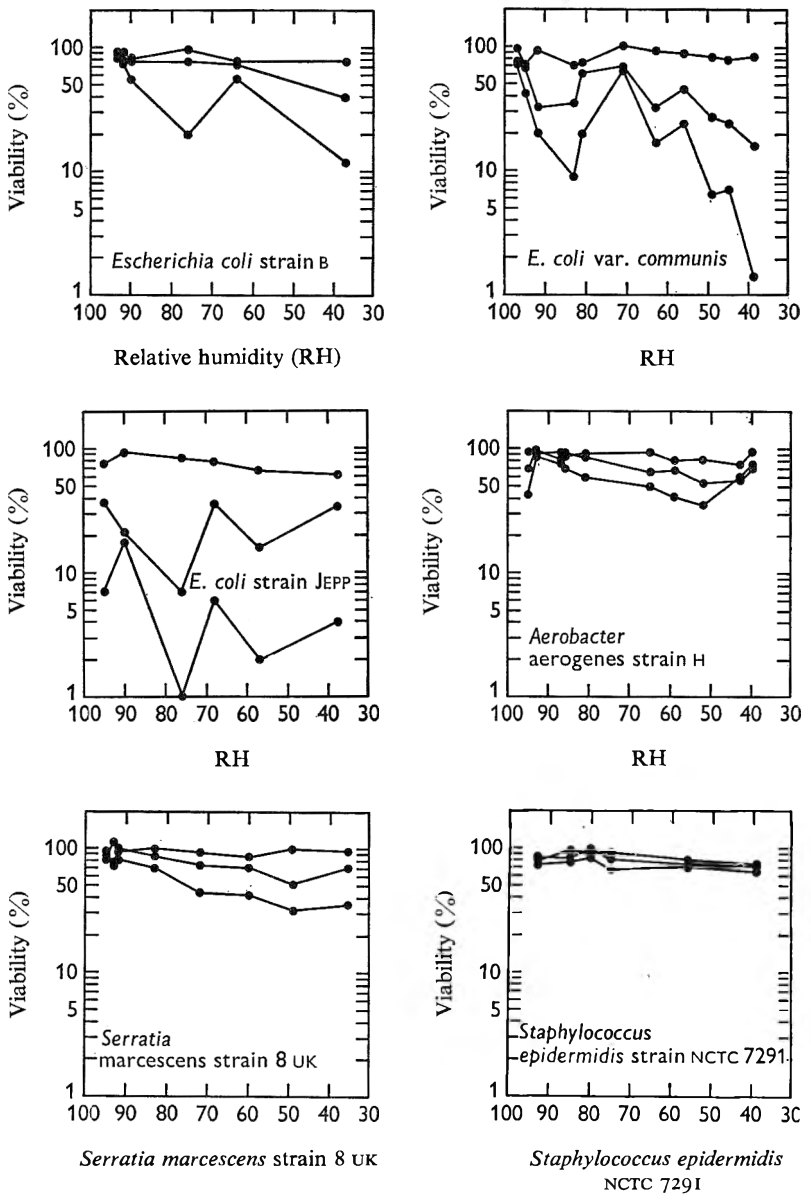

Fig. 1. The effect of relative humidity (RH) and aerosol cloud age upon the survival of various bacteria. In each case the plots represent (reading downwards) survival of populations recovered from aerosols into impingers at 1·2 sec., 5 and 30 min. after generation.

tracer and the unlabelled ion of the fluid into which the bacteria were recovered. Although the kinetics of [^{43}K] loss by *E. coli* strain B have already been described in detail certain results obtained with this strain will be reiterated here for this comparative survey.

The 'initial' loss of [^{43}K] from aqueous bacterial suspensions which were diluted in phosphate buffer+alginate collecting fluid was comparatively small and was not

greatly increased by violent agitation in the spray pot for 3 min. during the course of each experiment (Table 2). By contrast, 'initial' [^{43}K] losses observed from populations recovered from aerosols were much larger and might be a result of aerosolization. A correlation was therefore looked for between 'initial' [^{43}K] retention, the survival of organisms and the age and relative humidity of the bacterial cloud.

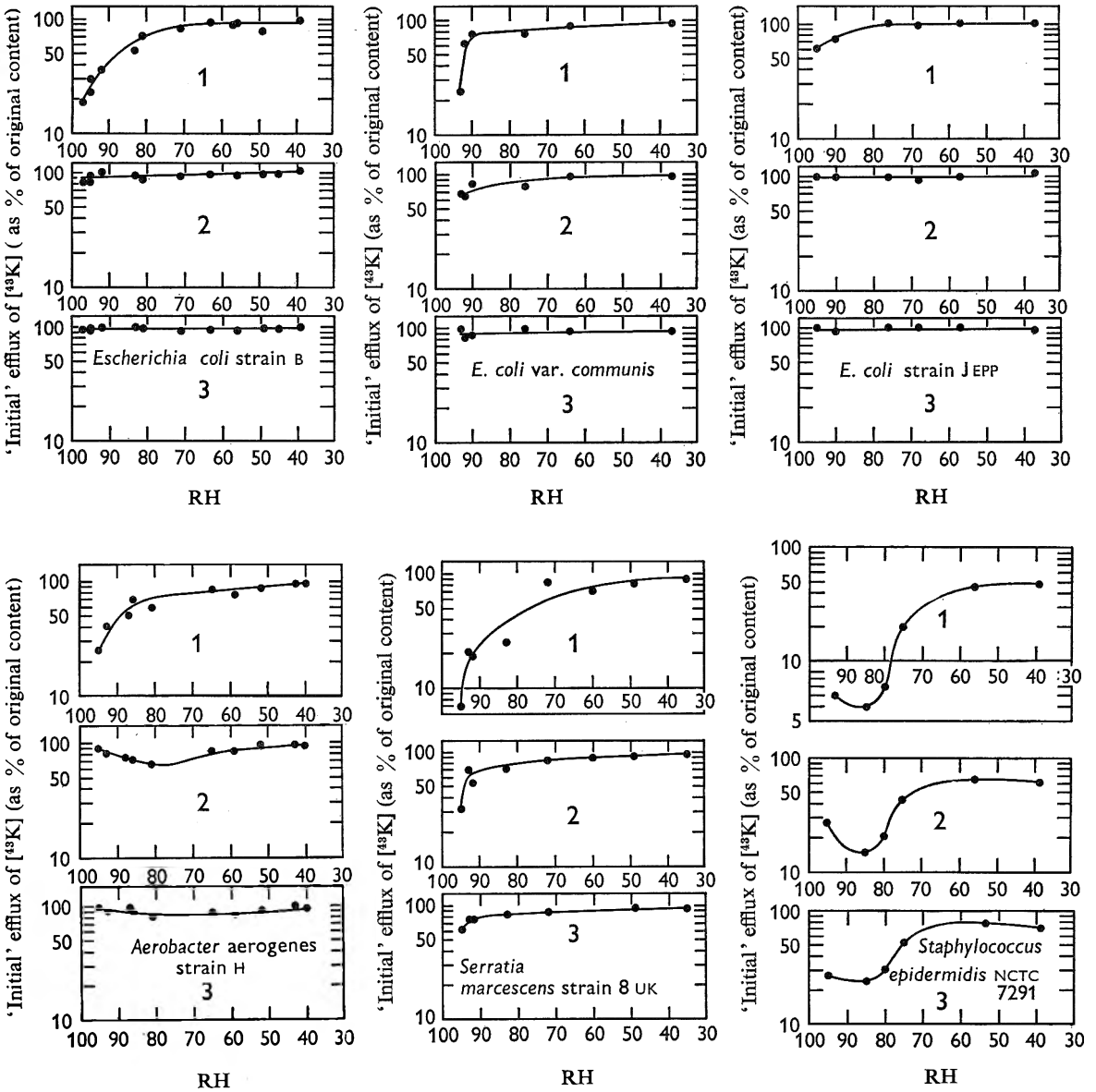

Fig. 2. The effect of relative humidity (RH) and aerosol cloud age upon the 'initial' efflux of [^{43}K] from labelled populations of different bacteria. For each organism plots 1, 2 and 3 represent respectively results obtained with populations recovered from aerosols into impingers at 1·2 sec., 5 and 30 min. after generation.

Fig. 1. summarizes the effect of relative humidity and holding time in the aerosol upon the survival of aerosolized bacteria. Aerosols of *Escherichia coli* strain B and of other organisms may be particularly unstable at certain narrow ranges of relative humidity values (evidence reviewed by Anderson & Cox, 1967). The apparent absence of such zones of instability for the organisms described in Fig. 1 may be due to dif-

Table 3. *Evidence for a correlation between the 'initial' efflux of [^{43}K] from bacteria recovered from aerosols and the relative humidity of the bacterial cloud*

Organisms (number of separate relative humidity values studied in parentheses)	Relative humidity range (%) studied	*Correlation coefficients of plot of [^{43}K]retention (100 − 'initial' efflux) versus relative humidity (%) for populations recovered from aerosol clouds at the following times after generation	
		1·2 sec.	5 min.
Escherichia coli strain B (12)	39–95	0·91 (0·1 %)	0·62 (5 %)
E. coli strain *communis* (6)	37–93	—	0·84 (5 %)
E. coli strain JEPP (6)	38–95	—	—
Aerobacter aerogenes (10)	40–95	0·91 (0·1 %)	0·69 (5 %)
Serratia marcescens (8)	35–95	0·90 (1 %)	0·84 (1 %)
Staphylococcus epidermidis (6)	39–93	0·94 (1 %)	0·88 (5 %)

* Significance levels are shown in parentheses; other correlation coefficients, although positive in most cases, were found to be not significant and are not shown.

Table 4. *Summary of the significant results of an investigation into a correlation between the extent of the 'initial' loss of [^{43}K] and the survival of organisms recovered from aerosols*

Organism	Age of aerosol cloud (min.)	*Correlation coefficients of plot of [^{43}K]retention (100 − 'initial' loss) versus viability (%) for populations recovered from aerosol clouds
Escherichia coli strain B	5	0·74 (10 %)
E. coli strain *communis*	—	—
E. coli strain JEPP	30	0·88 (5 %)
Aerobacter aerogenes	5	0·84 (1 %)
Serratia marcescens	5	0·78 (5 %)
	30	0·97 (0·1 %)
Staphylococcus epidermidis	5	0·84 (5 %)

* Significance levels in parentheses; correlation coefficients not shown were found to be not significant.

ferences in cultural conditions or to the comparatively large increments of relative humidity at which survival values have been determined. In view of these uncertainties the survival patterns shown in Fig. 1 have not been represented as smooth curves, and may only be regarded as a rough indication of the relative stability of the organisms.

Figure 2 summarizes the effect of relative humidity and holding time in the aerosol upon the extent of 'initial' [^{43}K] loss at points corresponding to those of Fig. 1. The

general pattern of the results suggests that the extent of the 'initial' [^{43}K] loss increases with decreasing relative humidity.

Since loss of [^{43}K] was practically complete at 30 min., correlation coefficients between the plot of [^{43}K] retention and relative humidity were generally only significant for the 1·2 sec. and 5 min. samples (Table 3); [^{43}K] loss from *Escherichia coli* strain JEPP were so rapid that the corresponding correlation coefficients were not significant even for the 1·2 sec. sample; at the other extreme the same correlation coefficients (0·88 each) for *Serratia marcescens* and *Staphylococcus epidermidis* were significant (1% and 5% levels, respectively) even for the 30 min. sample. The proven dependance of [^{43}K] efflux upon the age and relative humidity of the aerosol cloud shows that bacterial ion-control mechanisms were damaged as a result of changes which were initiated in the aerosol but which might, or might not, be expressed until the bacteria were recovered into aqueous media.

Since all the organisms were substantially viable when sampled from aerosols at 1·2 sec. after generation, and most organisms sampled from aerosols at 30 min. suffered an almost total 'initial' loss of [^{43}K] upon collection, a significant correlation between [^{43}K] retention and viability was only demonstrated over a limited range of conditions (Table 4; note the unsatisfactory significance level for *Escherichia coli* strain B). The partial dependence of survival upon [^{43}K] retention confirms that survival was not absolutely dependent upon the integrity of processes which retain potassium (Anderson & Dark, 1967). Nevertheless, failure of such processes would undoubtedly contribute to death and imply a breakdown of mechanisms for the retention of other ions and substrates in all the different organisms examined.

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A Staphylococcal Toxic Complex Affecting Particular Areas of the Mitochondrial Electron Transport System

By I. LOMINSKI, C. G. GEMMELL AND J. P. ARBUTHNOTT
Department of Microbiology, The University, Glasgow

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SUMMARY

Mitochondrial respiration can be impaired *in vitro* by a toxic complex, termed succinic oxidase factor (s.o.f.), produced by 70% of coagulase-positive staphylococci. The oxidation of succinate is most sensitive, cytochrome oxidase less so while succinic dehydrogenase is resistant. The complex consists of at least two components, differing in degree of heat sensitivity. The more heat-resistant component impairs electron transfer in the region of ubiquinone (ubiquinone can reverse the impairment), while the other component impairs electron transfer in the region of cytochrome *c* (cytochrome *c* can reverse the impairment). It is thought that the components of s.o.f. may be of enzymic nature, acting on the phospholipids responsible for the integrity of the electron transport chain.

INTRODUCTION

It has been reported that crude toxin from a number of strains of *Staphylococcus pyogenes* impairs succinic oxidase activity of mouse liver mitochondria. The effect is due neither to alpha toxin nor to other major staphylococcal products; the factor was termed succinic oxidase factor (s.o.f.) (Lominski *et al.* 1964; Lominski, 1966). Apart from impairing mitochondrial respiration, s.o.f. lowers the optical density of a mitochondrial suspension, affects mitochondrial morphology, and is produced by 70% of pyogenic staphylococci.

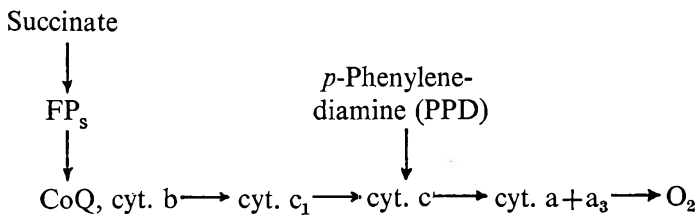

Fig. 1.

The present study was carried out to determine the sites of action of the toxin within the respiratory chain. For this purpose the whole chain of succinic oxidase (see Fig. 1), as well as two parts of it, i.e. succinic dehydrogenase and cytochrome oxidase, were assayed, adopting as model the scheme of Ernster & Lee (1964); it is of course realized that the mouse liver mitochondrion may differ in some respects from this model.

Early experiments showed that at least two sites in the chain were affected by s.o.f. Moreover, later results suggested that these two sites were attacked by two separate components present in crude s.o.f. For simplicity, however, we shall continue to refer to the complex as s.o.f.

METHODS

Strains. Three strains of coagulase-positive staphylococci of human origin, designated nos. 1, 2 and 3 in a previous study (Lominski *et al.* 1964) were used. Most experiments reported in the present study were carried out with strain no. 2 (NCTC 7121, WOOD 46) phage type 6/47/53/54/81.

Media. For most experiments semi-solid agar containing 2·5 g. Oxoid Nutrient Broth no. 2 and 1·0 g. Oxoid Blood Agar Base in 100 ml. water (referred to later as medium no. 1) was used as a culture medium. Five additional media varying in composition were compared with the standard medium in one experiment.

Preparation of succinic oxidase factor. Semi-solid agar plates were seeded with 1 ml. of a suspension of staphylococci (*c.* 6×10^8 organisms) from overnight 5% horse blood (Oxoid) agar cultures, and incubated for 72 hr at 37° in an atmosphere of 20% (v/v) CO_2 (adapted from the method of Burnet, 1930). The plates were frozen to −20°, allowed to thaw at room temperature, the extruded fluid collected, spun at 10,000 ***g*** for 15 min., distributed in small amounts in bijou bottles, stored at −20° and opened only once for use.

Control fluids were obtained by freezing and thawing uninoculated semi-solid agar plates or by autoclaving s.o.f.

Preparation of mitochondria. The bulk of the work was done with mouse liver mitochondria freshly prepared every day by a modification of the method of Schneider (1948). Porton white mice (35–45 g.) were killed by cervical dislocation and exsanguinated. The livers were placed in ice-cold suspending fluid (see below) and after removal of connective tissue by gentle teasing-out homogenized in a Griffith's glass tube by applying 10 strokes of a matching plunger. The homogenate was suspended in fluid at the rate of 1 g. per 10 ml. and first spun at 600 ***g*** for 10 min.; the sediment was discarded and the supernatant spun at 8500 ***g*** for 10 min. The mitochondrial sediment was washed in 10 ml. of suspending fluid, spun again at 8500 ***g*** for 10 min., finally resuspended in 5 ml. and stored for 2 hr; centrifugation and storage was at 4°. Immediately prior to use the mitochondrial suspension was diluted 1/3 with the same fluid.

Suspending fluids. As a rule the same suspending fluid was used for the preparation of mitochondria, for subsequent dilution in the assay and for dissolving all reagents; in most experiments this was 0·25 M-sucrose containing 0·023 M-phosphate buffer (Hendry, 1948) pH 7·3. However, in some experiments mitochondria were either prepared and assayed in 0·25 M-sucrose containing 0·033 M-tris/HCl (Gomori, 1946), pH 7·4, or in 0·44 M-sucrose containing 0·02 M-disodium citrate (Witter, Watson & Cottone, 1955), pH 6·2.

Assays. An interaction period of 30 min. at 37° was allowed between s.o.f. or control fluid and mitochondria before tipping the substrate.

All assays were done in duplicate; if values in a pair differed by more than 5%, the experiment was discarded. Where comparison of two enzymic activities, i.e. succinic oxidase and cytochrome oxidase was done, the same batch of mitochondria

was used for both assays in order to avoid day-to-day variation of mitochondrial preparations.

Succinic dehydrogenase was assayed both manometrically as described by Slater, (1949*b*), and in Thunberg tubes in the following way: 0·5 ml. of mitochondrial suspension was mixed with 1·4 ml. of sucrose/phosphate buffer and 0·5 ml. of toxin or control fluid. The material was then transferred into Thunberg tubes; 0·3 ml. of 0·2 M-sodium succinate and 0·3 ml. of 0·001 M-methylene blue were introduced into the side-arm. The tubes were evacuated for 5 min. with an Edwards High Vacuum Pump (Model 2SC50) with constant shaking to remove air bubbles, closed, and the contents of the side-arm tipped into the main vessel. The time taken to decolorize *c.* 90% of the dye (visually assessed) at 37° was taken as a measure of dehydrogenase activity.

Succinic oxidase (s.o.) was determined as previously described (Lominski *et al.* 1964), modified from Slater (1949*b*).

Cytochrome oxidase (c.o.) was assayed manometrically using paraphenylenediamine (PPD) as substrate by the method of Slater (1949*a*); the mitochondrial suspension oxidized this substrate in the absence of added cytochrome *c*. In the assay 0·5 ml. mitochondrial suspension, 1·7 ml. suspending fluid and 0·5 ml. of s.o.f. or control fluid were mixed. After 30 min. 0·3 ml. of PPD (0·15 M) was added from the side-arm.

Succinic oxidase factor activity was measured by comparing the oxygen uptake of s.o.f.-treated and control mitochondria; an impairment of 10% or more was taken as significant.

Effect of cytochrome c. Either at zero time or during the assay of succinic oxidase and cytochrome oxidase systems, 0·3 ml. aqueous cytochrome *c* (crystalline, iron content 0·43%, Koch-Light Ltd.) was added to both control and s.o.f.-treated mitochondria from a second side-arm to give a final concentration of 2×10^{-5} M.

Effect of ubiquinone. 5 mg. of ubiquinone (Koch-Light Ltd.) was dissolved in 1 ml. ethanol and further diluted 1/5 in sucrose/phosphate buffer. Either at zero time or during the assay of succinic oxidase, 0·3 ml. of this suspension was added from the second side-arm to both control and s.o.f.-treated mitochondria giving a final concentration of 33 μg./ml. The addition caused an immediate upset of equilibrium within the manometer due to the evaporation of ethanol. This was rectified by opening the manometer stop-cock, resetting the fluid level to zero and allowing 1 min. for equilibration. The rate of oxygen uptake thereafter was measured in the usual way.

Heating of succinic oxidase factor. The pH of s.o.f. was adjusted by adding to four parts of toxin one part of 0·15 M-phosphate buffer (Hendry, 1948), pH 7·3, the mixture heated in a water bath at 60° for 15 or 30 min., and subsequently diluted for assay to the required strength in sucrose/phosphate buffer.

RESULTS

Crude preparations of s.o.f. impaired succinic oxidase and cytochrome oxidase. Typical results of the experiments are given in Figs. 2 and 3.

Succinic dehydrogenase, previously reported (Lominski *et al.* 1964) to be resistant to s.o.f. when assayed both by manometry and by methylene blue reduction was again found to be resistant; 24 hr treatment with s.o.f. gave no impairment.

The experiments shown below were carried out in sucrose/phosphate buffer. Since phosphate is a well-known mitochondrial swelling agent the effect of s.o.f. on mitochondria, suspended in two other buffers commonly used for the assay of mitochondrial activity, i.e. sucrose/tris/HCl and sucrose/citrate (see Methods) was examined; in both, mitochondrial respiration was impaired.

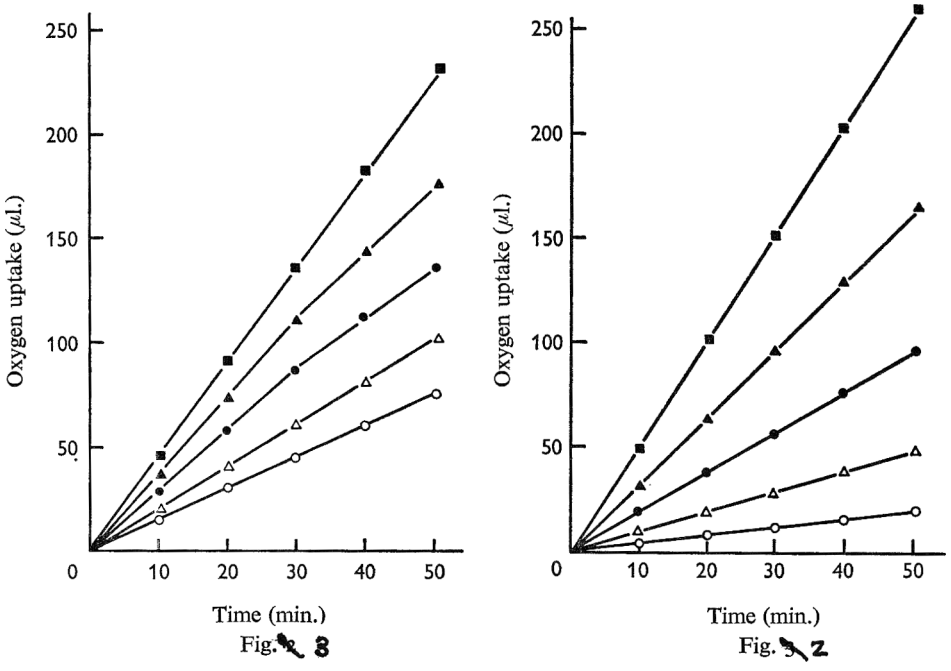

Fig. 2. The impairment of succinic oxidase: the effect of varying concentrations of s.o.f. 1/60 dilution ○——○; 1/300 dilution, △——△; 1/600 dilution, ●——●; 1/1500 dilution, ▲——▲; control (no s.o.f.), ■——■.

Fig. 3. The impairment of cytochrome oxidase: the effect of varying concentrations of s.o.f. 1/60 dilution, ○——○; 1/300 dilution, △——△; 1/600 dilution, ●——●; 1/1200 dilution, ▲——▲; control (no s.o.f.), ■——■.

When a strain (Wood 46) was grown on our standard medium (see Methods) the ratio of the percentage impairment of succinic oxidase and of cytochrome oxidase at a constant dilution of s.o.f. varied very little from experiment to experiment (1·0–1·3). However, when this strain was grown on media of differing composition it yielded preparations of s.o.f. with impairment ratios ranging from 1·10 to 2·78 (see Table 1). Moreover, when two strains were tested in parallel at two dilutions, the ratios became widely different (see Table 2), revealing a quantitative difference between the two toxins in their ability to impair succinic oxidase and cytochrome oxidase. It can be seen that at a dilution of 1/60 the ratio in strain 1 increased to 1·66, while in strain 3 it fell to 0·82.

Finally, different strains of staphylococci grown on one and the same medium yielded s.o.f. with widely differing impairment ratios. The findings strongly suggested that s.o.f. consists of more than one component. More conclusively this was shown by the following experiments.

It has previously been reported (Lominski *et al.* 1964) that crude s.o.f. heated at a high pH to 60° for 30 min. lost almost completely its activity. An attempt was now made to ascertain whether one component of s.o.f. could not be selectively inactivated by the application of heat under carefully controlled conditions. This proved successful: it was found that heating of s.o.f. buffered to pH 7·3, for 15–30 min., inactivated

Table 1. *The ratios of impairment of succinic oxidase and cytochrome oxidase by s.o.f. prepared from Staphylococcus aureus strain* WOOD *46 on different culture media at a constant dilution of toxin of 1/36.*

Medium no.	Percent impairment of		Ratio of impairment of s.o. to that of c.o.
	s.o.	c.o.	
1	87	80	1·10
2	75	29	2·58
3	84	58	1·45
4	89	66	1·35
5	93	82	1·14
6	64	23	2·78

Note: Medium 1: Oxoid Nutrient Broth no. 2, 2·5 g.; Oxoid Blood Agar Base no. 2, 1 g.; water 100 ml. Medium 2: Oxoid Nutrient Broth no. 2, 2·5 g.; Oxoid Agar no. 3, 0·3 g.; water, 100 ml. Medium 3: Oxoid Blood Agar Base, 1·0 g.; saline 100 ml. Medium 4: Bacto Tryptone, 1·0 g.; Bacto Agar 0·6 g.; saline 100 ml. Medium 5: Oxoid Tryptone, 1·0 g.; Oxoid Agar no. 3, 0·3 g.; saline 100 ml. Medium 6: Oxoid Nutrient Broth no. 2, 2·5 g.; Oxoid Blood Agar Base no. 3, 0·3 g.; water 100 ml.

Table 2. *The ratios of impairment of succinic and cytochrome oxidase by s.o.f. from strains 1 and 3 tested at different dilutions*

	Dilution tested	Percentage impairment		Ratio of impairment of s.o. to impairment of c.o.
		s.o.	c.o.	
Strain 1	1/18	55	43	1·30
	1/60	55	33	1·66
Strain 3	1/18	68	50	1·36
	1/60	27	33	0·82

Table 3. *Effect of heat on components of s.o.f.*

Heat treatment	Percentage impairment of oxygen uptake	
	Succinic oxidase	Cytochrome oxidase
None	75	70
60°/15 min.	62	33
60°/30 min.	51	7

more of the component affecting cytochrome oxidase than of that affecting succinic oxidase. In only some experiments were we successful in completely inactivating the component which impaired cytochrome oxidase; the thermal resistance of the two components is probably very close. Table 3 shows a typical experiment.

The question arose whether the two components acted on one and the same site in the electron transport chain or whether more than one site was involved. The answer was provided by the following experiments. When mitochondrial succinic oxidase and cytochrome oxidase activities were impaired by s.o.f. to about 70% an aqueous solution of cytochrome *c* (0·3 ml., 2×10^{-4} M) was added to the reaction vessel from the side-arm. In both systems respiration was for a time restored to a rate close to that of the control mitochondrial preparation. However, the restoration of succinic oxidase was shorter-lived and less complete than that obtained with cytochrome oxidase (see Figs. 4*a* and *b*). In some experiments the rate of oxygen uptake of restored cytochrome oxidase showed distinct tailing off 20 min. after the addition of cytochrome *c*. This, for the time being, remains unexplained although there may be yet another point of attack in the cytochrome oxidase chain.

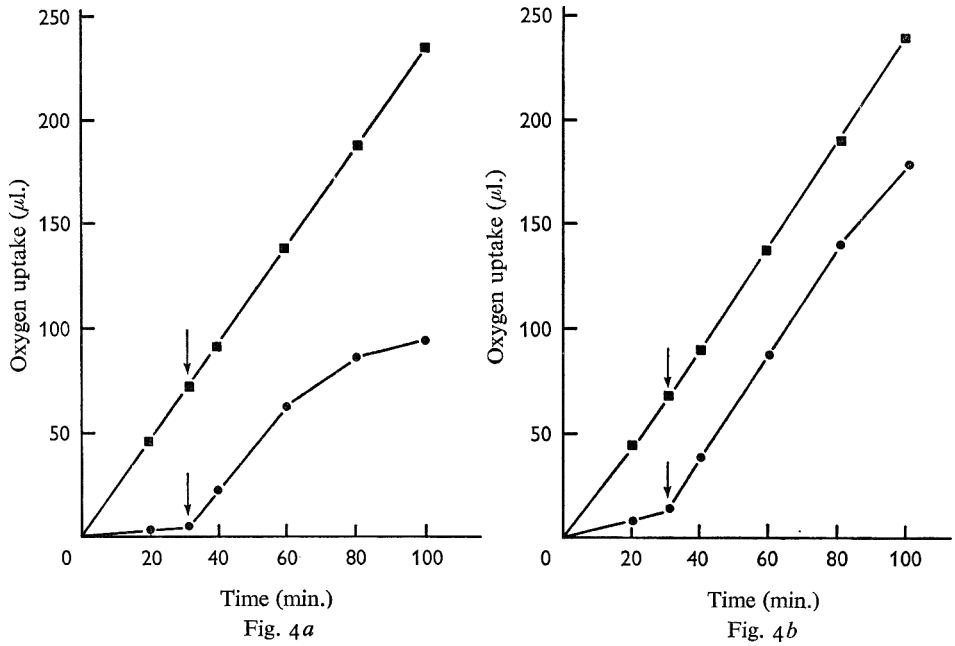

Fig. 4. Restoration of activity to s.o.f.-impaired mitochondria by cytochrome *c*: (*a*) succinic oxidase; (*b*) cytochrome oxidase. s.o.f.-treated, ●——●; control (no s.o.f.), ■——■. Cytochrome *c* added at point indicated by arrows.

The difference in efficacy of restoring the two respiratory systems by cytochrome *c*, an electron carrier common to both, suggested that an additional site involved in the oxidation of succinate might be attacked. With this in mind the effect of another component of the electron transport chain, i.e. ubiquinone (not involved in cytochrome oxidase), was examined in a similar way. Ubiquinone, as expected, did not restore cytochrome oxidase activity but was effective in partially restoring impaired succinic oxidase (see Fig. 5*a*) though not as completely as did cytochrome *c*. When cytochrome *c* and ubiquinone were both added simultaneously to s.o.f.-impaired mitochondria, succinic oxidase was almost fully restored (see Fig. 5*b*).

Heating experiments (see Table 3) showed that the component acting on cyto-

chrome oxidase is more heat-labile than that acting on succinic oxidase. On the other hand, it is known (Fig. 5*a*) that ubiquinone restores s.o.f.-impaired succinic oxidase. It seemed logical therefore to assume that the heat-resistant component is the one which attacks the ubiquinone site in succinic oxidase. This indeed was shown by the following experiment: succinic oxidase, impaired by heated s.o.f., could be well restored by ubiquinone (see Fig. 6*a*) but no more by cytochrome *c* (see Fig. 6*b*). It follows that the component attacking the cytochrome *c* locus was almost totally destroyed by heating.

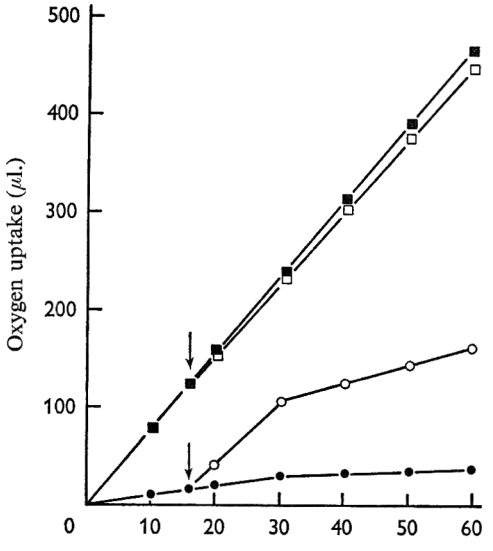

Fig. 5*a*

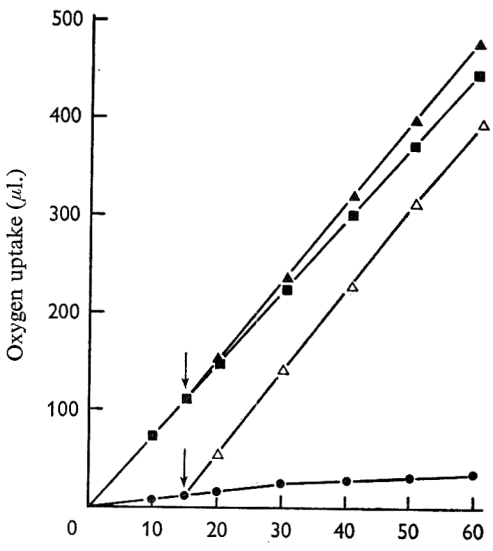

Fig. 5*b*

Fig. 5. Restoration of activity of s.o.f.-impaired succinic oxidase by (*a*) ubiquinone and (*b*) ubiquinone plus cytochrome *c*. s.o.f.-treated succinic oxidase, ●——●; s.o.f.-treated succinic oxidase after addition of (*a*) ubiquinone, ○——○; or (*b*) ubiquinone plus cytochrome *c*, △——△; control succinic oxidase, ■——■; control succinic oxidase after addition of (*a*) ubiquinone, □——□; or (*b*) ubiquinone plus cytochrome *c*, ▲——▲. Ubiquinone (*a*) and ubiquinone plus cytochrome *c* (*b*) added at points indicated by arrows.

It thus appears that s.o.f. contains a heat-labile component acting on the cytochrome *c*, and a heat-resistant one acting on the ubiquinone locus. Attack on the cytochrome *c* locus causes impairment of both cytochrome oxidase and succinic oxidase—attack on the ubiquinone locus only of succinic oxidase.

The fact that ubiquinone and cytochrome *c* restore respiratory activity to s.o.f.-impaired mitochondria prompted the question whether these components themselves were the substrates of s.o.f. attack and as such were modified *in vitro*. The following experiments were accordingly carried out. Cytochrome *c* and ubiquinone were treated with s.o.f. for 30 min. at 37°; their ability to restore respiratory activity to s.o.f.-impaired mitochondria was then compared with that of untreated cytochrome *c* and ubiquinone. The experiments showed that treatment with s.o.f. did not modify either of these substances: s.o.f.-treated cytochrome *c* and ubiquinone were as active in restoring impaired mitochondrial respiration as were untreated control preparations.

It has accordingly to be postulated that s.o.f. exerts its effect in the mitochondrion on components other than cytochrome *c* or ubiquinone, but esential for their intra-mitochondrial function. These, from preliminary experiments, may be phospholipids.

Finally, in a few experiments (to be reported elsewhere) an attempt was made to study the kinetics of s.o.f.: the results showed that s.o.f. which has acted consecutively on four aliquots of fresh mitochondria quantitatively retained its ability to impair respiration.

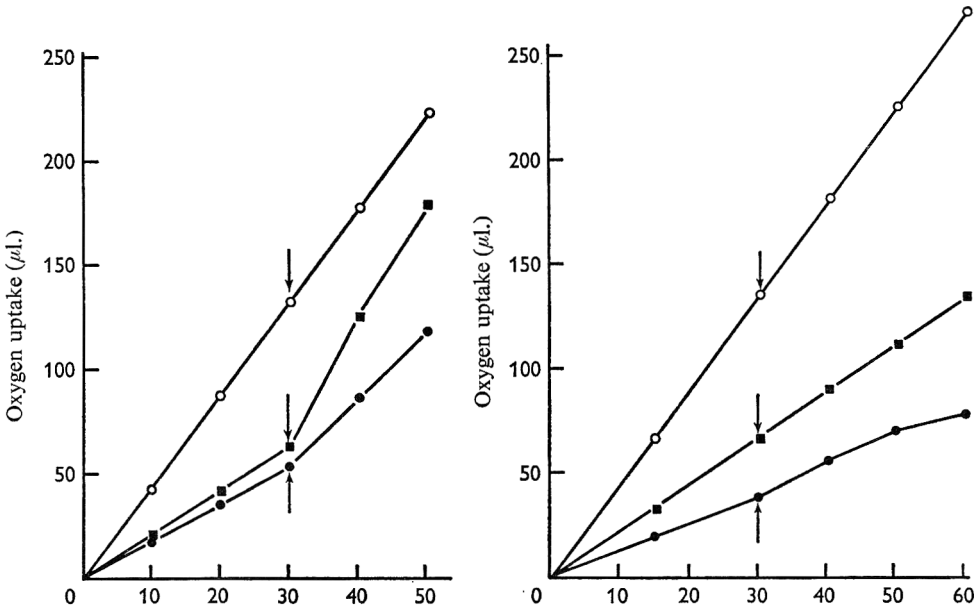

Fig. 6. Effect of (*a*) ubiquinone and (*b*) cytochrome *c* on succinic oxidase impaired by heated s.o.f. Unheated s.o.f.-treated succinic oxidase, ●——●; heated s.o.f.-treated succinic oxidase, ■——■; control (no s.o.f.) succinic oxidase, ○——○. Ubiquinone (*a*) and cytochrome *c* (*b*) added at points indicated by arrows.

DISCUSSION

The greater impairment of succinic oxidase than of cytochrome oxidase by s.o.f. can be accounted for by the finding that s.o.f. attacks two sites in the former and only one in the latter.

The heat sensitivity of s.o.f. and the fact that it is not used up during several successive contacts with fresh mitochondria suggest that the complex is enzymic rather than a blocking agent.

Likely enzymes would be the phospholipases, many of which are known to impair mitochondrial activity: phospholipase A from snake venom (Braganca & Quastel, 1953; Nygaard & Sumner, 1953; Edwards & Ball, 1954), phospholipase C from *Clostridium welchii* (Wooldridge & Higginbottom, 1938; Macfarlane & Datta, 1954; Edwards & Ball, 1954), and phospholipase D from cotton seed (Tookey & Balls, 1956)

act by destroying mitochondrial phospholipids. Experiments in progress have shown that s.o.f. attacks both phospholipids and lipids extracted from mitochondria.

The close association of phospholipids with the protein components of the electron transfer chain and their importance to mitochondrial function have been well established (Lester & Fleischer, 1959, 1961; Fleischer, Klouwen & Brierley, 1961; Fleischer, Brierley, Klouwen & Slautterback, 1962; Brierley & Merola, 1962; Green, 1962; Green & Fleischer, 1963). Disruption of the phospholipid-protein bonds by detergents such as cholate, deoxycholate and various alcohols produces submitochondrial particles of limited, specific activity (Crane & Glenn, 1957; Hatefi, Haavik & Jurtshuk, 1961). Harsher treatment (acetone, octanol and ether) abolishes respiration (Fleischer *et al.* 1962; Jacobs & Sanadi, 1955; Lester & Fleischer, 1961; Szarkowska, 1966), which can only be restored to these particles by adding exogenous cytochrome *c*, ubiquinone or phospholipids.

Succinic oxidase factor attacks phospholipids and lipids extracted from mitochondria, cytochrome *c* and ubiquinone restore activity to s.o.f.-impaired mitochondria, though neither is modified *in vitro*. We believe at present that s.o.f., by impairing the function of mitochondrial phospholipids, causes dislocation of ubiquinone and cytochrome *c*: addition of excess exogenous cytochrome *c* and ubiquinone may either provide by-passes around s.o.f.-affected areas or replace ubiquinone and cytochrome *c* lost from essential sites of electron transport.

Toxins impairing mitochondrial respiration have hitherto only been found in frankly pathogenic micro-organisms, e.g. the alpha toxin of *Clostridium welchii* (Macfarlane & Datta, 1954), the plague murine toxin of *Pasteurella pestis* (Kadis, Ajl & Rust, 1963; Kadis, Cohen & Ajl, 1965) and the NAD-splitting enzyme formed by Group A streptococci (Carlson, Kellner & Bernheimer, 1956); s.o.f. was demonstrated in 70% of pathogenic coagulase-positive staphylococci and only exceptionally in coagulase-negative strains.

There is as yet no evidence that highly purified s.o.f. passes through intact cell membranes. However, it is unlikely that s.o.f. is formed *in vivo* in the absence of alpha toxin, an agent well known to increase membrane permeability (Artenstein, Madoff & Weinstein, 1963; Cooper, Madoff & Weinstein, 1964; Rahal, Madoff & Weinstein, 1964); even strains apparently deficient in alpha toxin *in vitro* do synthesize it *in vivo* (Foster, 1963). In the presence of alpha toxin s.o.f. does indeed cross cell membranes; the consequent impairment of electron transport, of oxidative phosphorylation and of energy release could account for some of the damage caused by staphylococci to the tissues of the host.

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Identification of Mycoplasmas of Human Origin

By HAVA HAAS, T. G. SACKS AND S. RAZIN
Department of Clinical Microbiology, The Hebrew University-Hadassah Medical School, Jerusalem, Israel

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SUMMARY

Fifty-five strains of the genus *Mycoplasma* were isolated from sputam of 95 patients suffering from various respiratory tract diseases. The polyacrylamide gel electrophoretic method was used to identify the isolated strains, in addition to the conventional biochemical methods and growth-inhibition by antisera. The electrophoretic patterns of the isolated strains were compared with those of known human mycoplasmas. The electrophoretic patterns obtained were species specific and highly reproducible. The results of the identification of the isolated strains by the gel electrophoretic method correlated well with those of the growth inhibition test. It is suggested that polyacrylamide gel electrophoresis of whole cell proteins might become a useful routine method for identification of mycoplasmas.

The most prevalent Mycoplasma found in the sputa was *Mycoplasma salivarium*, only four strains were identified as *M. orale* type 1 and one strain as *M. pneumoniae*. One strain was similar to, but not identical with, *M. salivarium*. The three oral anaerobic mycoplasmas (*M. salivarium*, *M. orale* types 1 and 2) showed a certain similarity when examined by the gel electrophoretic method, they may be genetically related to each other.

INTRODUCTION

Since it was established that the aetiological agent of primary atypical pneumonia associated with cold agglutinins is not a virus but a mycoplasma (Chanock, Hayflick & Barile, 1962), interest in these organisms has markedly increased. Recent reports suggest that mycoplasmas may be associated with some diseases of as yet unknown aetiology. Hayflick & Koprowsky (1965) reported the recovery of a mycoplasma directly from human leukaemic bone marrow, and their isolation has been reported from patients with Reiter's syndrome, rheumatoid arthritis and systemic lupus erythematosus (Bartholomew, 1965). There are indications that certain mycoplasmas, the T-strains, may be a cause of non-gonococcal urethritis (Csonka, Williams & Corse, 1966; Shepard, 1967). Several Mycoplasma species are common inhabitants of the human oral cavity. *Mycoplasma salivarium* was isolated from the gingival sulci of almost all persons having natural dentitions, and it was concluded that this mycoplasma is a normal constituent of the anaerobic flora of the human oral cavity (Razin, Michmann & Shimshoni, 1964). A strain more recently classified as *M. orale* type 1 is also a common inhabitant of the oropharynx; less common are *M. orale* types 2 and 3 and *M. hominis* type 1 (Taylor-Robinson, Canchola, Fox & Chanock, 1964; Taylor-Robinson, Fox & Chanock, 1965; Purcell & Chanock, 1967). The relationship of these strains to respiratory tract diseases has been investigated, but there is as yet no con-

clusive evidence that they are able to produce disease in man (Glezen, Thornburg, Chin & Wenner, 1967). The pathogenicity of *M. pneumoniae*, on the other hand, has been proved beyond doubt and it is rarely recovered from the oropharynx of healthy people (Chanock, 1965). *M. pneumoniae* also differs from all the other human mycoplasmas in its biochemical characteristics and in its antigenic structure.

The species identification of Mycoplasma strains, and in particular those isolated from the human oropharynx, is a frequent laboratory problem. The few biochemical tests available are of little help in species identification and at present this is based mainly on serological methods, which are time consuming and some of which frequently yield non-specific results. Nucleic acid homology techniques recently applied to Mycoplasma classification (Reich, Somerson, Rose & Weissman, 1966) are still too complicated to be done in most laboratories. Rottem & Razin (1967) showed that the membrane proteins of animal and saprophytic Mycoplasma strains produce electrophoretic patterns in polyacrylamide gels which are species specific and reproducible and can be used for identification purposes. Further studies (Razin & Rottem, 1967) have shown that the electrophoretic patterns of whole-cell proteins can serve the same purpose, simplifying the procedure considerably. The present report deals with the results of a survey of the Mycoplasma species isolated from sputa of patients suffering from various respiratory diseases, with special emphasis on the identification of the isolates by the new electrophoretic method as compared with the conventional biochemical and serological methods.

METHODS

Organisms. Mycoplasma pneumoniae, 15531; *M. fermentans*, 15474; *M. hominis*, type 1, 15056; *M. salivarium*, 14277; *M. orale* type 1, 15539 were obtained from the American Type Culture Collection (Rockville, Md.). *Mycoplasma orale* type 2 was kindly provided by R. M. Chanock (National Institutes of Health, Bethesda, Md.). Locally isolated strains were obtained as described below.

Cultural conditions. The medium used for the isolation and growth of the mycoplasmas was that of Chanock, Hayflick & Barile (1962). Sputum specimens collected from 95 patients with respiratory tract infections, mainly bronchitis, were inoculated on two agar plates; one was incubated aerobically and the other anaerobically at 37°. The plates were inspected microscopically for Mycoplasma colonies every 2 days for up to 21 days. For polyacrylamide gel electrophoretic studies the reference and some of the isolated strains were grown in 200–500 ml. of liquid medium, and incubated for 5–10 days at 37°. In those cases where the local strains did not appear to be pure cultures on the basis of disc growth-inhibition tests, pure cultures were obtained from single colonies. The organisms were harvested by centrifugation at 13,000 *g* for 20 min., washed three times and resuspended in 1 to 2 ml. of 0·25 M-NaCl. The amount of cell protein was determined according to Lowry, Rosebrough, Farr & Randall (1951). One mg. of cell protein was sufficient for polyacrylamide gel electrophoresis.

Biochemical characters. Glucose fermentation was examined on agar plates containing 1 % (w/v) glucose and 0·0025 % (w/v) phenol red. Reduction of 2,3,5-triphenyltetrazolium chloride (TTC) and growth inhibition by methylene blue were tested according to Kraybill & Crawford (1965). Haemolytic activity of the mycoplasmas was tested by the method described by Somerson, Taylor-Robinson & Chanock (1963).

Serological characterization. Immune sera against the six reference Mycoplasma strains were prepared in rabbits by the method of Clyde (1964). All sera were stored at $-20°$ until used. Growth inhibition tests were done with impregnated filter-paper discs (Clyde, 1964). Each isolate was tested for growth inhibition by antiserum against each of the reference strains. Inhibition was recorded when colony-free zones were observed around the filter-paper discs; these zones usually measured 5–12 mm. from the edge of the disc.

Polyacrylamide gel electrophoresis. Washed whole organisms were dissolved in phenol + acetic acid + water (2 + 1 + 0·5, by vol.) and the dissolved material was run in polyacrylamide gels containing 35 % acetic acid and 5 M-urea (Rottem & Razin, 1967).

RESULTS

Fifty-five of the 95 sputum specimens, were positive for Mycoplasma (57·9 %). One of the isolated strains grew aerobically, fermented glucose, reduced TTC aerobically, haemolysed guinea-pig erythrocytes and was not inhibited by 0·02 % methylene blue,

Table 1. *Mycoplasma strains isolated from sputa of patients with various respiratory diseases as identified by growth-inhibition with monospecific antisera*

Species	No. of strains
Mycoplasma salivarium	48
M. orale type 1	4
M. pneumoniae	1
identification doubtful	2
Total	55

presenting the biochemical characters of *Mycoplasma pneumoniae.* All the other 54 isolates grew only anaerobically and did not show any of the characters described above. Table 1 summarizes the results of the growth inhibition tests by immune antisera. Forty-eight of the isolates (87 %) were identified by this method as *M. salvarium* and only four isolates (7·2 %) as *M. orale* type 1. The strain which presented the biochemical properties of *M. pneumoniae* was inhibited by the antiserum against *M. pneumoniae* only. Two strains could not be definitely identified because the zones of inhibition which they showed with antiserum against *M. salivarium* were very small compared to that obtained with the reference *M. salivarium* strain. They were not inhibited at all by any of the other immune sera.

The electrophoretic patterns of the cell proteins of the six reference strains were compared. Each of the species exhibited a characteristic pattern. Although the patterns of *Mycoplasma salivarium* and those of the *M. orale* type 1 and type 2 showed a certain similarity, a well-defined band near the top of the gel of *M. salivarium* which is characteristic for this species did not appear in either of the latter two (Fig. 1). Nineteen of the local isolates were selected and their electrophoretic patterns compared to those of the reference strains. Thirteen locally isolated strains which were inhibited by *M. salivarium* antiserum were examined by polyacrylamide gel electrophoresis and showed patterns identical to each other and to that of *M. salivarium.* All the isolates inhibited by *M. orale* type 1 antiserum showed a pattern identical with that of the reference *M. orale* type 1 strain. The electrophoretic patterns of *M. orale* type 1

always lacked the well-defined upper band characteristic of *M. salivarium*. Of the two Mycoplasma isolates that were only slightly inhibited by antiserum against *M. salivarium*, one exhibited a pattern identical with that of the reference strain of *M. salivarium*, whereas the other showed a somewhat different pattern. The electrophoretic pattern of the isolate identified biochemically and serologically as *M. pneumoniae* was identical to that of the reference *M. pneumoniae* strain.

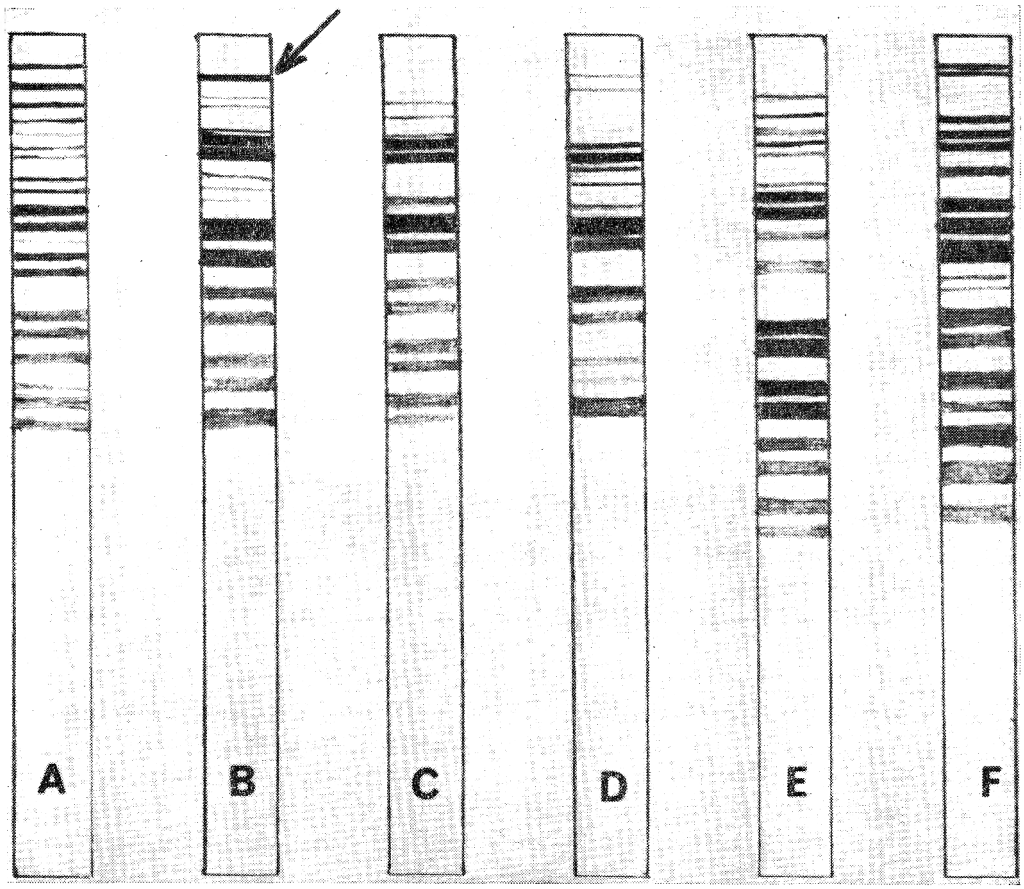

Fig. 1. Schematic representation electrophoretic patterns of the cell proteins of type strains of human mycoplasmas. A = *M. pneumoniae*, ATCC 15531; B = *M. salivarium*, ATCC 14277; C = *M. orale* type 1, ATCC 15539; D = *M. orale* type 2 (Chanock); E = *M. fermentans*, ATCC 15474; F = *M. hominis* type 1, ATCC 15056. The arrow indicates the characteristic upper band of *M. salivarium*.

The polyacrylamide gel electrophoresis patterns of the various mycoplasmas were reproducible in that identical patterns were obtained when the electrophoresis was repeated several times with different pools of the same strains.

DISCUSSION

The isolation of Mycoplasma strains other than *Mycoplasma pneumoniae* from the respiratory tract in health and disease has often been reported, but their role in

producing disease is as yet unconfirmed. *Mycoplasma hominis* type 1, however, was found to produce exudative pharyngitis in volunteers (Mufson *et al.* 1965). The mycoplasmas most frequently found in the oropharynx are *M. salivarium* and *M. orale* type 1. According to some authors, *M. orale* type 1 is found much more frequently than *M. salivarium* (Glezen, Thornburg, Chin & Wenner, 1967; Clyde, 1964), while others (Del Giudice, Robillard & Carski, 1967) found, as we did, a higher incidence of *M. salivarium* than of *M. orale* type 1. *Mycoplasma pneumoniae* is rarely found in the normal oropharynx. Its isolation is usually associated with disease. The strain isolated in the present study exhibited all the characteristics of *M. pneumoniae*. The person from whom it was isolated had complement-fixing antibodies against *M. pneumoniae* in a serum dilution of 1/100, but the diagnosis of primary atypical pneumonia was not confirmed clinically.

It is likely that the strains of *Mycoplasma salivarium* and *Mycoplasma orale* present in the sputum are salivary contaminants. The fact that they require an anaerobic or micro-aerophilic atmosphere for growth suggests that they might form part of the normal anaerobic flora of the gingival sulci. Evidence for this suggestion was presented before (Razin *et al.* 1964). Organick (1967) showed that the percentage of Mycoplasma isolates in bronchial swabs obtained by bronchoscopy was much lower than in pharyngeal swabs. Since the Mycoplasma isolates belonged to the same species in both cases, they suggested that the mycoplasmas occurring in the bronchial swabs resulted from contamination by pharyngeal secretions. Organick (1967) also claimed that the mycoplasmas do not play any causative role in chronic bronchitis.

Growth inhibition by specific antiserum yields highly specific results in the identification of Mycoplasma species (Hayflick & Chanock, 1965). Unfortunately this method has some disadvantages. It requires the preparation of potent immune sera and the results of the growth inhibition depend to some extent on the size of the inoculum. The polyacrylamide gel electrophoretic method used in this study seems to be adequate for the rapid identification of new isolates and eliminates the need for expensive antisera. The results obtained by the gel electrophoretic method are reproducible and correlate well with those obtained by the growth inhibition test. The electrophoretic pattern of *Mycoplasma salivarium* and *M. orale* types 1 and 2 resembled each other and formed a distinct group in comparison with the other human mycoplasmas. This finding suggests that the oral anaerobic mycoplasmas may be genetically related to each other, since the composition of cell proteins may reflect genetic relatedness. Complement-fixation tests showed a high level one way cross reaction between *M. salivarium* and *M. orale* (Taylor-Robinson *et al.* 1964). On the other hand, the few available results of nucleic acid homology determinations showed only a low degree of cross-reaction between the anaerobic oral mycoplasmas (Reich *et al.* 1966). The one strain which could not be definitely identified as *M. salivarium* by the growth inhibition test and which also differed from *M. salivarium* in its electrophoretic pattern seems nevertheless to be more closely related to *M. salivarium* than to the other human mycoplasmas. The possibility that this was a mixed culture cannot be ruled out, although the disc growth inhibition test did not indicate the presence of more than one type. These findings suggest the existence of different types of *M. salivarium* as was found for *M. hominis* type 1 (Reich, Somerson, Rose & Weissman, 1966). Further study is needed before any conclusion can be reached in this matter.

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Free Endotoxin and Non-toxic Material from Gram-negative Bacteria: Electron Microscopy of Fractions from *Escherichia coli*

By D. G. MARSH* AND P. D. WALKER

Departments of Bacteriology and Anaerobic Bacteriology, Wellcome Research Laboratories, Beckenham, Kent

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SUMMARY

Toxic and non-toxic fractions, isolated from culture supernatant fluids or from organisms of *Escherichia coli* O78K80, were examined with the electron microscope after negative staining with phosphotungstate. Preparations of endotoxin from the supernatant fluids ('free endotoxin') contained large numbers of rod-like particles, considered to be the native undegraded endotoxic lipopolysaccharide-protein complex. Phenol-water extraction of either free endotoxin particles or whole organisms caused aggregation of the complex, leading to chain-like structures. Both 'rods' and 'chains' were observed in an endotoxin preparation extracted from organisms by aqueous ether. A non-toxic predominantly polysaccharidic preparation from *E. coli* was not visible after negative staining; but this fraction, and to a lesser extent the toxic fractions derived from culture supernatant fluids, were found to contain well-defined fragments believed to be derived from the bacterial cell wall.

INTRODUCTION

An appreciable quantity of 'free endotoxin' is secreted into the culture fluid of a Gram-negative bacterium grown to a high population density under intensive growth conditions (Crutchley, Marsh & Cameron, 1967*a*). The isolation and properties of toxic and non-toxic lipopolysaccharide-protein complexes from the culture supernatant of a strain of *Escherichia coli*, O78 K80, have been described in detail (Marsh & Crutchley, 1966; Marsh & Crutchley, 1967; Crutchley, Marsh & Cameron, 1968*b*).

Purified free endotoxin (Fraction D2.S1; for detailed nomenclature of fractions see Marsh & Crutchley, 1967) was found to be indistinguishable biologically from conventional cell-wall endotoxin obtained by extracting Gram-negative bacteria with aqueous phenol according to the procedure of Westphal, Lüderitz, Eichenberger & Keiderling (1952) or with aqueous ether (Ribi, Haskins, Landy & Milner, 1961). Fraction D2.S1 had a much higher peptide content than purified cell-wall endotoxin preparations which had been deproteinated with phenol-water at 65° (Westphal *et al.* 1952; Ribi *et al.* 1964). The ratios of lipid to polysaccharide for all preparations of endotoxin from *Escherichia coli* were similar.

The highly purified free non-toxic fraction (D1.S1) had similar properties to the

* Present address: Gates and Crellin Laboratories of Chemistry, California Institute of Technology, Pasadena, California, U.S.A.

so-called 'native hapten', a protoplasmic component of *Escherichia coli* extracted by Anacker *et al.* (1964). These workers originally proposed that this substance was a structural element of endotoxin; but later work by Rudbach *et al.* (1967) clearly invalidated this hypothesis, in full agreement with the findings of Marsh & Crutchley (1967). Rudbach *et al.* (1967) redesignated this non-toxic product 'native protoplasmic polysaccharide' (NPP).

The present electron microscopic examination of fractions from *Escherichia coli* was undertaken with the aim of comparing the structures of 'free' and 'cell-wall' endotoxins, and also to investigate whether any structural relationship between non-toxic and toxic fractions could be observed. It was not possible to make this latter comparison because of failure to obtain pictures of the non-toxic fraction. However, electron microscopy revealed the presence of large fragments, believed to be derived from the cell wall, in many of the fractions examined.

METHODS

Lipopolysaccharide preparations. Escherichia coli O 78 K 80 was grown in a vigorously aerated casein sucrose medium and the resulting supernatant solution fractionated to yield non-toxic and toxic lipopolysaccharide-protein complexes, D 1 . S 1 and D 2 . S 1 respectively (Marsh & Crutchley, 1967). Loosely bound protein was removed from both fractions by a modification of the procedure of Westphal *et al.* (1952).

A 90 % phenol in water mixture (5 ml.) was added to a solution of fraction D 2 . S 1 or D 1 . S 1 (20 mg.) in water (5 ml.). The resulting mixture was shaken for 30 min. in a water bath at 65–68° and centrifuged at 4°. The aqueous layer was removed, dialysed against distilled water and freeze-dried (yields: 15–17 mg.).

Purified cell-wall endotoxic fractions P 1 and E, from aqueous phenol and aqueous ether extracts of bacteria harvested from the aforementioned culture of *Escherichia coli*, were prepared as described by Marsh & Crutchley (1967), by using modifications of earlier methods of Westphal *et al.* (1952) and Ribi *et al.* (1961).

Electron microscopy. Lipopolysaccharide preparations were examined by negative staining with phosphotungstate. A 1 % (w/v) solution of phosphotungstic acid, adjusted to pH 5·3 with 2 N-NaOH, was mixed with an equal volume of a 0·1 % aqueous solution of the lipopolysaccharide under investigation. The mixture was applied as a drop to a carbon-coated copper microscope grid and excess liquid carefully removed by blotting with filter paper. The sample was examined under a Phillips Model EM 200 electron microscope at a magnification of 71,000.

Preliminary experiments, with uranyl acetate as an alternative negative staining agent, showed that structural details on the complex molecules could be distinguished with a similar degree of resolution as with the phosphotungstate stain.

RESULTS AND DISCUSSION

Endotoxins

Plate 1, fig. 1, shows an electron micrograph of the crude material precipitated from the culture supernatant fluid by adding ammonium sulphate to saturation point. The rod-like particles visible in this picture were prominent in the purified preparation of free endotoxin derived from the crude precipitated material (Fraction

D2.S1; Pl. 1, fig. 2); these 'rods' are considered to be the toxic lipopolysaccharide-protein complex. Measurement of six well-defined rods, lying with their axes parallel to the plane of the grid, showed their average dimensions to be 295 ± 15 Å $\times 65 \pm 10$ Å. Substructural details, visible on certain particles (arrowed in Pl. 1, fig. 2), suggest that they may be composed of three segments. If one assumes the particles to be cylindrical with a partial specific volume, $\bar{V} = 0{\cdot}6$ ml./g. (see Schramm, Westphal & Lüderitz, 1952), a particle weight of about 1 million may be calculated. This figure is in good agreement with an estimate made from sedimentation studies on the complex (Marsh & Crutchley, 1967).

Extraction of Fraction D2.S1 with phenol–water produced linear aggregates of various degrees of polymerization (Pl. 2, fig. 3). Electron micrographs of this extract resemble those of the purified phenol–water extract (P1) from the parent intact bacterial cells (Pl. 2, fig. 4), although substructural details of the chains are not so well defined in this cruder preparation. Published electron micrographs of phenol-extracted endotoxin preparations from other serotypes of *Escherichia coli*, examined after heavy metal shadowing (Schramm *et al.* 1952; Weidel, Frank & Martin, 1960) or negative staining (Taylor, Knox & Work, 1966) show similar chain-like structures.

The aqueous ether extract (E) of the bacteria contained a heterogeneous mixture of rods and chains of various lengths (Pl. 3, fig. 5).

It has been postulated that the mild techniques used to extract and purify free endotoxin (Fraction D2.S1) yield essentially unaltered native endotoxin complexes, whereas chemical extraction of bacteria leads to degradation and aggregation of the native material (Marsh & Crutchley, 1967). A comparison of the electron micrographs of different preparations of endotoxin (Pls. 1–3) show these assumptions to be justified experimentally. Rod-like particles considered to be the native lipopolysaccharide-protein complex of endotoxin appeared to be the same in both crude and highly purified preparations of free endotoxin (Pl. 1, fig. 1, 2), showing that no apparent structural alteration took place during purification. On the other hand, aggregated material was produced by the removal of protein from the endotoxin complex during phenol–water extraction of purified free endotoxin (Pl. 2, fig. 3) or of bacteria (Pl. 2, fig. 4). 'Conventional aqueous ether endotoxin' (Pl. 3, fig. 5), prepared by a somewhat milder procedure, contains many rods together with some aggregated material. Ribi *et al.* (1964) showed that this type of preparation still contained a significant amount of protein. It therefore seems most probable that degradation, accompanied by the subsequent tendency towards aggregation, results mainly from the removal of protein from the endotoxin complex.

Schramm *et al.* (1952) showed that the degree of aggregation of the protein-free phenolic extract was affected significantly by altering the pH value of the solution: at highly alkaline pH values particularly marked aggregation occurred, whilst at more nearly neutral pH values monomeric 'pearls' were observed.

All phenol-extracted endotoxins from *Escherichia coli* O78 K80, prepared according to the method of Marsh & Crutchley (1967), were found to be aggregated when examined after negative staining at pH 5·3. At higher values (pH 9–11) aggregated material was also observed, although the quality of the stained preparations was greatly inferior to those examined at pH 5·3. At pH 5·3 a purified phenol-extracted endotoxin from *E. coli* O111 prepared by Professor Westphal (supplied through the courtesy of Dr C. G. Pope and D. Gall) was found to consist entirely of monomeric

'pearls' (Pl. 3, fig. 6) similar to those found in a comparable preparation from *E. coli* O8 (Schramm *et al.* 1952). At greater pH values both of these preparations were aggregated. Differences in the tendency of different preparations of phenol-extracted endotoxin to aggregate may be a feature of their different bacteriological serotypes or of variations in the preparation procedures, leading to products differing in their extent of degradation.

Schramm *et al.* (1952) concluded that the aggregated material possessed a 'pearl chain-like' structure. In certain of the aggregates examined in the present study (arrowed in Pl. 2, fig. 3) there is evidence of 'pearl chains', although the resolution is insufficient to demonstrate such structures unequivocably.

Endotoxin complexes have recently been described as 'rods and hollow spheres of varying sizes' (Knox, Cullen & Work, 1967); both the rods and the spheres have been described to have 'a bimolecular leaflet-like structure' (Shands, Graham & Nath, 1967; Rothfield & Horne, 1967). No such 'leaflets' were observed in the present study, probably due to differences in the endotoxin preparations examined. Indeed, it is clear from the recent studies by many workers that endotoxin complexes may present widely different aspects under the electron microscope, depending on their method of preparation. Evidence has been presented here that the endotoxin complex from a virulent strain of *Escherichia coli*, O78 K80, when released into the supernatant culture fluid, consists of a rod-like particle of molecular weight about 1 million. This entity can be further degraded by physical and chemical treatments to produce the complex structures often observed when cell-wall endotoxin preparations are examined with the electron microscope.

Non-toxic fraction

All attempts to reveal the non-toxic substance (Fraction D1.S1) by negative-staining proved to be unsuccessful. This finding is in agreement with experiments of Drs Ribi, Anacker, Brown, Haskins, Malmgren, Milner & Rudbach, reported by Rudbach *et al.* (1967), who have shown that their non-toxic fraction, NPP, extracted from bacteria, was a long narrow rod-like molecule (1300 Å × 16 Å).

Cell-wall fragments

Despite the lack of success in detecting the non-toxic fraction (D1.S1) under the electron microscope, all preparations of this substance were found to contain a very small percentage of larger fragments, heterogeneous in particle size, but exhibiting considerable structural similarity within themselves. These fragments were also detected in the unfractionated culture supernatants and occasionally in the toxic fraction (D2.S1). Photographs of some well-defined fragments are shown in Pl. 4, fig. 7, 8, 9, 10. Along their major axes, the fragments show a ridged structure, not generally penetrated by the staining agent. The ridges were occasionally observed to divide into two or more branches (e.g. lower inset, Pl. 4, fig. 7). It appears that the ridges constitute the main external supporting structure of the fragments and assist in containing the amorphous internal material, which is more easily penetrated by the stain (Pl. 4, fig. 10). The width of the ridges varies between about 14 and 30 Å. Plate 4, fig. 7 (lower inset), illustrates a branching of the 'ridges', the width of which lies between 14 and 17 Å. These dimensions suggest that the ridges may, in this case, be composed of a single-branched macromolecule.

Most probably the fragments are produced by the detachment from the bacteria of 'finger-shaped elements' which emanate from the cell wall of *Escherichia coli* (Bayer & Anderson, 1965). These authors also reported that 'sausage-shaped bodies' and 'droplets' were easily detached from the cell walls, particularly by exposing the ghosts to distilled water or 0·9 % NaCl solution.

The overall dimensions of the particles usually observed in the present work were 1250–1750 Å long × 150–230 Å. One complex particle (Pl. 4, fig. 8) was found to be 4110 Å in length, and diameters of about 80 and 300 Å have occasionally been observed. These are similar to those measured by Bayer & Anderson (up to 1500 Å long × 100–250 Å). In the latter case no structural detail was observed on the fragments. Bayer & Anderson (1965) considered the fragments to be lipoprotein in nature. Should this be the case, it may partly account for the small percentage of lipid and protein (1–2 % of each) found in the non-toxic fraction D1.S1 (Marsh & Crutchley, 1967) in which most of the fragments have been found in the present study.

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EXPLANATION OF PLATES

Preparations from cultures of *Escherichia coli* O78 K80

All preparations were negatively stained with phosphotungstate at pH 5·3. Horizontal lines shown in the bottom left-hand corners of photographs represent 1000 Å, except that in Pl. 4, fig. 10, which represents 250 Å.

PLATE 1

Fig. 1. Fraction precipitated from the culture supernatant fluid by saturated ammonium sulphate. × 213,000.

Fig. 2. Toxic fraction D2.S1, purified from the crude fraction shown in fig. 1 by chromatography on DEAE-cellulose and Sephadex columns. Substructure of rods indicated by arrow. × 213,000.

PLATE 2

Fig. 3. Toxic fraction D2.S1 after treatment with phenol water at 65°. Possible pearl chain-like structure indicated by arrow. × 213,000.

Fig. 4. Toxic fraction P1, obtained by purifying a crude phenol water extract of bacteria on Sephadex G100. × 213,000.

PLATE 3

Fig. 5. Toxic fraction E: conventional aqueous ether extract of bacteria. × 213,000.

Fig. 6. Westphal's toxic fraction purified from a phenol water extract of bacteria. × 213,000.

PLATE 4

Figs. 7, 8. Fragments considered to be derived from cell wall. × 213,000.

Fig. 9. Fragment from cell walls observed in a crude ammonium sulphate precipitate of the culture supernatant of *E. coli* O78 K80. × 213,000.

Fig. 10. Fragment shown in Fig. 9. × 710,000.

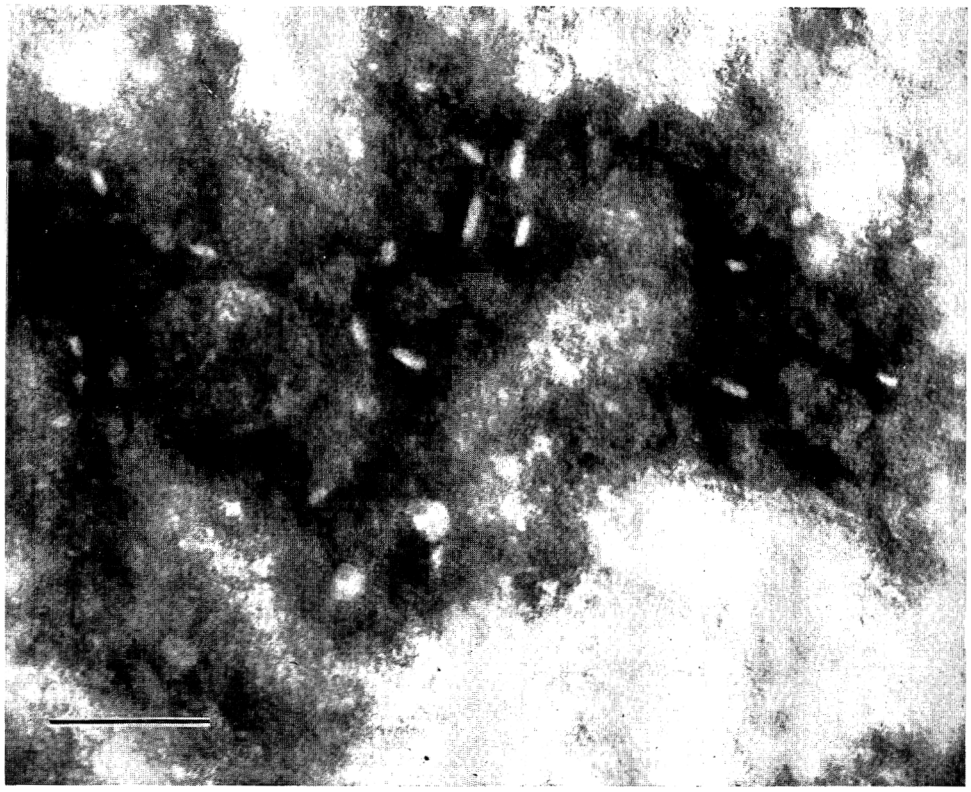

Fig. 1

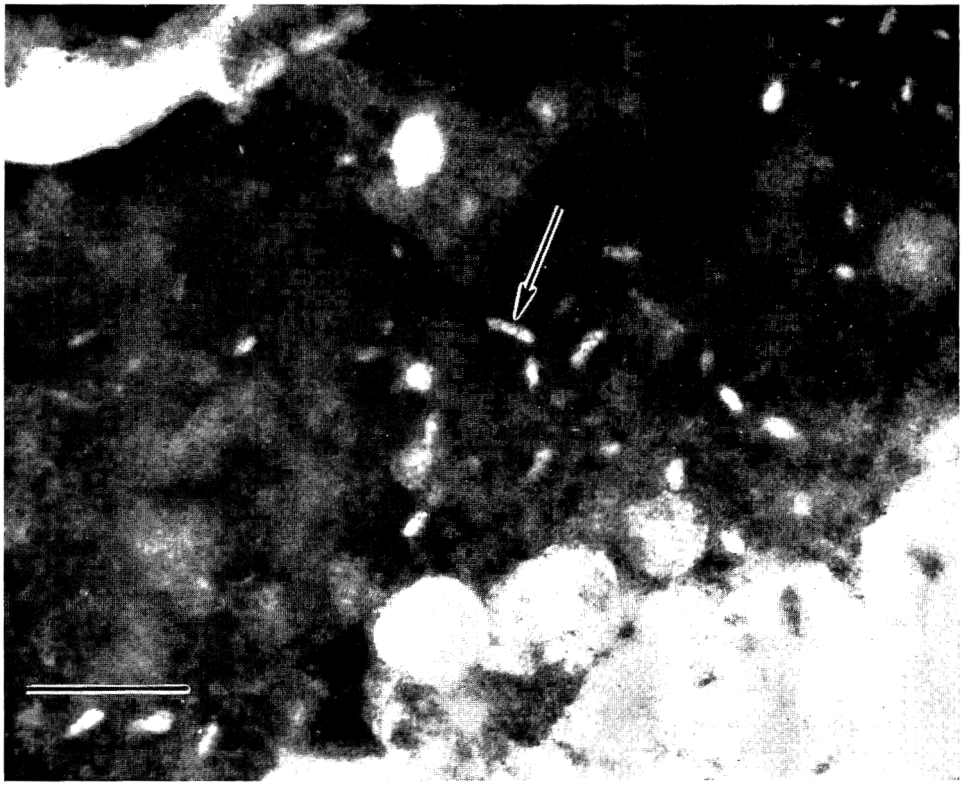

Fig. 2

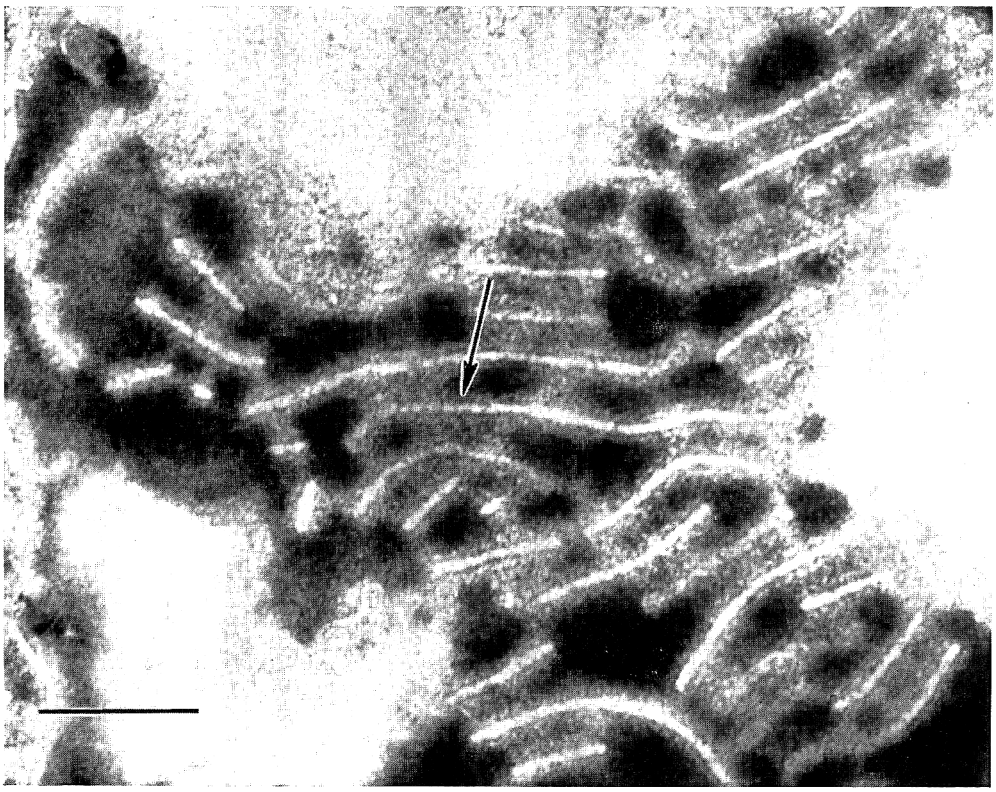

Fig. 3

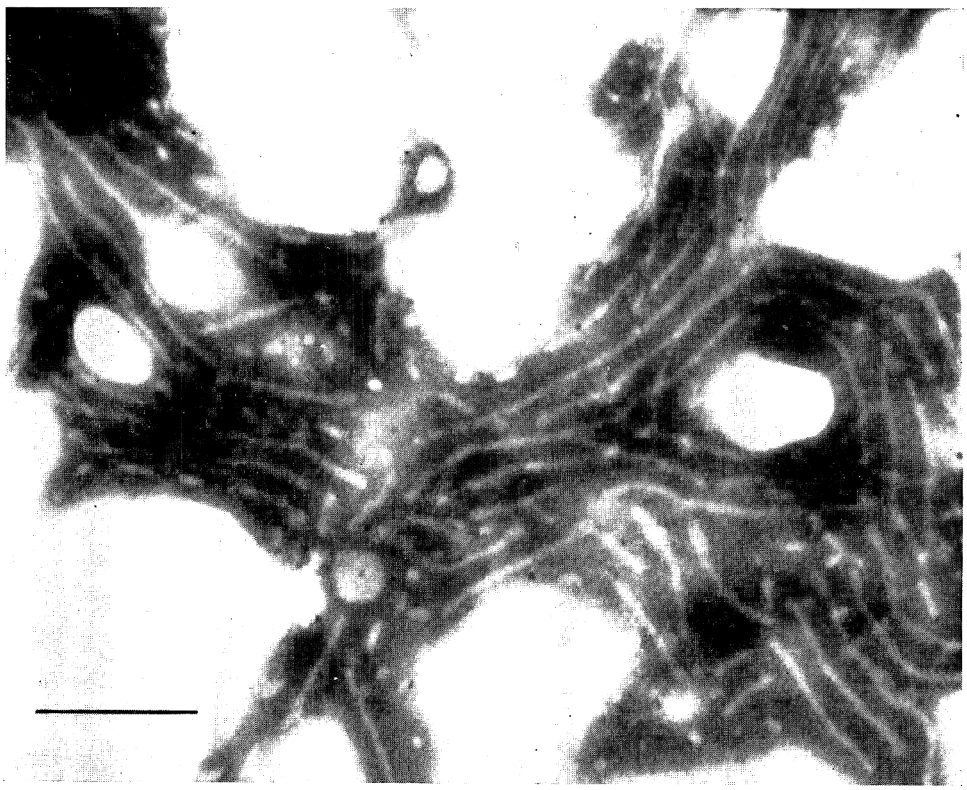

Fig. 4

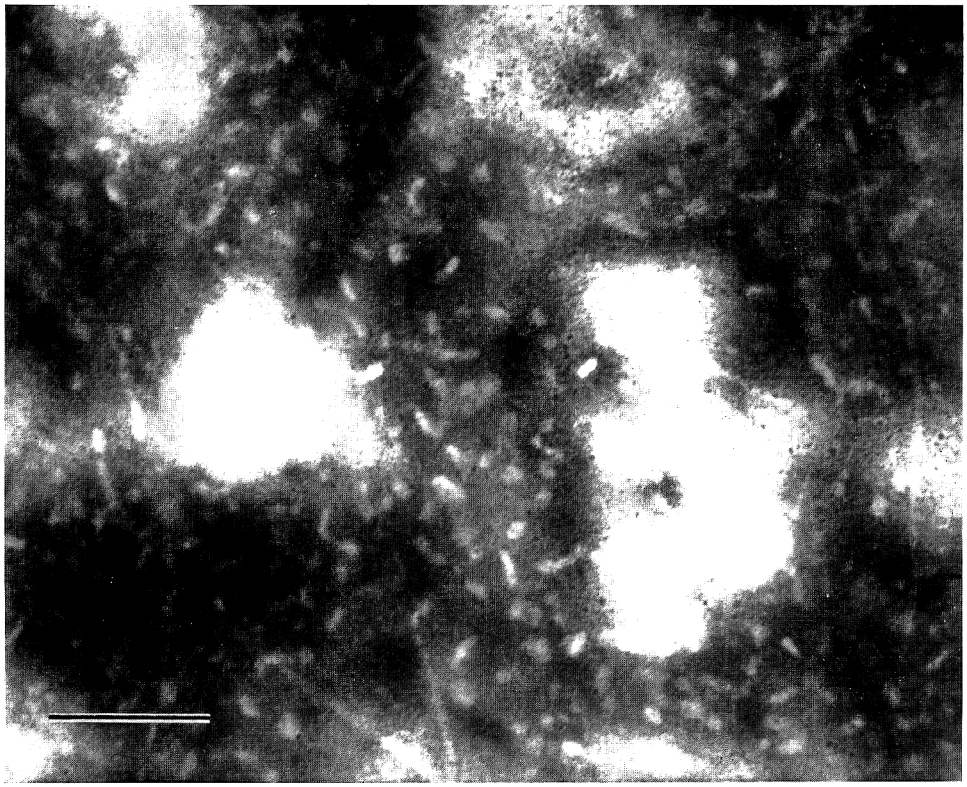

Fig. 5

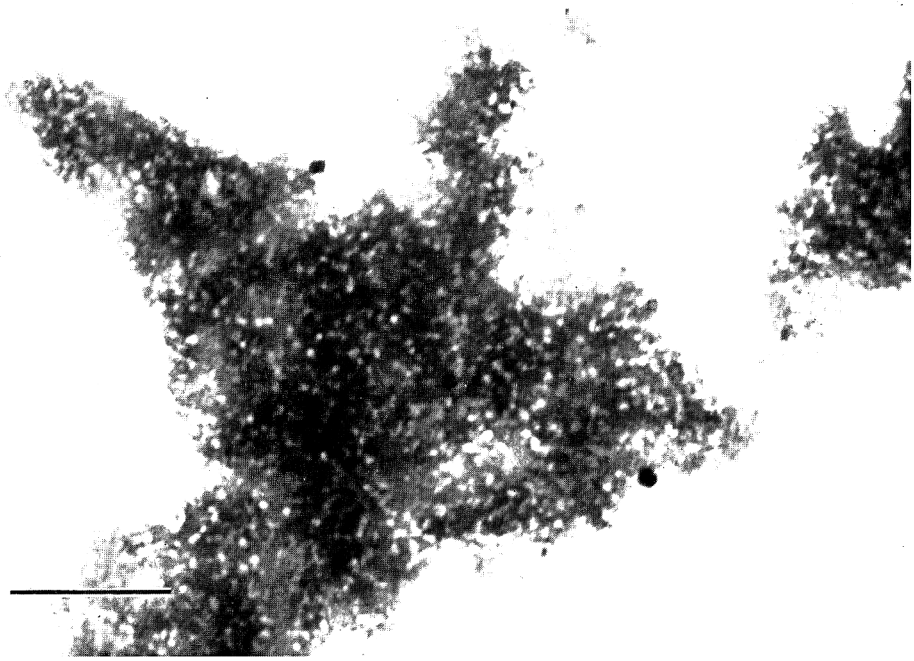

Fig. 6

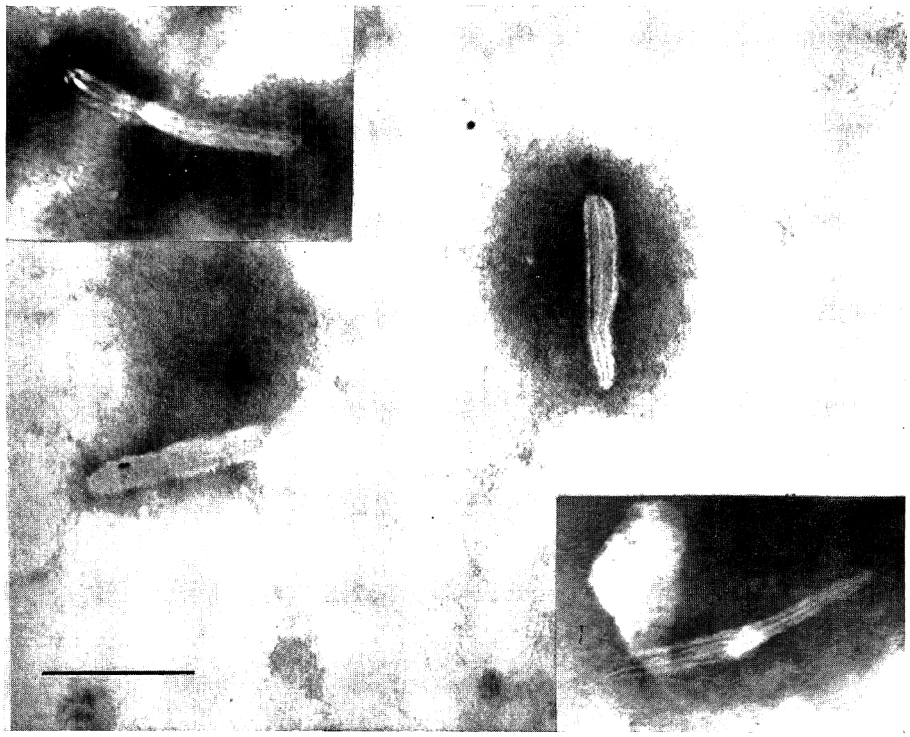

Fig. 7

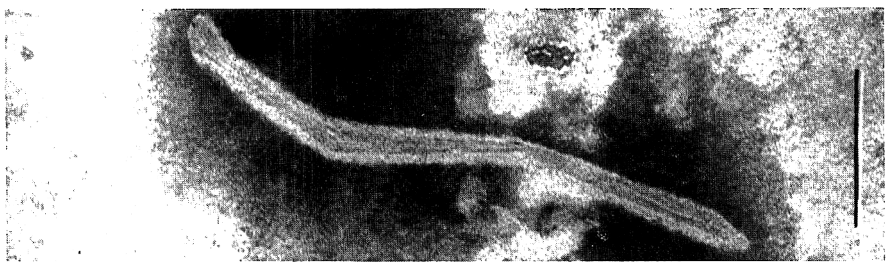

Fig. 8

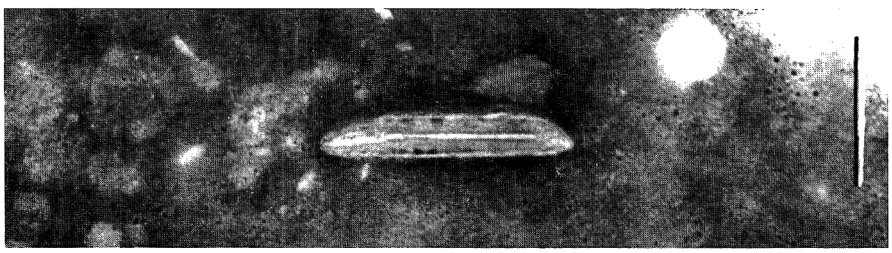

Fig. 9

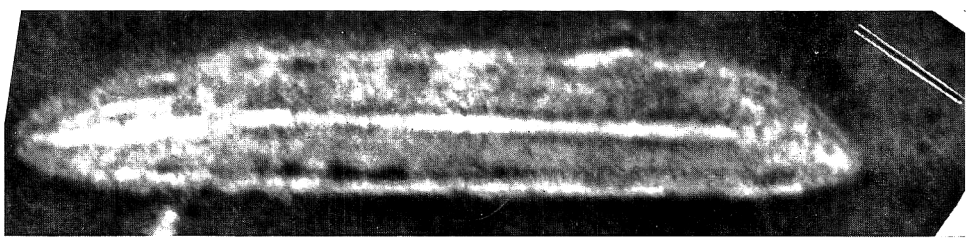

Fig. 10

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The Catabolism of Acyclic Polyols by Yeasts

BY J. A. BARNETT*
Low Temperature Research Station, Cambridge

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SUMMARY

Eight polyols were employed in turn as sole carbon source for aerobic growth tests with sixteen yeasts. The yeasts studied varied from those using none to others using all the polyols. Mean generation times in aerated, liquid, shaken medium for five yeasts and four polyols, were from 2 to 7·5 hr.

With seven yeasts, oxygen uptake was measured for different polyols as sole carbon source, of washed, starved yeast cells, harvested in the exponential phase of growth. No yeast respired a substrate on which it did not grow, or vice versa. Except when glucose, erythritol or galactitol (dulcitol) were employed, respiration rates were not greatly affected by the carbon source for growth.

Coenzyme-linked polyol dehydrogenase activity was measured with crude cell-free extracts of four yeasts and several polyols or sugars. The enzymes had a low affinity for their substrates. In certain cases the polyol oxidation products were examined chromatographically.

The polyol dehydrogenases of four yeasts were separated from crude cell extracts by gel electrophoresis, and detected on the gels by their activity with different polyols, and with NAD^+ or $NADP^+$. One strain of *Torulopsis candida* appears to synthesize at least eight polyol dehydrogenases which differ in their specificity for polyols, coenzyme or inducer. Similarly, four polyol dehydrogenases were found in a strain of *Candida utilis*. Most of these enzymes were inducible.

There was no evidence that phosphorylation was the first step in polyol catabolism.

From experiments with ^{14}C-labelled polyols, it seems that *Candida utilis* and *Pichia membranaefaciens* do not utilize D-glucitol (sorbitol) or D-mannitol because these substrates do not enter the yeast cells.

INTRODUCTION

Differences between yeasts in their ability to use polyols as sole carbon sources for aerobic growth have been employed for classifying and identifying yeasts (e.g. Wickerham, 1951; Kudriavzev, 1954; Kreger-van Rij, 1964; Poncet & Arpin, 1965).

Work on the metabolism of the acyclic polyols has been reviewed by Edson (1953), Touster & Shaw (1962), Hollmann & Touster (1964), Lewis & Smith (1967*a*) and Barnett (1968). The following three kinds of enzymatic reaction are capable of initiating a metabolic attack on polyols.

(*a*) Polyol + NAD^+ (or $NADP^+$) → ketose + NADH (or NADPH) + H^+.
(*b*) Polyol + $NADP^+$ → aldose + NADPH + H^+.
(*c*) Polyol + ATP → polyol phosphate + ADP.

* Present address: Agricultural Research Council Food Research Institute, Earlham Laboratory, Recreation Road, Norwich, Norfolk.

The polyol phosphate, formed in reaction (*c*), may be oxidized to give a sugar phosphate by reactions analogous to (*a*) and (*b*). The sugars or sugar phosphates formed from the polyols are probably metabolized by the Embden–Meyerhof pathway or the pentose cycle.

All-or-none differences in the ability of yeasts to utilize polyols presumably reflect the presence or absence of (i) enzymes necessary to metabolize the polyols, or (ii) mechanisms for the entry of the polyols into the yeast cells. The present paper reports investigations of biochemical systems which may underlie differences between yeasts in their utilization of polyols.

The aim of this work was to study a number of yeasts and to relate differences in the behaviour of whole organisms to differences between their internal mechanisms. There are, however, special difficulties in showing why one yeast uses a particular substrate for growth and respiration, and another does not. For example, *Pichia delftensis* can grow on ribitol, D-glucitol or D-mannitol, whilst *P. membranaefaciens* cannot grow on any of the polyols (Table 1). Beech (1965) used this difference to distinguish these two species. Now, although succinate-grown *P. delftensis* may be compared with succinate-grown *P. membranaefaciens*, one or more enzymes essential for the catabolism of, say, D-glucitol may be inducible so that it is necessary to examine D-glucitol-grown *P. delftensis* in order to find out how the yeast metabolizes D-glucitol. For such reasons, a single strain of *Torulopsis candida* (NCYC 576), chosen for its polyol-using versatility (Table 1), was studied extensively.

Candida utilis (LTS 23) was also studied for two reasons. First, *C. utilis* contrasted with *Torulopsis candida* in using no polyols (Table 1). Secondly, Horecker and his colleagues (Chakravorty *et al.* 1962) had already examined the polyol metabolism of this strain using enzyme purification methods, and it was considered that their results would be useful for comparison with those obtained here with unfractionated extracts.

A possible reason for a yeast's inability to use a polyol is the absence of enzymes capable of catabolizing the product of the oxidation of the polyol. For example, Horecker (1962*a*) drew attention to the absence of kinase activity with either ribose or *erythro*-pentulose in both *Candida utilis* and *C. albicans*. Neither species can utilize ribose for growth (Dr B. L. Brady, 1965: personal communication), yet extracts of both yeasts could interconvert D-ribose and D-*erythro*-pentulose via ribitol (Horecker, 1962*a*):

$$\text{D-ribose} \rightleftharpoons \text{ribitol} \rightleftharpoons \text{D-}erythro\text{-pentulose}$$

Provided that ribitol enters these yeasts, lack of kinases may be said to block the utilization of ribitol.

METHODS

Yeasts

The following organisms have been used in this work: *Candida utilis* NCYC 359 and LTS 23 (received from B. L. Horecker); *Debaryomyces phaffii* (CBS 4346); *D. vanriji* NCYC 577 (received from V. I. Kudriavzev as *D. konokotinae*); *Hansenula anomala* CBS 1981; *Kluyveromyces* (*Saccharomyces*) *drosophilarum* CBS 2896 (received from V. I. Kudriavzev as *Zygofabospora krasilnikovi*); *Pichia delftensis* LTS 24 (received from F. W. Beech); *P. membranaefaciens* NCYC 54; *Saccharomyces acidifaciens* CBS 749; *S. cerevisiae* NCYC 231; *S. rouxii* CBS 732; *S. rouxii* var. *polymorphus* CBS 713; *Torulopsis candida* NCYC 576 (or *T. famata*, received from V. I. Kudriavzev as

Table 1. *Auxanographic growth tests on sixteen yeasts*

The yeast was washed off a slope into 10 ml. of water. For each auxanogram, 0·5 ml. of this suspension was added to a tube containing 12 ml. of melted agar medium without carbon source, at 47°. After mixing, the medium was poured into a Petri dish, cooled, and the surface dried in an incubator. Test carbon sources were placed on the agar surface of each plate, which was incubated and examined daily for 4 days. The results are given in terms of + (growth) and − (no growth); n, means not tested.

Yeast	Substrate															
	Erythritol	Ribitol	D-Arabinitol	L-Arabinitol	Xylitol	D-Glucitol	Galactitol	D-Mannitol	L-Arabinose	D-Arabinose	D-Xylose	D-Ribose	L-Sorbose*	D-Tagatose	Succinate	Acetate
Saccharomyces cerevisae (NCYC 231)	−	−	−	−	−	−	−	−	n	n	n	n	−	−	−	+
S. rouxii v. *polymorphus* (CBS 713)	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	n
Candida utilis (NCYC 359)	n	−	−	−	−	−	−	−	−	−	+	−	−	−	+	+
C. utilis (LTS 23)	−	−	−	−	−	−	−	−	−	−	+	−	−	−	+	+
Pichia membranaefaciens (NCYC 54)	−	−	−	−	−	−	−	−	−	−	−	−	+	−	+	−
S. rouxii (CBS 732)	−	−	−	−	n	+	−	+	n	n	n	n	−	−	+	n
Kluyveromyces drosophilarum (CBS 2896)	−	−	−	−	+	+	−	+	−	−	−	−	n	−	+	+
Trigonopsis variabilis (NCYC 378)	−	−	−	−	+	−	−	+	−	−	−	−	n	−	+?	n
S. acidifaciens (CBS 749)	−	+	+	−	−	+	−	+	−	−	−	−	−	−	−	n
Hansenula anomala (CBS 1981)	+	−	+	−	−	+	−	+	−	−	−	−	−	−	+	+
P. delftensis (LTS 24)	−	+	+	−	−	+	−	+	−	−	−	−	n	−	+	+
Torulopsis capsuligenus (CBS 4736)	−	+	+	−	+	+	+	+	−	−	−	−	+	−	+	n
T. pseudaeria (LTS 19)	−	+	+	+	−	+	+	+	n	n	+	n	n	−	−	n
T. candida (NCYC 576)	+	+	+	+	+	+	+	+	+	−	+	+	+	−	+	n
Debaryomyces vanriji (NCYC 577)	+	+	+	+	+	+	+	+	+	−	+	+	+	−	+	n
D. phaffii (CBS 4346)	+	+	+	+	+	+	+	+	−	−	+	−	+	−	+	n

* L-*xylo*-Hexulose.

Debaryomyces mucosus); *Torulopsis capsuligenus* CBS 4736; *T. pseudaeria* LTS 19 (received from J. Zsolt); *Trigonopsis variabilis* NCYC 378.

Each strain of yeast was taken from a single colony isolated after streaking out twice on Y–M agar in a Petri dish (*Topley & Wilson's Principles*, 1964). After incubation, stock slopes of each strain were kept at +1° and subcultured not less often than every 6 months (cf. Kirsop, 1954). The identity of each strain was checked at least by (i) microscopic examination, and (ii) auxanographic tests of its ability to use certain carbon compounds (e.g. Lodder & Kreger-van Rij, 1952).

Media

The following media were used to cultivate the yeasts: (i) Difco Bacto Y–M agar (pH 6·2); (ii) A2 (pH 4·8) a chemically defined, carbon-free medium (Wickerham & Burton, 1948; modified by Barnett & Ingram, 1955), plus a source of carbon usually at 50 mM. Succinate was added as the sodium salt in solution at pH 4 and acetate as ammonium acetate. Vitamins and glucose were filter-sterilized and added aseptically to the autoclaved medium (120° for 15 min.). Autoclaved carbon compounds were sterilized separately. 2 % (w/v) Difco Special Agar-Noble was added to certain media.

Growth rate

For determination of growth rates, T-tubes (Kay & Fildes, 1950; Barnett & Ingram, 1955) of 40 ml. total capacity, containing 10 ml. A2 medium with 0·5 mmole test substrate, were inoculated with 0·1 ml. yeast suspension (about 10 μg. dry weight of yeast). The suspension was prepared by suspending the growth from an A2 agar slope to a suitable density. The tubes were rocked at 1 cycle/sec. at 25°. Growth was followed with either an E.E.L. nephelometer or automatically with a recording nephelometer (Dr H. Laser, private communication) and the readings converted to mg. dry weight by means of a standard curve.

'Induction or selection' experiments

For each strain, a culture was grown for about 18 hr in 10 ml. medium A2 with D-glucose as carbon source, in a T-tube as described above. The yeast, treated aseptically, was then washed twice in 10 ml. 0·1 M-KH_2PO_4 and resuspended in 100 ml. 0·1 M-KH_2PO_4. Plates containing 15 ml. A2 agar, with 750 μmoles of a polyol or glucose, were inoculated from serial dilutions of this suspension in 0·1 M-KH_2PO_4, and colonies were counted after 2 days at 25°.

Auxanograms

The auxanographic methods used were similar to those described by Barnett & Kornberg (1960).

Manometry

The methods used for these experiments correspond closely to those used by Barnett & Kornberg (1960). Yeast from a 1-day-old slope was washed into 100 ml. medium A2 (+appropriate carbon-source) in a 1 l. Pyrex Fernbach culture flask. The flask was rocked at 1 cycle/sec. through an angle of 12° (6° either side of horizontal) for 20 hr. During this time air, passed through 5 % (v/v) H_2SO_4 and sterilized by cotton-wool filters, was passed through the upper part of the flask at 5 l./hr.

Aseptically, the contents of the Fernbach flask were centrifuged and the yeast

washed and re-suspended in 100 ml. 0·1 M-potassium phosphate buffer (pH 5·8). To reduce the endogenous rate of oxygen uptake the suspension was replaced in a Fernbach flask and rocked and aerated for 4 hr. The yeast cells were then centrifuged and re-suspended in 0·1 M-potassium phosphate buffer (pH 5·8) at a final concentration of about 2 mg. dry wt/ml.

Oxygen uptake on addition of substrates was measured with single side arm Warburg manometers containing 2 ml. yeast suspension in the main compartment, 20 μmoles substrate in 0·2 ml. water in the side arm, and a strip (3·5 × 1 cm.) of Whatman no. 40 filter paper and 0·2 ml. 2 N-KOH in the centre well. The gas phase in the manometers was air and the flasks were equilibrated at 25° for 10–20 min. before the contents of the side arms were mixed with those of the main compartments. After each experiment the contents of every flask were examined microscopically to ascertain that there were no bacterial contaminants.

Substrate penetration experiments

For experiments on the penetration of a substance into a yeast which did not use it, the yeast was grown on a suitable carbon source in the presence of that substance (0·025 M). This procedure allowed for cases where the mechanism for translocation might be inducible, as for maltose with *Saccharomyces cerevisiae* (Harris & Thompson, 1961). Otherwise growth, harvesting and starving the yeast suspension were performed exactly as described for the manometric experiments.

Incubation. Nine ml. of the yeast suspension (about 3 mg. dry weight of yeast/ml.) in 0·1 M-potassium phosphate buffer (pH 5·8) were placed in each of two 100 ml. Erlenmeyer flasks (A and B) containing a glass-covered rod magnet. Flask A was held in a water bath at 30° and flask B in a vessel packed with ground ice. The second flask allowed for radioactive material adsorbed by the cell walls and outside the cell membranes (Cirillo, 1961). Both flasks were over magnetic stirrers to keep the suspensions mixed and aerated. After 10 min. equilibration, to each flask were added at zero time either: (i) 0·1 ml. M-D-glucitol and 1·0 ml. [U-^{14}C]D-glucitol (10 μC, 1·2 μmoles/ml.), or (ii) 0·1 ml. M-erythritol and 0·5 ml. [U-^{14}C]erythritol (10 μC, 0·5 μmoles/ml.). Samples (1·0 ml.) were taken from each flask as near as practicable to zero time (within 20 sec.) and thereafter at 15 min. intervals for 1 hr. Each sample was sucked quickly through a membrane filter (MF 50, Membranfilter GmbH, Göttingen, diam. 30 mm.). The retained yeast was quickly washed twice with 1 ml. water at 1° (cf. Cirillo & Wilkins, 1964; Kotyk & Kleinzeller, 1963) and the membrane, with the yeast *in situ*, was removed immediately and transferred to a counting vessel containing 15 ml. toluene with 0·3 % (w/v) PPO (2,5-diphenyloxazole) at 0°. The counting vessels were glass bottles (27 mm. diam. 48 mm. height) with plastic screw caps (Wheaton Vials, Ekco Electronics Ltd.). Samples were stored at 0° until counted with a Packard Tri-carb coincidence liquid scintillation spectrometer (55–66 % maximum efficiency) and were counted at least twice for 30 min. at approx 2°. No corrections were made for quenching or for coincident disintegrations.

Cell-free extracts

For preparation of cell-free extracts 1 litre A2 medium, containing a suitable source of carbon, was inoculated with 100 ml. of a culture in a similar medium and

incubated at 25° in a rotating (100 to 110 rev./min.) 10 l. flask (Mitchell, 1949). After 20 hr, the yeast was harvested by centrifugation at about 10,000 *g* for 30 min. at −3° and stored at −20°.

Extracts were prepared with a Nossal disintegrator (Nossal, 1953). A closed stainless steel capsule of 20 ml. capacity contained 2–4 g. wet weight of frozen yeast, 10 g. washed ballotini no. 12 (Jencons Ltd.), 100 μmoles tris/g. of yeast and 7·5 ml. water. For some experiments 1 mmole sodium phosphate (pH 7·0 at 18°) was used in place of tris. The capsule was shaken reciprocally at room temperature for 30 sec. at about 6000 cycles/min., 15 mm. travel. During shaking, the temperature of the capsule's contents rose from 0° to between 5° and 10°. The capsule was carried to and from the disintegrator in ice. The suspension of disintegrated material was at about pH 7.

The suspension was centrifuged at about 10,000 *g* for 15 min. at −3° and the supernatant fluid centrifuged again in a refrigerated preparative ultracentrifuge (Spinco) at about 100,000 *g* for 2 hr. Without such severe centrifugation, electrophoresis was unsatisfactory; material, perhaps nucleoprotein, accumulated at the origin slot and interfered with the free-running of the protein.

The supernatant was dialysed for 4 hr. at 1° against 1000 ml. of 2 mM-tris HCl (pH 7·8), to which 1 mole of sucrose was added (final concentration 0·82 M) when the extract was prepared for electrophoresis. For certain experiments, 5 mM-sodium phosphate (pH 7·0) was used instead of tris buffer.

Estimation of protein

For routine purposes the concentration of protein in the cell-free extract was estimated by the method of Warburg & Christian (Layne, 1957), though in certain cases by that of Lowry, Rosebrough, Farr & Randall (1951).

Measurement of dry weight

For yeast suspensions in phosphate buffer, samples of from 2 to 10 ml. (as large as practicable) of the suspension were pipetted into weighed tubes or beakers and dried at 103–105° to constant weight. For yeast suspensions in growth medium with carbon source, a similar procedure was followed except that the samples were pipetted into weighed centrifuge tubes in which they were finally dried. Before drying, the samples were centrifuged, washed twice and re-suspended in buffer.

Measurements of enzyme activity

Coenzyme reduction and oxidation by crude extracts in the presence of polyols, ketoses or aldoses, were followed spectrophotometrically by measuring changes in extinction at 340 mμ with a Beckman automatic recording spectrometer (DK 2). For most experiments, the assay mixture contained 200 μmoles buffer [glycine pH 9·0 for NAD(P) reduction or phosphate pH 6·8 for NAD(P)H oxidation], 1·0 mmole substrate (polyol, ketose or aldose), 0·1 ml. enzyme-containing extract (about 0·4 mg. protein), 4·5 μmoles $NAD(P)^+$ or 0·45 μmoles NAD(P)H, and water to 3·0 ml. The reference cuvette contained water only. Reactions were started by addition of coenzymes. Recordings were begun within 10 sec. of the addition, and were usually continued for 3 min. In every experiment, control recordings were made without added substrate, without added coenzyme and without added extract. Assays were

carried out at 30° using a constant temperature cell and $E_{340m\mu}$ values were converted to NAD(P)H concentrations using $\epsilon_{mM} = 6{\cdot}22$.

Products of polyol oxidation

The reactions were carried out in T-tubes containing 750 μmoles tris buffer (pH 8·0), 0·5 μmoles polyol, 2·0 ml. crude extract (approx. 10 mg. protein), 5·0 μ-moles NAD^+ or $NADP^+$, 200 μmoles sodium pyruvate, about 400 units crystalline lactate dehydrogenase (capable of oxidizing about 0·2 μmoles NADH/min. at 25° and pH 7·5) and water to 10 ml. Extract or coenzyme was omitted from control tubes. Pyruvate and lactate dehydrogenase were included to reoxidize the coenzyme (cf. Fossitt, Mortlock, Anderson & Wood 1964). The tubes were covered and shaken gently for 3 hr. at 30°.

At the end of the incubation the mixture was checked for absence of microbial contamination, acidified to pH 5·5 or 6·0 with about 10 μl. acetic acid, heated for 2 min. at about 100°, cooled, and centrifuged at about 30,000 *g* for 30 min. The clear supernatant fluid was put through about 2 g. cation exchange resin (200–400 mesh AG 50 W-X 8 Bio Rad H^+ form) and the effluent dried under reduced pressure in a rotary evaporator at 40–50°.

Chromatography. The residue was taken up in about 1 ml. water and applied in 1 μl. quantities to a silica-gel layer, made up in 0·1 M-boric acid and spread on glass as a layer 250 μ thick. Authentic sugar samples were also applied in different positions. The chromatograms were run vertically for a distance of 150 mm. with one of the following solvents: ethyl acetate + pyridine + water (10 + 4 + 3, by vol.), *n*-butanol + acetone + water (4 + 5 + 1, by vol.), *n*-butanol + ethanol + water (4 + 1 + 5, by vol.), or water-saturated phenol. The chromatograms were dried at laboratory temperature in a strong stream of air and were usually run again in the same solvent exactly as before. They were then treated to locate carbohydrates with the periodate-benzidine reagent of Gordon, Thornburg & Werum (1956) or with slightly modified naphtho-resorcinol or aniline phthalate reagents of Hollmann & Touster (1964) in which ethanol and water-saturated *n*-butanol were replaced with an equal volume of methanol or methanol + water (80 + 20, v/v) respectively. Boric acid, when present in the silica-gel layer, interfered with the action of these reagents (cf. Lewis & Smith, 1967*b*) and methanol prevents this by removing boric acid as its volatile methyl ester (Wolfrom & Thompson, 1963). At least two (usually three) solvents were used for each identification. In two experiments (oxidation of D-glucitol by extracts of succinate- and D-glucitol-grown *Torulopsis candida*), chromatographic identification of fructose and *xylo*-hexulose was confirmed by means of the cysteine-carbazole reaction of Dische & Borenfreund (1951) and the cysteine-sulphuric acid reaction of Dische & Devi (1960).

Gel electrophoresis

The best separations of the polyol dehydrogenases were obtained by vertical starch gel electrophoresis (Smithies, 1959), using a modification of the discontinuous buffer system of Poulik (1957) at pH 7·8. The use of M-urea to stabilize the starch gels at low temperatures and also operation at pH 8·6 each led to inactivation of the polyol dehydrogenases. The buffer discontinuity was achieved by using two different gel-buffers. The two end gel blocks were of borate buffer (Scopes, 1963), between which

was poured an inner gel containing tris-DETPA (diethylenetriamine penta-acetic acid) buffer (Scopes, 1964). This inner gel included the sample slots.

Inner gel buffer (pH 7·8 at laboratory temperature, i.e. about 18°) consisted of 2 mM-DETPA + 6·7 mM-tris. The solution of DETPA was prepared by the method of Scopes (1964).

Outer gel buffer (pH 7·8) consisted of 0·2 M-boric acid + 0·053 M-tris + 2 mM-HCl.

The top (cathode) tray buffer (pH 8·6) contained 0·2 M-boric acid + 0·05 M-NaOH + 0·03 M-NaCl.

The bottom (anode) tray buffer (pH 8·6) was 0·1 M-tris + 0·025 M-H_2SO_4.

Most of the extracts were also run in an alternative continuous buffer system, composed of *gel buffer* (pH 7·0): 3 mM-Na_2HPO_4 + 0·4 mM-citric acid (cf. Fine & Costello, 1963) and *tray buffer* (pH 7·0) of 30 mM-Na_2HPO_4 + 4 mM-citric acid.

The starch gels were prepared as follows. *Outer gel*: 25 g. hydrolysed potato starch were mixed with 250 ml. outer gel buffer in a 1000 ml. conical flask. The buffer and starch were heated over a Bunsen burner with continuous agitation. The gel first thickened and then liquefied. Heating was continued until the gel was almost boiling, and the dissolved air was removed by boiling under reduced pressure for about 30 sec. The two end-blocks of the gel tray were then filled with the gel, the latter was prevented from filling the centre part by pieces of perspex. After 30 min., the pieces were removed and the inner gel was poured. *Inner gel*: 21·5 g. starch were mixed with 180 ml. inner gel buffer, heated as described for the outer gel, and poured into the tray between the two outer gels. The slot-former was immediately put in place towards one end of the inner gel. Usually 16 slots were used of 3 mm. width (capacity 10 μl.), 4 mm. apart; but 10 slots of 7 mm. width (capacity 25 μl.), 4 mm. apart were used for some experiments. After 30 min., the whole gel was covered with a sheet of polythene to prevent drying; it was kept overnight at 10°, and put in a room at 1° for 30 min. before the extracts were inserted.

Dialysed, concentrated extract (about 15 mg. protein/ml.) was inserted into each slot as a free solution and the slots sealed with liquid petroleum jelly, mixed with CCl_4 to lower the melting point. Bovine serum albumin, stained with excess naphthalene black and then dialysed against distilled water at 1° to remove excess stain, was added to the concentrated extracts at a final concentration of 0·05 mg./ml. extract. The stained albumin acted as a visible marker throughout electrophoresis and served as a guide when cutting the gel into strips and as a reference substance for the identification of enzyme bands by their relative positions.

To prevent evaporation during electrophoresis, the gel was covered with a thin sheet of polythene held in position by petroleum jelly. Electrophoresis was carried out vertically for 3·5–5 hr in a constant temperature room at 1° at about 10 V/cm. Initially the current was about 24 mA, falling to about 18 mA during electrophoresis. The temperature of the gel did not rise above 10°.

Polyacrylamide gels were prepared as described by Raymond (1964) and Lund (1965) and were used with the starch-gel buffers at half the above concentrations.

Buffers

The recipes used for buffers were based on those given by Gomori (1955) and by Datta & Gryzbowski (1961). The buffers are listed below; the concentrations in which they were used are given with the specific experimental methods:

pH range	Buffer system
1·5–2·0	Hydrochloric acid + potassium chloride
2·1–3·5	Glycine + hydrochloric acid
3·6+4·5	Formic acid + sodium formate
5·0–5·6	Potassium hydrogen phthalate + sodium hydroxide
5·0–6·0	Citric acid + *di*-potassium hydrogen orthophosphate
5·8–7·5	Potassium dihydrogen orthophosphate + *di*-potassium hydrogen orthophosphate
7·6–8·6	Tris (hydroxymethyl) aminomethane + hydrochloric acid
8·8–10·5	Glycine + sodium hydroxide

Chemicals, enzymes and coenzymes

Most of the chemicals were obtained from British Drug Houses; and, where possible, they were of Analar grade. D-mannitol was recrystallized twice from water. D-glucitol was recrystallized twice from the pyridine-glucitol complex (Strain, 1934, 1937), and then recrystallized twice from methanol. Galactitol was recrystallized twice from water. L-iditol was a gift from Dr F. A. Isherwood. Ribitol and erythritol were recrystallized twice from ethanol, then twice from water by the addition of acetone. Xylitol was prepared by reduction of D-xylose with sodium borohydride and was recrystallized twice from methanol (Hayward & Wright, 1963). D- and L-Arabinitol (obtained from L. Light and Co. Ltd.) were recrystallized twice from methanol. The melting-point and optical rotation were checked on each preparation.

Uniformly labelled polyols [U-^{14}C]D-glucitol and [U-^{14}C]erythritol, were obtained from the Radiochemical Centre, Amersham. D-mannitol 1-phosphate and D-glucitol 6-phosphate were prepared by the sodium borohydride reduction of D-mannose 6-phosphate and D-glucose 6-phosphate, respectively (Wolff & Kaplan, 1956).

D-*Erythro*-pentulose was prepared by reacting its *o*-nitrophenylhydrazone (obtained from Calbiochem Ltd.), with benzaldehyde (Schmidt & Treiber, 1933). D-*threo*-Pentulose, and L-*glycero*-tetrulose were gifts from Dr R. J. Ferrier, and D-erythrose was obtained from Sigma Chemical Co.

Sodium pyruvate was prepared by (i) redistilling pyruvic acid three times, and (ii) preparing the sodium salt according to the method of Price & Levintow (1952).

Starch for gel-electrophoresis was obtained from the Connaught Medical Research Laboratories, Toronto. Silica Gel G for thin layer chromatography came from E. Merck, A.G., Darmstadt.

Alcohol dehydrogenase, lactate dehydrogenase, NAD^+, $NADP^+$, NADH and NADPH were obtained from the Sigma Chemical Co.

RESULTS

Polyol utilization by growing cells

The results of the auxanographic growth tests (Table 1) agreed generally with other published work on the growth of yeasts on polyols (e.g. Wickerham, 1951; Kudriavzev, 1954; Kreger-van Rij, 1964). The number of polyols employed as test substrates by

those authors was extended in the present work to include xylitol and D-and L-arabinitol. The following points are of particular interest: (i) The yeasts varied from those like *Saccharomyces cerevisiae* that used no polyols as sole carbon-source to others such as *Torulopsis candida* which used all that were offered. (ii) All yeasts that used any polyol used D-mannitol. (iii) No yeast used D-*lyxo*-hexulose (D-tagatose), an expected oxidation product of galactitol (Shaw, 1956, 1962), not even those able to grow on galactitol. (iv) Most of the yeasts could use succinate, and some were found able to use acetate. Hence these compounds could be employed as carbon substrates for growing yeasts in order to study their constitutive polyol dehydrogenases. (v) The identity of the strain LTS 23 was confirmed as that of *Candida utilis* by its responses to the growth tests. These were identical to the reactions given by *C. utilis* strain NCYC 359. Some strains of this species have been reported to use certain polyols; unpublished results of van Uden (B. L. Brady, personal communication, 1965) show that of 13 strains tested one used erythritol, four used ribitol and five used D-mannitol. L-*xylo*-Hexulose, D- and L-arabinose, D-ribose, galactitol and D-glucitol were not used by any strain, whereas D-xylose was used by all 13. Hence *C. utilis* appears to use D-xylose characteristically but not L-arabinose, although *C. utilis* has been reported as able to use L-arabinose for growth (Chakravorty, Veiga, Bacila & Horecker, 1962; Horecker, 1962*a*, pp. 21, 30). (vi) Growth tests with *Pichia delftensis* confirmed the observations of Beech (1965) that it can use ribitol, D-mannitol and D-glucitol, but not erythritol. The strain tested also used D-arabinitol, though not xylitol, L-arabinitol or galactitol (Table 1).

Table 2. *Rates of growth on polyols and glucose as substrates by five yeasts*

Cultures in T-tubes were rocked and incubated at 25°. Growth was measured nephelometrically every 30 min. and recorded automatically. n means not measured. In each case, mean generation (doubling) time was estimated from the exponential phase of growth (cf. Fig. 1).

	D-Glucose	D-Glucitol	D-Mannitol	Galactitol	Erythritol
Torulopsis candida (NCYC 576)	121	134	141	147	163
Debaryomyces vanriji (NCYC 577)	132	404	451	153	234
D. phaffi (CBS 4346)	115	128	n	139	180
Hansenula anomala (CBS 1981)	163	152	257	n	168
Kluyveromyces drosophilarum (CBS 2896)	102	210	160	n	n

Rates of growth during the exponential phase, in terms of mean generation (doubling) time, are given in Table 2 for five yeasts. These rates varied from about 2 to 7·5 hr. The growth of *Torulopsis candida* on four polyols or D-glucose was measured in terms of dry weight (Fig. 1). The inoculum, the same for each substrate, was grown in liquid culture on succinate, and harvested and washed before the end of the exponential phase of growth. The lag phases were particularly long for erythritol, galactitol and D-mannitol. The length of the lag phase could be reduced in each case by growing the inoculum on the same substrate as that on which the yeast's growth was to be measured. Generally, it was possible to harvest the yeasts just before the end of the exponential phase (about 20 hr after inoculation) with a yield of from 3 to 5 mg./ml.

Table 3. *Rates of respiration on polyols and sugars as substrates by intact cells of seven yeasts*

Manometer flasks contained approx. 5 mg. dry wt of washed, starved yeast in 2 ml. of 0·1 M-potassium phosphate buffer (pH 5·9) in the main compartments; 20 μmoles of substrate (as indicated) in 0·2 ml. of water in the side arms; 0·2 ml of 2 N-KOH with filter paper in each centre well. Incubation was in air at 25°, and substrates were added at zero time. 20 hr cultures were used. The figures for Q_{O_2} have not been corrected for endogenous respiration. n means not tested.

Carbon source for growth	Respiratory substrate: Glycerol	Erythritol	Ribitol	D-arabinitol	L-arabinitol	D-glucitol	D-mannitol	Galactitol	L-sorbose*	D-glucose	Endogenous
	Q_{O_2} (μl. O_2 consumed/mg. dry wt yeast/hr)										
Torulopsis candida											
D-Glucose	29	8	11	10	8	12	10	8	36	64	8
Succinate	58	4	16	12	4	42	28	4	45	70	3
Glycerol	79	7	9	36	6	57	35	6	67	83	4
Erythritol	60	65	15	40	4	50	24	4	68	67	4
Ribitol	60	20	14	31	10	40	17	9	56	65	3
D-Glucitol	59	5	11	49	9	60	27	11	64	80	5
D-Mannitol	54	5	10	31	8	46	45	4	59	80	4
Galactitol	56	9	33	36	40	39	28	76	59	60	5
Debaryomyces vanriji											
Erythritol	17	24	9	12	12	17	15	7	18	32	4
Ribitol	14	6	9	12	8	16	16	10	10	32	6
D-Glucitol	16	8	10	14	9	16	17	7	16	27	5
D-Mannitol	10	6	10	10	6	16	13	6	10	35	5
Galactitol	13	6	12	15	n	18	17	42	17	55	6
Hansenula anomala											
D-Glucose	n	6	6	4	5	9	7	3	6	30	4
Succinate	n	5	3	5	4	21	21	4	5	65	3
Glycerol	60	6	4	3	2	17	16	3	3	45	2
Erythritol	84	73	11	3	3	27	17	5	5	66	3
D-Mannitol	47	7	14	6	4	25	16	6	8	39	5
Saccharomyces acidifaciens											
D-Glucose	20	4	3	2	2	6	3	2	4	74	2
Acetate	22	4	n	6	3	76	15	2	3	38	2
Ribitol	32	4	25	28	10	53	28	4	7	48	5
D-Glucitol	32	3	n	7	3	66	25	3	5	61	2
D. phaffii											
Erythritol	25	40	30	28	10	40	33	16	45	60	5
D-Glucitol	16	10	39	41	11	63	44	19	60	68	6
Galactitol	9	5	14	21	n	25	16	84	n	70	5
Pichia delftensis											
Succinate	n	5	6	6	5	17	4	4	n	47	5
D-Glucitol	n	17	48	65	16	69	60	17	n	102	16
D-Mannitol	n	4	23	36	2	60	59	3	n	55	2
Kluyveromyces drosophilarum											
Succinate	n	5	5	5	5	5	6	5	5	100	5
D-Glucitol	n	9	13	40	10	57	80	9	62	163	9
D-Mannitol	n	4	4	21	3	39	34	4	52	126	3

* L-*xylo*-Hexulose.

Nature of polyol utilization by intact yeasts: induction or selection?

Is the ability to utilize polyols a function of a majority of yeast cells or, is it due to the growth of yeast cells of a minority genotype (cf. Mortlock & Wood, 1964)? Surface colony counts were made with inocula from serial dilutions, for *Torulopsis candida* (NCYC 576) and *Pichia delftensis* (LTS 24) growing on D-glucose or a polyol. There was no appreciable difference between the numbers of yeast cells that grew on the different substrates. Hence polyol utilization in these two yeasts is probably not attributable to the selection of a minority genotype.

Oxidation of polyols by suspensions of washed yeast cells

The rates of oxidation of various polyols, L-*xylo*-hexulose and D-glucose by seven strains of yeast are shown in Table 3. Starvation generally lowered the endogenous rate to a Q_{O_2} of about 5 μl. O_2/mg. dry wt. of yeast/hr; so that rates of at least $Q_{O_2} = 15$ in the presence of a polyol clearly indicated that it was utilized.

No yeast respired a substrate upon which it could not grow, or vice versa. With certain exceptions, the rates of respiration of the polyols were little affected by the nature of the growth substrate. Notable exceptions were provided by glucose, erythritol and galactitol.

The respiration of polyols by yeasts grown on D-glucose was usually much slower than that by yeasts grown on other substrates, even on succinate which, presumably, does not induce the formation of polyol dehydrogenases. For example (Table 3) rates (Q_{O_2}) for D-glucitol by *Torulopsis candida* (NCYC 576) were: (i) D-glucose-grown 12 (endogenous 8), (ii) succinate-grown 42 (endogenous 3) and (iii) D-glucitol-grown 60 (endogenous 5). This effect, also observed with *Hansenula anomala* and with *Saccharomyces acidifaciens*, is not surprising, since glucose is known to inhibit the formation of a number of enzymes in yeasts (MacQuillan & Halvorson, 1962) and in bacteria (e.g. Magasanik, 1957). However the polyol dehydrogenase activity of glucose-grown yeast was investigated only with *Candida utilis*. For this yeast, the level of inducible polyol dehydrogenase activity was about as low in glucose- as in acetate-grown cells (Table 6). The activity of the NADP-specific polyol dehydrogenase of the same strain is also low in glucose-grown cells (Scher & Horecker, 1966*a*).

Only yeasts grown on erythritol could respire erythritol. Similarly, only yeasts grown on galactitol respired galactitol. But the ability to respire other polyols did not depend on prior growth on a specific polyol (Table 3). *Torulopsis candida* respired L-arabinitol much faster when grown on galactitol than on the other substrates (Table 3). The rate for D-arabinitol was higher for that yeast when polyol-grown than when grown on succinate or D-glucose. On the other hand, the rates at which *T. candida* respired D-glucitol or D-mannitol varied much less with the growth substrate (succinate or polyol) than the rates at which other polyols were respired. These rates for D-glucitol and for D-mannitol were, however, fastest when the yeast was grown on the respiratory substrate itself.

Respiration rates were generally constant for the first 2 hr with no pronounced initial lag, though D-mannitol oxidation by *Torulopsis candida* grown on D-mannitol provided a striking exception (Fig. 2). The rate of respiration of this polyol gradually increased to a maximum at about 1·5–2 hr. after zero time.

Polyol dehydrogenase activities of cell-free extracts

Crude cell-free extracts oxidized several polyols at the expense of NAD^+ or of $NADP^+$. Similarly, the extracts reduced certain ketoses and aldoses in the presence of reduced coenzyme. Extracts of *Torulopsis candida*, however, did not oxidize either D-glucitol 6-phosphate or D-mannitol 1-phosphate in the presence of NAD^+ or $NADP^+$.

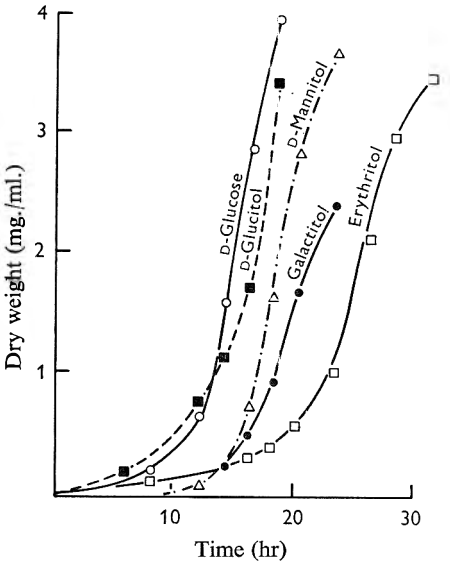

Fig. 1

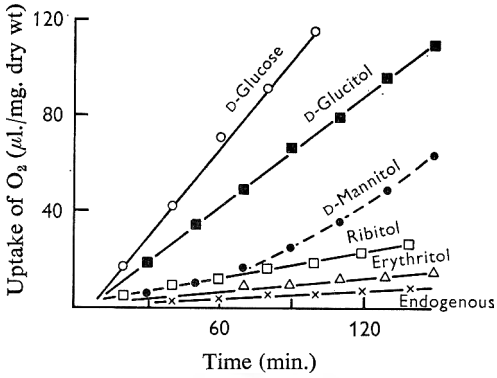

Fig. 2

Fig. 1. Growth of *Torulopsis candida* (NCYC 576) on polyols or glucose. T-tubes contained initially 10 ml. of medium A 2, 0·5 mmole of substrate (as indicated), and about 10 µg. dry wt of succinate-grown, washed yeast. Each point on the curves corresponds to an analysis of a single tube. The T-tubes were rocked and incubated at 25° and sterile air was passed through the upper part of the tube at 5 l./hr.

Fig. 2. Oxidation of polyols by intact cells of *Torulopsis candida* (NCYC 576) grown on D-mannitol as the sole source of carbon. Warburg manometer flasks contained: 7·4 mg. dry wt of washed, starved yeast and 20 µmoles of substrate as indicated. Other contents were as in Methods. Incubated in air at 25°; substrate added at zero time.

Effect of pH value. The effect of pH on polyol oxidoreductase activity of crude extracts of yeasts was studied for: (i) *Torulopsis candida*, D-glucitol $\rightleftharpoons$ D-fructose (NAD) (see Fig. 3), D-glucitol $\rightleftharpoons$ L-sorbose (NADP); (ii) *Pichia delftensis*, D-glucitol $\rightleftharpoons$ D-fructose (NAD); (iii) *Candida utilis*, D-glucitol $\rightarrow$ D-fructose (NAD^+), D-fructose $\rightarrow$ D-glucitol + D-mannitol (NADH) (cf. Chavravorty *et al.* 1962). In each case, maximum rates for the polyol oxidation were found at or above pH 9, and between pH 5 and 7 for the ketose reduction. Buffers at pH values near these optima were used for further experiments. Different pH optima for oxidation and reduction are characteristic for the pyridine coenzyme-linked dehydrogenases, e.g. for L-iditol dehydrogenase (Smith, 1962). By contrast, the cytochrome-linked mannitol dehydrogenase of *Acetobacter suboxydans* catalyses the oxidation of polyols with an optimum at about pH 5 (Arcus & Edson, 1956).

Effect of substrate concentration. Lineweaver–Burk plots of 1/v against 1/s (Dixon & Webb, 1964) were linear, though high concentrations of L-*xylo*-hexulose or D-fructose caused marked inhibition. With concentrations above 1·2 M-D-fructose, for example, there was a sharp fall in the rate of oxidation of NADH. The affinities for various substrates (Table 4) were low, as is not unusual with polyol dehydrogenases (e.g. Horecker, 1962*a*; Scher & Horecker, 1966*a*). Hence it was impracticable to compare activities at optimum substrate concentrations.

Table 4. *Michaelis constants for polyol dehydrogenase activity of crude extracts of yeasts*

Standard assay conditions as described in Methods

Crude extract from	Reaction studied	K_m
Succinate-grown *Torulopsis candida* (NCYC 576)	D-Glucitol $\underset{\text{NADH}}{\overset{\text{NAD}^+}{\rightleftharpoons}}$ D-fructose	0·26 M-D-glucitol 0·86 M-D-fructose 0·16 mM-NAD^+
	Ribitol $\xrightarrow{\text{NAD}^+}$ ribulose	0·060 M-ribitol
	L-*xylo*-Hexulose $\xrightarrow{\text{NADH}}$ L-iditol	1·3 M-L-*xylo*-hexulose
D-glucitol-grown *Pichia delftensis* (LTS 24)	D-Glucitol $\xrightarrow{\text{NAD}^+}$ D-fructose + L-*xylo*-hexulose	0·33 M-D-glucitol

Substrate and coenzyme specificities of crude cell-free extracts. The rates of NAD- and NADP-linked oxidations of polyols and reductions of sugars by crude extracts were nearly linear for at least 2 min. after the addition of coenzyme. The activities varied according to the substrate on which the yeast was grown (Tables 5, 6). With *Torulopsis candida* erythritol was oxidized only by erythritol-grown material; and the only NAD^+-linked oxidation of D-mannitol occurred with D-glucitol-grown yeast. For *Candida utilis*, the polyol dehydrogenase activity of extracts of D-*xylose*-grown cells was about ten times that of cells grown on acetate or glucose (Table 6).

The present observations on the polyol dehydrogenase activity of *Candida utilis* are compared in Table 6 with those made by Horecker and his colleagues (Chakravorty *et al.* 1962; Chakravorty & Horecker, 1966; Horecker, 1961; 1962*a*, *b*) with a partly purified enzyme (×35) obtained from the same strain of yeast (presumably D-xylose-grown, see Horecker, 1962*b*) by fractionation with ammonium sulphate and acetone (Chakravorty *et al.* 1962; Chakravorty & Horecker, 1966). The last two columns show that the relative rates of NAD^+-linked polyol oxidation were similar for the crude extracts of D-xylose-grown yeast used in the present experiments and for the fractionated preparation of Chakravorty *et al.* (1962). This similarity is particularly remarkable in view of differences between the two sets of experiments (*a*) in concentrations of polyol and of NAD^+, and (*b*) in pH.

Even larger differences were found between the activities of extracts of succinate-grown *Pichia delftensis* (LTS 24) and of the same yeast grown on D-glucitol (Table 5). There was much greater NADP-linked polyol dehydrogenase activity in extracts of *Torulopsis candida* than in those of the Pichia species.

No direct evidence was found of coenzyme-linked oxidation of galactitol or of L-arabinitol by extracts of *Torulopsis candida* (Table 5), even when the yeast had been grown on galactitol. Extracts of D-glucitol-grown *T. candida*, however, reduced both D-galactose and L-arabinose in the presence of NADPH (Table 5). From their configura-

Table 5. *Polyol dehydrogenase activity by crude extracts of four yeasts*

Activity was measured by following change of $E_{340m\mu}$ as described in Methods. Rates were calculated from the ΔE_{340} between 15 and 45 sec. after addition of coenzyme. Control cuvettes without alcohol or sugar gave negligible rates. n means not measured. o means < 0·005.

Specific activity of extracts (μmoles of co-enzyme reduced or oxidized/mg. of protein/min.)

	Torulopsis candida (NCYC 576)								*Pichia delftensis* (LTS 24)			*P. membranae-faciens* (NCYC 54)		*Saccharomyces acidifaciens* (CBS 749)	
	Succinate-grown		D-Mannitol-grown		D-Glucitol-grown		Erythritol-grown		D-Glucitol-grown		Succinate-grown	Succinate-grown		D-Glucitol-grown	
Enzyme substrate	NAD^+	$NADP^+$	NAD^+	$NADP^+$	NAD^+	$NADP^+$	NAD^+	$NADP^+$	NAD^+	$NADP^+$	NAD^+	NAD^+	$NADP^+$	NAD^+	$NADP^+$
D-Glucitol	0·11	0·17	0·09	0·08	0·43	0·41	0·72	0·21	1·8	0	0·020	0·013	0·007	1·2	0·31
D-Mannitol	0	0·22	0	0·10	0·09	0·71	0	0·33	3·1	0·060	0·033	0·006	0	0	0·53
L-Iditol	0·04	0	0·04	0	0·07	n	0·51	0·01	0·81	n	n	n	n	0·70	n
Ribitol	0·08	0	0·04	0	0·23	0·01	0·43	0·01	0·92	0	0	0·23	0	0·78	0
Xylitol	0·20	0·04	0·06	0·01	0·70	0·10	0·57	0·04	1·4	n	0·012	0·020	n	1·1	0·08
D-Arabinitol	0	0·07	0	0·02	0·33	0·16	0·01	0·07	1·6	n	0·10	0·034	0·066	0	0·06
L-Arabinitol	0	0	0	0	0	0	0	0	0	n	0	0	n	0·01	0
Erythritol	0	0	0	0	0	0	0·06	0	1·3	n	0·019	0·16	0·007	0	0
Ethanol	0	0	0	0	0	0	0	0	0·51	n	0·74	0·85	n	n	n
Galactitol*	0	0	0	0	0	0	0	0	0	n	n	0	n	0	0
	NADH	NADPH	NADH	NADPH	NADH	NADPH	NADH	NADPH	NADH		NADH	NADH		NADH	
D-Fructose	0·27	0·43	0·25	0·16	0·28	0·81	0·35	0·40	3·4		0·51	0·11		2·3	
L-*xylo*-Hexulose	0·14	0·40	0·10	0·12	0·13	0·71	0·18	0·34	0·84		0·044	0·031		0·99	
D-Glucose	0	0·02	0	0·01	0	0·10	n	n							
D-Mannose	0	0	0	0	0	0·07	n	0·03							
D-Galactose	0	0·03	0	0·01	0	0·20	n	n							
D-Ribose	0	0·06	0	0·02	0	0·23	n	n							
D-Xylose	0	0·12	0	0·05	0·01	0·35	n	n							
D-Arabinose	0	0	0	0	0	0·05	n	n							
L-Arabinose	0	0·13	0	0·05	0	0·38	n	n							

*0·1 M-galactitol

tions, the respective products of these two reductions were presumably galactitol and L-arabinitol, though this assumption was not checked experimentally. D-*lyxo*-Hexulose, an expected oxidation product of galactitol, was not reduced either by NADH or by NADPH in the presence of extracts of *T. candida*.

Oxidation of ribitol by cell-free extracts. An apparent NAD-linked oxidation of ribitol by extracts from acetate-grown *Candida utilis* was traced to small amounts of ethanol (< 0·3 %, w/w) in ribitol recrystallized from ethanol. These extracts did not oxidize ribitol recrystallized from water. *C. utilis* extracts catalysed a very active NAD-linked oxidation of ethanol. No spurious activity with ribitol was observed with preparations from *Torulopsis candida*, which appeared to lack NAD- or NADP-linked ethanol dehydrogenase activity.

Table 6. *Rates of NAD reduction by crude extracts of Candida utilis (LTS 23) in the presence of various polyols or ethanol*

The yeast was grown as indicated on acetate, D-glucose or D-xylose, and the reaction mixtures were as described in Methods. Rates were estimated from ΔE_{340} between 15 and 45 sec. after addition of NAD^+. Rates for the controls without substrate have been deducted from each observed rate. E_{340} readings were converted into NADH concentrations from $\epsilon_{mM} = 6{\cdot}22$. For comparison, the final column gives the relative rates obtained with a partly purified enzyme from the same yeast by Chakravorty *et al.* (1962). Note: both preparations oxidized L-iditol.

Enzyme substrate	Specific activity of extracts (μmoles of polyol oxidized/mg. protein/min.) Growth substrate: Acetate	Glucose	Xylose	Rates relative to that of D-glucitol. Growth substrate: Acetate	Glucose	Xylose	Purified enzyme
D-glucitol	0·058	0·061	0·82	100	100	100	100
Xylitol	0·070	0·047	0·75	121	77	92	115
D-arabinitol	0·063	0·061	0·19	109	100	23	8
D-mannitol	0·072	0·053	0·40	124	87	49	26*
Ribitol	0	.	0·17	0	.	20	26
L-arabinitol	.	.	0·027	.	.	3	1
Erythritol	.	.	0·018	.	.	2	1
Ethanol	4·4	2·2	2·3	7600	3600	280	

* Or 59 (Horecker, 1962*a*, Table 3).

Comparison of activities of extracts with those of whole organisms. Generally, yeasts that could respire any given polyol yielded extracts which oxidized that polyol: the only exception was that of *Torulopsis candida* which could utilize galactitol and L-arabinitol for growth but yielded extracts that were inactive towards galactitol and L-arabinitol. Extracts of erythritol-, but not succinate-, D-mannitol- or D-glucitol-grown *T. candida* oxidized erythritol (Table 5); this was true also for the oxidation of erythritol by intact organisms (Table 3).

There were a number of instances of a polyol oxidized by an extract of a yeast, but not utilized for growth by the corresponding intact organisms. Examples included *Candida utilis* (D-glucitol) and *Pichia membranaefaciens* (erythritol).

Products of polyol oxidation by cell-free extracts

The products of coenzyme-linked dehydrogenase activity of cell-free extracts were examined chromatographically and were identified as ketoses and aldoses, correspond-

Table 7. *Results of chromatographic and colorimetric identification of products of polyol oxidation by crude extracts of three yeasts*

The methods are described in the text. nr means no reaction. n means not done. ? indicates uncertainty owing to very weak reaction (low yield). Extracts of erythritol-grown *T. candida*, with NAD^+, oxidized erythritol to *glycero*-tetrulose.

		Product with extract from				
		Torulopsis candida (NCYC 576) Grown on		*Pichia delftensis* (LTS 24) Grown on		*Pichia membranaefaciens* (NCYC 54). Grown on
Polyol oxidized	Co-enzyme	Succinate	D-Glucitol	Succinate	D-Glucitol	succinate
D-Glucitol	NAD^+	Fructose	Fructose + *xylo*-Hexulose	Fructose	Fructose + *xylo*-Hexulose	Fructose
	$NADP^+$	*xylo*-Hexulose	*xylo*-Hexulose + glucose	nr	nr	n
D-Mannitol	NAD^+	nr	Fructose	Fructose	Fructose	n
	$NADP^+$	Fructose	Fructose + mannose	n	n	nr
L-Iditol	NAD^+	*xylo*-Hexulose	n	n	n	n
Ribitol	NAD^+	*erythro*-Pentulose	n	nr	n	n
	$NADP^+$	nr	Ribose	nr	nr	nr
Xylitol	NAD^+	*threo*-Pentulose	*threo*-Pentulose	n	n	n
	$NADP^+$	n	Xylose + *threo*-Pentulose	n	n	n
D-Arabinitol	NAD^+	nr	*threo*-Pentulose	*threo*-Pentulose	*threo*-Pentulose + ? *erythro*-Pentulose	?*threo*-Pentulose
	$NADP^+$	*threo*-Pentulose	Arabinose + *threo*-Pentulose	n	n	n
Erythritol	NAD^+	nr	nr	n	*glycero*-Tetrulose	*glycero*-tetrulose

ing to the polyol oxidized (Table 7). The R_F values agreed with those of authentic substances run on the same chromatogram to within 3 %. In certain experiments, two sugars were detected, suggesting that more than one dehydrogenase was acting on the polyol.

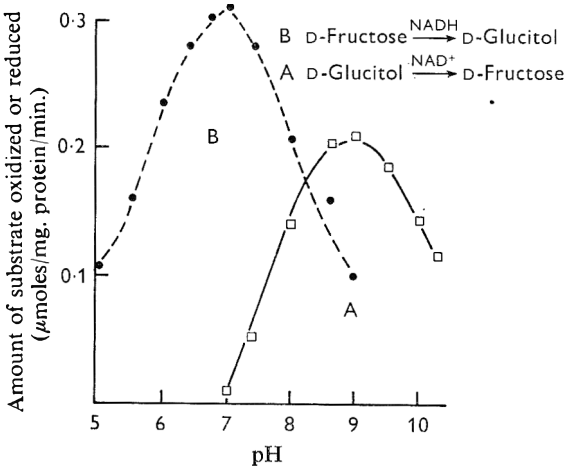

Fig. 3

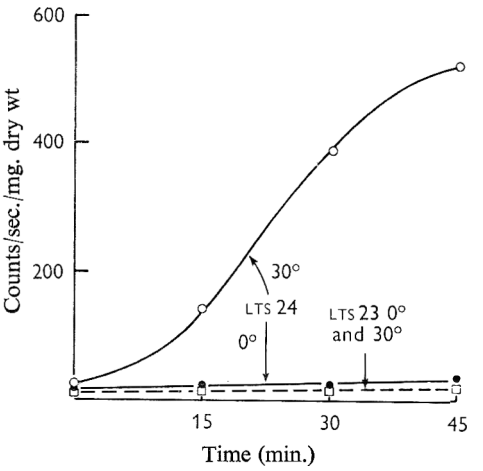

Fig. 4

Fig. 3. Effect of pH on NAD: polyol oxidoreductase activity of crude extracts of succinate-grown *Torulopsis candida* (NCYC 576). Reaction mixtures as described in Methods, using 200 μmoles of buffer as follows: citrate/phosphate (pH 5·0–6·0), potassium phosphate (pH 6·1–7·5), tris/HCl (pH 7·6–8·6), glycine/NaOH (pH 8·8–10·5). Rates of oxidation and reduction were estimated from ΔE_{340} between 15 and 45 sec. after addition of coenzyme. Rates obtained without added substrate were deducted from each reading. Rates were calculated by converting E_{340} readings into NADH concentrations from $\epsilon_{mM} = 6{\cdot}22$.

Fig. 4. The uptake of [U-^{14}C]D-glucitol by intact cells of *Pichia delftensis* (LTS 24) and *Candida utilis* (LTS 23). LTS 24 was grown on D-glucitol and LTS 23 on D-xylose in the presence of 0·025 M-D-glucitol.

Entry of polyols into yeast cells

D-*Glucitol: comparison of Pichia delftensis with Candida utilis.* Intact *C. utilis* (LTS 23) did not use D-glucitol for growth (Table 1), though extracts from cells grown on D-xylose contained an enzyme which oxidized D-glucitol to D-fructose (cf. Chakravorty *et al.* 1962) and the yeast could use D-fructose for growth and for respiration. Hence exogenous D-glucitol probably does not penetrate intact cells of this strain (cf. Cirillo, 1961). This inference was confirmed when comparing the uptake of [U-^{14}C]D-glucitol by *C. utilis* and by *Pichia delftensis* (which could use D-glucitol), assuming that the polyols are not modified before entering the yeast cells. Within 15 min. after the addition of labelled substrate, there was a much greater uptake by *P. delftensis* at 30° than at 0° (Fig. 4). For *C. utilis*, however, there was no significant difference between the radioactivity at 30° and at 0°, even after 45 min. incubation; the uptake at both temperatures by this yeast was lower than that of *P. delftensis* at 0°.

Erythritol: aerobic and anaerobic uptake by Torulopsis candida. In a similar experiment, the uptake of [U-^{14}C]erythritol was studied with erythritol-grown *Torulopsis candida* (NCYC 576). The approximate rate of aerobic uptake was found to be 640

counts/sec./mg. dry wt/hr, equivalent to at least 0·69 μmole of erythritol/mg. dry wt/hr. This rate was of the same order as the rate of erythritol oxidation: $Q_{O_2} = 65$ (Table 3) ≡ 0·64 μmole of erythritol/mg. dry wt/hr (assuming the complete oxidation of erythritol).

Entry of sugars into yeast cells has usually been measured either (i) with non-metabolizable substrates, for example the penetration of L-*xylo*-hexulose into *Saccharomyces cerevisiae* (e.g. Cirillo, 1961), or (ii) by using inhibitors of metabolism, such as iodoacetate (e.g. Burger, Hejmová & Kleinzeller, 1959). Since the specificity of inhibitors is often in doubt (cf. Davies, 1964) an advantageous technique is to study the entry of metabolites into a completely aerobic yeast under anaerobic conditions (Kotyk & Höfer, 1965). Yeasts do not appear to use polyols anaerobically, so the technique should be applicable to the study of polyol translocation with yeasts generally. Labelled erythritol entered the cells of *Torulopsis candida* under both aerobic and anaerobic conditions, though much more slowly anaerobically. (200 as against 640 counts/sec/mg. dry wt./hr). Further experiments with the same strain of *T. candida* have shown that D-glucitol, D-mannitol and erythritol are all concentrated by the cells (A. Kotyk & J. A. Barnett, unpublished observations). This 'uphill' transport of those polyols occurs even anaerobically or in the presence of 0·5 mM-iodoacetate, which stops oxygen uptake with these substrates and lowers the rate of endogenous respiration.

Pichia membranaefaciens and Pichia delftensis. In aerobic experiments such as those described above, succinate-grown *P. membranaefaciens* was found to be impermeable both to D-glucitol and to erythritol, and D-glucitol-grown *P. delftensis* to erythritol. In each case the yeast was grown in the presence of 0·025 M-substrate against which it was to be tested.

Number and identity of polyol dehydrogenases in crude extracts

Kinetic experiments. Activity against several polyols of certain extracts from *Torulopsis candida* suggested the presence of more than one NAD-linked polyol dehydrogenase in each extract. Tests for the presence of more than one polyol dehydrogenase in extracts of succinate-grown *T. candida* were done by adding two oxidizable polyols, of different configurations simultaneously (cf. Dixon & Webb, 1964). A mixture of D-glucitol and ribitol was oxidized a little more slowly (0·057 μmole of NADH formed/min.) than D-glucitol alone (0·062 μmole/min.) Similarly, a mixture of D-fructose and L-*xylo*-hexulose was reduced more slowly (0·111 μmole of NADH oxidized/min.) than D-fructose alone (0·122 μmole/min.). Since the experiment provided near saturation conditions for substrates, an additive effect would have been expected if two enzymes were present.

Separation of polyol dehydrogenases by gel electrophoresis

Enzymes of Torulopsis candida. Extracts of *T. candida* grown on succinate, D-mannitol, D-glucitol or erythritol, were subjected to starch-gel electrophoresis. The results of incubating the gels with different polyols and NAD^+ or $NADP^+$ are shown in Fig. 5. The polyol dehydrogenase activity was associated with nine distinct fractions (Fig. 5; Table 8), suggesting that there were nine distinct enzymes. These enzymes varied in specificity towards the polyols, and in coenzyme specificity. Also the presence or absence of some of the enzymes was affected by the carbon source on which the

yeast was grown. The enzymes have been numbered 1 to 9, no. 1 migrating farthest towards the anode during electrophoresis. Enzymes 1 to 5 were NAD-linked, 6 to 9 were NADP-linked.

Extracts of succinate-grown *Torulopsis candida* gave three bands of polyol dehydrogenase activity, numbered 1, 3 and 9 (Fig. 5; Table 8). Bands 1 and 3 were possibly isoenzymes since: (i) they are the only pair of all the nine bands which appear to be

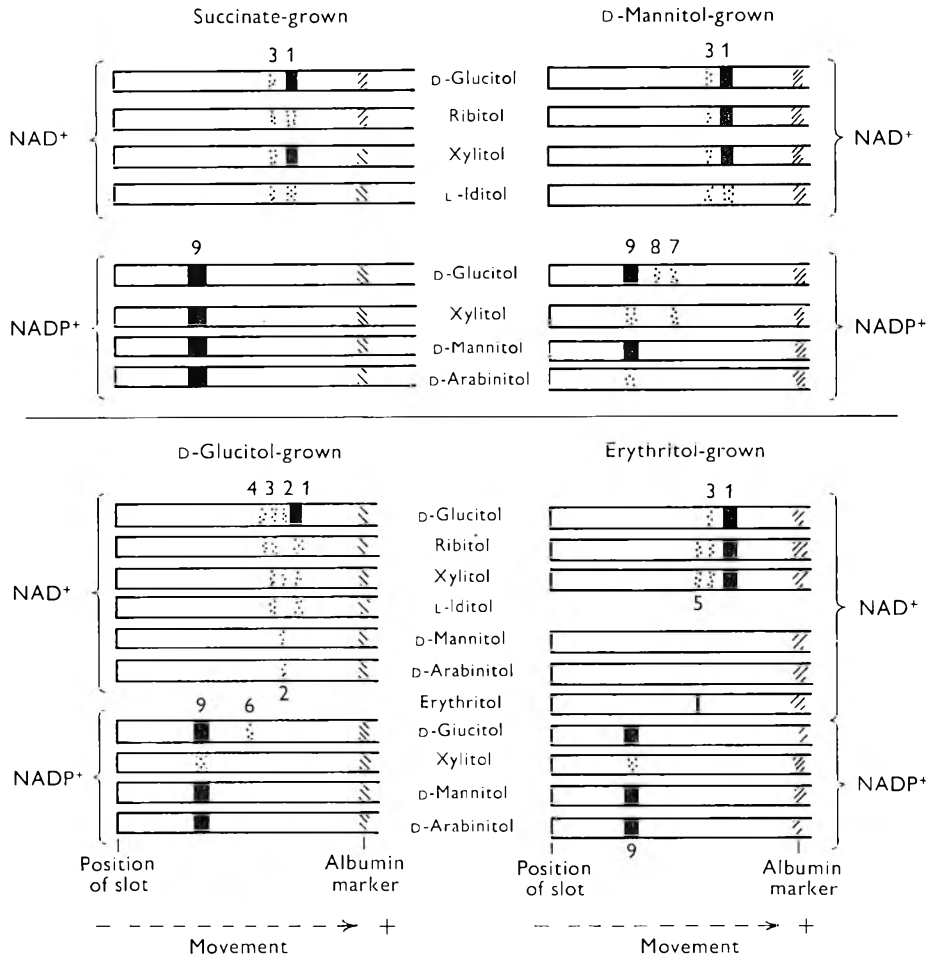

Fig. 5. Location of NAD^+- and $NADP^+$-linked polyol dehydrogenase activity on starch gel after electrophoresis of crude extracts of *Torulopsis candida* (NCYC 576). See Methods for experimental details. Polyol dehydrogenase activity was located as formazan bands, in the presence of various polyols. The intensity of each band is represented by the degree of shading. The bands have been numbered in accordance with the scheme shown in Table 8.

identical in their specificity for polyols and coenzymes; and (ii) they are both constitutive. On the other hand, the relative intensities of the two bands varied with different substrates (Fig. 5). That bands 1 and 3 have the same characteristics fits the conclusion from the kinetic evidence, already discussed, that the succinate-grown material contained only a single NAD-linked polyol dehydrogenase.

The specificity of these two constitutive NAD-linked enzymes corresponded with that of L-iditol dehydrogenase (L-iditol: NAD oxidoreductase [EC 1.1.1.14] see Dixon & Webb (1964), Touster & Shaw (1962); and Smith (1962)), these enzymes oxidized ribitol to D-*erythro*-pentulose, xylitol to D-*threo*-pentulose, D-glucitol to D-fructose and L-iditol to L-*xylo*-hexulose. On the other hand, unlike the enzymes 1 and 3, L-iditol dehydrogenase also oxidizes D-mannitol, L-arabinitol and galactitol, though very slowly under most conditions of assay.

Table 8. *Scheme for nine polyol dehydrogenases separated by starch-gel electrophoresis from crude extracts of Torulopsis candida* (NCYC 576)

This table summarizes the information given in Fig. 5, the caption of which gives the methods used. The enzymes are arranged in the order they were detected on the gels, no. 1, farthest from the slot. +, substrate oxidized; −, substrate not oxidized. An asterisk indicates that the corresponding enzyme was detected in yeast grown on the carbon-source indicated. Enzymes 1–5 were NAD-linked; 6–9 were NADP-linked. None of the enzymes oxidized L-arabinitol or galactitol (the latter used at 0·1 M).

Direction of movement

Slot → anode										
9	8	7	6	5	4	3	2	1	Enzyme no.	
+	+	+	+	−	+	+	+	+	D-Glucitol	Enzyme substrate
+	−	+	−	+	−	+	+	+	Xylitol	
−	−	−	−	+	+	+	−	+	Ribitol	
−	−	−	−	?	−	+	−	+	L-Iditol	
+	−	−	−	−	−	−	+	−	D-Mannitol	
+	−	−	−	−	−	−	+	−	D-Arabinitol	
−	−	−	−	+	−	−	−	−	Erythritol	
*	.	.	.	.	.	*	.	*	Succinate†	Carbon source for growth of yeast
*	.	.	*	.	*	*	*	*	D-Glucitol	
*	*	*	.	.	.	*	.	*	D-Mannitol	
*	.	.	.	*	.	*	.	*	Erythritol	
← NADP →				← NAD →					Coenzyme	

† Enzymes found in the succinate-grown yeast were also present in that grown on polyols.

The constitutive, NADP-linked polyol dehydrogenase (band 9) oxidized D-glucitol, xylitol, D-mannitol and D-arabinitol, but not ribitol, L-iditol or erythritol. Enzyme 9 gave a single band, showing this specificity, both with the tris-DETPA discontinuous gel-buffer (pH 7·8) and with the phosphate buffer system (pH 7·0). In oxidizing xylitol, this enzyme differed from the NADP-linked D-mannitol dehydrogenase of *Acetobacter suboxydans* (Bygrave & Shaw, 1961; Kersters, Wood & De Ley, 1965).

The $NADP^+$-linked polyol dehydrogenase activity of succinate- or erythritol-grown yeast was always associated with a single band (9), on both starch and polyacrylamide gels. However, the relative intensity of band 9 on different substrates varied with the source of extract; for example, the intensity with xylitol relative to that with D-glucitol, D-mannitol or D-arabinitol was greater for succinate-grown *Torulopsis candida* than that for D-mannitol-, D-glucitol- or erythritol-grown yeast (Fig. 5). This would not be expected if band 9 represented a single enzyme. Furthermore, it oxidized xylitol which does not possess the configuration common to D-glucitol, D-mannitol and D-arabinitol (*cis*-vicinal hydroxyls on carbon atoms 2 and 3, with the hydroxyl on carbon atom 4 in the *trans* position). Chromatographic evidence indicated that $NADP^+$-linked activity of extracts from succinate-grown *Torulopsis candida* formed fructose from D-mannitol, *xylo*-hexulose from D-glucitol and *threo*-pentulose from

D-arabinitol. Such extracts, it is true, were capable of NADPH-linked reductions of L-arabinose, D-xylose, D-ribose, D-galactose and D-glucose (Table 5), but no other sign of these aldose-reductase activities was detected in extracts from the succinate-grown material. This apparent contradiction between NADP-linked oxidative and reductive behaviour (for example, L-arabinose and D-ribose were reduced with NADPH, but neither L-arabinitol nor ribitol was oxidized with $NADP^+$ and extracts from succinate-grown *T. candida*) was not resolved.

The marked difference between the mannitol- and succinate-grown *Torulopsis candida* was the presence in the former of the $NADP^+$-linked bands 7 and 8. Perhaps one or both of these bands represented some of the missing aldose reductase activity discussed above. They were not investigated.

The NAD^+-linked enzymes 2 and 4 (Fig. 5; Table 8) appeared only in extracts of *Torulopsis candida* which were grown on D-glucitol. Enzyme 2 may be responsible for the NAD^+-linked activity with D-mannitol and D-arabinitol, which was not found with succinate- or D-mannitol-grown material (Table 5). The polyol-specificity of enzyme 2 corresponded exactly with that of the $NADP^+$-linked enzyme 9 (Table 8) which was discussed above.

The $NADP^+$-linked enzyme 6, which appeared only to oxidize D-glucitol, differed from enzymes 7 and 8 of the mannitol-grown yeast. Electrophoresis of mixtures of extracts from mannitol- and glucitol-grown yeast produced all three bands separately, in relative positions and with specificities corresponding to those shown in Fig. 5.

Erythritol-grown yeast was the only material in which the oxidation of erythritol was observed. The pattern of specificity was such that this NAD^+-linked enzyme (5) oxidized only the *meso*-polyols, i.e. xylitol, ribitol and erythritol: it was not tested against L-iditol.

Enzymes of Candida utilis. The NAD-linked polyol dehydrogenase activity of *C. utilis* (LTS 23) was difficult to resolve electrophoretically. The difficulties were mainly due (i) to the presence in the crude extracts of an NAD-linked ethanol dehydrogenase, (ii) to the presence of ethanol in the commercial starch preparation used for making the gel. After electrophoresis of extracts of *C. utilis*, starch-gel strips incubated with NAD^+, PMS and NBT, but without substrate, developed about 20 formazon bands. When 0·1 M-ethanol was added to the incubation mixture, bands in corresponding positions appeared more rapidly and were darker than those that developed without added substrate. This failure of the negative controls with starch-gel electrophoresis of extracts of certain yeasts did not occur when polyacrylamide was used in place of starch. A comparable effect for the starch-gel electrophoresis of extracts of mammalian tissue has also been attributed to the presence of alcohol dehydrogenase (Shaw & Koen, 1965).

A commercial preparation of yeast alcohol dehydrogenase subjected to starch-gel electrophoresis gave bands which developed without the addition of ethanol to the incubation mixture; and these bands did not increase in number when ethanol was added. After starch-gel electrophoresis of liver alcohol dehydrogenase, C. J. R. Thorne (personal communication, 1966) also found strong bands without added substrate. He estimated enzymically that one batch of starch contained 3·6 g. ethanol/100 g. starch (cf. Beutler, 1967).

Polyacrylamide gels also presented some difficulty. Crude extracts of *Candida utilis* oxidized ribitol in the spectrophotometric assays (Table 6), but no activity against

ribitol was detected in polyacrylamide gels, either with tris-DETPA (pH 7·8) or with phosphate (pH 7·0) gel buffers, even if the gels were washed to remove catalyst and unpolymerized acrylamide (Tombs, 1965). These gels revealed: (i) a single band of NAD^+-polyol dehydrogenase active against D-glucitol, D-mannitol, D-arabinitol and xylitol, and (ii) a band of ethanol dehydrogenase.

These two difficulties (the failure of the negative controls with starch and the apparent enzyme-lability in polyacrylamide) were side-stepped as follows. After only about 15 min. of incubation (before the bands evoked by ethanol appeared, but after those associated with the polyols had done so), the starch-gel strips were removed from the incubation-mixture and placed in 5% aqueous acetic acid. The result of this procedure can be seen in Fig. 6. The specificity of enzyme 1, oxidizing D-glucitol,

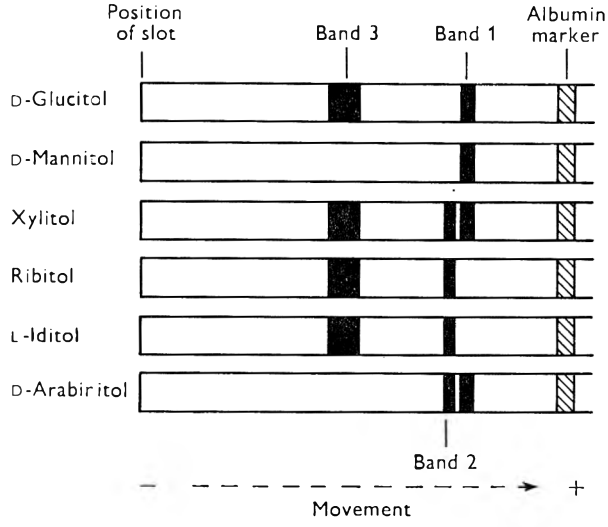

Fig. 6. Location of NAD^+-linked polyol dehydrogenase activity on starch-gel after electrophoresis of crude extract of D-xylose-grown *Candida utilis* (LTS 23). Vertical starch-gel electrophoresis was run for 5 hr under the conditions described in Fig. 5, except that formazan development was for 15 min. only, and the coenzyme was NAD^+ only. The albumin marker was at about 10 cm. from the origin-slot of each strip in the direction of the anode.

D-mannitol, D-arabinitol and xylitol, was comparable with that of enzyme 2 of *Torulopsis candida* (see Table 8). Enzyme 3 showed a specificity similar to that of the L-iditol dehydrogenase-like enzyme (1 and 3) of *T. candida*.

Candida utilis enzyme 1 (Fig. 6) was detected after starch-gel electrophoresis of an extract from the same yeast grown on acetate. It was not practicable to identify other enzymes from this material owing to interference from a very active alcohol dehydrogenase (see Table 6).

Starch-gel electrophoresis of xylose-grown *Candida utilis* revealed a single band which appeared when the gel-strip was incubated with $NADP^+$ and D-glucitol, xylitol or L-arabinitol, but not with $NADP^+$ and D-mannitol or D-arabinitol. This band probably corresponds with the enzyme described by Scher & Horecker (1966*a*, *b*).

Enzymes of Pichia species. Only weak NAD^+-linked polyol dehydrogenase activity was found on the starch-gels after electrophoresis of extracts of *P. delftensis* grown on D-glucitol or of *P. membranaefaciens* grown on succinate. Material from succinate-

grown organisms of both species yielded two or three rather indistinct bands with D-glucitol as substrate. D-glucitol-grown *P. delftensis* yielded one definite band with D-glucitol or D-mannitol, one with D-arabinitol only and another with D-mannitol only.

In general, there was a good agreement between the spectrophotometric observations, the results of starch-gel electrophoresis and the chromatographic analysis of the products of polyol oxidation. These results are considered together in the Discussion.

DISCUSSION

Identity of polyol dehydrogenases

Fifteen distinct polyol dehydrogenases have been described above: eight for *Torulopsis candida*, four for *Candida utilis* and three for *Pichia delftensis*. These enzymes varied in their substrate-, coenzyme-, and inducer-specificities. However, identity with a previously reported enzyme has been suggested only for the constitutive NAD-linked dehydrogenase of *T. candida*, corresponding with enzyme 3 of *C. utilis*. Too few substrates were used for identifying these enzymes; and, furthermore, the presence of more than one polyol dehydrogenase in the crude extracts usually made impracticable the measurement of relative rates of activity of a single enzyme. Attempts to elute the enzymes from the gels failed.

Polyol dehydrogenases of Torulopsis candida

Extracts of succinate-grown yeast oxidized D-glucitol to fructose in the presence of NAD^+ and to *xylo*-hexulose with $NADP^+$. From the configuration of D-glucitol, the two products were presumably D-fructose and L-*xylo*-hexulose (though the configurations were not checked experimentally); both these hexuloses were reduced by the extracts (Table 5).

The only constitutive dehydrogenase found to oxidize D-mannitol was the NADP-linked enzyme 9. Extract of succinate-grown yeast, with $NADP^+$, oxidized D-mannitol to D(?)-fructose only. Probably, therefore, enzyme 9 catalyzed the above oxido-reduction. L-Iditol, on the other hand, was oxidized only by enzymes 1 and 3; this polyol, when incubated with crude extracts in the presence of NAD^+, formed L(?)-*xylo*-hexulose. Similarly, NAD-enzymes 1 and 3 appeared to oxidize ribitol to *erythro*-pentulose. Since the specificity of this enzyme seemed to correspond to that of L-iditol dehydrogenase, the *erythro*-pentulose probably had the D-configuration (see Touster & Shaw, 1962; Smith, 1962). Similarly, xylitol was probably oxidized to D-*threo*-pentulose by enzymes 1 and 3 and to L-*threo*-pentulose by 9.

With D-glucitol-grown *Torulopsis candida*, more than one enzyme specific for each coenzyme was present (Table 8) and, correspondingly, more than one polyol oxidation product was formed (Table 7).

The failure to detect the oxidation of both galactitol and L-arabinitol, despite evidence of their formation from corresponding aldoses, may have been because the affinity of the appropriate dehydrogenase for these polyols was very low, or the experimental conditions (e.g. pH) were unsuitable for this reaction. However, the catabolism of galactitol and L-arabinitol was not investigated further. None the less, it is worth drawing attention to additional evidence for the association between the catabolism of galactitol and of L-arabinitol. All the yeasts which grew on L-arabinitol used galactitol too (Table 1). This was not true for D-arabinitol. Possibly, therefore,

an NADP-linked aldose reductase may initiate the catabolism of galactitol and L-arabinitol; and this enzyme may be specific for polyols of the L-*threo*-configuration adjacent to the primary alcohol group, as Shaw (1956, 1962) found for the ketose-forming galactitol: NAD oxidoreductase of a pseudomonad. Alternatively, the galactitol-specific enzyme of *Torulopsis candida* may prove comparable with the NADP-linked (aldose-forming) polyol dehydrogenase of *Candida utilis* (Scher & Horecker, 1966*a*, *b*) or even with that of *Candida albicans* (Veiga, Bacila & Horecker, 1960), though the latter enzyme was not tested against galactitol or galactose.

Where two sugars were detected as products of activity of cell-free extracts, these sugars are interpreted here as direct products of polyol oxidation, though they could have been formed by the action of isomerases. This suggestion applies most obviously to the mixture of mannose and fructose found after incubation of an extract of D-glucitol-grown *Torulopsis candida* with D-mannitol and $NAPD^+$. The mannose could have been formed from fructose by the action of a mannose isomerase (Palleroni & Doudoroff, 1956). Similarly, a xylose isomerase (Slein, 1962) could have formed D-xylose from D-*threo*-pentulose. Although such possibilities must be borne in mind, extracts of D-glucitol-grown yeast showed high aldose-reductase activity (Table 5) and controls incubated without coenzyme formed no sugars.

Table 9. *Scheme for specificities of four polyol dehydrogenases of Torulopsis candida* (NCYC 576)

Data from Tables 5, 7 and 8. The basis of this scheme is explained in the text. The enzyme numbers are those used in Table 8.

	Products with enzymes from yeast			
	Succinate-grown		D-Glucitol-grown	
Polyol oxidized	Enzymes 1 and 3 (NAD^+)	Enzyme 9 ($NADP^+$)	Enzyme 2 (NAD^+)	Enzyme 6* ($NADP^+$)
D-Glucitol	D-Fructose		L-*xylo*-Hexulose	D-Glucose
D-Mannitol	None		D-Fructose	D-Mannose
L-Iditol	L-*xylo*-Hexulose		None	None
Ribitol	D-*erythro*-Pentulose		None	D-Ribose
Xylitol	D-*threo*-Pentulose		L-*threo*-Pentulose	D-Xylose
D-Arabinitol	None		D-*threo*-Pentulose	D-Arabinose

* The specificities shown for enzyme no. 6 were not substantiated by the electrophoretic results (Table 8). N.B. the D- and L-forms were not identified experimentally (see text).

Table 9 summarizes the above and additional inferences on the specificities of the constitutive enzymes, from succinate-grown *Torulopsis candida* (1, 3 and 9) as well as two enzymes found in D-glucitol-grown yeast (2, 6). Extracts from D-glucitol-grown *T. candida* in the presence of $NADP^+$ formed certain aldoses in addition to those formed by succinate-grown yeast (Table 7), and only one additional $NADP^+$-linked enzyme (6) was detected on the gels for D-glucitol-grown material (Table 8). Although it appeared to react with D-glucitol only, the aldose-producing activity has been tentatively attributed to enzyme 6 (Table 9), assuming that its reactions with D-mannitol, ribitol, xylitol and D-arabinitol were too weak to detect on the gels. None of the other enzymes can be considered in this way, because the products with extracts from yeast grown on other substrates were not examined chromatographically.

NAD-linked polyol dehydrogenases of Candida utilis

In the present work, three different polyol dehydrogenases were separated from the crude extracts of *Candida utilis* by starch-gel electrophoresis. The purified preparation of Chakravorty *et al.* (1962) probably contained enzyme 1 (Fig. 6), since it oxidized mannitol. Since the preparation also oxidized L-iditol and ribitol, it probably contained also at least one of the other enzymes. Alternatively, the oxidation of the last two polyols may have been only apparent. The reduction of NAD^+ could have been due, not to the oxidation of ribitol or L-iditol, but to the presence of both (i) NAD-linked alcohol dehydrogenase in the purified preparation and (ii) traces of ethanol present in recrystallized polyols, as was experienced in the present work with crude extracts of acetate-grown *C. utilis*.

Although eight separate polyol dehydrogenases were detected by starch-gel electrophoresis in extracts of *Torulopsis candida*, and only four such enzymes in *Candida utilis*, the effective difference between these yeasts in their abilities to use polyols is probably one of penetration: that is to say, polyols do not enter the cells of this strain of *C. utilis*. As can be seen from the rates shown in Table 5, the specific activities of the NAD^+-linked dehydrogenases against a number of the polyols were of the same order for the crude extracts of both yeasts. Most of the polyol dehydrogenase activity of *Pichia delftensis* seemed to be NAD^+- rather than $NADP^+$-linked (Table 5). Further the NAD^+-linked activity was about 100 times greater in D-glucitol- than in succinate-grown *P. delftensis*; that of the latter was comparable to the activity of succinate-grown *P. membranaefaciens*.

As for *Candida utilis*, certain polyols were oxidized at appreciable rates by extracts of these yeasts, although the same polyols were not respired by the corresponding intact cells. Thus extracts of D-glucitol-grown *Pichia delftensis* oxidized xylitol and erythritol, and extracts of succinate-grown *P. membranaefaciens* oxidized ribitol and erythritol (Table 5); whereas corresponding intact cells did not respire these substrates. Hence, on this evidence alone, it seemed likely that permeability barriers would be found to be deciding factors of utilization or non-utilization of most of the polyols by these yeasts. This suggestion was corroborated by the results of experiments with labelled substrates. Probably, therefore, *P. delftensis* and *P. membranaefaciens* are distinguished from each other by some polyol translocation mechanism (or mechanisms) present in the first species and not in the second.

Only in the case of erythritol for *Torulopsis candida* was the presence and absence of an enzyme clearly associated with the capacity of the yeast to use the enzyme's substrate Most of the evidence in this study has been of an abundance of polyol dehydrogenase activity in extracts of the yeasts (mainly NAD^+-linked and ketose producing) whether or not the intact yeast could use the polyols which its enzymes oxidized. Active $NADP^+$-linked enzymes were also found particularly for the oxidation of mannitol by *T. candida*. Scher & Horecker (1966*a*, *b*) have described the NADP-linked polyol dehydrogenase of *Candida utilis*.

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