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THE JOURNAL OF GENERAL MICROBIOLOGY

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

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

The Editors wish to emphasize ways in which contributors can help to avoid delays in publication.

(1) Papers must be written in English 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.

(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, for many reasons, to wait until a concise and comprehensive paper can be written.

(3) Authors should state the objects they had in view when the work was undertaken, the means by which they carried it out and the conclusions they draw. A section labelled 'Discussion' should be strictly limited to discussing, if this be necessary, and not to recapitulating. Many papers when first sent to the *Journal* are too long for the crucial information they contain. It is unnecessary to describe preliminary or abortive experiments.

(4) Figures and tables should be selected to illustrate the points made, to summarize, or to record important quantitative results. Well-designed tables or graphs should need little explanatory letterpress. Photographs or drawings should not be submitted unless they illustrate facts that cannot be conveniently described in the text.

(5) Authors should remember that in preparing their typescript they are giving instructions to the printer (about layout, etc.), as well as attempting to convey their meaning to their readers. The latter object will be the better attained the more carefully authors consider how their typescripts will be converted to the printed page. Ink corrections on a typescript greatly prolong the type-setter's work; the final version of a paper must if necessary be retyped to provide a clean copy for the printer. Typescripts which do not conform to the conventions of the *Journal* will be returned to authors for revision.

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Communications. Papers submitted for publication should be sent to A. F. B. Standfast (*The Journal of General Microbiology*), Lister Institute of Preventive Medicine, Elstree, Hertfordshire, England. Communications about offprints should be addressed to The University Press, Cambridge.

General. Submission of a paper to the Editors will be held to imply that it reports unpublished work, that it is not under consideration for publication elsewhere, and that if accepted for the *Journal* it will not be published again in the same form, either in English or in any other language, without the consent of the Editors.

Form of Papers Submitted for Publication. The onus of preparing a paper in a form suitable for sending to press lies in the first place with the author. Authors should consult a current issue in order to make themselves familiar with the *Journal's* typographical and other conventions, use of cross-headings, layout of tables, etc.

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A paper should be submitted in double-spaced typing (top copy) with a 1½ in. left-hand margin, and on paper suitable for ink corrections. The paper should in general be divided into the following parts in the order indicated: (*a*) Summary: brief and self-contained; (*b*)

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Symbols and Abbreviations. Authors should refer to current issues of *The Journal of General Microbiology* for information in this connection. Attention is particularly drawn to the following points: *c.* = circa or approximately; degrees Centigrade are written, e.g. 100°, not 100° C.; hr, min., sec. (singular and plural); M = molar; m (milli-) = 10^{-3} and μ (micro-) = 10^{-6}; ml. (millilitre) should be used instead of c.c., and μg. (microgram) instead of γ; N = normal (of solutions); No. or no. = number. Ratios should be written 1:10; dilutions, 1/10.

Chemical Formulae. These should be written as far as possible on one line. The chemical nomenclature adopted is that followed by the Chemical Society (*J. chem. Soc.* 1936, p. 1067). With a few exceptions the symbols and abbreviations are those adopted by a committee of the Chemical, Faraday, and Physical Societies in 1937 (see *J. chem. Soc.* 1944, p. 717). Care should be taken to specify exactly whether anhydrous or hydrated compounds were used, i.e. the correct molecular formation should be used, e.g. $CuSO_4$, $CuSO_4.H_2O$ or $CuSO_4.5H_2O$.

Description of Solutions The concentrations of solutions are preferably defined in terms of normality (N) or molarity (M). The term '%' must be used in correct sense, i.e. g./100 g. of solution. For 'per cent of volume', i.e. ml./100 ml., the term '% (v/v)' should be used, and for weight of a substance in 100 ml. of solution, the term '% (w/v)'.

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Descriptions of new species of cultivable microbes should not be submitted unless an authentic specimen of a living culture has been deposited in a recognized culture collection.

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

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

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

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

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

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Growth of a Leptothrix Strain in a Chemically Defined Medium

By G. A. MORRISON and C. J. EDWARDS
Edward Davies Chemical Laboratory, Aberystwyth, Wales

(*Received* 6 *March* 1964)

SUMMARY

A Leptothrix strain was found to be a motile aerobe which did not ferment sugars but utilized some of them for growth. It required cystine and a source of molybdenum or vanadium, and preferred ornithine or arginine as its N-souree. Liquid media of defined chemical composition have been developed for its cultivation, and growth characteristics in these media determined.

INTRODUCTION

Little is known about the metabolism of the organisms of the genus *Leptothrix* (Bergey, 1957). The object of the present work was to develop a chemically defined medium for the growth of one of these organisms in preparation for a study of its metabolism.

METHODS

The strain chosen was obtained through the courtesy of Professor B. W. Lacey (Westminster Hospital School of Medicine), who isolated it in 1947 and classified it as a Leptothrix; it probably originated as an airborne contaminant (private communication). Since it grew moderately well at 25°, without the long delay caused by higher temperatures, on the surface of nutrient agar slopes buffered to pH 7·0, this temperature was used throughout the investigation. All media were prepared from the best available quality of chemicals in demineralized water of minimum resistance 2×10^6 ohm/cm. Exploratory experiments in liquid cultures were made in 6 in. × $\frac{5}{8}$ in. Pyrex tubes and the amount of growth estimated by an 'EEL' nephelometer calibrated to give results in equiv. mg. dry-wt. organism/ml. More exact results were obtained when necessary by measurements of the absorption of light (583 mμ) by killed samples from 50 ml. cultures in 7 in. × 1 in. Pyrex tubes, in a 'Unicam 500' spectrophotometer calibrated to give results in equiv. mg. dry-wt. organism/ml. Filtered and sterile air (Morrison, Griffiths & Harris, 1955) passed through a glass tube drawn out at the end of a fine capillary was used to aerate liquid media.

RESULTS

Characteristics of the organism

On the surface of nutrient agar (Oxoid containing KH_2PO_4, 5·4 g./l., adjusted to pH 7·0 with NaOH) the initial smooth round colonies gave way later to a spreading growth typical of a motile organism; motility was observed in a hanging drop. In liquid peptone media (various) the organism grew only on the surface with the

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formation of a pellicle. When the liquid peptone media were aerated there was a small amount of growth distributed evenly throughout the culture which was not increased by repeated subcultivation. The peptone cultures contained long slender threads and short rod-like forms, the former predominating in older cultures and the latter in younger ones. The Gram-staining technique gave variable results. Polychrome methylene blue stained the sheaths of filaments, the organisms within the sheaths and the individual shorter rods; the stained organisms exhibited a characteristic granular appearance and had parallel sides with characteristic oval or spherical terminal bodies which stained more strongly and there seemed to be protoplasmic 'bridges' between organisms in a sheath. The presence of granules or spores was not shown by Neisser's or Moeller's methods; the organism was not acid-fast according to the Ziehl–Neelsen method (Fairbrother, 1953).

When subjected to the usual biochemical tests by the media and methods recommended by the Oxoid Division of Oxo Ltd., and/or by Difco Laboratories, the organism (within 6 weeks) did not produce gas from any of the test sugars. It produced acid only from glucose, sucrose, lactose and dulcitol. It did not produce indole from tryptophan, hydrogen sulphide from sulphur-containing amino acids, acetylmethylcarbinol from glucose, nor did it grow on, or produce alkali from, media containing ammonium citrate as the sole carbon and energy source. It possessed a urease and a catalase. The production of acid from glucose, sucrose, lactose and dulcitol was confirmed in the conventional Warburg apparatus, which showed also a simultaneous uptake of oxygen.

The organism did not grow autotrophically on the solid or liquid media based on manganese salts used for *Leptothrix ochracea* (Lieske, 1919), in the iron bicarbonate liquid medium used for *Spirophyllum ferrugineum* (Lieske, 1911), nor did it grow heterotrophically in the medium formulated for the filamentous sheathed iron bacterium *Sphaerotilus natans* (Stokes, 1954); the sources of carbon tested were sucrose, glucose, lactose, acetate, succinate, malate, fumarate, citrate, pyruvate. It produced thin spreading colonies which developed brown edges on Mn-agar (Mulder, 1964).

Simplification of the composition of semi-solid media

During the early work excellent growths were obtained on the surface of 'S.I.M. medium' (Difco) which contains (%, w/v): 0·3 Difco Beef Extract, 0·02 Peptonized Iron, 3·0 peptone, 0·0025 sodium thiosulphate and 1·5 agar. The addition of 0·5 % (w/v) Difco yeast extract to this medium yielded luxuriant growth. The separate ingredients of this reinforced medium were tested individually and in all mixtures (the sample of peptonized iron was obtained through the courtesy of Difco Laboratories). Thiosulphate had no effect on growth, the peptonized iron alone yielded poor growth; but beef extract + peptone + peptonized iron + yeast extract were needed together to obtain luxuriant growth. Cultivation of the organism on slopes containing progressively less of the beef extract, until this was eliminated, yielded a substrain (I) which grew abundantly on peptone + peptonized iron + yeast extract. A similar procedure eliminated the peptonized iron to produce substrain II which grew abundantly on peptone + yeast extract. The yeast extract was similarly eliminated to yield substrain III which grew well on semi-solid peptone agar; the amount of growth declined when the concentration of peptone was less than 1 % (w/v); nine different peptones were equally effective.

Growth in liquid media

Substrain II grew in aerated liquid media containing (g./l.): 5·4 KH_2PO_4; 0·4 $MgSO_4.7H_2O$; 30·0 peptone; 5·0 yeast extract; adjusted to pH 7·0. Substrain III grew in a corresponding liquid medium from which the yeast extract had been omitted. Both strains were cultured in liquid media in which casein hydrolysate (prepared in the laboratory by Dr D. E. Griffiths and containing only amino acids) progressively replaced the peptone. Substrain II yielded substrain IVA which grew readily in the aerated buffered solution of casein hydrolysate + yeast extract. Substrain III did not grow when the concentration of casein hydrolysate exceeded the equivalent of one-third of the peptone; only when yeast extract was re-introduced was it possible to obtain substrain IV B which grew readily on the aerated buffered solution of casein hydrolysate + yeast extract. Substrains IVA and IVB were indistinguishable. Substrain IV grew abundantly in aerated media with casein hydrolysate + yeast extract, moderately in media with peptone or yeast extract alone, and not at all in media with only casein hydrolysate. Liver extract could replace the yeast extract but gave dark-coloured media. Thus substrain IV required a factor or factors provided by yeast extract, liver extract or peptone, but growth on one of these alone was limited by a shortage of amino acid present in the casein hydrolysate. The substrain which was used in the subsequent work can be preserved by cultivation on media containing (g./l.): 5·4 KH_2PO_4; 0·4 $MgSO_4.7H_2O$; 10·0 casein hydrolysate; 5·0 yeast extract; 1·5 agar; adjusted to pH 7·0 with NaOH.

Table 1. *Amount of growth of Leptothrix at 25° and composition of the medium*

Concentration of casein hydrolysate (%, w/v)	Concentration of yeast extract (%, w/v)		
	0·1	0·2	0·5
	Growth equiv. (mg. dry-wt. organism/ml.)		
No glucose			
0·00	0·14–0·16	0·26–0·29	0·55–0·60
0·50	0·38–0·42	0·54–0·58	0·67–0·74
1·00	0·62–0·68	0·73–0·80	0·89–0·99
1·2 % (w/v) glucose			
0·00	0·23–0·26	0·55–0·60	0·63–0·70
0·50	0·40–0·44	0·71–0·79	0·83–0·92
1·00	0·70–0·78	0·92–1·01	1·06–1·16

Medium also contained (g./l.): 5·4 KH_2PO_4; 0·4 $MgSO_4.7H_2O$: adjusted to pH 7·0.

The amount of growth in aerated liquid media with yeast extract alone was almost linearly dependent on the concentration of the yeast extract and reached the equivalent of 0·55–0·60 mg. dry-wt. organism/ml. at 0·5 % (w/v) yeast extract. The addition of casein hydrolysate, or of glucose, increased the response of growth to concentration of yeast extract. The two effects were additive (see Table 1). Other additions of carbon and energy sources had a similar effect to that of glucose in media containing 0·5 % (w/v) casein hydrolysate: 0·5 % (w/v) acetate or glutamate was as effective as 1·2 % (w/v) glucose, 0·5 % (w/v) aspartate was 70 % as effective, and

various sugars at 1·2 % (w/v) and dicarboxylic acids at 0·5 % (w/v) are from 15 to 50 % as effective.

Attempts were made to replace the factor(s) provided by yeast extract, but without success. These tests included single peptides, partially hydrolysed casein, acetylated amino acids, NAD, NADP, purines, pyrimidines and the vitamins known to be present in yeast extract. The material required is unusual and fractionation of the yeast extract was needed.

Fractionation of yeast extract

Colouring matter was adsorbed from aqueous solutions of yeast extract by dry alumina (activated by heating to 150° for 2 hr), exhaustively dried silica and activated charcoal. The charcoal adsorbed also amino acids and the material required particularly efficiently from cold solutions at pH 3·5–4·5, but subsequent preferential elution of the active material was not obtained. The cation exchange resins Amberlite IR120 and IRC50 in the hydrogen form retained the active material with some amino acids; the more strongly acidic IR120 (H) being the more efficient. The impure active material was recovered from resin IR120 by elution with M-ammonia solution. The amount of amino acid present in this eluate was decreased either by a long period of dialysis or by acetylation of the amino acids (Cheronis, 1954) and a second retention of the active material on IR120 (H), but acetylation caused some loss of activity and both procedures were too cumbersome for routine use.

Unlike non-polar solvents the partially water-miscible alcohols extracted all the active material together with some colouring matter and some amino acids from aqueous solutions of yeast extract. The extraction was most efficient from slightly acidic cold solutions. The pronounced tendency for emulsion formation and the need for large volumes of alcohol were overcome by slow extraction in the apparatus devised by Kutscher & Steudel (Umbreit, Burris & Stauffer, 1951); modified by the insertion of an extra condenser to ensure that the extracting alcohol was cold, and rates of flow adjusted so that emulsion was not returned to the boiler. The active material in 100 g. yeast extract was removed by continuous extraction in this way for 16 days. The extract in *n*-butanol stored overnight at 0° deposited a light brown powder which contained three-quarters of the active material but the remainder could be recovered only as a syrup which was difficult to fractionate further. The light brown powder dissolved readily in water. When ice-cold acetone was added slowly to an ice-cold 10 % (w/v) aqueous solution adjusted to pH 2 with nitric acid, a buff-coloured precipitate containing nucleotides (Le Page & Mueller, 1949) formed which was removed by a no. 3 sintered-glass filter. The washed and dried precipitate, which accounts for 20–24 % by weight of the material extracted by *n*-butanol, had no growth-promoting properties. The supernatant liquor adjusted to pH 7 and evaporated *in vacuo* yielded a yellowish active powder. A concentrated aqueous solution of this powder yielded a further inactive precipitate when diluted slowly with absolute ethanol until the solution contained 95 % (v/v) ethanol. Evaporation of the supernatant liquor *in vacuo* yielded an active pale orange powder, which accounted for about 6 % of the original weight of yeast extract. Chromatographic analysis showed the presence of norvaline, norleucine, alanine, serine, together with traces of other amino acids. This fraction was not an adequate source of nitrogen-

containing nutrients for the organism. The fraction was soluble in *n*-butanol and in ethanol and dry activated alumina removed the active material from solutions in these solvents, but none of the active material was recovered when the extraction was from *n*-butanol and only traces were recovered when it was from ethanol. This refined butanol-extracted material was a satisfactory source of the material needed to permit an investigation of the amino acid requirements of the Leptothrix used. This investigation, reported in detail below, showed that L-arginine and DL-cystine are necessary nutrients, and a new basal medium containing (g./l.): 12 glucose; 1 L-arginine hydrochloride; 0·1 DL-cystine; 5·4 KH_2PO_4; 0·4 $MgSO_4.7H_2O$; adjusted to pH 7·0, was used for testing further fractions of the yeast extract for the active material.

Molecules of *M* value less than 3500–4000 can enter the particles of the dextran Sephadex G 25 (Pharmacia, Uppsala, Sweden) swollen with water. When an aqueous solution is allowed to percolate into the gel, molecules larger than *M* 3500–4000 remain in the void volume of the gel and are removed immediately by washing. The molecules which have penetrated the gel particles are eluted in fractions according to the ease with which they pass in and out of the particles. The concentration of solution that can be used is limited solely by viscosity. In separate experiments 40 g. Difco yeast extract in 200 ml. of (*a*) demineralized water, (*b*) demineralized

Table 2. *Fractionation of yeast extract by elution from Sephadex G-25*

Solvent	Volume of eluate before the arrival of active material including void volume (ml.)	Active eluate	
		Volume (ml.)	% of original yeast-extract
Water	570	380	3·8–4·2
Dilute aqueous NH_3	570	380	14–16
Dilute aqueous HCl	495	250	17
5 % (v/v) aqueous ethanol	495	250	21

water adjusted to pH 9·0 with ammonia, (*c*) demineralized water adjusted to pH 3·0 with acetic acid, (*d*) 5 % (v/v) ethanol in demineralized water, were allowed to enter a column 1½ in. in diameter and 19 in. long containing 50 g. (dry) Sephadex G25 swollen with the solvent. The gels were eluted slowly with solvents of the same composition, the void volumes (294 ml.) being collected as an initial fraction, whereas the subsequent eluates were collected in 5 ml. fractions automatically. The fractions containing active material were located by testing whether 0·1 ml. added to 10 ml. of basal medium permitted growth of the organism. The active fractions were combined, evaporated to dryness and the weights of solid material determined (Table 2). The distribution of the active material in the fractions when water alone was used showed an early and late peak of concentration suggesting that the material occurred in more than one form.

Although water was the most efficient solvent, in practice the gel became pocketed with bubbles of CO_2 and its surface grew moulds. Consequently in routine productions dilute aqueous ammonia was used since this left the column ready for use again after the elution. The active material present in 40 g. yeast extract was recovered in 5·6–6·4 g. of dry powder in 49 hr but it was mixed with amino acids

which were nutrients of the Leptothrix. This powder is referred to subsequently as material I.

The active material and much of the colouring matter in material I was adsorbed on DEAE Sephadex A 50 fine-grade dextran when 2 g. of material I dissolved in 10 ml. of water percolated through a column of 5 g. (dry wt.) of this dextran swollen with water. This dextran is an anion exchanger in the chloride form with a capacity of 3–4 m-equiv./g. (dry wt.). Elution with water removed the active material in a 20 ml. fraction which yielded 0·46 g. of almost colourless solid when evaporated *in vacuo* (material II).

Alternatively, material I was acetylated in 6 g. portions (Cheronis, 1954). The active material was retained by a column of 40–50 ml. of Amberlite CG 120 (H) 16–50 mesh (twice the amount needed to retain the sodium ions present) from the acetylated product dissolved in 40 ml. water, and recovered by elution with aqueous ammonia (M). After a repetition of the process, the product (0·7 g. (dry wt.)) contained active material but did not give a positive test with ninhydrin. This material dissolved in 5 ml. water was allowed to percolate into a column of 5 g. DEAE Sephadex A 25 dextran (medium grade corresponding to A 50) swollen with water. The remaining colouring matter was retained strongly at the top of the column and on elution with water, after the void volume had been displaced, the active material was recovered in 25–30 ml. solution. Vacuum distillation and exhaustive drying over anhydrous $CaCl_2$ yielded 0·03 g. of a faintly yellow solid which was strongly hygroscopic and dissolved readily in water but not in absolute ethanol. It was obtained as a fine powder by precipitation from aqueous solution by ethanol, recovery being complete when the liquid contained 90% (v/v) ethanol. One mg. of this material (material III) permitted growth in 30 l. of basal medium to a population equiv. 0·5 mg. dry-wt. organism/ml.

Analysis of the isolated material III

When the isolated material III was heated to dryness with concentrated nitric acid a white crystalline powder was obtained which amounted to 48·88% of the original weight. Analysis of an aqueous solution of this powder by standard procedures (Morrison, 1961) isolated appreciable quantities of molybdenum, which according to the *Biochemist's Handbook* (1961 *a*) accounts for $5\text{–}9 \times 10^{-6}$% of the dry weight of baker's yeast and $9\text{–}13 \times 10^{-5}$% of brewer's yeast. Microtitration with lead nitrate, with diphenylcarbazide as indicator (Evans, 1939), established that the ash contained 16·22% (w/w) Mo. corresponding to 7·93% (w/w) Mo in material III. The content of Mo in material I was 2·31% (w/w).

The amino acid requirements of the Leptothrix

The organism was grown aerobically in a basal medium (containing (g./l.): 5 KH_2PO_4; 0·4 $MgSO_4.7H_2O$; 12 glucose; 5 material I; adjusted to pH 7·0), basal medium + 0·1% (w/v) nitrogenous material supplements (casein hydrolysate and individual amino acids). The sizes of the populations produced are compared in Table 3. Only DL-cystine had an effect comparable to that of casein hydrolysate, and only DL-cysteine was inhibitory. With the latter a few cultures showed a slight deposit after 800 min. (the usual period of lag) and then a slow growth after 26–33 hr to a population equiv. 0·31–0·36 mg. dry-wt. organism/ml. When the tests

were repeated at 0·5% (w/v) of supplement, L-arginine gave a maximum growth equiv. 0·25 mg. dry-wt. organism/ml. The effects of lower concentrations of DL-cystine are shown in Table 4. DL-Cystine at 0·0005–0·001% (w/v) was required for maximal growth and when its concentration was below 0·0005% (w/v) the rate of growth decreased.

Table 3. *Effect of casein hydrolysate and single amino-acids on the growth of Leptothrix at* 25°

Medium	Maximum growth equiv. mg. dry-wt. organism/ml.
Basal	0·15
Basal + casein hydrolysate	0·56
Basal + DL-cystine	0·38
Basal + L-ornithine	0·23
Basal + L-arginine or tyrosine	0·21
Basal + DL-cysteine	Trace
Basal + any one of others present in the hydrolysate	0·14–0·18

Basal medium (g./l.): 12 glucose, 5 purified butanol extract of yeast extract, 5·4 KH_2PO_4, 0·4 $MgSO_4.7H_2O$; buffered to pH 7·0. Nitrogenous material supplements at 0·1% (w/v).

Table 4. *Effect of cystine on the growth of Leptothrix at* 25°

Initial concentration of DL-cystine (%, w/v)	Maximum growth equiv. mg. dry-wt. organism/ml.	Mean generation time (min.)
0	0·15	420
0·00001	0·16	420
0·0001	0·23	400
0·0005	0·36	220
0·001	0·38	200
0·002–0·020	0·38	200

Basal medium as for Table 3.

The other amino acids at 0·1% (w/v) were retested in media to which 0·01% (w/v) DL-cystine had been added (Table 5). Citrulline and urea were tested also since with arginine and ornithine (which increased the amount of growth markedly) they are members of the ornithine cycle (Krebs, 1934). Whenever the addition was of an amino acid containing two nitrogen atoms per molecule the culture showed two successive phases of logarithmic growth at different rates, and all cultures showed a lag of about 13 hr. Mixtures of the amino acids which extended growth were tested; rates of growth as well as amounts of growth being measured (Table 6). Of the seven media which gave large populations, L-ornithine was present in four, L-arginine, L-proline and L-glutamate in three, and L-hydroxyproline in one (when with L-ornithine). L-Ornithine was present in the four media which gave the most rapid growths in phase one, but L-arginine was present in the four media which gave the most rapid growths in phase two. The fastest growths in both phases were in media containing L-ornithine and L-arginine. Different samples of L-ornithine gave slightly different results; chromatographic analysis detected traces of L-proline in some of the samples. Consequently L-arginine was preferred as a N-source.

Table 5. *Effect of amino acids on the growth of Leptothrix at 25° in media containing cystine*

Medium	Amount of growth equiv. mg. dry-wt. organism/ml.
Basal	0·38
Basal plus L-arginine	0·63–0·75
Basal plus L-ornithine	0·60–0·75
Basal plus L-proline	0·57–0·69
Basal plus L-glutamate	0·42–0·50
Basal plus L-hydroxyproline	0·39–0·44
Basal plus any one of others present in the hydrolysate	0·38–0·41
Basal plus citrulline	0·29
Basal plus urea	0·38

Basal medium as for Table 3 plus 0·01 % (w/v) cystine.

Table 6. *Growth of Leptothrix at 25° in media containing at* 0·1 % *(w/v)* L-*arginine* L-*ornithine,* L-*hydroxyproline,* L-*proline and* L-*glutamate either singly or two at a time*

Amino acids present	Maximum growth equiv. mg. dry-wt. organism/ml.	Mean generation times (min.) Phase 1	Mean generation times (min.) Phase 2
None	0·37–0·41	200	—
L-Arginine	0·75	200	260
L-Arginine plus L-ornithine	> 0·80	110	230
L-Arginine plus L-proline	> 0·80	170	350
L-Arginine plus L-hydroxyproline	0·76	180	430
L-Arginine plus L-glutamate	> 0·80	150	280
L-Ornithine	0·75	130	420
L-Ornithine plus L-proline	> 0·80	150	390
L-Ornithine plus L-hydroxyproline	> 0·80	150	520
L-Ornithine plus L-glutamate	> 0·80	130	500
L-Proline	0·69	210	770
L-Proline plus L-hydroxyproline	0·69	210	860
L-Proline plus L-glutamate	> 0·80	210	660
L-Glutamate	0·50	190	—

Basal medium as for Table 3: +0·01 % (w/v) DL-cystine.

Carbohydrate requirements

The abilities of a number of substances at 0·5% (w/v) to replace glucose in the medium were re-examined. Acetate, DL-malate, fumarate or α-ketoglutarate gave the same rate of growth as glucose (mean generation time, m.g.t., 200–210 min. for the first phase of logarithmic growth), whereas oxaloacetate gave a slower one (m.g.t. 320 min.) compared with the rate in absence of an added C-source (m.g.t. 230 min.). Glucose or acetate produced slightly greater amounts of growth than the other compounds, but all, with the exception of citrate, which was inhibitory, yielded denser populations than were obtained in absence of an added C-source.

Chemically defined media

The sodium salts chromate, manganate, molybdate, selenate, selenite, tellurate, tellurite, tungstate, and the chlorides chromous, chromic, manganous, were tested as

alternatives to the yeast extract, or materials I, II or III, needed to complete the basal medium ((g./l.) 12 glucose; 1 L-arginine hydrochloride; 0·1 DL-cystine; 5·4 KH_2PO_4; 0·4 $MgSO_4.7H_2O$; adjusted to pH 7·0). Only sodium molybdate and sodium vanadate allowed growth to take place. The effective ranges of concentration of these materials were very different; and whereas with molybdate the medium was colourless (unless the molybdate was in excess, when the production of phosphomolybdate caused an intense yellowing), with vanadate the cultures were pale yellow-green initially, then transitionally purple and finally dingy brown as growth ceased. Because of these colourings the amounts of growth in the presence of higher

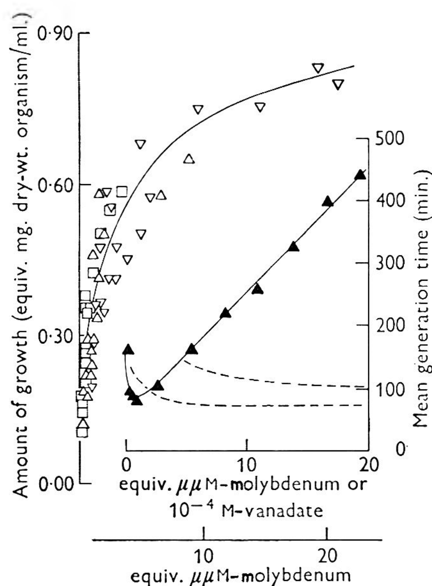

Fig. 1

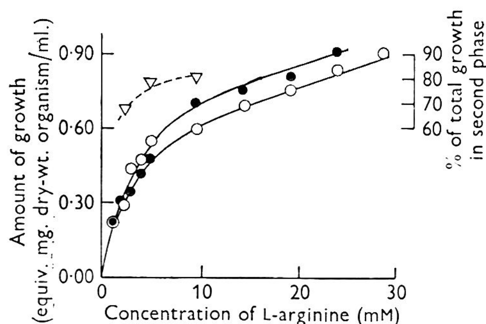

Fig. 2

Fig. 1. Growth of Leptothrix at 25° and availability of molybdenum or vanadium. Medium contained (g./l.): 30·0 glucose; 1 L-arginine hydrochloride; 0·1 DL-cystine; 5·4 KH_2PO_4; 0·4 $MgSO_4.7H_2O$; adjusted to pH 7·0. Molybdenum supplied as: molybdate, ▽; material I, △; material III, □. Rates of growth as mean generation times: all sources of molybdenum between the broken lines; vanadate, ▲.

Fig. 2. Growth of Leptothrix at 25° and the concentrations of nutrients in the medium. Medium contained (g./l.): 0·1 DL-cystine; 5·4 KH_2PO_4; 0·4 $MgSO_4.7H_2O$; adjusted to pH 7·0: plus glucose (mM): ○, 30; ●, 66. Percentage of total growth by the second phase in media containing 66 mM-glucose, ▽.

concentrations of molybdate, and all concentrations of vanadate, were not measured. The relations between rate of growth and concentration of (1) material I, (2) material III, (3) sodium molybdate, (4) sodium vanadate, and the relations between amount of growth and the first three of these supplements are shown in Fig. 1.

The rates of growth of cultures containing sodium molybdate were not highly reproducible, but the cultures that started to grow with a mean generation time of 80 to about 130 min. clearly had a second phase of logarithmic growth with a mean generation time of 160–200 min. Below approximately 5 μμM the concentration of molydate controlled the initial rate of growth. In the amount and rate of growth the

organism showed no preference for any of the forms of molybdenum tested in the basal medium. Molybdate was very much more effective than vanadate, which had a critical concentration above which it progressively decreased the rate of growth. In subsequent work 1 g./l. sodium molybdate was the supplement used to complete the medium.

Amount of growth and the concentration of nutrients

In the chemically defined medium DL-cystine was a negligible source of nitrogen, carbon and energy as compared with the L-arginine and glucose. The sole N-source, L-arginine, was also a possible source of carbon and energy and thus the availability of L-arginine may have affected the utilization of glucose and vice versa. The dependence of amounts of growth on the initial concentration of glucose in the presence of different initial concentrations of L-arginine hydrochloride, and on the initial concentration of L-arginine in the presence of different concentrations of glucose, are shown in Figs. 2 and 3. Without glucose there was negligible growth but in its presence L-arginine caused a considerable increase in the ratio of cell material produced to glucose consumed. This is explicable if the arginine is used as a source of C but not as a source of energy. Glucose has a much smaller sparing action on the utilization of L-arginine. Unlike the cases of coliform organisms growing in glucose + ammonium salt + buffer media (Dagley, Dawes & Morrison, 1951) the amounts of growth of the Leptothrix were not linearly dependent on the initial concentrations of nutrients. Particularly with respect to L-arginine the production of Leptothrix was more efficient when the amount of nutrient severely limited the amounts of growth. These cultures showed two logarithmic phases of growth and the proportion of the total amount of growth which occurred in the first phase was greater when the total growth was limited severely by a low initial concentration of L-arginine. This is shown also in Fig. 2 for media containing 66 mM-glucose.

Effect of serial subcultivation in the defined medium on the behaviour of the Leptothrix strain

After serial subcultivation in the defined medium cultures of the organism showed only one phase of growth, but there was some dependence of the rate of growth on the concentrations of glucose and L-arginine (Table 7). The amounts of growth were not detectably different from those shown in Figs. 2 and 3. Thus serial subcultivation had increased the rate of the second phase of growth and the two phases merged.

Table 7. *Effect of glucose and L-arginine concentrations on growth of Leptothrix at* 25°

Conc. glucose (mM)	66	66	30	30
Conc. arginine (mM)	47·5	4·75	47·5	4·75
Mean generation time (min.)	84	96	102	102

Basal medium (g./l.): 0·1 DL-cystine; 1 $Na_2MoO_4.2H_2O$; 5·4 KH_2PO_4; 0·4 $MgSO_4.7H_2O$; adjusted to pH 7·0.

The initial pH value may affect the amount of growth (Fig. 4). Growth took place in the media initially at pH values greater than 5·1 and less than 8. When the medium contained initially only 30 mM-glucose and 4·75 mM-L-arginine the amount of growth was not dependent on the initial pH value when this was between 5·6 and 7·7, but in media containing 66 mM-glucose and 47·5 mM-L-arginine the amount of growth

was dependent on the initial pH value being greatest at pH 7. Thus in the less nutritious medium initially between pH 5·6 and 7·7 the concentrations of nutrients were limiting whereas in the richer medium growth ceased for a reason which was related to the initial pH value.

Effect of temperature on growth of the Leptothrix

The organism was subcultivated in the defined medium six times at each of a number of progressively higher temperatures. After each change of temperature the lag phase was prolonged in the first two subcultivations but then decreased progressively; after acclimatization to 37° the lag phase was decreased to about 2 hr. The rates of growth were still erratic but showed an overall increase with increase of temperature, the shortest mean generation times recorded being (min.): 83 at 25°; 64 at 30°; 48 at 34°; 37 at 37°. After six subcultivations at 37° the organism had the same nutritional requirements as at 25°.

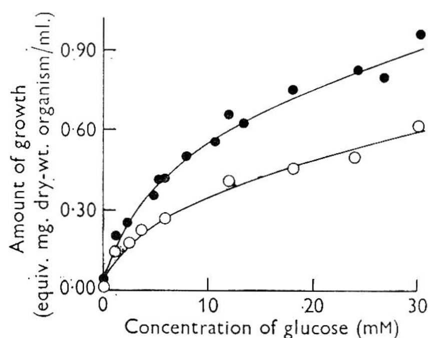

Fig. 3

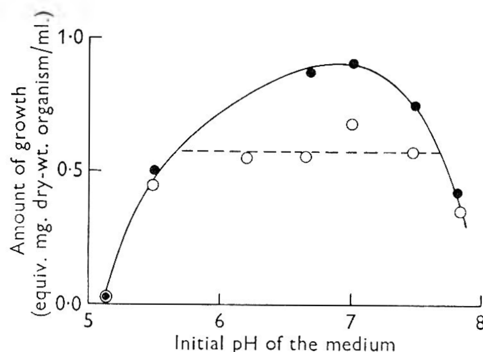

Fig. 4

Fig. 3. Growth of Leptothrix at 25° and the concentrations of nutrients in the medium. Medium contained (g./l.): 0·1 DL-cystine; 5·4 KH_2PO_4; 0·4 $MgSO_4.7H_2O$; adjusted to pH 7·0: plus L-arginine (mM): ○, 4·75; ●, 47·5.

Fig. 4. Growth of Leptothrix at 25° and the initial pH of the medium. Medium contained (g./l.): 0·1 DL-cystine; 5·4 KH_2PO_4; 0·4 $MgSO_4.7H_2O$: pH adjusted with NaOH: plus: ●, 66 mM-glucose + 47·5 mM-L-arginine; ○, 30 mM-glucose + 4·75 mM-L-arginine.

The role of molybdenum and cystine

Measurements of the consumption of oxygen by suspensions of the Leptothrix (equiv. 1·5 mg. dry-wt. organism/ml. and washed twice by isotonic buffer) in glucose + buffer solutions showed that molybdate and cystine both enhanced the rate of oxidation and that these effects were additive. In the absence of added molybdate the oxidation was of short duration, but in its presence continued to the limit measurable by the Warburg apparatus. It has not yet been possible to demonstrate an inability to consume oxygen in the absence of a source of molybdenum but this may be because of the extremely small amount of molybdenum required.

Similarly measurements (Friedemann & Haugen, 1943) of the aerobic production of pyruvate from glucose established that: in the absence of a source of molybdenum no pyruvate was produced in 12 hr; in the presence of sodium molybdate, or of

material III, pyruvate production became significant after 5 hr and thereafter accelerated rapidly to a maximum steady rate; in the presence of yeast extract, material I or material II, the maximum rate was established after 2 hr. Thus the source of molybdenum was required for oxidative processes and the active form is not molybdate itself though the Leptothrix can utilize molybdate to supply its needs.

DISCUSSION

The strain of Leptothrix examined was exacting in two respects: (i) it required for growth a Mo-containing compound and cystine; (ii) it was a strict aerobe, incapable of fermenting sugars. It has been established that the need for molybdenum was connected with oxidative processes since there was stimulation of oxygen uptake in glucose + buffer solutions when molybdenum was provided, and vanadate, which could replace molybdate, showed progressive valency changes as the cultures grew. In particular the need for Mo was concerned with the oxidative production of pyruvate from glucose. A requirement for Mo is known for the reductive systems of Neurospora, soya bean and probably *Escherichia coli*, where it is also thought to act as an electron carrier: the relevant literature has been reviewed by Nason (*Biochemists' Handbook*, 1961*b*). Other biological uses of Mo are known: it affects the nitrogen metabolism (Mulder, 1948) and the growth (Mulder, 1954) of some higher plants and micro-organisms; molybdate is metabolized by Azotobacter (Keeler, 1958) and by certain farm animals (Stewart, Farmer & Mitchell, 1946). The concentration of molybdenum (mμM) required by Azotobacter (Burk, 1934) are not as small as with the present Leptothrix and vanadium can replace molybdenum (Horner, Burk, Allison & Sherman, 1942) in the case of Azotobacter more easily. The strain of Leptothrix, growing in a chemically defined medium, provides an opportunity to study mechanisms which may be of widespread importance.

In the defined medium the Leptothrix showed no preference for the materials isolated from yeast extract over molybdate when growth rates were the criterion. However, this was probably because the rate of growth was not controlled by a step in the part of the mechanism which required Mo: the measurements of pyruvate production showed a clear preference for the form of Mo occurring in the yeast extract, and that the acetylation procedure used in the isolation of material III had been detrimental. The need for Mo may be exhibited generally by organisms of the Sphaerotilus–Leptothrix group since all the media used by Mulder (1964) in his survey of them contained either yeast extract (Difco) or a basal culture solution containing (mg./l.) 0·05 $Na_2MoO_4.2H_2O$.

As the Leptothrix's requirement for Mo was almost specific this strain might provide the basis of a micro-biological assay for extremely low concentrations of this element.

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Further Studies of the Genus *Pediococcus*

By ELISABETH COSTER* and HELEN R. WHITE
Microbiology Department, Queen Elizabeth College (University of London), London, W. 8

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SUMMARY

The cultural, physiological and serological characters of new Pediococcus isolates have been studied. Suggestions are made for the amendment of the definition of the genus *Pediococcus*. The genus is considered to contain the four species *P. cerevisiae*, *P. parvulus*, *P. damnosus* and *P. halophilus*.

INTRODUCTION

The genus *Pediococcus* is now well established by the systematic studies of Mees (1934), Pederson (1949), Pederson, Albury & Breed (1954), Jensen & Seeley (1954), Nakagawa & Kitahara (1959), and Günther & White (1961*a*, *b*). There is, however, conflict of opinion among various workers regarding the definition of various species. The description of the type species *P. cerevisiae* Balcke given by Pederson (1949) and followed by Jensen & Seeley and Günther & White is not accepted by Nakagawa & Kitahara who consider that this name is valid only for cultures isolated from beer and apply the name *P. pentosaceus* Mees to strains fitting Pederson's description.

Beer was the original source of Balcke's strain (Balcke, 1884) and many workers have obtained cultures from this habitat. These cultures have been named *Pediococcus cerevisiae* (Nakagawa & Kitahara, 1959); *P. damnosus* (Claussen, 1903) with varieties *salicinaceus* and *perniciosus* (Mees, 1934); *Streptococcus damnosus* with varieties *salicinaceus*, *pentosaceus*, *viscosus* and *limosus* (Shimwell, 1949), and with variety *diastaticus* (Andrews & Gilliland, 1952). Günther & White (1961*a*) examined three cultures labelled *P. damnosus* (originally from Mees and Claussen) and concluded that they form a distinct physiological group.

Pediococcus halophilus Mees differs from other pediococci in its absolute requirement for sodium chloride for growth (Günther & White, 1961*a*). The work of Sakaguchi (1959) on bacteria associated with the fermentation process of soya sauce is of interest here. He isolated salt-requiring pediococci which he designated *P. soyae* (Sakaguchi, 1958). Later these isolates were found to resemble closely *P. halophilus* strain TC1 and were renamed *P. halophilus* by Nagakawa & Kitahara (1959).

Other species of pediococci have been proposed by Nakagawa & Kitahara (1959). *Pediococcus acidi lactici* Lindner was the name given to strains having an optimum temperature of 40°, and *P. urinae equi* Mees to organisms with the ability to grow in media of pH 9.

Views vary on the taxonomic position of aerococci. Williams, Hirch & Cowan

* Present address: Bacteriology Department, University of Melbourne, Australia.

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(1953) described a large number of strains and proposed *Aerococcus* as a separate genus, but Deibel & Niven (1960) regarded such strains as pediococci forming the species *Pediococcus homari*. Some of these cultures have been included in the present study and their taxonomic position is further considered.

In their preliminary survey of the serology of the pediococci and allied genera, Günther (1958) and Günther & White (1961 *b*) showed the presence of precipitins not shared with members of the genera *Streptococcus*, *Leuconostoc*, *Staphylococcus* and generally not with aerococci. Separate precipitins characteristic of *Pediococcus cerevisiae* (group I), *P. parvulus* (group II), and *P. damnosus* (group IV) were also demonstrated and could be considered analogous to the 'group' antigens of streptococci. The detection of other precipitins and agglutinins indicated closer serological inter-relationships within the pediococci. However, Günther & White's group III strains did not appear to possess an antigen-antibody system (detectable by the precipitin reaction) in common with *P. cerevisiae*, *P. parvulus*, *P. damnosus*, *P. halophilus* or *Aerococcus viridans*.

The present study extends the work of Günther & White (1961 *a*, *b*). The examination of the physiological and serological characters of fresh cultures was used to assess the validity of previous classifications. Repetition of Dr Günther's physiological work was avoided except where necessary for comparative purposes or where it became evident that new characteristics might be used to define the genus and species in a more exact manner. The aim of the serological investigation was the more detailed study of *P. parvulus* and group III organisms.

METHODS

Cultures

Sources of cultures. Cultures were freshly isolated from a variety of habitats of both animal and vegetable origin. Tomato juice agar (Oxoid), Rogosa SL agar (DIFCO) and 'Beer' agar (Williamson, 1959) were used as primary selective media. Strains representative of named species were also included. Details of all isolates examined are given in Table 1.

Maintenance of stock cultures and culture media used. The method of Günther & White (1961 *a*) was employed in the maintenance of stock cultures. Tomato juice (TJ) broth or agar with the addition of Tween 80 (0·1 %, v/v), pH adjusted to 6·6, were used in the maintenance, preparation of inocula and all experimental investigation of cultures with the exception of: (*a*) strains labelled *Pediococcus halophilus* where sodium chloride was added to all media, 7 % (w/v) for strains D2, D12, and D8, and 5 % (w/v) for TC1; (*b*) strains BE-121, BE-125, BE-126, BE-127, AG, P1 and PD isolated from beer, in future referred to as the 'brewing strains', which were grown on a beer medium (Williamson, 1959) modified by using bottled pale ale to replace unhopped beer with 'Panmede' liver digest (1 %, w/v) added as liver extract source; (*c*) *Pediococcus homari* FP-2, S-53A, strains CH-3, SF3, KT-1 and KT-2 which were cultured on APT agar or broth (Evans & Niven, 1951).

Media for growth of organisms other than pediococci, used in the serological investigation were as follows: *Aerococcus viridans*, ATP agar or broth; staphylococci, nutrient agar or broth (Günther & White, 1961 *a*); and streptococci, 'Oxoid' cooked meat medium for storage and glucose Yeastrel (GY) agar or broth (Günther & White,

1961*a*) for routine subculture. Seven strains of aerococci and staphylococci were obtained from the National Collection of Type Cultures, London, and streptococci representing Lancefield's serological groups A to S from Wellcome Research Laboratories, Beckenham, Kent.

Table 1. *Details of cultures examined*

Code numbers	Species name (as received)	Habitat	Source
NZ-1, NZ-3, NZ-6, NZ-R2, NZ-R3, NZ-T1, E-1	—	Cheddar cheese	—
SA-88, SA-90, SA-92, SA-96, SA-100, SA-103	—	Sauerkraut	—
F-35, F-98	—	Faecal material from turkeys	—
TO-111, TO-118, TO-119	—	Tinned tomatoes	—
BE-121, BE-125, BE-126, BE-127	—	Guinness beer	—
BE-1, BE-2	—	Lager beer yeast	(1)
AG	*P. damnosus*	Beer	(2)
P1	*P. limosus*	Beer	(2)
PD	*P. diastaticus*	Beer	(2)
PP	*P. pentosaceus* (Kitahara & Nakagawa, 1959)	—	(3)
PA	*P. acidi lactici* (Kitahara & Nakagawa, 1959)	Mash, type not specified	(3)
D2, D12, D8	*P. halophilus* (Kitahara & Nakagawa, 1959)	Soy sauce brewing mashes	(3)
FP-2	*P. homari*	Ham curing brine	(4)
S-53A	*P. homari*	Discoloured bacon	(4)
CH-713, CH-778, CH-797, CH-819, CH-867, CH-3SF3	—	Vacuum packed bacon	(5)
KT-1, KT-2	—	Discoloured cooked hams	(6)
NCIB 8559	*Streptococcus cremoris*	Beer	(7)
L-16, L-20, L-22, L-24, L-92, L-95, L-148, L-171 L-223, L-345, L-347, L-351, L-352, L-354	Group III	Silage	(8)

It may be noted that the species name of some cultures listed above was altered as a result of this investigation.

(1) Dr D. H. Williamson, The Brewing Research Foundation, Nutfield, Surrey.
(2) Dr R. B. Gilliland, Arthur Guinness, Son and Co. Ltd., Dublin.
(3) Dr K. Sakaguchi, Noda Institute for Scientific Research, Noda-shi, Chiba-ken, Japan.
(4) Dr C. F. Niven, Jr., A.M.I.F., University of Chicago, Illinois, U.S.A.
(5) Mr J. J. Cavett, Unilever Research Laboratory, Sharnbrook, Bedford.
(6) Mr M. G. Read, Kearley and Tonge Ltd., Southall, Mddx.
(7) National Collection of Industrial Bacteria, Torry Research Station, Aberdeen.
(8) Dr H. L. Günther, Microbiology Department, Queen Elizabeth College, University of London, W. 8.

Preparation of inocula and conditions of incubation. Transfers for preparing inocula were made every 24 hr except for *Pediococcus halophilus*, BE-1, BE-2 and *Streptococcus cremoris* 8559 where transfers were made every 48 hr; these strains will in future be referred to as the 'slower growing' strains. The 'brewing strains' were subcultured fortnightly. For the examination of most cultural and biochemical characters 0·02 ml. of inoculum/5 ml. of medium was used.

All cultures were incubated aerobically at 30° except strains BE-1, BE-2, and 8559

which were incubated at 22°. The 'brewing strains' were incubated in an atmosphere of 95% (v/v) hydrogen + 5% (v/v) carbon dioxide at 22°.

Physiology

In these studies new strains were compared as closely as possible with cultures studied by Günther & White (1961*a*, *b*), in particular the proposed type strains *Pediococcus cerevisiae* ATCC 8081 (Günther & White, 1962) and *P. parvulus* S-182 (Günther, Coster & White, 1962).

Morphological examination. The methods of Günther & White (1961*a*) were used to observe morphology, Gram reaction and motility except that Rowland's method was employed to detect capsules (Rowland, 1914). The slower growing strains were examined after 48 hr and brewing strains after 7 and 14 days of incubation.

Cultural characters. In general the methods of Günther & White (1961*a*) were again used. Brewing cultures were examined after 7 and 14 days of incubation. Colony size was determined by measuring the diameter of ten colonies picked at random. The presence of growth on Rogosa SL agar was observed for all cultures after 24 hr, 48 hr and 7 days of incubation, brewing strains were examined after 7, 14 and 28 days.

Factors affecting growth. Again the methods of Günther & White (1961*a*) were followed except for using pH levels of 4·4 and 8·6, and testing some strains for growth at the 10% (w/v) sodium chloride level. All strains were grown in the liquid medium used for their routine cultivation and examined after 1, 2 and 7 days of incubation. Brewing strains were examined after 7, 14 and 28 days of incubation.

Biochemical reactions. The methods of Günther & White (1961*a*) were employed for the following tests; utilization of ammonium salts as sole nitrogen source, hydrolysis of gelatin and aesculin; production of carbon dioxide from glucose; final hydrogen-ion concentration in GY broth; production of acetylmethylcarbinol from glucose; haemolytic reaction (brewing strains incubated for 14 days), and production of ammonia from arginine (brewing strains tested after 21 days of incubation). Günther & White's (1961*a*) method was also used to determine the reaction in litmus milk, bearing in mind that not all strains appear to require folinic acid for growth (Günther & White, 1961*a*). A fairly large inoculum was used (0·04 ml. of T.B.) so some carry-over may have occurred.

The presence of catalase was observed after cultivation for 24 hr using the method and medium of Günther & White (1961*a*). As some strains grew poorly in this medium all cultures were also tested after subculturing for 24 hr (7 days for brewing strains) on YTG agar (Felton, Evans & Niven, 1953) modified by the addition of Tween 80 (0·1%, v/v), $FeSO_4$ (0·04%, w/v), $MgSO_4$ (0·08%, w/v) and $MnCl_2$ (0·014%, w/v).

The method devised by Rogosa (1961) was employed to test for the reduction of nitrates. Cultures were grown in the indole nitrite medium (BBL) with $MgSO_4$, $MnSO_4$ (0·05%, w/v) and with and without added glucose (0·01%, w/v).

The type of lactic acid produced from glucose by pediococci was examined using the medium and method of Camien & Dunn (1954). The medium was modified by the addition of $MgSO_4$ and $MnSO_4$ (0·05%, w/v) and Tween 80 (0·1%, v/v). Cultures were incubated for 1 month. The optical rotation was determined polarimetrically on a 5% (w/v) aqueous solution.

The general method of Günther & White (1961*a*) was followed in determining carbohydrate fermentation reactions apart from a modification of the basal medium by the addition of Tween 80 (0·1 %, v/v).

No record of lipolytic enzymes in pediococci was found in the literature. The observation that the presence of Tween 80 stimulated growth (Garvie & Gregory, 1961; Coster, 1963) might indicate the presence of lipolytic enzymes. The method of Sierra (1957) was employed to test for the presence of these enzymes in some pediococcus cultures using TJ agar as the basal medium.

Serology

Organisms used in the preparation of antisera. Antisera were prepared against 14 strains of pediococci, 11 of these being strains already examined physiologically by Günther & White (1961*a*). *Pediococcus cerevisiae* (group I) was represented by strains 8081 and BP-1; *P. parvulus* (group II) by strains SS-101, HY-22S, S-182 and S-344; group III by cultures L-24, L-92, L-345, L-351 and L-352. Strains NZ-R3, F-98 and *P. damnosus* PD were also used to prepare antisera. An antiserum was prepared against *Aerococcus viridans* NCTC 8251.

Preparation of antisera. All antisera except antiserum *P. damnosus* PD were prepared by intravenous injection of whole living organisms into rabbits known to be free of naturally occurring antibodies. Cells were grown in 40 ml. glucose Lemco broth containing as %, w/v; glucose, 1·0; lab Lemco, 1·0; peptone (Evans), 1·0; NaCl, 0·5; $MgSO_4$, 0·05; $MnSO_4$, 0·05; Tween 80, 0·1 (v/v). The methods of Günther & White (1961*b*) were followed regarding inoculation and incubation of the medium, and preparation of cells for animal injection.

Great difficulty was experienced in preparing antisera against strains L-24 and L-92. Potent antisera were obtained after five courses of injections and antibodies detected for only a week in the animal before loss in potency occurred. Two injections of 2 ml. each were given always a week before these antisera were withdrawn.

Due to the lack of reactivity noted in preliminary precipitin tests of cultures considered to resemble the species *P. damnosus* Mees with *P. cerevisiae* antisera, a disintegrated cell suspension (prepared with the Mickle tissue disintegrator) was used to obtain a potent antiserum against strain PD by the method of Shattock (1949) who found this method successful for refractory Lancefield group D streptococci. Shattock's method was also used by Günther & White (1961*b*). Cells for injection were grown in beer broth for 14 days.

Sera were stored at 4° without preservative.

Commercial antisera of streptococci Lancefield's groups A to Q were obtained from Burroughs Wellcome and Co. Ltd.

Preparation of extracts. The HCl extraction method of Lancefield (1933) as modified by Sharpe (1955*a*) and Günther & White (1961*b*) was employed. Duplicate extracts were prepared of new isolates and of most of the pediococci in Dr Günther's collection (Günther & White, 1961*a*, Table 1), and also of representative aerococcus, staphylococcus, leuconostoc and streptococcus strains.

Precipitin tests. The method of Sharpe (1955*a*) was used, observations being made at intervals up to 1 hr.

Precipitin–absorption tests. The techniques outlined by Sharpe (1955*a*) and Günther & White (1961*b*) were followed.

Tube-agglutination tests. Tests were carried out in Dreyer's agglutination tubes in 0·9 % (w/v) saline using a suspension of washed cells, opacity approximately equivalent to Brown's tube no. 3, grown for 16 hr in TJ broth. The tubes were read after overnight incubation at 50°.

RESULTS

Physiology

All new isolates resembled each other in their morphological arrangement, the cells occurring singly, in pairs, tetrads, regular and irregular clusters and short chains of 4 to 5 cells. All cultures were Gram-positive, non-motile, without spores or capsules. The formation of a turbid growth and slightly viscid sediment in broth was typical of all strains except the strain *Pediococcus limosus* (P1) which could form slime. No strains were able to hydrolyse gelatin, utilize ammonium salts as sole nitrogen source, reduce nitrates to nitrites or nitrogen, or produce carbon dioxide from the fermentation of glucose. Lipolytic enzymes were not detected.

On comparing the characters shown by new isolates with those described by other workers it was evident that all new cultures closely resembled previously defined species. In this section the detailed consideration of characters of new strains is therefore presented under species headings.

Strains fell into two major physiological groups, those resembling pediococci as defined by Pederson (1949), Jensen & Seeley (1954), Günther & White (1961*a*, *b*); and those similar to *Pediococcus homari* (Deibel & Niven, 1960) and *Aerococcus viridans* (Williams *et al.* 1953). The latter strains have been labelled aerococci in Table 2 which summarizes the physiological characters of the strains studied.

Pediococci. The majority of isolates belonged to this group and resembled strains previously placed in the four species *Pediococcus cerevisiae*, *P. parvulus*, *P. damnosus* and *P. halophilus*.

Pediococcus cerevisiae. Seventeen strains belonged to this species, NZ-1, NZ-3, NZ-6, NZ-R2, NZ-R3, NZ-T1, E-1, TO-111, TO-118, TO-119, CH-713, CH-788, CH-797, CH-819, CH-867, and the two cultures received as *P. pentosaceus* (PP) and *P. acidi lactici* (PA). Cultures were easily recognized by their profuse growth in TJ broth and on Rogosa SL agar in 24 hr; surface agar colonies having a diameter between 0·25 mm. and 1·2 mm.; ability to initiate growth at pH 8·6 after incubation of 5 days; the production of ammonia from arginine, a low pH value in GY broth, and the formation of acid, usually with coagulation, in litmus milk. All isolates grew well in the presence of 4 % and 6·5 % (w/v) sodium chloride in 24 hr except for one strain where growth was delayed at the 6·5 % level, only half showed poor growth at the 10 % level. Cultures placed in this species showed growth at the widest temperature range, the optimum occurring between 30° and 37°, some strains growing at 45° and 50°. Catalase was present in all strains on first testing (except PA), but could not be detected in one culture (NZ-6) on repeating the test 18 months later. A narrow zone of beta haemolysis was observed in 10 cultures on first isolation. This character was lost in all but 4 cultures on retesting a year later. Lactic acid isolated from one culture (NZ-R3) was optically inactive.

Pediococcus parvulus. Eight cultures SA-88, SA-90, SA-92, SA-96, SA-100, SA-103, F-35, F-98, formed a distinct entity based on the following characters: moderate growth in TJ broth; moderate growth after 48 or 72 hr on Rogosa SL agar; colony

size ranging from 0·25 mm. to 0·7 mm.; inability to grow at pH 8·6; inability to grow at 45° or in the presence of 10 % (w/v) sodium chloride. All organisms, except one, grew in the presence of 4 % (w/v) sodium chloride within 24 hr. Growth was considerably delayed at a concentration of 6·5 %, one strain did not grow at this sodium chloride concentration. Catalase was not detected, ammonia was not produced from arginine nor acetylmethylcarbinol from glucose. Litmus milk showed a weak acid reaction. Lactic acid obtained from two strains (SA-100, F-35) was optically inactive. The two strains isolated from turkey faeces (F-35 and F-98) differed from the other cultures in this group in showing poor growth at 37° and in the presence of 4 % sodium chloride, with no growth at 40°.

Pediococcus damnosus. The ten cultures isolated from beer and beer yeast, BE-1 BE-2, BE-121, BE-125, BE-126, BE-127, NCIB 8559, AG, PD and P1 were characterized by their low optimum temperature of 22°, no growth at 37° nor in the presence of 4 % (w/v) and 6·5 % sodium chloride. Growth in broth cultures was moderate to poor, and it is obvious that if a better growth medium could be devised these results might be modified and the subgrouping amended. Catalase was absent and no ammonia was formed from arginine. (More variation in physiological characters as between strains occurred in this species than in the species *P. cerevisiae* and *P. parvulus*.) Two main divisions were recognized within *P. damnosus*.

Three strains (BE-121, BE-126, AG) were distinct in having transparent colonies on beer agar, some punctiform in shape with a diameter varying from 0·15 mm. to 0·9 mm.; giving poor growth in broth culture; no growth at 10°, 30°, pH 4·4 or 8·6, or in the presence of 4 % (w/v) and 6·5 % sodium chloride. No acetylmethylcarbinol was formed from glucose. Growth was stimulated by anaerobic incubation. These cultures formed group (*a*).

The remaining strains except strain P1 formed group (b) and showed the off-white circular colonies characteristic of pediococci, and moderate growth in broth culture. Growth took place at 10°, 30°, and pH 4·4. On further examination strains BE-1, BE-2 and NCIB 8559 were found to have a colony size varying between 0·2 mm. and 0·7 mm., they also formed acetylmethylcarbinol from glucose and fermented trehalose. Strains BE-125, BE-127 and PD attained a larger colony size of up to 1·2 mm. The culture received as *P. limosus* P1, although showing the characters shared by group (b) cultures, was distinctive in forming slime in beer and TJ broth under anaerobic conditions, giving good growth in the liquid media used and having a larger colony size of up to 2 mm. diameter.

Pediococcus halophilus. Cultures D2, D12 and D8 were distinctive in their absolute requirement for sodium chloride in all media used during this study. Growth was present but delayed at 1 % (w/v), 2 %, 3 %, 4 %, 15 % and 20 %, maximum growth was obtained after 48 hr in 7 % and 10 % sodium chloride. Temperature growth range was from 10° to 40°, strains were able to initiate growth at pH 8·6 after 5 days of incubation but not at pH 4·4. Catalase was not detected, ammonia was not formed from arginine and no growth occurred on Rogosa SL agar.

Aerococci. Cultures *Pediococcus homari* FP-2, S-53A; KT-1, KT-2, CH-3SF3 were separated from strains considered to be pediococci on the basis of the following characters; inability to grow well in TJ broth, on Rogosa SL agar and at pH 4·4; inhibition of growth under anaerobic incubation and in stab culture, growth occur-

Table 2. *Comparison of physiological characters of pediococci and aerococci*

	Pediococci							
			P. damnosus					
	P. cerevisiae	*P. parvulus*	Group (a)	Group (b)	P1	Group III*	*P. halophilus*	Aerococci
Number of strains	17	8	3	7	1	14	3	5
Growth in TJB (pH 6·6)	+ + +	+ +	+	+	+ +	+ +	+ +	−
Optimum temp. 22°	−	−	+	+	+	−	−	−
Optimum temp. 30°	+	+	−	−	−	+	+	+
Growth at 10°	+	+	−	+	+	+	+ (2)	+ + +
37°	+ + +	+ +	−	−	−	+ +	+ +	+ + +
40°	+	+ (6)	·	−	·	+ +	+	+ +
45°	+ (8)	−	·	−	·	−	−	−
Growth in NaCl 4 %, w/v	+ + +	+ +	−	−	−	+ +	+ +	+ + +
6·5 %	+ +	+ (7)	−	−	−	− (11)	+ +	+ + +
10 %	+ (9)	−	·	·	·	·	+ +	+ + +
Growth at pH 4·4	+ + +	+ +	−	+	+	− (4/4)	−	−
8·6	+ + +	−	−	−	−	− (4/4)	+ +	+ + +
Growth on RSLA	+ + +	+ +	+	+	+	+	−	−
Slime production	−	−	−	−	+	−	−	−
Catalase reaction	+ (15)	−	−	−	−	−	−	− (4)
NH_3 from arginine	+ (14)	−	−	−	−	−	+	−
Aesculin hydrolysis	+	+ (4)	+ (2)	+ (3)	−	+	+	+
AMC from glucose	+	−	−	+ (3)	+	−	+ (2)	−
Final pH in GYB	3·2–4·2	3·74–4·02	5·41–5·94	3·95–6·62	4·13	4·4–5·0	4·52–4·64	4·72–5·26
Litmus milk								
Acid reaction	+	+ (weak)	−	−	−	+ (8)	−	−
Dye reduction	−	−	−	−	−	−	−	−
Coagulation	+ (15)	−	−	−	−	−	−	−
Acid from								
Glucose	+	+	+ (2)	+ (5)	−	+	+	+
Xylose	− (12)	−	−	−	−	− (13)	−	−
Arabinose	+	−	−	+ (2)	−	− (13)	+	−
Fructose	+	+	− (2)	+ (2)	−	+ (12)	+	+
Sucrose	−	+ (1)	−	−	−	−	+	+
Lactose	+ (14)	− (5)	−	−	−	− (12)	+ (2)	−
Maltose	+ (16)	+ (7)	+ (2)	−	−	+ (8)	+	+
Trehalose	+	+ (7)	− (2)	+ (3)	−	−	+	−
Raffinose	−	−	−	−	−	−	− (2)	+
Inulin	−	+ (1)	−	−	−	−	−	−
Dextrin	−	−	−	−	−	+	−	+ (2)
Glycerol	+ (11)	−	−	−	−	−	+	−
Mannitol	−	+ (3)	−	−	−	−	+	+ (3)
Sorbitol	−	− (7)	−	−	−	−	+	−
Salicin	+	+	− (2)	− (6)	−	− (8)	+	+

+ + +, Profuse growth; + +, good growth; +, moderate to poor growth, or a positive reaction; −, no growth or reaction.
TJB = tomato juice broth; RSLA = Rogosa SL agar; GYB = glucose Yeastrel broth; AMC = acetylmethylcarbinol.
Figures in parentheses denote number of strains giving indicated reaction: if expressed as a fraction denotes number of strains giving reaction/number tested.
* Most results taken from table 2, Günther & White (1961 *a*).

ring along the upper half only of the inoculation; good growth in the presence of 4 % (w/v), 6·5 % and 10 % sodium chloride but not at 15 % or 20 %; profuse growth at pH 8·6.

Serology

The antiserum prepared against *Pediococcus cerevisiae* 8081 used as a reference serum gave similar results in the precipitin reactions to the antiserum prepared against the same culture by Günther & White (1961 *b*) but showed less specificity in the agglutination tests.

Cultures of *Pediococcus damnosus* M-1, 8519, 8520 examined by Günther & White (1961 *a*) are included in our *P. damnosus* group (*b*) of this species. Difficulty was experienced in obtaining potent extracts of some cultures placed in the species *P. damnosus* on physiological evidence which requires further investigation.

No formation of the Tween antibodies noted by Middlebrook & Dubos (1947) for the tubercle bacillus was observed in any of our antisera which thus resembled lactobacilli antisera (Sharpe, 1955 *b*).

Precipitin tests. The results are summarized in Table 3. All antisera reacted with their homologous extract and most of the heterologous extracts of the same species or physiological group. Antisera prepared against *Pediococcus cerevisiae* reacted with most extracts of *P. cerevisiae*. Antisera against *P. cerevisiae* 8081 and BP-1 reacted with approximately half the extracts of *P. parvulus* cultures while antiserum NZ-R 3 precipitated consistently with more extracts of *P. cerevisiae* and *P. parvulus* strains.

Pediococcus parvulus and *P. damnosus* antisera reacted with the majority of *P. parvulus* and *P. cerevisiae* extracts. *Pediococcus damnosus* antiserum reacted with strains *P. damnosus* BE-125, BE-127 and P 1 but not with strains placed in group (a) or other group (b) strains. Extracts of this species showed little reactivity with other antisera, group (a) not reacting with any antisera.

The antisera prepared against group III strains showed many cross-reactions with *Pediococcus cerevisiae*, *P. parvulus* and a few *P. damnosus* extracts. Group III extracts did not react with antisera prepared against strains other than group III, except for a few isolated reactions which proved to be non-specific on absorption of the antiserum with homologous cells. Cross-reactions were also observed between the antiserum prepared against *Aerococcus viridans*, and *Pediococcus cerevisiae* and a few *P. parvulus* extracts, but none occurred between extracts of aerococci and pediococcal antisera except one strain (FP-2) which formed a heavy precipitate.

Extracts of *Pediococcus halophilus* strains did not react with any antisera except that of *P. cerevisiae* NZ-R 3; precipitated with one culture (TC 1) with *A. viridans* antiserum.

No cross-reactions were noted between a leuconostoc or staphylococcus except for *Staphylococcus aureus* and the group III antiserum L-351. This antiserum also precipitated with extracts of Lancefield's group B and L streptococci. A cross-reaction was seen between antiserum *P. cerevisiae* NZ-R 3 and a Lancefield group D streptococcus. On testing commercial streptococcal antisera against extracts of representative strains of pediococci, positive precipitin reactions were obtained with *P. cerevisiae*, *P. parvulus* and *P. damnosus* (b). Similar results were observed with a group D serum obtained from the Institut Pasteur, Paris. No other reactions occurred between pediococcal antisera and extracts of streptococci or commercial streptococcal antisera and extracts of pediococci.

Table 3. *Results of precipitin tests using pediococcal and aerococcal antisera*

Antisera...	*P. cerevisiae*		*P. parvulus*			Group III					*P. damnosus*	*Aerococcus viridans*
	8081*	NZ-R3	F-98	S-182	S-344	L-24	L-92	L-345	L-351	L-352	PD	8251
P. cerevisiae 8081	++	++	+	+	+	+	±	+	++	++	+	+
NZ-R3	−	++	±	−	−	±	±	−	±	−	+	−
Other strains	+30/37	+34/37	+28/35	+32/37	+21/37	+28/35	+26/35	+28/35	+26/35	+28/35	+20/25	+23/34
P. parvulus F-98	−	±	++	+	−	±	−	−	−	±	+	±
S-182	−	−	+	++	+	−	−	−	−	−	+	−
S-344	−	±	±	+	++	±	−	−	−	−	+	−
Other strains	+12/35	+23/35	+28/35	+28/35	+18/35	+18/35	−27/35	−28/35	−29/35	−20/35	+14/17	−31/35
Group III L-24	−	−	−	−	−	+	±	+	±	+	−	−
L-92	−	−	−	−	−	+	+	±	±	+	−	−
L-345	−	−	−	−	−	±	±	++	+	++	−	−
L-351	−	−	−	−	−	±	+	++	++	++	−	−
L-352	−	−	−	−	−	±	±	++	+	++	+	−
Other strains	−9/9	−8/9	−7/9	−8/9	−9/9	+8/9	+8/9	+9/9	+9/9	+7/9	+5/9	−8/9
P. damnosus (b) PD	−	−	−	±	±	−	±	±	±	±	+	−
Other strains	−8/8	−7/8	−4/8	−4/8	−6/8	−8/8	−5/7	−7/8	−6/7	−5/7	−4/8	−8/8
P. damnosus (a)	−3/3	−3/3	−3/3	−3/3	−3/3	−3/3	−3/3	−3/3	−3/3	−3/3	−3/3	−3/3
P. damnosus var. *limosus*	−	+	+	+	+	±	−	+	+	+	+	−
P. halophilus	−4/4	+2/4	−4/4	−4/4	−4/4	−4/4	−4/4	−4/4	−4/4	−4/4	−4/4	+1/4
P. urinae equi	−	−	−	−	−	−	−	−	−	−	−	−
Aerococcus viridans 8251	−	−	−	−	−	−	−	−	−	−	−	++
Other strains	−10/10	−9/10	−10/10	−10/10	−9/10	−9/9	−10/10	−10/10	−10/10	−9/10	−8/8	+7/10

++, strong precipitate; +, good precipitate; ±, weak precipitate; −, no precipitate formation.
Fractions indicate no. of extracts giving reaction/total no. tested.
* Reference antiserum.

Precipitin-absorption tests. The results of the absorption of three group III antisera with homologous and heterologous cells are shown in Tables 4 and 5.

The presence of separate antigens in group III was shown on absorption of antisera prepared against L-351 and L-352 with cells of *Pediococcus cerevisiae* and *P. parvulus* where precipitin reactions indicated that antibody activity was removed for these two species but not for group III extracts. All antibody activity was lost in a similar experiment with the antiserum prepared against L-24.

When the antiserum against L-352 was absorbed with cells of L-92, the absorbed antiserum did not react with extracts of L-92 and L-24, but did react with homologous and other extracts of group III, indicating the presence of a different antigen.

Table 4. *Absorption of antiserum* L-352 *(group III) with cells of pediococci and an aerococcus*

		Antiserum absorbed with							
		Group III			*P. cerevisiae*			*P. parvulus*	*Aerococcus viridans*
Antigen	Unabsorbed	L-352	L-92	L-92 + L-352	8081	F-166	NZ-T1	S-188	8251
Group III									
L-352	++	–	+	–	+	+	+	+	+
Other strains	+	–	+	–	+	+	+	+	+
			–*		–*	±*		–*	
P. cerevisiae	+	+	–	–	–	–	–	–	+
P. parvulus	+	(±)	(±)	–	–	(+)	–	–	+
Aerococcus sp. FP-2	+	–	–	–	–	–	–	.	–

., Not tested.
* = Result of reaction with extracts L-24 and L-92.
Symbols in parentheses indicate majority of extracts giving reaction, other symbols as Table 3.

Table 5. *Absorption of antisera* L-24 *and* L-351 *(group III) with cells of P. cerevisiae, P. parvulus and group III*

	Antiserum							
	L-24				L-351			
		Absorbed with				Absorbed with		
		Group III	*P. cerevisiae*	*P. parvulus*		Group III		*P. cerevisiae*
Antigen	Unabsorbed	L-24	NZ-R3	S-290	Unabsorbed	L-351	L-345	CH-867
Group III								
L-24	+	–	–	–	±	–	–	–
L-351	±	–	.	.	++	–	–	+
Other strains	+	–	–	–	+	–	–	±
P. cerevisiae	+	±	–	–	+	–	–	–
P. parvulus	±	±	–	–	+	–	–	–
P. damnosus P1	.	.	.	.	+	–	–	–

Symbols as Table 4.

On absorbing the same batch of antiserum with homologous cells to remove antibody activity for all group III extracts, all antibody activity was lost.

The antibodies present in antisera L-24 and L-352 reacting with extracts of *Pediococcus cerevisiae* and *P. parvulus* were not removed on absorption with homologous cells. Absorption might not have been complete.

Table 6. *Results of agglutination tests*

	Antisera							
	P. cerevisiae		*P. parvulus*		Group III			
Antigen	8081	NZ-R3	F-98	S-182	L-24	L-92	L-345	L-352
P. cerevisiae								
8081	**1280**	40	20	40	20	—	20	20
NZ-R3	80	**640**	80	160	80	320	80	40
P. parvulus								
F-98	40	160	**160**	80	20	20	80	80
S-182	80	160	80	**1280**	—	20	80	80
Group III								
L-24	—	—	—	—	**320**	640	—	—
L-92	—	—	—	—	80	**160**	20	—
L-345	80	160	—	160	80	160	**1280**	1280
L-352	160	160	•	160	40	160	1280	**1280**

Figures indicate reciprocals of the highest titres read.
—, no agglutination reaction observed, > 20.

On absorption of antiserum L-352 with an aerococcus no antibodies were removed except those reacting with extracts of strain FP-2 thereby giving additional evidence that this strain resembled aerococci. Extracts of *Pediococcus damnosus* strain P1 showed no reaction with antiserum L-351 absorbed with *P. cerevisiae* and group III cells. It would appear that no separate antigen was involved.

Tube-agglutination tests. The results are summarized in Table 6. Half the antisera gave titres of 1/1280 with homologous cells. Titres did not exceed 1/80 with heterologous cells of the same species except for several group III strains. This group showed some heterologous titres of the same value as homologous titres.

Cross-reactions were present between antisera of *Pediococcus cerevisiae* and cells of *P. parvulus* and group III except strains L-24 and L-92. The same was true for sera prepared against *P. parvulus*.

The group III sera prepared against L-24 and L-92 reacted with all strains of group III as well as some strains of *Pediococcus cerevisiae* and *P. parvulus* to titres of 1/20, 1/80 and one of 1/320. It was noted that a higher titre was obtained between antiserum L-92 and cells of L-24 than with homologous cells. However, the agglutinating titres were proportionally the same as those observed with antiserum L-24 and cells of L-92. Sera prepared against L-345 and L-352 did not react with cells of L-24 of group III, and antiserum L-345 only agglutinated with strain L-92 to a titre of 1/20. These two antisera reacted with all strains of *P. cerevisiae* and *P. parvulus* included in this experiment and would appear to share a common type antigen.

DISCUSSION

In this study most of the pediococcus cultures examined resembled the species *Pediococcus cerevisiae* Pederson and would appear to be more 'typical' of the genus than the cultures obtained from brewing sources.

It will be noted that *Pediococcus cerevisiae* Pederson may be found in a wide variety of habitats and thus may be more suitable as the type species than the less common strains from breweries, although these were the first pediococci described. The strains the Japanese workers studied were mostly of brewing origin, and although they applied the name *P. cerevisiae*, in our opinion these strains should be classed as *P. damnosus*.

For classification purposes the definition of species in the genus *Pediococcus* given by Nakagawa & Kitahara (1959), based primarily on growth at different pH levels, was found difficult to apply to strains from a variety of sources and we prefer to separate the species by consideration of a number of different characters.

No strains having a high optimum growth temperature were found. The culture received as *Pediococcus acidi lactici*, separated from other pediococci by Nakagawa & Kitahara (1959) on the ability to grow at higher temperatures, was not observed to be different in its temperature growth range from some other strains identified as *P. cerevisiae*. The good general agreement in physiological and serological findings between new isolates from sauerkraut and faecal material, and strains defined as *P. parvulus* Günther & White adds further weight to the acceptance of this new species in the genus *Pediococcus*. There does appear to be some physiological variation within the species, verifying the results of Günther & White (1961*a*, *b*). New *P. parvulus* cultures showed a greater tolerance to sodium chloride than Günther & White's strains, possibly due to the presence of Tween 80 in the medium thus rendering their criterion 'growth in 4% NaCl' unsatisfactory (Günther & White, 1961*a*, Table 1). It is suggested that the criterion 'growth at pH 8·6' be included to separate *P. cerevisiae* from *P. parvulus*. Colony size was larger in the new strains again possibly due to the incorporation of Tween 80 in the medium. The definition of *P. parvulus* may have to be amended on this point. No marked serological differences were noted.

Organisms isolated from brewing sources emerged as a distinct group. The species name of *Pediococcus damnosus* Mees is accepted as applicable to such cultures. The species may be divided into several groups. The distinctive physiological features of *P. damnosus* group (a) and lack of antibody activity with *P. damnosus* PD antiserum is evidence for the proposal of this group as a variety. In sensitivity toward temperature and in stimulation of growth by anaerobic incubation, group (a) resembles the *P. cerevisiae* strains described by Nakagawa & Kitahara (1959). Their proposed varietal names of this species, *diastaticus*, *salicinaceus* for salicin fermenting isolates, and *mevalovorus* for cultures requiring mevalonic acid for growth do not adequately define group (a) strains. We therefore suggest the name *P. damnosus* var. *damnosus*. The description of *Streptococcus damnosus* var. *diastaticus* (Andrews & Gilliland, 1952) the name originally applied to the culture received as *P. diastaticus* fitted the general character of group (b) strains and 'diastaticus' is proposed as the varietal name. Cultures *P. damnosus* M-1, 8519, 8520 examined by Günther & White (1961*a*) resembled group (b) isolates BE-1, BE-2 and NCIB 8559 in colony size, acetylmethyl-

carbinol production and fermentation of trehalose. Further work may show these cultures to be a distinct entity. It should be noted that NCIB 8559 was received as *Streptococcus cremoris*. We consider it to be a *P. damnosus*. The most accurate taxonomic position of the culture *P. limosus*, pending further work with slime-forming strains, is considered to be as a further variety of *P. damnosus*, *P. damnosus* var. *limosus* Shimwell. Thus there may be three variants of *P. damnosus*.

The results obtained with salt-requiring strains would indicate that they can be separated from other pediococci at the species level. The nutritional studies of *Pediococcus halophilus* and other pediococcal strains by Sakaguchi (1960) and Nakagawa & Kitahara (1959) which showed that *P. halophilus* required more vitamins but less amino acids for growth than the other strains, affords additional evidence of specific differences. The ability to grow in high salt concentrations involving the withstanding of high osmotic pressures, and the type of colony growth distinguishes *P. halophilus* from *P. homari* Deibel & Niven and *Aerococci viridans*. Characters shared with aerococci such as sensitivity towards acid conditions, ability to ferment sucrose and mannitol, and formation of optically active lactic acid (Sakaguchi, 1958) places this species in an intermediate position between aerococci and other pediococcal species.

The numerous cross-reactions shown by group III antisera with extracts of other species or serological groups was also experienced by Swartling (1951) and Briggs & Newland (1952) with *Streptococcus cremoris* cultures where absorption experiments were required to verify the possession of the Lancefield group N antigen. Similarly, with our group III antisera, precipitin-absorption experiments indicated the presence of separate antigens for group III, and *Pediococcus cerevisiae* and *P. parvulus*. This evidence together with the ability to form dextrorotatory lactic acid (detected in two cultures, Coster, 1963; Garvie, personal communication) is characteristic of these organisms which thus may be considered as a definite group certainly at variety or perhaps at species level. They resemble *P. urinae equi* in the formation of dextrorotatory lactic acid (Garvie, personal communication) but no serological relationship was found. We had hoped to be able to assign these group III organisms to a new species but did not feel justified in so doing at this stage as no new isolates were obtained during the course of the present work and 14 strains only were available from Dr Günther's collection. Further work, preferably with fresh isolates, may establish the differences at species level. Günther & White (1961 *a*) considered that these strains resembled the published description of *Streptococcus damnosus* var. *diastaticus* (Andrews & Gilliland, 1952), but the present work on the species 'damnosus' shows that they are closer to *P. cerevisiae* than to *S. damnosus* var. *diastaticus*.

The position with regard to the culture received as *Pediococcus urinae equi* (PUE) remains obscure. Günther & White (1961 *b*) reported serological reactions between PUE extracts and *P. cerevisiae* sera but we were unable to confirm these results—PUE reacted with none of the sera (of whatever group) that we tested. In view of this, we did not feel it reasonable to assign this organism to group III despite the resemblance shown in such characters as the formation of dextrorotatory lactic acid.

The five new isolates from meat sources resembled aerococci and *Pediococcus homari* and formed a distinct group easily distinguished from other strains by their sensitivity towards acid and oxygen tension, tolerance to sodium chloride and lack

of common antigens. Such marked differences can be expressed by division at the specific or generic level. The placing of these strains in the species *P. homari* would entail widening the definition of the genus to include such strains. Much evidence is present on which to base a separation of these organisms from other pediococci at generic level, thus retaining *Aerococcus* as a distinct genus.

In the light of recent work, it is suggested that the definition of the genus as given in *Bergey's Manual* (1957) be widened to include the formation by some strains of dextrorotatory lactic acid, and of slime under defined cultural conditions. The requirement of carbon dioxide for good growth (Nakagawa & Kitahara, 1959) by some strains should also be included.

Proposed classification of the genus Pediococcus

Species name	Synonym	Reference
P. cerevisiae Balcke	*P. pentosaceus*	(1)
	P. acidi lactici	(1)
var. *dextrinicus*	Group III	(2)
var. *urinae equi*	*P. urinae equi*	(1)
P. parvulus Günther & White		(2)
P. damnosus Claussen	*P. damnosus*	(3)
	P. cerevisiae	(1)
var. *diastaticus*	*Streptococcus damnosus* var. *diastaticus*	(4)
var. *damnosus*	*Streptococcus damnosus*	(5)
var. *limosus*	var. *limosus*	
P. halophilus Mees	*P. soyae*	(6)
Aerococcus viridans Williams, Hirch & Cowan	*P. homari*	(7)

References

(1) Nakagawa & Kitahara (1959)
(2) Günther & White (1961*a*)
(3) Mees (1934)
(4) Andrews & Gilliland (1952)
(5) Shimwell (1949)
(6) Sakaguchi (1958)
(7) Deibel & Niven (1960)

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Restoration of *Escherichia coli* Strain B after γ-irradiation

BY A. J. FORAGE AND N. E. GILLIES

Department of Biology as Applied to Medicine, Middlesex Hospital Medical School, London W. 1

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SUMMARY

The fraction of bacteria of a proline-requiring auxotrophic strain of *Escherichia coli* strain B which was able to originate macrocolonies on a defined nutrient medium after exposure to γ-rays under anoxic conditions was markedly increased when the organisms were deprived of proline or were treated with chloramphenicol for the initial period after irradiation. Either treatment was equally effective and the maximum degree of survival which was obtained was above that observed when the cells had been incubated throughout on a proline + inorganic salts + glucose medium. The depression in survival caused by including NaCl in the defined nutrient medium, on which the irradiated bacteria were grown, was completely eliminated by both treatments. 'Rescue' appears to depend on the temporary inhibition of protein synthesis.

INTRODUCTION

The amount of damage which becomes apparent in irradiated micro-organisms may be modified by a variety of treatments applied to the organisms either before or after irradiation. For example, the survival of *Escherichia coli* strain B after exposure to ultraviolet (u.v.) or ionizing radiation depends on the nature of medium on which they are plated (Robert & Aldous, 1949; Alper & Gillies, 1958*a*, *b*, 1960). Alper & Gillies (1958*b*, 1960) showed that there was good correlation in this strain between high survival and slow growth after irradiation. Thus, the survival of the irradiated organisms was higher on an inorganic salts glucose medium, on which the rate of growth was slow, than on nutrient medium on which the bacteria grew more rapidly. However, survival on the nutrient medium can be markedly increased when growth of the bacteria is inhibited by chloramphenicol for an interval immediately after irradiation (Gillies & Alper, 1959). Chloramphenicol is believed to inhibit protein synthesis in bacteria specifically (Gale & Foulkes, 1953; Wisseman, Smadel, Hahn & Hopps, 1954). It appears, therefore, that the survival of that fraction of organisms which can be rescued is dependent on the inhibition of protein synthesis during the initial post-irradiation period. Further data in support of this conclusion were obtained from experiments on u.v.-irradiated auxotrophic mutants of *E. coli* B, which showed that considerable rescue could be effected when utilization of an amino acid, but not utilization of thymine, was inhibited immediately after irradiation (Gillies, 1961). The same effect was observed in a proline-requiring strain of *E. coli* B deprived of proline or treated with chloramphenicol for the initial period after X-irradiation (Gillies, 1963). The present paper describes a series of experiments made to determine in more detail the conditions under which the proline-requiring

strain *E. coli* B33 could be rescued after exposure to ionizing radiation. These experiments were:

(*a*) Determination of the effect on survival of depriving the bacteria of proline for an initial period after exposure to γ-rays, by incubating the irradiated bacteria for various periods on a nutrient medium of defined composition which lacked proline, before transferring them to the defined nutrient medium containing proline.

(*b*) Comparison of the effect of the above treatment with that of incubating irradiated bacteria on the defined nutrient medium containing chloramphenicol, before transferring them to fresh defined medium without chloramphenicol.

(*c*) Determination of the effect of incorporating NaCl into the defined nutrient medium on the extent of rescue brought about by proline deprivation or by chloramphenicol treatment. Previously chloride was found to decrease the number of irradiated bacteria of *Escherichia coli* B able to form macrocolonies on a nutrient medium (Alper & Gillies, 1958*b*).

METHODS

Media. The defined glucose medium used was that devised by Davis (quoted by Lederberg, 1950). The defined nutrient medium, called medium M19, consisted of the defined salts glucose medium supplemented with amino acids and nitrogenous bases; its composition was the same as that of the medium N of Gillies (1961) except that it lacked isoleucine and inosine. Proline (10 μg./ml.), chloramphenicol (5 μg./ml.) and NaCl (5 g./l.) were added as required. When examining the effect of chloramphenicol proline was always included in the growth medium. Strain *Escherichia coli* B33, a proline-requiring mutant, was isolated after induction with u.v. radiation from the strain of *E. coli* B used in previous experiments (Alper & Gillies 1958*a*, *b*, 1960; Gillies & Alper, 1959). The methods used in isolating and identifying the auxotrophic mutant were as previously reported (Gillies, 1961).

Preparation of bacterial suspensions for irradiation. The strain of *Escherichia coli* B33 was grown in Oxoid nutrient broth, supplemented with proline (10 μg./ml.), at 37° for 18 hr. The bacteria, which were then in the stationary phase of growth, were harvested by centrifugation, washed 3 times in M/15 phosphate buffer (pH 7) and finally resuspended in buffer to a concentration of about 6×10^6 bacteria/ml.

Irradiation of bacteria. Bacteria were exposed to radiation from a ^{60}Co source (Vickers-Armstrong's MK. IV Hotspot Irradiation Unit) in the vessel shown in Fig. 1. Oxygen-free N_2 was bubbled vigorously through the suspension during irradiation, since previous experiments by Alper (1961), Alper & Gillies (1958*a*) and Gillies & Alper (1959) had shown that more rescue of irradiated bacteria could be obtained if they had been irradiated in anoxic rather than in oxygenated conditions. The dose rate, as measured by ferrous sulphate dosimetry (Miller & Wilkinson, 1952), was 2·2 Kr./min.

Treatment of bacteria after irradiation. Suitable dilutions of the suspension of irradiated bacteria and also samples of un-irradiated organisms were dispensed on to cellophan carriers lying on the surface of the growth media previously warmed to 37°. This technique, which has been described (Alper & Gillies 1958*b*), allows bacteria to be transferred rapidly from one medium to another. Bacteria were incubated at 37° overnight and the macrocolonies counted.

RESULTS

The γ-ray sensitivity of the proline-requiring *Escherichia coli* strain B33 was the same as that of the parent strain *E. coli* B, and it also responded to the conditions of growth after γ-irradiation in the same manner as *E. coli* B. It was concluded, therefore, that the mutagenic treatment had not caused changes in radiobiological characteristics other than those specifically concerned with the proline requirement. As was found for *E. coli* B (Alper & Gillies, 1960), the survival of irradiated *E. coli* strain B33 on defined medium and on defined medium + NaCl was the same, and no effect of NaCl was observed. As shown in Fig. 3, however, a marked decrease in the number of irradiated bacteria able to form colonies was observed when NaCl was included in the M19 medium.

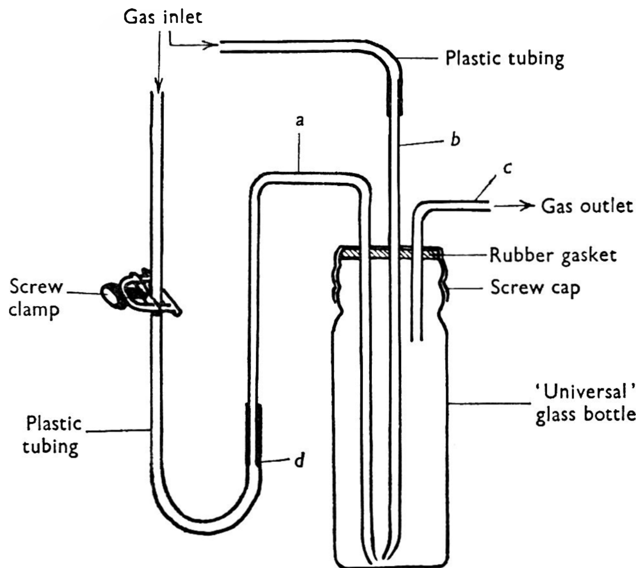

Fig. 1. Diagram of vessel in which bacterial suspensions were irradiated. *a*, *b* and *c* are glass tubes (4 mm. diam., 2 mm. bore). The bottle is half-filled with the bacterial suspension and gas is bubbled through tubes *a* and *b*. To remove a sample from the vessel, the screw clamp is closed and the sample is collected at *d*. Tube *c* is then opened, the plastic tubing replaced on tube *a*, and the clamp opened so that bacterial suspension remaining in *a* is forced back into the vessel. In this way the gas conditions are kept constant during sampling.

The effect of inhibiting the growth of irradiated *Escherichia coli* strain B33 on M19 medium with or without NaCl, either by deprivation of proline or by treatment with chloramphenicol, is illustrated in Fig. 2. It can be seen that considerable and equal rescue of irradiated bacteria was effected by deprivation of proline or by treatment with chloramphenicol. Inhibition of the growth of the bacteria for about 5 hr on a medium lacking NaCl was required to produce maximum rescue after a dose of 22 krads of γ-radiation under anoxia. When bacteria were exposed to smaller doses of γ-radiation a shorter period of post-irradiation treatment was sufficient to effect maximum rescue. However, when NaCl was included in the medium, the growth of

the bacteria must be inhibited for a longer period, about 8 hr after exposure to 22 krad, to increase survival to a maximum value. Despite the fact that there was a tenfold difference in the fraction of bacteria which survived, depending on whether NaCl was included in the growth medium or not, there was little difference in the maximum survival obtainable after growth had temporarily been inhibited. Thus, after a dose of 22 krads of γ-radiation approximately a 50-fold increase in survival (from 0·5 to 30 %) was effected on medium containing NaCl, and sixfold increase (from 6 to 35–45 %) on a medium lacking NaCl.

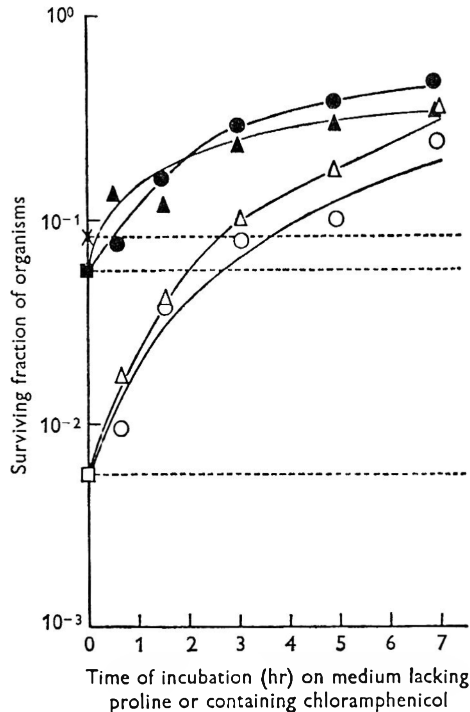

Fig. 2. Survival of stationary-phase Escherichia coli strain B 33 after exposure to 22 krads γ-radiation under anoxia.
Level of survival of bacteria incubated throughout on: ×, defined salts glucose medium + proline; ■, M19 medium + proline; □, M19 medium + proline + NaCl. Survival of bacteria incubated on first medium for the intervals indicated before transfer to second medium.

1st medium		2nd medium
●	M19 →	M19 + proline
○	M19 + NaCl →	M19 + proline + NaCl
▲	M19 + proline + choramphenicol (CMP) →	M19 + proline
△	M19 + proline + NaCl + CMP →	M19 + proline + NaCl

The points on the figure indicate the mean value of 6–18 separate observations for individual treatments. To preserve clarity, experimental errors have been omitted, but the 95 % confidence limits of the majority of the points fall within ± 20 % of the mean, with a few as high as ± 30 %.

Figures 3 and 4 show dose-effect curves for rescued and unrescued cells. Figure 4 illustrates in more detail the initial portions of the survival curves shown in Fig. 3 and it can be seen that the extrapolation number (Alper, Gillies & Elkind, 1960) of the

curve for rescued bacteria was greater than 1, whereas the extrapolation number of the curves for unrescued cells was unity. The number of unirradiated bacteria which originated macrocolonies was not altered by incubating them on different growth media, by temporary deprivation of proline or by treatment with chloramphenicol.

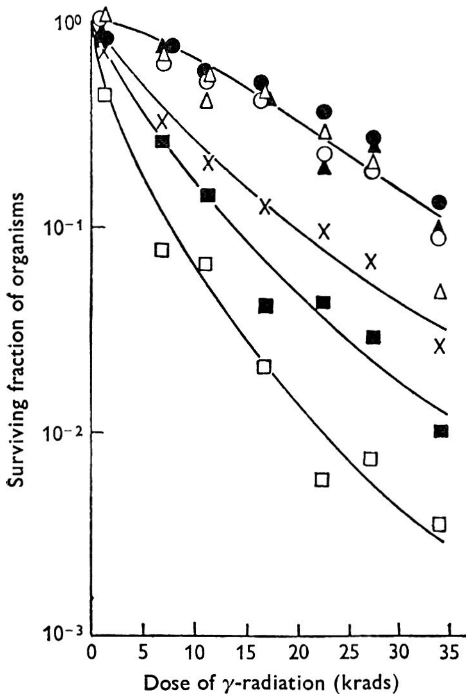

Fig. 3

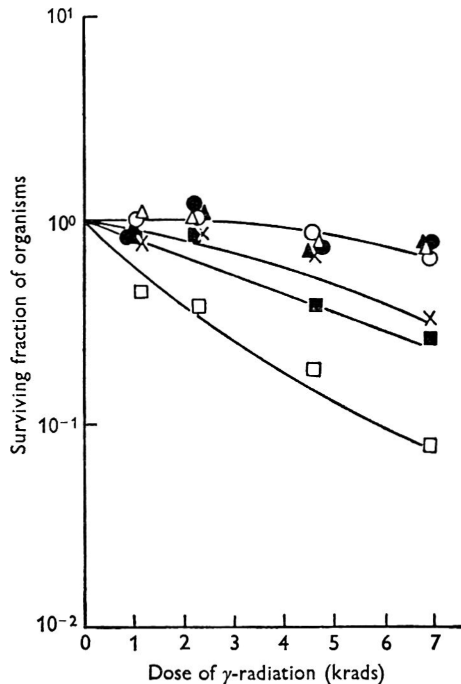

Fig. 4

Fig. 3. Survival curves of stationary phase Escherichia coli strain B33 incubated under different conditions after exposure to γ-radiation under anoxia.
Survival of bacteria incubated throughout on: ×, defined salts glucose medium + proline, ■, M19 medium + proline; □, M19 medium + proline + NaCl.
Maximum level of survival of bacteria incubated on first medium before transfer to second medium.

1st medium		2nd medium
● M19	→	M19 + proline
○ M19 + NaCl	→	M19 + proline + NaCl
▲ M19 + proline + CMP	→	M19 + proline
△ M19 + proline + NaCl + CMP	→	M19 + proline + NaCl

Fig. 4. The initial portions of the survival curves of stationary-phase Escherichia coli strain B33 shown in Fig. 3 but with the dose scale expanded by a factor of 5. Treatments are denoted by the same symbols as used in Fig. 3.

DISCUSSION

The results confirm that the fraction of γ-irradiated bacteria of *Escherichia coli* strain B33 which are capable of originating macrocolonies can be markedly increased when the bacteria are prevented from utilizing proline or are treated with chloramphenicol for an interval after irradiation. It seems probable that this 'rescue' is a consequence of inhibiting protein synthesis in the organisms. The modes of action by

which the two treatments inhibit protein synthesis, however, are not likely to be identical. Proline deprivation will lead to inhibition of synthetic reactions which involve proline, whereas chloramphenicol appears to act by preventing the attachment of messenger RNA to ribosomal RNA (Jardetzky & Julian, 1964). The fact that the extent of rescue effected by the two methods is approximately the same may be fortuitous, for the efficiency and pattern of rescue obtained by chloramphenicol is dependent on the concentration of inhibitor used (Gillies; unpublished observation). Data presented by Gillies (1963) suggested that treatment with chloramphenicol (5 μg./ml.) was more effective in promoting rescue than was deprivation of proline, but this finding is not supported by the present work in which incubation without proline was as efficient in increasing survival as was chloramphenicol treatment. The reason for this is unknown, but the sensitivity of *E. coli* strains to various antibiotics appears to vary over prolonged periods. Also, the rescue which can be effected by deprivation of proline increased survival above that observed when the organisms had been incubated throughout on the defined salts + glucose medium.

The presence of NaCl in the post-irradiation nutrient medium was previously found to decrease the fraction of bacteria able to form colonies. However, inhibition of division for a limited period appears to eliminate completely the effect of NaCl. At the same time NaCl had little effect on the extent and pattern of rescue of the irradiated bacteria, although the time required to effect maximum rescue was longer when NaCl was included in the incubation medium.

Previous work has shown that a considerable fraction of u.v.-irradiated organisms of amino acid requiring mutants of *Escherichia coli* B may be rescued if they are deprived of the essential amino acid for the initial post-irradiation period (Gillies, 1961). It is therefore tempting to suggest that inhibition of protein synthesis prevents the expression of damage which is common to u.v.-, X- and γ-irradiation. U.v.-radiation will be selectively absorbed in nucleic acids and their precursors. Barner & Cohen (1956) postulated that cell death may be due to an imbalance in cytoplasmic and nuclear synthesis after irradiation. When protein synthesis is temporarily inhibited the development of a lethal imbalance in synthesis may be temporarily prevented. However, the nature of such an imbalance is obscure and, as pointed out by Okagaki, Tsubota & Sibatani (1960), is not simply a matter of protein synthesis continuing in the absence of DNA synthesis.

The present results support previous suggestions (Alper, 1961; Alper & Gillies, 1958*a*, *b*) that damage which is expressed in *Escherichia coli* B is the summation of at least two types of damage, one of which may be modified by post-irradiation treatment, while the other cannot. Alper (1961) postulated that these different types of damage may be due to absorption of radiation energy in at least two different sites in the cell. Although it may be that the site in which damage can be modified is associated with DNA, there are no clues which might indicate the nature of the other site.

The recent demonstration that the property of resistance to radiation action can be transferred genetically from one strain of *Escherichia coli* to another (Adler & Copeland 1962; Howard-Flanders, Boyce, Simson & Theriot, 1962) suggests that rescue of *E. coli* organisms may be dependent on the action of cellular reactivating enzymes after irradiation. However, the present work does not easily lend support to this interpretation, since conditions which inhibit protein synthesis are unlikely

to bring about rescue by such a mechanism. It is possible that reactivating enzymes are continuously synthesized in normal cells and that they only act after exposure to radiation, but it would be very unusual for an enzyme to exist in the absence of its substrate.

We are grateful to Miss Barbara Porter for skilful technical assistance. One of us (A. J. F.) is indebted to the Medical Research Council for a studentship.

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Factors Affecting the Germination of Spores of *Clostridium bifermentans*

By P. A. GIBBS
The Wellcome Research Laboratories (Biological Division), Beckenham, Kent

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SUMMARY

The germination of spores of *Clostridium bifermentans* in media based on acid hydrolysates of casein has been studied. Germination of spores was estimated by observing the decrease in turbidity of a spore suspension and confirmed by observation with the phase contrast microscope and by the increase in permeability to stains. The optimum conditions for germination of spores suspended in phosphate buffer were incubation at 37° within the range pH 6·0–7·8 following heat shock at 85° for 10 min. Anaerobic conditions were not necessary for germination, and addition of mercaptoacetate decreased the rate of germination. The minimum requirements of compounds needed for the germination of spores of *C. bifermentans* was the presence of L-α-alanine, L-phenylalanine and lactate. L-phenylalanine could be replaced by L-leucine but the rate of germination was slower. L-α-Alanine was essential for germination and was not replaced by any other amino acid examined.

INTRODUCTION

Though there is now a considerable volume of published work on the optimum conditions for and specific stimulants of germination of spores of the aerobic genus *Bacillus*, there is comparatively little information of this type about the spores of the anaerobic genus *Clostridium*. Such information as is available appears to be largely confined to those obligate anaerobes which have economic and pathological importance. Much of the work is also restricted in that the major emphasis has been on the destruction of these spores or the prevention of germination. *Clostridium bifermentans*, a non-pathogenic obligate anaerobe, has apparently not been studied with respect to the germination of its spores and this dearth of information directly prompted the present investigation. The work reported here is in two sections: (1) the determination of the optimum conditions for germination; (2) the elucidation of the compounds specifically responsible for breaking of dormancy.

METHODS

Organism. Clostridium bifermentans CN 1617 (Wellcome Research Laboratories Culture Collection Catalogue number) was used throughout. This organism has been clearly differentiated from the closely related pathogenic species *C. sordellii* by biochemical (Brooks & Epps, 1958) and immunological methods (Walker, 1963).

Preparation of spore suspensions. The contents of an ampoule of dried culture were suspended in 20 ml. Brewer's thioglycollate (mercaptoacetate) medium (Brewer, 1940) and incubated at 37°. After 18 hr, subcultures (1 ml.) were transferred to

nutrient broth containing 0·25 % glucose, and incubated at 37° under anaerobic conditions for 72 hr. The resulting growth, consisting of 85–90 % phase-bright spores, was harvested by centrifugation and washed with sterile 0·1 M-phosphate buffer (pH 7·4). During the washing procedure the upper white layer of the deposit, consisting largely of cellular debris and vegetative organisms, was suspended in the buffer solution and discarded (Long & Williams, 1958). After washing five times with buffer, the suspension was aerated by vigorous shaking and stored overnight at 4° to allow any remaining vegetative forms to lyse. The spores were then washed five more times with phosphate buffer and the volume of the final suspension adjusted to have an extinction value of 0·35–0·4 at 680 mμ (15 mm. diam. tube; Hilger 810 Biochem. Absorptiometer), corresponding to approximately 10^7 spores/ml.

Media. A medium (CMB) based on that described by Smith & Douglas (1950) was used and had the following composition: acid-hydrolysed casein, 2 % (w/v; Oxoid, London); KH_2PO_4, 0·5 % (w/v); biotin, nicotinamide, pyridoxal and calcium pantothenate, 10 μg./ml. each; $MgSO_4.7H_2O$, 1·6 mg./ml.; $MnSO_4.4H_2O$, 80 μg./ml.; $FeSO_4.7H_2O$, 80 μg./ml. The medium was adjusted to pH 8·0, boiled, filtered, readjusted to pH 7·4, and sterilized by autoclaving at 121° for 20 min.

Preparation of vitamin-free acid hydrolysed casein. This was prepared in order to study a possible vitamin requirement for the germination of spores. Commercial casein (British Drug Houses Ltd., vitamin- and fat-free) was extracted with methanol and hydrolysed by the method described by Barton-Wright (1961), by using litharge to decrease the concentration of chloride in the final product. The final solution was freeze-dried and solutions of this hydrolysate were prepared as detailed above, with or without the salts of magnesium, manganese and iron.

Yeast extract. A 25 % solution (YE) was prepared by the method of Phillips & Gibbs (1961).

Amino acids. For investigations of the amino acid requirements for germination of spores of *Clostridium bifermentans*, aqueous solutions of amino acids (5 mg./ml.; L. Light and Co.) were pasteurized by heating at 100° for 20 min., and stored at 4° until required for use; 0·5 ml. volumes of amino-acid solutions were added to 5 ml. spore suspension.

Carboxylic acids. For work on the specific stimulants of germination of spores of *Clostridium bifermentans* aqueous solutions of the following carboxylic acids were used: lactic acid (0·5 %, v/v; B.P., C_3H_6O 89·3 %); oxalosuccinic acid Ba salt, DL-isocitric acid lactone, *cis*-aconitic acid anhydride, oxaloacetic acid, α-oxoglutaric acid and L-malic acid (from L. Light and Co.); succinic acid, citric acid (British Drug Houses Ltd., Analar). Aqueous solutions of these acids (5 mg./ml.) were stored at 4°.

Heat shock of spore suspensions. Samples (5 ml.) of spore suspensions in sterile plugged tubes (150 × 15 mm.) were heated in water baths at selected temperatures ($\pm 1°$) for 10 min., after which the tubes were cooled rapidly in chilled water.

Adjustment of pH value of spore suspensions. Amounts of acid or alkali to adjust spore suspension to required pH values were calculated by reference to a titration curve of the 0·1 M-phosphate buffer in which the spores were suspended.

Estimation of degree of germination. The germination of a spore suspension was followed by observing the decrease in extinction at 680 mμ. Samples of CMB

medium (1 ml.) or of YE solution (0·5 ml.) or both were added to 5 ml. of spore suspension, and measurements of extinction made at intervals of 10 min. (Hilger 810 Biochem. Absorptiometer; Powell, 1950). The extent of germination of a spore suspension was also determined by observation of films stained with 1 % aqueous methylene blue for 30 min. (Powell, 1950; Levinson & Sevag, 1953) and by phase-contrast microscopy (Pulvertaft & Haynes, 1951). Those spores which were completely stained blue or were completely phase-dark were counted as having germinated.

RESULTS

Smith & Douglas (1950) described the growth requirements of *Clostridium bifermentans* in a medium based on acid-hydrolysed casein. Since these workers used a washed spore suspension for inoculation, the medium they described was apparently sufficient to stimulate germination and to support vegetative growth and was therefore used in the investigations of the optimum conditions for the germination of spores of *C. bifermentans.*

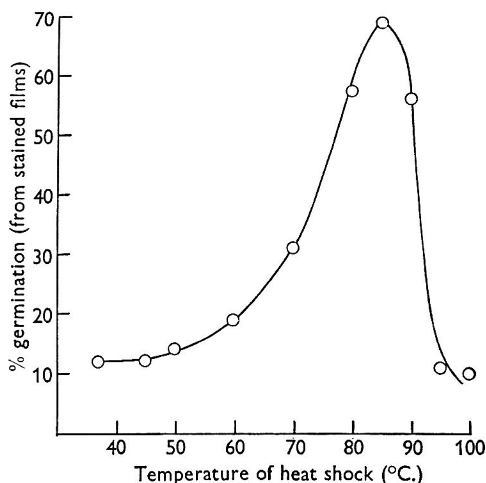

Fig. 1. The effect of temperature of heat shock for 10 min. on the germination of spores of Clostridium bifermentans in CMB medium + yeast extract.

Effect of glucose, tryptophan, cystine, thioglycollate and yeast extract on germination. Though glucose, tryptophan, cystine and thioglycollate are essential for vegetative growth, they were not required for germination in medium based on acid-hydrolysates of casein. Indeed the presence of thioglycollate, in concentrations between 0·005 and 0·1 % (v/v), markedly decreased the rate of germination as compared with that observed in the absence of thioglycollate. However, outgrowth and formation of a vegetative culture of *Clostridium bifermentans* occurred only when thioglycollate was present. The addition of yeast extract to acid-hydrolysates of casein increased the rate of spore germination.

Effect of heat shock on spore germination. Maximum germination in CMB medium + yeast extract at 37° was attained following a heat shock for 10 min. at 80–85° (Fig. 1). Little germination of a spore suspension occurred when heated at 70° or below, and temperatures of 90° and above prevented subsequent germination. Spores heated at 95° for 10 min. did not lose phase-brightness but did take up

methylene blue in the spore coats to a limited extent. Viable counts in agar media showed that 95° for 10 min. was lethal for 99·99 % of the spores. In all subsequent work, spores were heated at 85° for 10 min., these conditions of heat shock being optimal for subsequent germination.

Effect of pH value on germination. There appeared to be no sharp optimal pH value for germination of heat shocked spores in CMB medium + yeast extract incubated at 37°. Germination occurred within the range pH 6·0 – 7·8 with little difference in rate. At pH 5·0 and pH 8·5 no germination occurred. Incubation at pH 10·0 or pH 3·0 had little effect on the ability of spores to germinate on subsequent re-adjustment to pH 7·4.

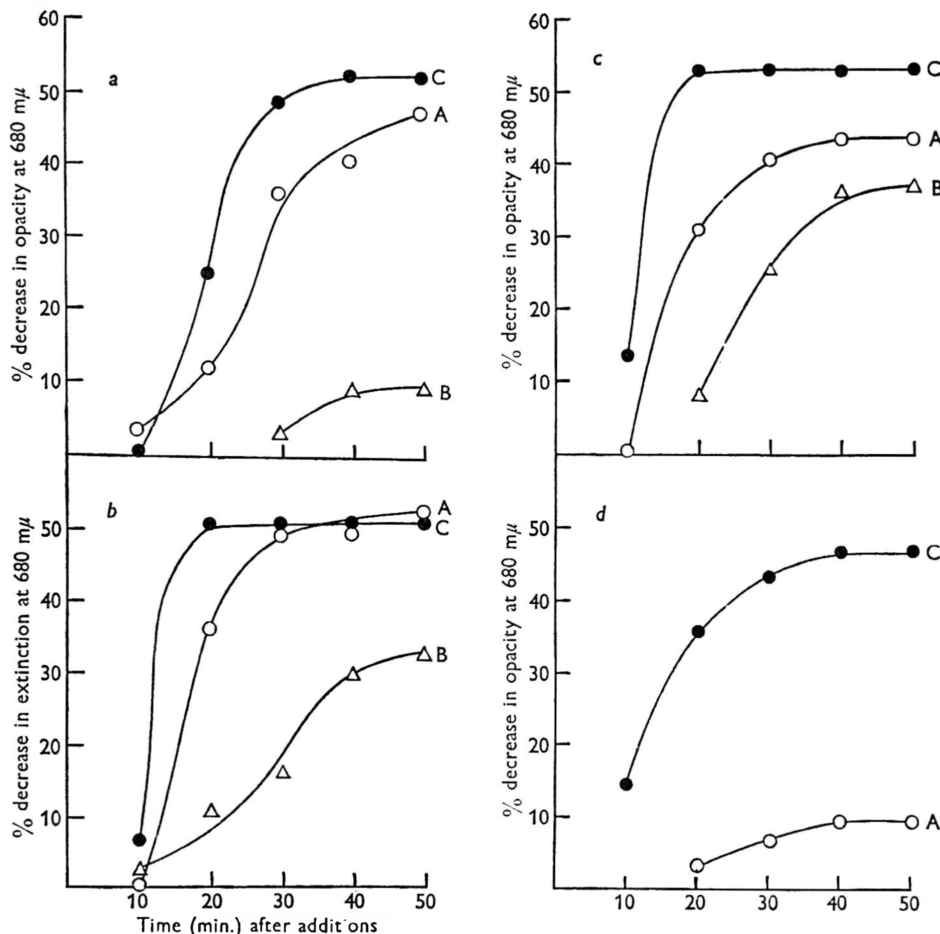

Fig. 2. The effect of temperature of incubation on the germination of suspensions of spores of Clostridium bifermentans. *a*, 25°: *b*, 30°: *c*, 37°: *d*, 45°. A, ○——○, +CMB medium: B, △——△, +yeast-extract: C, ●——●, +CMB+yeast extract. % decrease in opacity = $(E_0 - E_x/E_0) \times 100$ where E_0 and E_x are extinctions immediately after the addition of germinating agents and at time x respectively.

Effect of temperature of incubation. The germination of spores of *Clostridium bifermentans* in CMB and yeast extract both separately and as a mixture was investigated between 12 and 45°. At temperatures below 20°, little or no germination

occurred with 60 min. The maximum rate of germination at each temperature was attained in CMB medium + yeast extract (Fig. 2). At 45° germination was markedly decreased, particularly in CMB medium and was absent in yeast extract. The optimum temperature of incubation was 37° and all subsequent work was therefore carried out at this temperature.

Investigation of the compounds specifically responsible for the germination of spores of Clostridium bifermentans

Effect of vitamin- and fat-free casein hydrolysate. Medium prepared with vitamin- and fat-free casein hydrolysate did not stimulate the germination of spores, whereas commercial casein hydrolysate did. Addition of yeast extract to the vitamin-free casein hydrolysate stimulated germination to a degree comparable with that obtained with CMB medium + yeast extract. The addition of the four vitamins biotin, nicotinamide, pyridoxal and pantothenate, salts of manganese, magnesium and iron to the vitamin-free hydrolysate had no effect. Amino acid analysis (Beckman-Spinco, Model 120 Amino Acid Analyzer) of the commercial and vitamin-free casein hydrolysates revealed no major differences in amino acid composition and addition of the commonly occurring L-amino acids to the vitamin-free casein hydrolysate did not stimulate germination in the absence of yeast extract. It seemed likely therefore that amino acids alone could not stimulate the germination of spores of *Clostridium bifermentans*, and that an unknown factor present in yeast extract was required.

Amino acid requirement for germination in the presence of yeast extract. Twenty-seven L-amino acids were examined for their effect on germination in the presence of yeast extract. Of these L-phenylalanine, L-leucine and L-cystine markedly increased the rate of germination; glycine, L-valine, L-methionine and L-cysteine inhibited germination; the other amino acids tested were without effect (Fig. 3).

Replacement of yeast extract in the presence of vitamin-free casein hydrolysate. Since nucleosides have been implicated in the germination process of spores of *Bacillus* spp. (see Halvorson & Church, 1957), the effect of some purine and pyrimidine bases and ribosides as replacements for yeast extract was examined. The addition of thymine, uracil, uric acid, guanine, adenine, xanthine, hypoxanthine, adenosine and guanosine did not stimulate spore germination in vitamin-free casein hydrolysate. Ultraviolet absorption spectra of solutions of commercial and vitamin-free casein hydrolysates showed no significant differences at 260 $m\mu$, the region in which many purines and pyrimidines and their derivatives are strongly absorbing.

The other group of compounds of metabolic importance investigated included glucose and some of its degradation products, namely the carboxylic acids pyruvic, lactic, acetic, citric, *cis*-aconitic, isocitric, oxaloacetic, malic, succinic, α-oxoglutarie and oxalosuccinic acids. In the presence of commercial casein hydrolysate, lactate, pyruvate, malate, succinate and oxaloacetate stimulated germination, but in vitamin-free casein hydrolysate only lactate had a marked effect on germination, and pyruvate and oxaloacetate a slight effect; succinate and malate were inactive. In a mixture of amino acids which simulated vitamin-free casein hydrolysate, lactate stimulated rapid germination, but pyruvate and oxaloacetate were very much less active.

The specific stimulants of germination of spores of Clostridium bifermentans

Since germination of spores was obtained in a mixture of amino acids + lactate, an examination of those specific amino acids which were needed for the breaking of dormancy in the presence of lactate was made. A mixture of L-phenylalanine, L-leucine and L-cystine in the presence of lactate did not stimulate germination, but on addition of L-α-alanine to this mixture, rapid germination occurred; L-α-alanine was not replaced by any other amino acid tested. Further investigations showed that L-phenylalanine + L-α-alanine + lactate constituted the specific requirements for germination and that L-phenylalanine could be replaced by L-leucine, although the rate of germination was then decreased (Fig. 4).

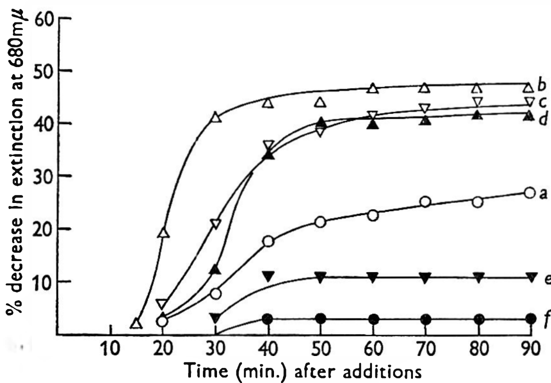

Fig. 3

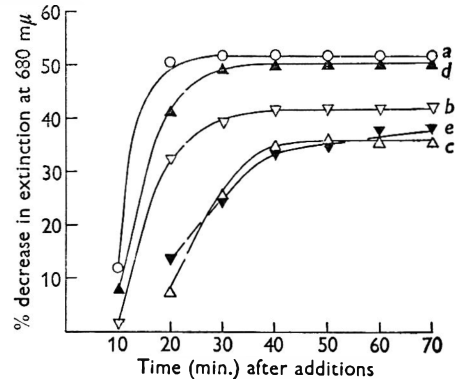

Fig. 4

Fig. 3. The effect of amino acids on the germination of suspensions of spores of Clostridium bifermentans in yeast extract (YE) solution. *a*, ○——○, +YE only: *b*, △——△, +YE+L-phenylalanine (50 μg./ml.): *c*, ▽——▽, +YE+L-leucine (50 μg./ml.): *d*, ▲——▲, +YE+L-cystine (50 μg./ml.): *e*, ▼——▼, +YE+L-methionine (50 μg./ml.): *f*, ●——●, +YE+glycine, or L-valine or L-cysteine (50 μg./ml.).

Fig. 4. The minimum requirements for the germination of suspensions of spores of Clostridium bifermentans. *a*, ○——○, +CMB medium+yeast extract: *b*, ▽——▽, +CMB medium: *c*, △——△, +yeast extract: *d*, ▲——▲, +L-phenylalanine (50 μg./ml.)+L-α-alanine (50 μg./ml.)+lactic acid (0·5 μl./ml.): *e*, ▼——▼, −L-leucine (50 μg./ml.)+L-α-alanine (50 μg./ml.)+lactic acid (0·5 μl./ml.).

DISCUSSION

Although the heat resistance of spores is usually regarded as the feature that most clearly differentiates them from the vegetative forms, spores may be clearly recognized by other properties. Thus, the heat sensitivity of spores of some aerobic and anaerobic bacilli is paralleled by loss of phase-brightness, increased permeability to stains and decrease in turbidity of spore suspensions, so making it possible to use the latter group of properties as criteria of germination. The criteria of germination used in the present work were the microscopic appearance and change in turbidity of suspensions of spores. In common with spores of the aerobic bacilli (Powell & Hunter, 1955) spores of *Clostridium bifermentans* were found to require activation by heat shock before rapid and complete germination ensued. This observation appears to distinguish the spores of *C. bifermentans* from those of *C. roseum* studied by Hitzman, Halvorson & Ukita (1957).

Although strictly anaerobic conditions are required for the vegetative growth of *Clostridium bifermentans*, these conditions are not essential for the germination of its spores. Indeed the addition of thioglycollate markedly inhibited germination as previously reported for spores of *C. botulinum* (Treadwell, Jann & Salle, 1958). The germination of spores of other species of the genus *Clostridium* is apparently not inhibited by thioglycollate, and anaerobic conditions are apparently required for germination to occur (Wynne, Mehl & Schmieding, 1954; Hitzman *et al.* 1957). By making experiments under aerobic conditions therefore, it was possible in the present work to differentiate between germination and subsequent outgrowth since the latter (see above) will only take place under anaerobic conditions. Thus it was possible to observe separately the three major phases in the transition from dormant spores to vegetative forms, namely activation, germination and outgrowth.

The compounds found specifically responsible for stimulating the germination of spores of *Clostridium bifermentans* included L-α-alanine and L-phenylalanine, compounds also required by spores of *C. roseum* (Hitzman *et al.* 1957). L-α-Alanine is also required by the spores of many aerobic bacilli (Thorley & Wolf, 1960), and it is possible that L-α-alanine is essential for the germination of spores of aerobic and anaerobic bacilli. However, lactic acid has apparently not been reported previously to be required for the germination of spores, although some dicarboxylic acids have been reported to stimulate the germination of spores of *C. botulinum* (Wynne & Foster, 1948).

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Back Mutation of Leucine-Requiring Auxotrophs of *Salmonella typhimurium* Induced by Diethylsulphate

By A. RONEN*

Department of Biology, University of California, San Diego, La Jolla, California, U.S.A.

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SUMMARY

Ten leucine-requiring mutants of *Salmonella typhimurium* were induced to revert with the alkylating agent, diethylsulphate (DES). The pattern of reversion was studied by following the appearance of leucine-independent colonies on minimal plates, and of turbid (revertant) tubes among tubes containing 10^5–10^6 treated bacteria in minimal medium. Five mutants gave high ($0{\cdot}6$–5×10^{-5}) frequencies of reversion. The other five gave lower (5×10^{-8}–$1{\cdot}3 \times 10^{-6}$) frequencies immediately after plating on minimal agar or dilution in minimal medium, but reversion continued to take place until a high frequency was attained. This delayed mutation was found to be independent of cell metabolism. The possibility is discussed that the different patterns of reversion reflect alkylation of different base pairs. It is suggested that mutants having guanine : cytosine (G : C) base pairs at the mutant site revert mainly by pairing errors made by alkylated guanine. Mutants with adenine : thymine (A : T) sites are assumed to revert through the production of apurinic gaps, formed by the hydrolysis of ethyladenine.

INTRODUCTION

It has been proposed that ethylating agents exert their mutagenic effects mainly through the production of 7-ethylguanine (EG) in the DNA molecule (Bautz & Freese, 1960). The other major product of alkylation, 3-ethyladenine (EA), is assumed to have a lesser role in ethylation-induced mutagenesis (Brooks & Lawley, 1962; Pal, 1962; Krieg, 1963*a*, *b*). Ethylation-induced mutation of phage exhibits a pattern of delayed mutation, in which mutant subclones appear to be formed after normal non-mutant gene copies have been made (Green & Krieg, 1961). Two interpretations of this phenomenon have been advanced, based on different base-pairing properties of ionized or tautomerized EG, or based on hydrolysis of EG (Green & Kreig, 1961; Freese, 1963; Krieg, 1963*a*). Experiments to test the latter interpretation did not support it and favoured the pairing-error interpretation of the delayed mutant subclones (Löbbecke & Krieg, cited in Krieg, 1963*b*).

In experiments with bacteria it is common practice to count revertant colonies at an early time (36–48 hr), to avoid counting slow-growing colonies which may be due to suppressor mutations (Margolin & Mukai, 1961). Yet it is common experience to find a continuous appearance of new colonies on the selective plates after plating bacteria treated with alkylating agents.

Two years ago an experiment was designed by Professor F. J. Ryan and the present author to study diethylsulphate (DES)-induced recombination in *Salmonella*

* Present address: Laboratory of Genetics, The Hebrew University, Jerusalem, Israel.

typhimurium. The results were indecisive because of a continuous reversion of some of the markers, although the treated bacteria were kept in buffer. This finding could not be accounted for by selection. Somewhat similar results were found by Strauss & Okubo (1960), who found increased reversion from auxotrophy to prototrophy upon incubation of DES-treated *Escherichia coli* at an elevated temperature before selective plating was done. In the present paper, a study of the pattern of mutant appearance after DES treatment is described. It shows that late appearance of revertant colonies after DES treatment is indicative of delayed mutation rather than slowly growing suppressor mutations, and is not due to a general 'physiological damage' of alkylation. It further shows that the different mutations can be grouped in two classes—delaying and non-delaying ones—which can be correlated with the pattern of mutation found after treatment with some base analogues.

METHODS

Organisms used. Leucine-requiring mutants of *Salmonella typhimurium* strain LT2 were used (we are indebted to Dr P. Margolin for these mutants) and are among 42 studied by Margolin & Mukai (1961) which were shown to be revertible by both diethylsulphate and 2-amino-purine (AP). They fell into two groups with respect to AP-induced reversion: those which showed a higher frequency of induced mutation when several cell divisions occurred after treatment (division-dependent, DD), and those whose frequency of induced reversion was not dependent on further cell division (division-independent, DI).

Media. Liquid minimal medium (MM) used in reversion experiments contained (g./l.): 10·5, K_2HPO_4; 4·5, KH_2PO_4; 1·0, $(NH_4)_2SO_4$; 0·98, Na-citrate; 8·0, NaCl; 2·0, glucose; 1 ml. M-$MgSO_4$; water to 1 l. Minimal agar (MA) contained, in addition, 20 g. agar/l. The same minimal agar, supplemented with 25 μg. leucine/ml., was used for viable counts. Tryptone broth contained (g./l.): 10, Bacto tryptone; 9, NaCl; 2, glucose. The buffer used for treatment as well as post-treatment incubation contained (g./l.): 5·8, Na_2HPO_4; 3, KH_2PO_4; 1, NH_4Cl; 8, NaCl; and 1 ml. M-$MgSO_4$; water to 1 l.

Treatment. Overnight cultures grown in 100 ml. tryptone broth were washed twice and resuspended in 10 ml. buffer. After further incubation for about 3 hr at 37°, diethylsulphate was added to 5 ml. of suspension. The treatment mixture was shaken at 37° on a shaker. Treatment was stopped by diluting 0·05 ml. of the mixture into 5 ml. buffer supplemented with $Na_2S_2O_3$ to a final concentration of 0·04 M. Whenever large numbers of treated bacteria were needed, 1·0 ml. of the treated suspension was diluted in 100 ml. of buffer supplemented with $Na_2S_2O_3$. After incubation at 37° for 1 hr, the bacteria were collected by centrifugation and resuspended in 5 ml. buffer containing 0·04 M-$Na_2S_2O_3$.

Plating experiments. Plates of minimal agar (MA) seeded with 10^6–10^9 bacteria were incubated at 37°, and revertant colonies marked and counted. In each experiment, a 36 hr colony from a control MA plate (seeded with untreated bacteria) was chosen as a 'reference colony' (and the plate kept in a refrigerator). Revertant colonies were counted only after attaining the size of that colony.

Tube experiments. For tube experiments, treated bacteria were further diluted in chilled liquid minimal medium (MM) and distributed in small tubes. The tubes were incubated at 37° without aeration and examined routinely for the appearance

of turbidity caused by overgrowth of revertants. Whenever it was necessary to follow the optical density in those tubes, 4 ml. samples were distributed; otherwise, each tube contained 2 ml. A Bausch and Lomb 'Spectronic 20' spectrophotometer was used for readings of optical density at 540 mμ.

RESULTS

Alkylation of mutant leu-524

The effects of diethylsulphate (DES) treatment on viability and mutation in the *Salmonella typhimurium* mutant, *leu-524*, are shown in Fig. 1. Samples (5 ml.) of buffer suspension of bacteria were treated with different DES concentrations for 20 min., or with 0·04 ml. (final concentration of 0·06 M) of DES for various lengths of time. When the whole range of diethylsulphate concentrations used was drawn so as to match the whole range of treatment time, the two curves closely resembled one another within the ranges of times and doses shown; they deviated, however, with more severe treatments. This was because of rapid decomposition of DES in aqueous solution and because, with higher concentrations of DES, the oily DES drops in the treatment suspension become very large and remain attached to the bottom of the tube. A 20 min. treatment with 0·04 ml. DES/5 ml. bacterial suspension was arbitrarily chosen as standard treatment in further experiments.

The reversion data shown in Fig. 1 were obtained by treating 5 ml. bacterial suspension with different concentrations of DES for 10 and 20 min. Colonies growing on minimal medium agar (MA) were counted after 5 days. After that time, there was little change in colony count from day to day. One can, however, count the colonies on the same reversion plates every day, and plot the additive numbers against time. When this was done with mutant *leu-524*, the results shown in Fig. 2 were obtained. Spontaneous revertants form colonies which reach the size of the reference colony on MA after 30–40 hr, and there is little or no increase in their numbers after 60 hr. DES-induced revertant colonies lag behind the spontaneous colonies, and their number continues to increase considerably for another 96 hr. *A priori*, this increase in colony count could be accounted for by some physiological variability, slow-growing suppressor mutants, or delay in back mutation of *leu-524* itself. On the other hand, the possibility exists that the DES curves in Fig. 2 finally levelled off only because of environmental changes that took place on the plates while they were being incubated (such as bacteria becoming devoid of nutrients as the plates became too dry).

To test these possibilities, the following experiment was made. Treated bacteria of strain *leu-524* were diluted in MM to approximately 10^5 bacteria/ml.; 2 ml. samples were distributed in small tubes and allowed to stand at 37° without aeration. Control bacteria were diluted in MM to about 10^7 bacteria/ml. (very slight turbidity). The results are shown in Fig. 3. Most of the control tubes remained clear for about 3 weeks. However, some tubes became turbid because of the overgrowth of leu$^+$ revertants. Pre-existing mutants required, under those conditions, about 48 hr for full overgrowth. After that time, no more mutants were found. (The rate of spontaneous reversion in *leu-524* is too low to become manifest at these conditions.) In the treated series, the number of turbid tubes continued to increase, although at a decreasing rate, throughout the experiment.

One can estimate, for any given time, the mean number (m) of mutant bacteria per tube (Ryan, 1955). After a time sufficient for full overgrowth (as made manifest by increase of turbidity), m represents the average number of pre-existing mutants originally distributed in the tubes. Thereafter, m also includes new mutants which

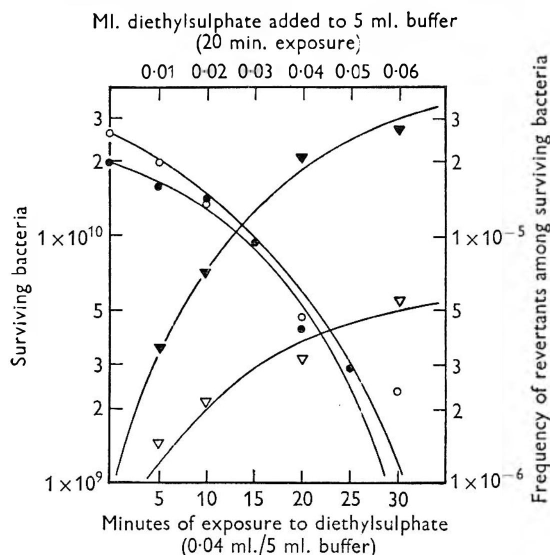

Fig. 1

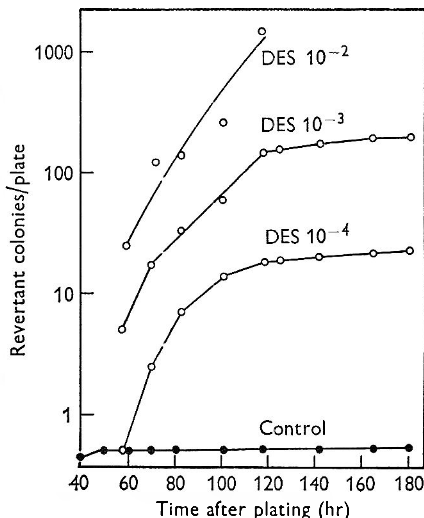

Fig. 2

Fig. 1. Viability and mutation frequency after various treatments with diethylsulphate. ● = Viability after treatment with 0·04 ml. diethylsulphate/5 ml. bacterial suspension for various lengths of time. ○ = Viability after treatment with various concentrations of diethylsulphate for 20 min. ▼ = The frequency of revertants among surviving bacteria after 20 min. treatment with various concentrations of diethylsulphate. ▽ = The frequency of revertants among surviving bacteria after 10 min. treatment with various concentrations of diethylsulphate.

Fig. 2. The number of revertant colonies growing on minimal medium agar, as recorded at different times after plating. A culture of c. 10^{10} leu-524 bacteria/ml. was treated with diethylsulphate and plated at final dilution of 10^{-2}, 10^{-3} and 10^{-4}. Control bacteria were plated undiluted.

originated after the bacteria were distributed in the tubes. m is estimated by determining the fraction of clear tubes among all the tubes (P_0), and applying it to the zero term of the Poisson distribution:

$$P_0 = e^{-m}. \tag{1}$$

In Fig. 3, typical m curves for control and DES-treated *leu-524* bacteria are shown. In the control m increased from 30 to 48 hr, after which there was no further increase. In the treated series m increased continuously for more than 400 hr.

'*Suppressor*' *mutations*

Inasmuch as some suppressor mutations can be distinguished from 'true' reversion by the slow growth rate of suppressor mutants, mutant *leu-524* gives, upon alkylation, mainly true revertants; only few slow-growing tubes were detected by routine measurements of optical density. Nor were many found in reconstruction

experiments. In such experiments, single bacteria were isolated from tubes which had become turbid at different times in the main experiment. This was done by diluting them (so as to give, on the average, less than one cell per tube) into tubes

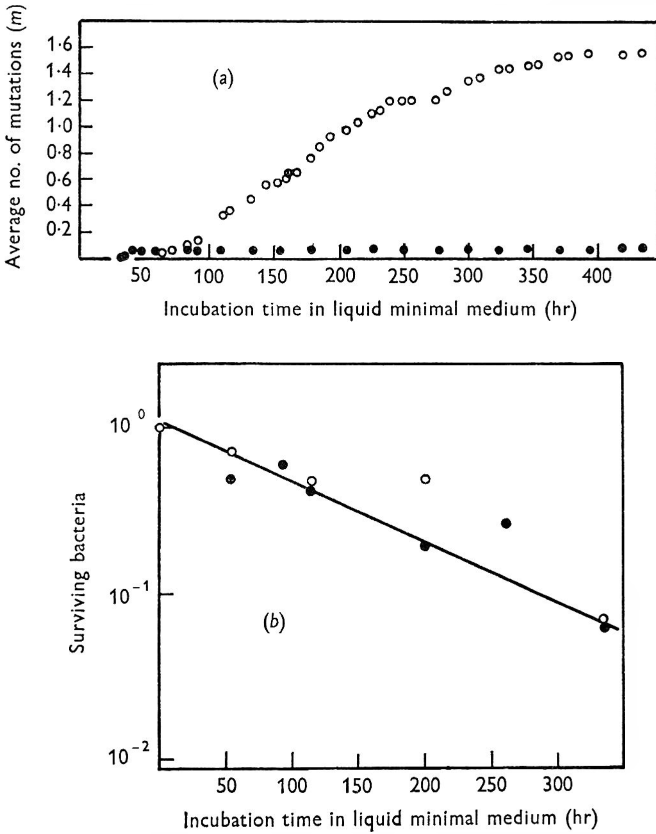

Fig. 3. (*a*) The average number of mutations (m) in control (closed circles) and diethylsulphate-treated (open circles) leu-524 bacteria. The total numbers of tubes were 285 and 492, respectively. (*b*) Survival of control (closed circles) and diethylsulphate-treated (open circles) in the above experiment.

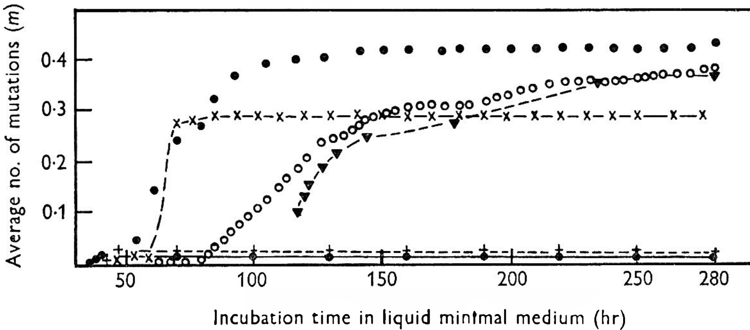

Fig. 4. The average number of mutations (m) in control (bottom curves) 524, +- - -+; 48 and 122, ●——●; and diethyl-sulphate-treated (upper curves) bacteria of mutants 48, ×——× ; 122, ● ●; 524, ○ ○; 524 slow, ▼——▼. 524 slow was the slow growing tubes from leu 524.

containing 2 ml. liquid minimal medium with or without a 'background' population of about 5×10^5 mutant (untreated) bacteria/ml. The time for overgrowth in each tube was measured. The same observation, namely, that 'early' and 'late' mutants grew at the same rate, was confirmed in later experiments by the use of the spectrophotometer: with few exceptions (considered as slow growers), it took 6–10 hr from the time tubes showed any measurable turbidity until they became fully turbid. However, by using the spectrophotometer it was possible to secure a sufficient number of slow-growing revertants. After 120 hr from the time of dilution, the m curve for slow growers followed closely the m curve obtained with the normal growers (Fig. 4).

Table 1. *Delayed mutation in various leucine mutants of Salmonella typhimurium*

	Frequency of spontaneous mutation		Frequency of diethylsulphate-induced mutation			
Mutant	At 48 hr	After 7 days	At 48 hr	After 7 days	Division-dependence*	Delayed mutation
leu-10	1.7×10^{-9}	2.3×10^{-9}	8.8×10^{-8}	$> 10^{-4}$	+	+
leu-25	3.0×10^{-9}	5.0×10^{-9}	5.0×10^{-8}	2.8×10^{-6}	+	+
leu-34	4.6×10^{-9}	6.3×10^{-9}	1.4×10^{-7}	8.9×10^{-5}	+	+
leu-44	1.2×10^{-9}	2.3×10^{-9}	2.0×10^{-5}	5.1×10^{-5}	−	−
leu-48	1.0×10^{-9}	2.0×10^{-9}	2.8×10^{-5}	3.9×10^{-5}	−	−
leu-122	4.2×10^{-9}	7.8×10^{-9}	5.4×10^{-6}	5.9×10^{-6}	−	−
leu-127	1.3×10^{-9}	4.5×10^{-9}	3.8×10^{-5}	4.0×10^{-5}	−	−
leu-130	4.3×10^{-9}	8.3×10^{-9}	2.4×10^{-5}	2.5×10^{-5}	−	−
leu-140	1.7×10^{-8}	3.9×10^{-8}	6.5×10^{-7}	1.3×10^{-5}	+	+
leu-524	6.4×10^{-10}	9.5×10^{-10}	1.3×10^{-6}	2.4×10^{-5}	+	+

* As observed by Margolin & Mukai (1961): + = division-dependent; − = division-independent.

Experiments with other leu mutants

Having thus established that delayed mutation in mutant *leu-524* was not an artifact caused by slow growth, it became desirable to see whether other *leucine* mutants would show the same phenomenon after treatment with diethylsulphate (DES), or whether the delayed mutation was peculiar to mutant *leu-524*. Nine other DES-revertible *leu* mutants were selected for this purpose, on the basis of their patterns of reversion with 2-aminopurine (Margolin & Mukai, 1961) (Table 1, column 6). Buffer suspensions of these mutants were treated with diethylsulphate as described for mutant *leu-524*, and the numbers of revertant colonies were recorded repeatedly.

The results obtained with all 10 mutants are summarized in Table 1 and in Fig. 5. It can be clearly seen that all mutants did not show the delay effect. Mutants *44*, *48*, *122*, *127* and *130* (namely, all division-independent mutants), after the appearance of the first burst of revertant colonies, show no further increase in the number of revertant colonies. On the other hand, all five division-dependent mutants did show such an increase for more than a week.

Delayed mutation on minimal medium agar and in buffer

A question arises about the relation between diethylsulphate-induced mutation and division-dependence after treatment with 2-aminopurine. To separate the time effect from the cell-replication effect, treated bacteria were kept in buffer before

plating was done on minimal medium agar. The results are shown in Fig. 6. *Leu-524* bacteria showed about a 40-fold increase in the number of revertant colonies over the first burst, when plated immediately after treatment. After incubation in buffer for 72 hr there was less than a 1·5-fold increase in the number of revertant colonies (Fig. 6*a*). This was even more pronounced in another experiment (Fig. 6*b*) where treated bacteria were kept in buffer for 140 hr before a tube test was made.

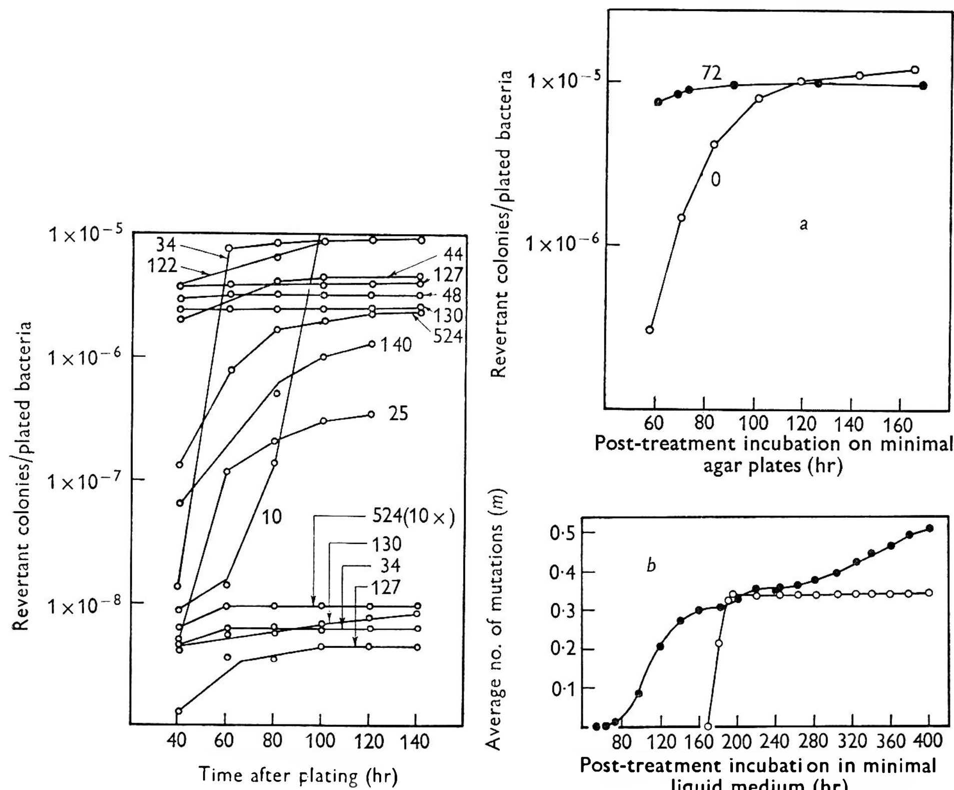

Fig. 5. Frequency of revertant colonies of the various mutants on minimal medium agar, as recorded at different times after plating. The frequencies of induced reversion of all 10 mutants are shown (10 upper curves). To make the figure more legible, only some of the control curves are shown. The others fall within the same limits and show the same pattern. The figures for leu-524 are magnified ten times.

Fig. 6. (*a*) Reversion frequency of leu-524 on minimal medium agar, as recorded at different times after plating. 0 = Cells plated immediately after treatment. 72 = Cells plated after 72 hr of post-treatment incubation in buffer. (*b*) The average number of mutants (*m*) among diethylsulphate-treated leu-524 bacteria in a tube experiment. ●——● = 0 cells distributed immediately; ○——○ = 140 cells distributed after 140 hr of post-treatment incubation in buffer.

In contrast with the gradual increase in the *m* curve obtained with bacteria which were distributed immediately after treatment, the rise in *m* values for the bacteria which had been kept in buffer was rather abrupt, with no further increase. In both cases, however, the actual frequency of revertants was the same whether or not the bacteria had been aged in buffer before selection.

DISCUSSION

Margolin & Mukai (1961) studied 2-aminopurine-induced reversion of some 42 leucine-requiring mutants of *Salmonella typhimurium*, including 9 of the mutants used in the present work. They were able to demonstrate a distinction between continuous (division-dependent) and a single burst (division-independent) mutation. Both theoretical considerations (Freese, 1959) and the patterns of mutation after 2-aminopurine and 5-bromouracil found in *Escherichia coli* (Strelzoff, 1962) strongly suggest that the division-dependent mutants, *10*, *25*, *34*, *140* and *524* have adenine–thymine base pairs at their mutant sites, while mutants *44*, *48*, *122*, *127* and *130* have guanine–cytosine sites.

The present work shows that mutants of the two groups are diethylsulphate revertible and have different mutation patterns after diethylsulphate treatment. In spite of some degree of intra-group variability, mutants that are division-dependent after 2-aminopurine treatment show delayed mutation after alkylation, while division-independent mutants do not. However, from the experiments with incubation in buffer it follows that this delay is dependent only upon time, and not on replication.

At present, little is known about mechanisms that can account for the different patterns of alkylation-induced mutation (see review by Krieg, 1963*b*). Alkylation of native DNA was found to give mainly 7-alkylguanine and 3-alkyladenine (Lawley & Brooks, 1963).

Krieg (1963*a*) found that *r*II mutants of bacteriophage T4 that were highly revertible by ethyl methanesulphonate gave only a small fraction of their induced revertants when plated directly on selective host bacteria. The high frequency of induced revertants was found only when the treated phages were first allowed to reproduce in a non-selective host. (Neither procedure involved post-treatment storage of phage to promote depurination.) He attributed this phenomenon, and the delayed mutations occurring during replication (Green & Krieg, 1961), to pairing errors of 7-ethylguanine. Other T4*r*II mutants, assumed to have adenine–thymine pairs at their mutant sites, gave much lower or no ethyl methanesulphonate-induced reversion. Schwartz (1963), treating *lac*⁻ mutants of *Escherichia coli* with ethyl methanesulphonate, failed to induce the reversion of ethyl methanesulphonate-induced mutants at a high frequency. He attributed it to the fact that ethyl methanesulphonate induces mainly guanine–cytosine to adenine–thymine transitions. However, in the present study diethylsulphate was capable of reverting both guanine–cytosine and adenine–thymine mutant sites. Since diethylsulphate acts as a monofunctional ethylating agent like ethyl methanesulphonate, their mutagenicity should be expected to be similar.

Time-dependent delayed mutation in alkylation-induced bacteria is not necessarily identical with replication-dependent delay in phages. It is not clear, *a priori*, which of the alkylated bases should be implicated in interpreting the pattern of delayed mutation reported here. Nearly equal amounts of 7-ethylguanine and 3-ethyladenine are released initially during post-treatment storage (Lawley & Brookes, 1963). The apurinic gaps formed in this manner may account for the delayed mutations, by any of three mechanisms: (1) by taking in a 'wrong' base prior to replication (Ryan, Nakada & Schneider, 1961); (2) by the incorporation of the

latter opposite the gap, during replication; (3) by being capable of coding for the 'right' messenger RNA, thus initiating growth and replication. Such processes are quite speculative, but the idea of an apurinic gap leading to mutation is supported by the mutagenic effects of treatment at low pH values on phage (Freese, 1959), and of heat to bacteria (Greer & Zamenhof, 1962).

It is also possible that the depurination-dependent process may be masked at guanine–cytosine sites, due to the 'erroneous' pairing of 7-ethylguanine with thymine (or uracil, in RNA synthesis) (Brookes & Lawley, 1962). These hypothetical mechanisms are summarized in Fig. 7. Such a mechanism would be facilitated by

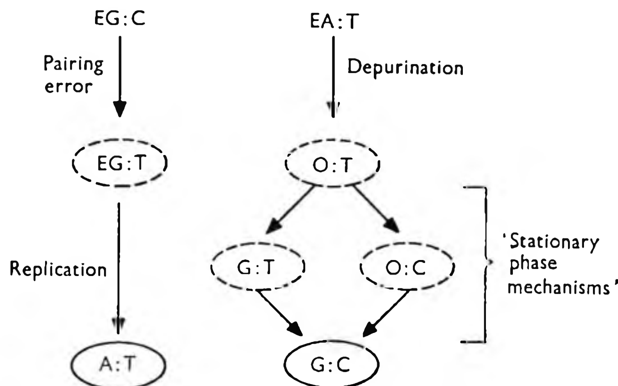

Fig. 7. Possible mechanisms of alkylation-induced reversion. Mutant 7-ethylguanine: cytosine (EG:C) sites are assumed to revert (immediately) through pairing errors during residual replication on minimal medium agar or in liquid minimal medium. 3-ethyl-adenine:thymine (EA:T) sites are assumed to revert through (delayed) depurination followed by insertion of guanine (G) in the apurinic gap, or by the polymerization of cytosine (C) opposite the gap. Of the other theoretical possibilities, delayed mutation through the production of apurinic gaps in EG:C sites is assumed to be masked by the process indicated here, while the ability of EA to pair with cytosine (Krieg, 1963*a*) has not been proven. ○ = Apurinic gap. Base pairs encircled by a broken line indicate a possible partial (phenotypic) reversion. Base pairs encircled by a continuous line indicate complete reversion.

the technically uncontrolled residual replication of the bacteria upon resuspension in minimal medium. Whatever the mechanism of delayed mutation, further complications may be expected to stem from indirect effects, such as the possible influence of neighbouring bases in the DNA molecule, on base substitution. It is not unlikely that similar mechanisms underlie spontaneous mutation in the stationary phase (Ryan, 1959) and of diethylsulphate-induced mutation. More direct evidence and better understanding of the role of base turnover in stationary-phase mutation (Ryan *et al.* 1961) seem necessary for the interpretation of alkylation-induced mutagenesis. Experiments bearing on the uptake by bacteria of bases alkylated *in vitro*, and the pattern of consequent mutation, are underway now.

The author dedicates this paper to the memory of the late Professor Francis J. Ryan, a teacher and a friend.

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An Effect of Light on Glucose Uptake by the Fungus *Blastocladiella britannica*

BY EVELYN A. HORENSTEIN AND E. C. CANTINO
Department of Botany and Plant Pathology, Michigan State University, East Lansing, U.S.A.

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When synchronized single-generation cultures of the unicellular water mould *Blastocladiella britannica* are grown in the presence of white light, they develop into nearly colourless thin-walled sporangia; in the dark they develop into brown pitted thick-walled resistant sporangia. Under these conditions, dry weight/cell increases exponentially at the same rate in light and dark. However, the capacity for uptake of glucose by cells of various ages grown in the dark exceeds that of light-grown cells. Furthermore, just as the course of development along either of the two morphogenetic pathways can be reversed by excluding or supplying light before their respective points of no return, so, too, the rise in their capacities for glucose uptake can be similarly reversed. However, the point of no return for glucose uptake precedes the point of no return for morphogenesis by several hours. The light-sensitive glucose uptake by *B. britannica* may be a factor in determining the ultimate morphology of this organism.

INTRODUCTION

The occurrence of alternate sporangial types among aquatic Phycomycetes is not an unusual state of affairs. The formation of a thick-walled resistant structure is often an integral stage—sometimes the result of sexual fusions and sometimes not—in their life histories, and it may serve as a protective device against unfavourable environments (Emerson, 1954; Sparrow, 1960). For the Blastocladiales, one of the triggering agents in such environments is CO_2 and/or bicarbonate (Emerson & Cantino, 1948; Cantino & Lovett, 1964). On the other hand, the developmental pattern of *Blastocladiella britannica* appears to be exceptional, for it does not respond to CO_2 or bicarbonate; instead, it is visible light which determines its ultimate structural form.

From the progeny of our original single-cell isolate of *Blastocladiella britannica* (Horenstein & Cantino, 1961) we selected a substrain (101B) with dual morphogenetic potentialities. When grown on agar media, it produces: (*a*) in the dark, about 90–100 % brown pitted thick-walled resistant sporangia (RS) with a generation time (g.t.) of about 65 hr; (*b*) in white light, nearly colourless thin-walled sporangia (TWS) with a g.t. of about 30–32 hr. However, neither the absence nor presence of light is required continuously throughout the entire growth period for the genesis of one or the other of these morphological forms. The organism's early stages of development are quite plastic; cells which start their growth in the light, and which are, therefore, on the TWS pathway, can be induced to revert to RS types by eliminating the light. Conversely, calls which start growth in the dark

(i.e. along the RS pathway) can be transformed into TWS types by exposure to light. In both instances, however, a stage in development is reached beyond which addition or withdrawal of illumination can no longer effect morphogenetic reversal. At this point of no return, the cells have become committed to one pattern or the other. Subsequently we succeeded in demonstrating: (*a*) the morphological response to light and dark; (*b*) the reversal of morphogenesis by alteration of the light-and-dark regime, using large populations of synchronous single generations of this strain in submerged liquid cultures (Horenstein & Cantino, 1962).

Up to a point just preceding the end of the generation time of a thin-walled sporangium (TWS), no morphological differences are discernible under the light microscope between it and a dark-grown developing resistant-sporangium (RS) at a corresponding chronological age. Yet, within the next 1–2 hr, the entire protoplast of a thin-walled sporangium is cleaved into hundreds of uninucleate uniflagellate motile spores, and this new generation of cells is then discharged. On the other hand, the thalli growing in the dark have reached only the half-way point in their ontogeny; they continue to enlarge for several hours thereafter and then gradually differentiate into mature resistant sporangia.

X-ray diffraction studies, electron microscopy and chemical analyses (Nabel, 1939; Frey, 1950; Aronson & Preston, 1960; Fuller, 1960) have established that the cell walls of the Blastocladiales consist predominantly of chitin, and that very little, if any, cellulose is present. Chemical analyses (Cantino, Lovett & Horenstein, 1957) also suggest that the thickened walls of the resistant sporangium of *Blastocladiella emersonii* results primarily (Lovett & Cantino, 1960*a*, *b*) from increased synthesis of chitin, as does that of *B. britannica* (Horenstein & Cantino, 1962). Thus, since metabolism of glucose or glucose derivatives must have been involved in the differentiation of the resistant sporangium of *B. britannica*, we undertook studies of glucose uptake to ascertain whether or not light exerted an effect on it.

METHODS

Growth and preparation of organisms. One-l. Erlenmeyer flasks containing 550 ml. Difco PYG broth (1·25 g. peptone, 1·25 g yeast extract, 3·0 g. glucose/l.) made up in citric acid ($3{\cdot}2 \times 10^{-3}$M) + Na_2HPO_4 ($7{\cdot}2 \times 10^{-3}$M) buffer (pH 5·8) were inoculated with 50 ml. of a spore suspension prepared according to Horenstein & Cantino (1962). In making up media allowance was made for dilution by the inoculum. The final population in the cultures, determined by plate counts, was kept between 7×10^4 and 10^5 cells/ml. These were incubated at 24° in a water bath and aerated with water-saturated air at 1500 ml./min. Light-grown cultures were illuminated from below with 500 f.c. fluorescent white light; dark-grown cultures were covered with aluminium foil.

The entire population in each flask was harvested by filtration, and the cells washed with 600 ml. of citric acid + phosphate buffer (see above). In the process, the fungus was concentrated into the bottom of the cone of a filter paper in a glass funnel. The latter was then placed over a calibrated cylinder, a small hole was poked through the bottom of the paper, and the cells (easily dislodged) washed quantitatively into the cylinder with buffer and made up to a desired volume. The density of the final suspension ranged from $0{\cdot}3 \times 10^6$ to $1{\cdot}4 \times 10^6$ cells/ml. Throughout

the preceding procedure, cells were readily maintained in suspension and unclumped. Replicate samples were removed for dry-weight determinations (24 hr, 70°, in vacuum oven).

Determination of glucose uptake capacity. Ten and 15 ml. samples of the above cell suspension were placed in test tubes at 24°, and the volume adjusted to 19 ml. with the citrate + phosphate buffer. At zero time, 1 ml. of buffer containing 50 μM glucose was added to each tube (this concentration of sugar was selected because higher concentrations yielded no increase in glucose consumption while lower concentrations tended to depress its uptake). A fine stream of air served to keep the contents agitated and the cells suspended throughout the 45 min. incubation. For this period, glucose uptake by such pre-formed cells was not affected by the presence or absence of light; therefore, these incubations were carried out under ordinary light conditions of the laboratory. At the end of the incubation, cells were filtered off and washed with water; cells and filtrates were then frozen.

Glucose was determined with glucose oxidase (Glucostat Reagent from Worthington Biochemical Corp., Freehold, New Jersey, U.S.A.) according to Washko & Rice (1961). The glucose uptake capacity (GUC), defined as μg. glucose consumed/cell during 45 min. incubation at 24°, was calculated from glucose concentrations in the filtrate before and after incubation.

RESULTS AND DISCUSSION

Effects of the light-dark regime

In synchronous single-generation cultures of *Blastocladiella britannica*, the generation time (g.t.) of light-grown thin-walled sporangia (TWS) is defined as that time when about 20 % of the population has developed discharge tubes; at 24°, with population densities between 3×10^4 and 10^5 cells/ml., it is about 30 hr (Horenstein & Cantino, 1962). Within 1–2 hr thereafter, all the rest of the cells develop discharge tubes and a few begin to release their spores. The g.t. for dark-grown resistant sporangia (RS) is defined as 65 hr; such cells never discharge spores in culture.

When cells are grown continuously in the dark from spores, they can still be induced to form thin-walled sporangia if they are transferred to an illuminated environment at any time up to a critical point of no return; this occurs at 28 hr. However, when illumination is delayed until the last possible moment—i.e. until this point of no return is reached—the generation time of the thin-walled sporangia is usually prolonged by a few hr; and, when it is delayed beyond this point, thin-walled sporangia are no longer formed while resistant sporangia appear instead. Thus, on the basis of the combinations tried so far, there are three different regimes of light and dark which yield only thin-walled sporangia, and three which yield only resistant sporangia (Fig. 1). In effect, there appear to be two non-overlapping periods (18–20 hr and 28–30 hr) in the life span of a thin-walled sporangium during which light exerts its sharp effect on morphogenesis. Light is obligatory for formation of thin-walled sporangia only during one or the other of these periods; or alternatively, light must be absent during either one of these two periods if resistant sporangia are to be formed.

Growth rates in light and dark

Although the first visible signs of photomorphogenesis are not detectable microscopically until the generation time of a thin-walled sporangium is reached, it was clear from previous reversal work (Horenstein & Cantino, 1962) and the results described above, that light modifies the cells of *Blastocladiella britannica* in some manner at some earlier stage in their ontogeny. Since a parameter associated with

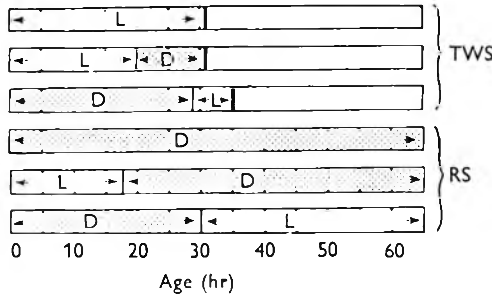

Fig. 1. The effect of exposure during growth to different light–dark regimes upon the nature of cell types finally formed (thin-walled sporangia, TWS, or resistant sporangia, RS) and their generation times (vertical heavy bars) at 24°. See text for details.

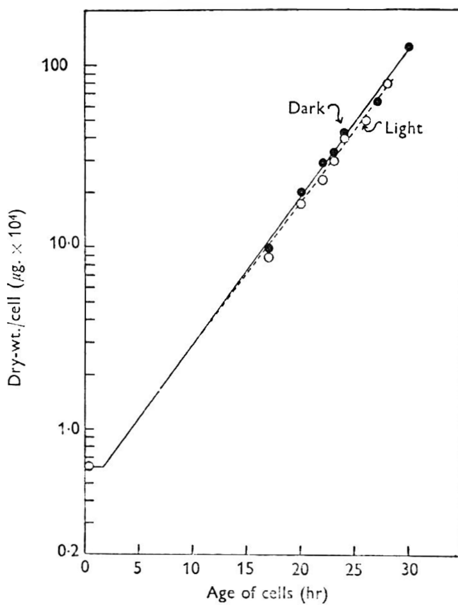

Fig. 2

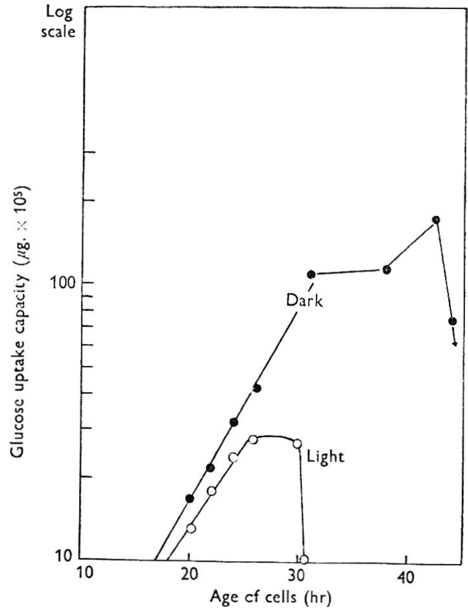

Fig. 3

Fig. 2. The exponential increase in dry wt./cell during growth in the light and dark at 24°. Each point is an average for at least three different cultures, twenty-nine of them light-grown and forty-two dark-grown. Population densities were within the ranges $3–15 \times 10^4$ cells/ml. medium.

Fig. 3. The glucose uptake capacity of light-grown and dark-grown cells at different stages in their development at 24°.

growth rates might have reflected these changes, a detailed search was made for possible differences in cell weights. However, in both light- and dark-grown cultures, the dry weight/cell increases exponentially at identical rates (following a 1–2 hr lag after spore inoculation) up to 30 hr; i.e. the pattern is not affected by illumination (Fig. 2). These data complement favourably an earlier report (Horenstein & Cantino, 1962) that the rate of exponential increase in the volume of a growing cell is also independent of light and darkness.

Glucose uptake capacity

Next, the glucose uptake capacity (GUC) was determined with cells of various ages. In striking contrast to the lack of effect of illumination on the cell dry weight and volume the glucose uptake capacity was affected. The previous history of the cell—i.e. whether it grew in light or dark—had a definite effect on its capacity to consume glucose under non-growing conditions (Fig. 3). The rate of increase of GUC was slightly greater for dark-grown than for light-grown cells; in both cases, it was exponential up to 26 hr (i.e. 81 % of the generation time for thin-walled sporangia, and 40 % of the generation time for resistant sporangia). At 26 hr, the glucose uptake capacity of a light-grown cell levelled off and then fell precipitously just before the completion of its generation-time. A dark-grown cell also exhibited a change in its glucose uptake pattern, although a different one. In this case, the glucose uptake capacity continued to increase up to 30 hr, after which it increased at a much lower rate up to about 65 % of the generation time of the resistant sporangia; then, it too decreased sharply.

Reversal of glucose uptake capacity

Experiments were done to ascertain whether the glucose uptake capacity of a dark-grown cell could be reversed (i.e. reduced) to the glucose uptake capacity of a light-grown cell, just as morphogenesis itself can be reversed. Cultures were grown in the dark for 18 hr (an 18 hr period was selected because this precedes by only a few hours the point of no return for resistant sporangium morphogenesis). The same cultures were then illuminated, and after various additional periods of growth (up to 30 hr) in the light, the cells were harvested and their glucose uptake capacity determined. Our thesis was this: if light-depressed glucose uptake capacity is a causal factor for the light-induced differentiation of a thin-walled sporangium, then the effect of light on glucose uptake capacity should occur before the photomorphogenetic response. The results of these experiments on reversal of glucose uptake capacity are shown in Fig. 4, where they are compared with the glucose uptake capacity values for control cultures grown continuously either in light or in dark. It is apparent that with relatively short exposures to light before the point of no return for resistant sporangium morphogenesis, values for glucose uptake capacity approached those of cells grown continuously in the dark for the same total period of time. But with increasing exposures to light, glucose uptake capacity values were progressively depressed and closely approached those of light-grown cells. It should be noted, however (see below), that complete reversal of glucose uptake capacity to the value associated with a thin-walled sporangium should not be expected, because it turned out that 18 hr is just a little beyond the point of no return for glucose uptake capacity itself.

The point of no return for glucose uptake capacity

The morphological point of no return for light-grown thin-walled sporangia and dark-grown resistant sporangia has been established (see: *Effects of the light–dark regime*). The following experiments were designed to define the point of no return for the accumulation in the cell of glucose uptake capacity itself. Cells were grown in the dark for different lengths of time and then transferred to light; after 30 hr of growth, all of them were harvested and their glucose uptake capacities determined.

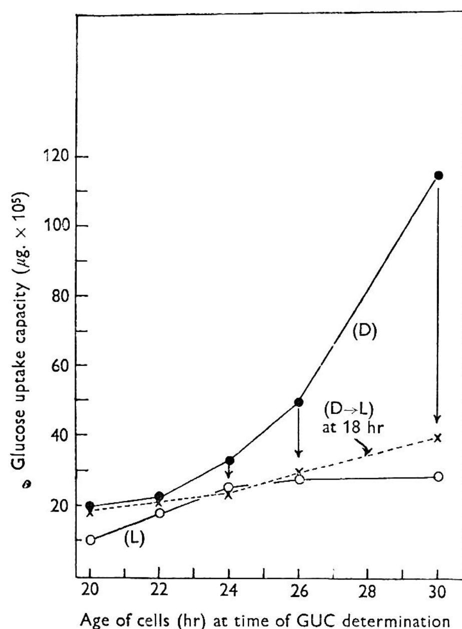

Fig. 4

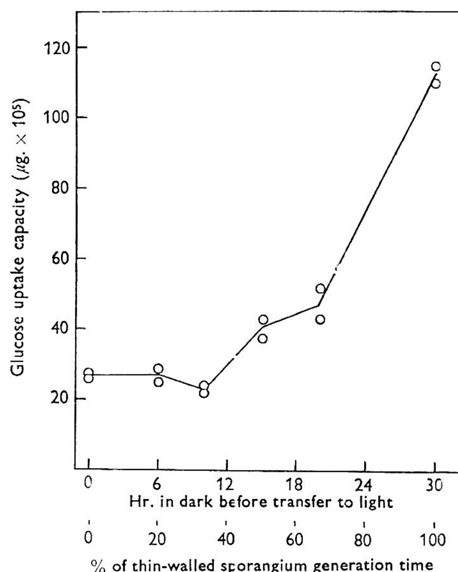

Fig. 5

Fig. 4. The effect of different exposures to light (following growth in the dark for 18 hr) upon the glucose uptake capacity of cells at different stages in their development at 24°. See text for details.

Fig. 5. The point of no return for the depressing effect of light upon glucose uptake capacity (GUC). See text for details.

The results (Fig. 5) show that when cells were grown from the outset in the dark and then illuminated before 12 hr had elapsed (40 % of the generation time of a thin-walled sporangium), they retained the same low glucose uptake capacity characteristic of cells grown continuously in the light for 30 hr. But once 40 % of the cell's generation time had elapsed, increased exposures to darkness, before providing the dosage of light, resulted in increased values of glucose uptake capacity; these values gradually approached the typically high glucose uptake capacity of a cell grown in the dark throughout its generation time.

The data provided in this paper do not, of course, offer proof that a cause-and-effect relationship exists between the magnitude of glucose uptake capacity and the differentiation of resistant sporangia *versus* thin-walled sporangia. However, the

data are consistent with our present hypothesis, namely: (*a*) that although there is considerable latitude in the glucose uptake capacity which permits a cell to develop into a thin-walled sporangium, a significantly higher minimum value of glucose uptake capacity must be attained by a cell before it can develop into a resistant sporagium; (*b*) that visible light in some fashion controls this value of the glucose uptake capacity. These notions are currently being tested.

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The Action of Ultrasonic Vibrations on Actinophages

By D. L. ANDERSON and S. G. BRADLEY
Department of Microbiology, University of Minnesota, Minneapolis, U.S.A.

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SUMMARY

High-frequency sonic oscillations destroyed the plaque-forming ability of actinophages. Among the 31 actinophage strains studied, the fraction of viral particles which survived an exposure for 1 min. to vibrations of 20 kcyc./sec. varied from 100 to 0·0002 %. Coliphage T2hr+ was more susceptible to ultrasonic treatment than were some actinophages and was more resistant than other actinophages. In general, large actinophage particles were more sensitive to sonic treatment than small particles. A 60 W. 20 kcyc./sec. M.S.E. Ultrasonic Disintegrator destroyed actinophages more rapidly than a 50 W., 9 kcyc./sec. Raytheon Sonic Oscillator. Electron micrographs of actinophage MSP8 treated with sonic vibrations showed progressive disruption of the viral particles. Susceptibility to sonic inactivation gave an additional criterion for grouping actinophages.

INTRODUCTION

Ultrasonic oscillations produced by vibrating crystals or diaphragms have been shown to inactivate various types of bacteriophages suspended in liquid media (Anderson, Boggs & Winters, 1948; Beumer & Beumer-Jochmans, 1950; Fadeeva, Rautenshtein & El'piner, 1959). Anderson and co-workers reasoned that if bacteriophages were more resistant to sonic vibration than were their hosts, early stages of phage multiplication could be studied by sonically-disrupting bacteria at intervals after infection. Anderson & Doermann (1952) used this method in studies on the intracellular development of coliphage T3. Beumer & Beumer-Jochmans (1950) found large differences in the sensitivities of staphylococcus phage 'Twort' and coliphages PF and pfi, to sonic vibrations of 1000 kcyc./sec. Fadeeva *et al.* (1959) found a phage of *Bacillus megaterium* to be much more sensitive to oscillations at a frequency of about 650 kcyc./sec. than were phages virulent for *Actinomyces olivaceus* (*Streptomyces olivaceus*) and *Actinomyces streptomycini* (*Streptomyces griseus*). Hydrogen and argon atmospheres had no protective effect against the inactivation of the *B. megaterium* phage.

We wished to determine whether ultrasonic treatment could be used to prepare actinophage 'ghosts', to separate phage heads from tails, and to determine whether actinophages with similar host ranges had similar sensitivities to sonic treatment. Susceptibility to ultrasonic vibrations has been generally correlated with other criteria for classifying actinophages. Electron microscopic examination of treated actinophage suspensions showed that mechanical disruption was correlated with loss of plaque-forming ability.

METHODS

Actinophages. The actinophages used and their hosts are given in Table 1. The origins of these actinophages were recorded previously (Anderson, 1961).

Actinophages were grown on the appropriate host in a medium composed of: 5 g. Difco peptone; 3 g. Difco yeast extract; 0·5 g. $Ca(NO_3)_2$; per litre of de-ionized water. The methods used for propagating and assaying the actinophages were described by Bradley, Anderson & Jones (1961). Phage lysates were filtered through unglazed porcelain filters to remove host debris, and the filtrates stored at 4°.

Ultrasonic treatments. Filtered phage suspensions were subjected to 18–20 kcyc./sec. generated by a 60 W. M.S.E. Ultrasonic Disintegrator (Measuring and Scientific Equipment Ltd., London) or to 9 kcyc./sec. from the 50 W. Model S102A Raytheon Oscillator (Raytheon Manufacturing Company, Waltham, Massachusetts, U.S.A.). Containers holding 10 ml. of phage suspension were immersed in an ice bath during the 12 min. treatment period. Samples (0·3 ml.) were removed at intervals; the numbers of infectious particles were enumerated on peptone yeast-extract agar by the soft agar overlay method (Jones & Bradley, 1962).

Electron microscopy. Suspensions of actinophage MSP8 to be observed by electron microscopy were purified on columns of G-75 coarse Sephadex (Pharmacia, Uppsala, Sweden) and ECTEOLA-cellulose (Bio-Rad Corp., Richmond, Calif., U.S.A.) or by several cycles of centrifugation (Kolstad & Bradley, 1964). Treated suspensions were dried on Formvar-coated specimen grids, shadowed with uranium, and observed in an RCA EMU-3F electron microscope.

RESULTS

Effects on infectivity

Actinophages markedly lost infectivity as measured by plaque forming ability when subjected to ultrasonic oscillations of 18–20 kcyc./sec. or 9 kcyc./sec. for various periods (Tables 1 and 2). The actinophage strains differed substantially in sensitivity to ultrasonic treatment. For example, 10 % of the infective units of phages MSP8, WSP1, and WSP3 survived 12 min. ultrasonic treatment, whereas the infective titre of other actinophages (e.g. MSP11) decreased from 10^8 particles/ml. to undetectable values after treatment for 2 min. Coliphage T2hr+ was more sensitive to ultrasonic treatment than were some of the actinophages and was more resistant than others. A comparison of phage inactivation produced by the M.S.E. Disintegrator (Table 1) and by the Raytheon Oscillator (Table 2) suggested that the former instrument was more effective. The M.S.E. apparatus destroyed more than 10 times as many particles of coliphage T2hr+ as did the Raytheon apparatus after treatment for 2 min., about 100 times as many particles after 4 min., and about 1000 times as many particles after 12 min. Similar results were obtained in comparing the susceptibilities of actinophages MNP8 and WSP3 to treatment in these two instruments.

To determine the effect of the initial phage concentration on the inactivation rate, the initial concentration of several phage suspensions was adjusted so that their titres differed by factors of 10 to 1000. In general, the rate of inactivation of separate suspensions of a particular phage was independent of initial phage concentration (Table 3).

For those actinophages whose size had been determined, large phages proved more sensitive to ultrasonic treatment than small phages. For example, actinophages MSP19, MNP8, MNP6, all of which were quickly inactivated by ultrasonic treat-

Table 1. *The effects of ultrasonic treatment at* 20 *kcyc./sec. on the infectivity of various actinophages and coliphage T2hr* +

Phage	Host	Log. initial titre	Log. Initial phage titre / Phage titre after ultrasonic treatment for (min.)					
			0·25	1	2	4	7	12
MSP8	*Streptomyces venezuelae* S13	8·8	0·1	0·3	0·4	0·5	0·7	1·0
MSP8	*S. griseus* S104	4·4	−0·1	0·1	0·1	0·4	0·3	0·9
MSP1	*S. griseus* S86	9·0	0·2	0·8	2·6	3·5	3·5	3·6
MSP2	*S. griseus* S86	7·7	0·2	0·3	0·4	0·8	1·3	1·7
WSP1	*S. griseus* S86	7·8	−0·1	−0·2	0·1	0·2	0·6	1·1
WSP3	*S. griseus* S104	6·3	−0·2	−0·1	0·2	0·1	0·5	1·1
MSP7	*S. violaceoruber* S199	8·3	0·4	1·3	2·8	4·5	5·7	5·8
MSP11	*S. violaceoruber* S199	8·1	2·6	4·3	—	—	—	—
MSP15	*S. violaceoruber* S199	7·5	0·7	2·4	4·3	5·1	6·5	6·0
MSP16	*S. violaceoruber* S199	7·2	1·5	2·8	3·4	3·6	6·2	—
MSP17	*S. violaceoruber* S199	7·7	2·3	3·7	4·2	—	—	—
MSP18	*S. violaceoruber* S199	9·4	1·6	3·0	4·1	5·1	6·2	6·4
MSP19	*S. violaceoruber* S199	7·0	4·0	5·3	—	—	—	—
MSP21	*S. vinaceus* S208	8·0	1·0	2·3	3·9	3·7	4·1	5·9
MSP22	*S. vinaceus* S208	7·9	1·3	3·1	4·5	—	—	—
MNP4	*S. griseus* S104	9·4	1·0	2·4	3·9	4·7	—	—
MNP3	*S. griseus* S104	9·6	0·3	1·1	2·3	2·6	3·5	4·4
MVP4	*S. cinnamomeus* 1285	7·7	0·5	1·6	3·2	4·3	4·7	6·7
MNP1	*Nocardia brasiliensis* N301	6·8	0·4	0·5	1·3	2·4	4·4	4·6
MNP2	*N. brasiliensis* N301	7·3	2·3	3·2	3·9	4·1	—	—
MNP6	*N. brasiliensis* N301	7·7	0·2	0·7	2·2	3·7	4·2	6·4
MNP7	*N. brasiliensis* N301	8·1	0·6	2·3	3·5	4·9	5·9	6·2
MMP1	*N. brasiliensis* N301	6·2	0·4	0·1	2·2	—	—	—
MJP1	*Jensenia canicruria* 1574L	8·9	0·8	2·1	3·0	3·7	4·7	5·3
MMP9	*Mycobacterium* sp. A405	5·6	0·2	1·7	—	4·2	—	—
MMP14	*M. friburgensis* A403	5·5	1·0	2·2	4·8	—	—	—
MMP13	*M. friburgensis* A403	5·1	0·6	1·9	4·1	—	—	—
MMP8	*M. rabinowitsch* A402	4·1	1·1	3·4	—	—	—	—
MMP10	*M. stercoides* A406	6·9	0·9	2·1	4·1	3·7	3·9	—
MMP7	*M. rabinowitsch* A402	6·9	4·0	5·7	—	—	—	—
T2hr+	*Escherichia coli* B	9·0	0·1	0·4	1·4	3·3	4·5	5·4
T2hr+	*E. coli* B	9·1	0·1	0·4	1·2	3·0	4·9	5·2

Table 2. *The effects of ultrasonic treatment at* 9 *kcyc./sec. on the infectivity of various actinophages and coliphage T2hr* +

Phage	Host	Log. initial titre	Log. Initial phage titre / Phage titre after ultrasonic treatment for (min.)						
			0·5	2	4	7	12	27	57
WSP3	*Streptomyces griseus* S104	8·2	—	—	—	0·2	0·4	0·5	0·6
MNP8	*Nocardia corallina* W5	5·2	−0·3	0·9	2·3	—	4·2	4·2	—
T2hr+	*Escherichia coli* B	9·1	−0·1	−0·1	0·9	0·7	1·8	3·7	4·4

ment, were longer than 300 mμ and had head dimensions of about 70–80 mμ (Anderson, 1961). Conversely, actinophages MSP2, WSP1, WSP3, which were highly resistant to ultrasonic treatment, were all about 250 mμ in overall length. Actinophages WSP1 and WSP3 had head dimensions of less than 60 mμ.

Table 3. *The effects of initial phage concentration on the ultrasonic inactivation rate*

Ultrasonic treatment by a 20 kcyc./sec. M.S.E. instrument.

Phage	Host	Log. initial titre	Log. (Initial phage titre / Phage titre after ultrasonic treatment for (min.)) 0·25	1	2	4	7	12
MSP10	*S. violaceoruber* S199	6·6	0·2	1·0	3·4	4·3	—	—
MSP10	*S. violaceoruber* S199	5·6	0·5	1·3	2·7	4·6	4·6	—
MNP8	*N. corallina* W5	7·9	0·9	2·7	5·0	6·4	—	—
MNP8	*N. corallina* W5	4·8	1·0	3·1	—	—	—	—

Table 4. *The relationship between mechanical disintegration of actinophage MSP8 and loss of plaque-forming ability*

Min. of treatment	Log. plaque forming units	Total heads (no.)	Whole phage (%)	Phage with broken heads (%)	Whole detached heads (%)	Broken detached heads (%)	Whole free tails (no.)
0	9·9	155	95	3	—	2	6
0·5	9·8	212	83	15	—	1	29
2	9·7	194	64	9	2	25	34
4	9·5	542	51	8	—	41	106
7	Undetermined	162	49	7	1	44	56
12	8·7	294	31	6	—	62	142
22	8·3	227	7	2	8	83	90
42	7·7	121	3	1	3	95	56

Effects on structure

Samples for electron-microscope examination were taken at intervals during ultrasonic treatment of actinophage MSP8, and the frequencies of whole particles, particles with broken heads, broken detached heads, and whole detached heads were determined (Table 4). Almost all the viral particles of the untreated control were found to be intact (Fig. 1). After a short period of treatment, some phage heads were broken and separated from their tails; other particles appeared to be undisturbed (Pl. 1, figs. 2, 3). As the treatment progressed, more particles were disrupted; after 42 min. of ultrasonic treatment no intact particles were found (Pls. 1, 2. figs. 4–8). Electron micrographs at this point showed masses of debris which included short tail fragments.

DISCUSSION

Fadeeva *et al.* (1959) showed that actinophages were inactivated by ultrasonic treatment at a frequency of about 650 kcyc./sec. Treatments of 45 min. to 6 hr. produced large decreases in infective titres. In the present work, exposure to ultrasonic treatment at a frequency of 18–20 kcyc./sec. for 1–12 min. caused a comparable loss of infectivity. Moreover, 20 kcyc./sec. was more effective in destroying the infec-

tivity of the phages studied than a frequency of about 9 kcyc./sec. The 650 kcyc./sec. treatment used by Fadeeva *et al.* (1959) apparently was not as effective in destroying actinophages as our 20 kcyc./sec. treatment; but meaningful comparisons cannot be made because their actinophages may have been quite different from those we studied. Moreover, our results cannot be compared directly with those of Beumer & Beumer-Jochmans (1950), who used a 120 W. instrument with a frequency of 1000 kcyc./sec. The intensity and the frequency of oscillation are undoubtedly both important factors in the destructive action of any ultrasonic treatment.

Anderson *et al.* (1948) suggested that small phages such as coliphage T3 probably escape the destructive cavitation which disrupts the larger phages such as coliphages T2, T4 and T6. In the present work, actinophages WSP1, WSP3 and MSP2 were inactivated less rapidly than the smaller coliphage T2hr+ (overall length 190 mμ). The head of coliphage T2hr+, however, is about 95 mμ long which is larger than the actinophage head structure. This suggests that susceptibility to ultrasonic treatment may be primarily dependent upon the dimensions of the head. Consistent with this view are the observations that coliphage T5 is far more susceptible to ultrasonic treatment than is coliphage T1 and that the head of coliphage T5 (65 mμ) is larger than the head of coliphage T1 (50 mμ). The tails of coliphages T5 and T1 are of about the same length (170 mμ and 150 mμ, respectively; Stent, 1963).

Mechanism of ultrasonic inactivation of actinophage MSP8. Electron-microscope examination of samples of an ultrasonically treated suspension of actinophage MSP8 taken at intervals showed that mechanical disruption was important in inactivation (Pls. 1, 2, figs. 1–8). No sharp sequence of destructive events was detected (for example, breaking of heads, separation of heads from tails, fragmentation of tails). Tails, however, did not appear to fragment extensively until the ultrasonic treatment was prolonged from 22 to 42 min. (Pl. 2, figs. 6, 7). Free tails consistently clumped after prolonged treatment (Pl. 2, fig. 8). If the time of irradiation were controlled, it might be possible to separate intact actinophage tails from the phage heads, and then purify each component by appropriate physical techniques.

Taxonomic implications. One of the objects of this work was to determine whether ultrasonic treatment data would provide one more criterion in grouping actinophages. The sensitivity pattern of actinophages to ultrasonic treatment agrees in general with the classification based upon host range (Bradley *et al.* 1961). For example, actinophages MSP15, MSP16, MSP17, MSP18, MSP19, which are closely related in host range, are all markedly susceptible to ultrasonic treatment. Similarly, actinophages WSP1 and WSP3, which are quite resistant to ultrasonic treatment, are indistinguishable in host range. Adams (1959) stated that one of the major objections to the use of inactivation of any type of phage as a taxonomic criterion is that phage strains often differ quantitatively rather than qualitatively; indeed, there is limited diversity in sensitivity among members of a group with similar host ranges. When the sensitivities of different groups are compared, there is overlapping in ranges of variation among the groups. Obviously no one type of inactivation can be used as a sole taxonomic criterion; inactivation characteristics should be correlatable with other criteria, such as morphology and serology.

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EXPLANATION OF PLATES

Plate 1

Fig. 1. Untreated particles of actinophage MSP8; ×25,000.

Fig. 2. Actinophage MSP8 treated with 20 kcyc./sec. ultrasonic vibrations for 30 sec.; ×25,000.

Fig. 3. Actinophage MSP8 after 4 min. treatment; ×25,000.

Fig. 4. Actinophage MSP8 after 7 min. treatment; ×26,818.

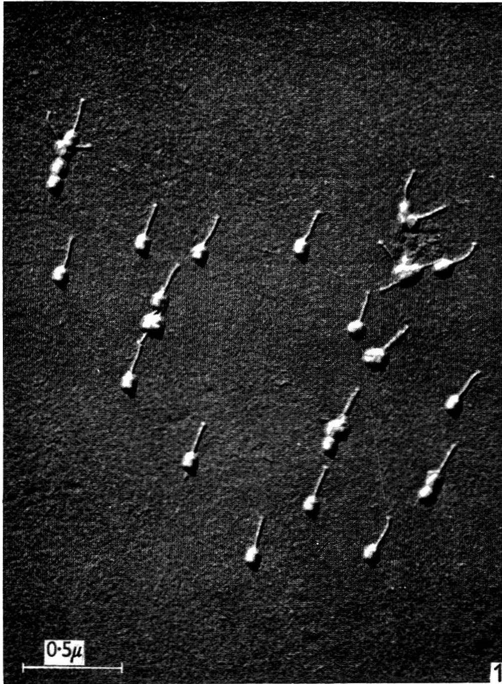
Plate 2

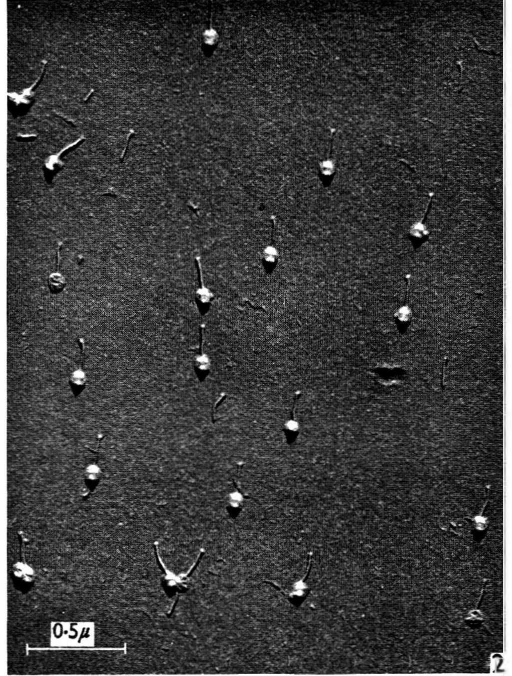
Fig. 5. Actinophage MSP8 particles and debris after 12 min. treatment; ×25,000.

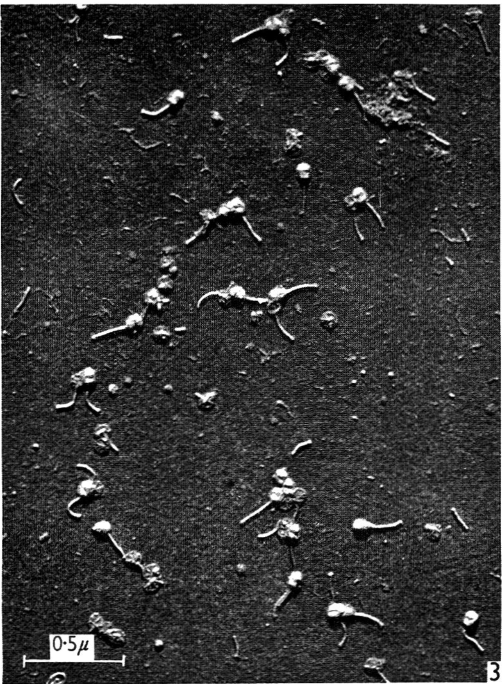
Fig. 6. Actinophage MSP8 particles and debris after 22 min. treatment; ×25,000.

Fig. 7. Head coats and debris from actinophage MSP8 particles after 42 min. treatment; ×25,000.

Fig. 8. Large clump of tails apparently held together by debris from actinophage MSP8 particles after 42 min. treatment; ×25,000.







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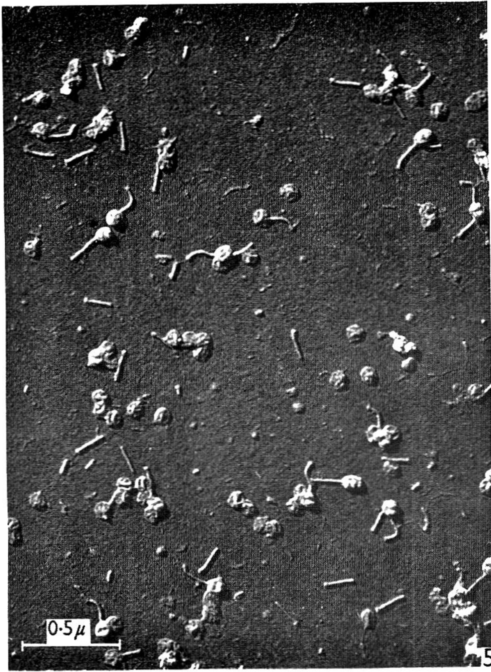

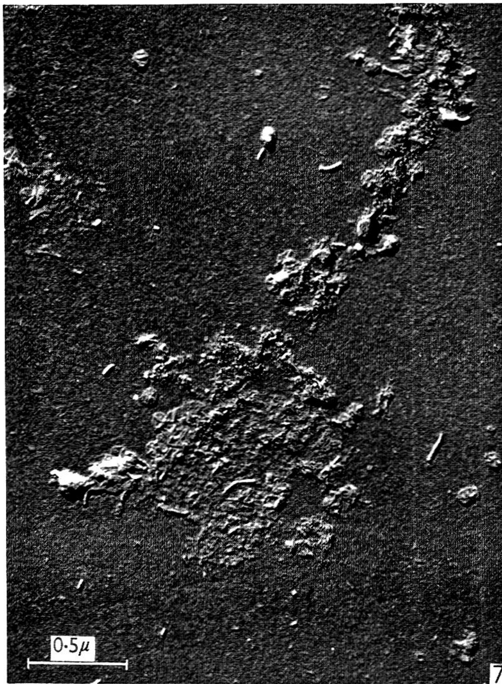

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β-Glucosidase Activity in Mycoplasma

By C. V. HENRIKSON and P. F. SMITH

Department of Microbiology, School of Medicine, University of South Dakota, Vermillion, South Dakota, U.S.A.

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SUMMARY

Strains of three groups of Mycoplasma with different nutritional requirements were analysed for β-glucosidase activity. The enzyme was found in the cell membrane of *Mycoplasma laidlawii* strain B and *M. gallisepticum* strain J, which metabolize carbohydrates; negligible β-glucosidase activity was found in *M. hominis* strain 07 which does not metabolize carbohydrates. β-Glucosidase in all strains was inactivated by heating at 56° for 30 min. or at 100° for 10 min. With *M. laidlawii* strain B as the test organism, β-glucosidase was found to possess the following characteristics: pH optimum, 6·8; optimum temperature, 30°; no dialysable cofactors required; phosphate ions had no effect on activity; enzyme was specific for substrates (glucosides) with β configuration; aglycon of the glucoside must be an aryl group; enzyme exhibited absolute specificity for carbon atoms number 4 and 5 of the glycon. The presence of β-glucosidase in *M. laidlawii* strain B and *M. gallisepticum* strain J is assumed to be involved in the synthesis and hydrolysis of carotenyl glucoside and sterol glucoside by these organisms.

INTRODUCTION

Mycoplasma organisms can be divided into three groups on the basis of nutritional requirements: *Mycoplasma laidlawii* strain B is representative of a group which does not require a sterol as a nutrient and which metabolizes carbohydrates; *M. gallisepticum* strain J belongs to a group which requires a sterol and metabolizes carbohydrates (Rothblat & Smith, 1961); *M. hominis* strain 07 belongs to a group which requires a sterol but does not metabolize carbohydrates.

Strains of the third group (*M. hominis* strain 07) contain free and esterified sterol (Smith, 1959). The group represented by *M. gallisepticum* strain J contains free and esterified sterol and steryl-glucoside. When cholesterol is the sterol source, the glucoside is a cholesteryl-β-D-glucoside. The amount of steryl-glucoside has been shown to be related to the amount of glucose added to the culture medium (Rothblat & Smith, 1961). The group represented by *M. laidlawii* strain B contains non-saponifiable lipids comprised of carotenoids which are synthesized *de novo*; however, these organisms are also capable of incorporating sterol which spares carotenoid synthesis (Smith, 1963*a*). This sparing action suggested a similarity in function of sterol and carotenoids.

One function of the non-saponifiable lipids in Mycoplasma is believed to be structural since sterol is incorporated into the cell membrane and is not metabolized. Another possible function is that the sterol or carotenol may act as carriers of metabolizable substrates into the cell and of metabolic end-products out of the cell

(Smith, 1963*b*). No direct demonstration of glucosidases in Mycoplasma has hitherto been made, although their presence was suspected since the organisms contain glucosides. The present work demonstrates β-glucosidase activity in the cell membrane of glucose-fermenting strains but a negligible activity in one strain which does not ferment glucose.

METHODS

Organisms. Six representative strains of Mycoplasma were examined. The saprophytic strains *M. laidlawii* strains A and B, *M. inocuum*, and the unclassified strain KHS of caprine origin which do not need a sterol as nutrient and utilize carbohydrates; *M. gallisepticum* strain J which requires sterol and utilizes carbohydrates; *M. hominis* strain 07 which requires sterol and does not utilize carbohydrates. All of the above strains have been maintained in this laboratory.

Media. Saprophytic strains were grown in a tryptose broth of the following composition: 2 % tryptose (Difco); 0·5 % sodium chloride; 0·5 % sodium acetate; 0·5 % glucose; pH 7·8. Sterol-requiring strains were grown in the medium described by Morton, Smith & Leberman (1951) supplemented with 1 % Bacto PPLO serum fraction (Difco). The medium for *M. gallisepticum* strain J was supplemented with 0·5 % glucose.

Cultivation. The organisms were grown and harvested as has been reported (Smith, 1955). The amounts of organisms were measured on the basis of total cellular N (Lecce & Morton, 1954). Thallium acetate 1/2500 (w/v) was used as a selective inhibitor of bacterial growth. The presence of this inhibitor had no effect on glucosidase activity. The organisms were resuspended in M/15 pH 7·5 phosphate buffer for most experiments although tris-hydroxymethylamino-methane-maleate buffer (tris-maleate; Gomori, 1955) was used for various determinations.

Enzymic activity. In most experiments, the enzymic activity was determined by spectrophotometric measurement at 400 mμ of the amount of *p*-nitrophenol released from *p*-nitrophenyl-β-D-glucoside (Duerksen & Halvorson, 1958). The activity of the enzyme toward *o*-nitrophenyl-β-D-galactoside as substrate was determined in a similar fashion. The procedure was modified by diluting the test samples in 0·1 N-sodium hydroxide instead of phosphate buffer (pH 11) to assure a maximum colour development. For substrates with non-coloured aglycons, the activity was determined by measuring the amount of reducing sugar released (Park & Johnson, 1949). Since intact organisms would metabolize the liberated sugar, the organisms were disrupted in a 10 Kc Raytheon oscillator (10 min.) and dialysed for 48 hr in 0·005 M tris-maleate buffer (pH 7·0) to eliminate cofactors. Essentially no loss of glucosidase activity occurred as a result of the sonic treatment (see Results).

Chemicals. The substrates used were: *p*-nitrophenyl-β-D-glucoside, *o*-nitrophenyl-β-D-galactoside, maltose, cellobiose, gentiobiose, α-methyl-D-glucoside, β-methyl-D-glucoside, β-methyl-D-arabinopyranoside, β-methyl-L-arabinopyranoside, β-methyl-D-xylopyranoside, α-methyl-D-xylopyranoside, 6-bromo-2-naphthyl-α-D-glucoside, 6-bromo-2-naphthyl-β-D-glucoside and α-methyl-D-mannopyranoside (California Corp. for Biochemical Research), amygdalin (Difco), salicin (Pfanstiehl Chemical Co.).

Enzyme localization. Distribution of enzyme activity was determined by use of

sonically treated suspensions of *Mycoplasma laidlawii* strain B and *M. gallisepticum* strain J and frozen-thawed suspensions (10 cycles) of *M. laidlawii* strain B. The broken suspensions were centrifuged in a Spinco Model L centrifuge (94,500 *g*) for 30 min. and the deposit resuspended in an equal volume of buffer. A fraction of intact organisms, sonically treated organisms, the centrifuged supernatant fraction, and the centrifuged deposit were each used as enzyme source.

Glucoside synthesis and transglycosidation. Analysis of reaction systems designed to demonstrate synthesis of glucosides and transglycosidation was done by chemical measurement of disappearance of substrate and by detection of glycosides on thin-layer chromatographs. The procedure for thin-layer chromatography was like that used by Gee (1963), with the difference that the developing solvent was chloroform + methanol + acetic acid + water (65 + 25 + 8 + 4, by vol.).

RESULTS

Glucosidase activity was found in *Mycoplasma laidlawii* strain B (Fig. 1) and *M. gallisepticum* strain J, but no activity was found in *M. hominis* strain 07 (Fig. 2). That this activity was enzymic was supported by its lability to heat and its dependence upon the concentration of organisms. *M. laidlawii* strain B, heated at 100° for 10 min. or 56° for 30 min., showed complete loss of enzymic activity (Fig. 1). Increase in glucosidase activity towards *p*-nitrophenyl-β-D-glucoside was shown upon increase of organism concentration (Table 1). All readings were taken at time intervals indicative of enzyme saturation with substrate.

Table 1. *Effect of cell concentration on β-glucosidase activity in Mycoplasma laidlawii strain B and M. gallisepticum strain J*

Reaction mixture; *p*-nitrophenyl-β-D-glucoside, 18 μmole; glutathione, 0·25 mg.; 0·067 M-phosphate buffer (pH 7·5); final volume, 3 ml. Reactions carried out 3 hr at 30° for *M. laidlawii* strain B and 37° for *M. gallisepticum* strain J.

Cell nitrogen (mg.)	*M. laidlawii* strain B	*M. gallisepticum* strain J
	p-nitrophenol formed (μmole)	
1	2·58	1·17
2	3·47	2·10
3	4·33	3·00

The major activity was found in the residues (cell membranes) in contrast to the supernatant fluid fractions (cytoplasm) (Table 2), whether derived by sonic treatment or by freeze-thaw. These results were indicative of a membrane location for the glucosidase.

The optimal temperature for activity was the same as that for growth, i.e. 30° for the saprophytic strains and 37° for the sterol-requiring strains. The pH optimum was 6·8 although this optimum was not sharply defined (Table 3). In most experiments with whole organisms, there was a 2 hr lag before activity became optimum (Fig. 1, 3). When the organisms were incubated at 30° or 37° for 24 hr, the lag period was eliminated (Fig. 3). Storage of *Mycoplasma laidlawii* strain B at 4° for 72 hr or at room temperature for 24 hr had little effect on glucosidase activity. Glucosidase activity of intact *M. laidlawii* strain B against *p*-nitrophenyl-β-D-glucoside in

0·048 M—tris-maleate buffer (pH 7·0) was neither augmented nor decreased by the addition of di-basic sodium phosphate in equimolar proportion to the substrate. Material from organisms after sonic treatment and also after subsequent dialysis showed about the same activity.

Table 2. *Distribution of β-glucosidase activity in Mycoplasma laidlawii, strain B and M. gallisepticum*

Reaction conditions same as Table 1 with the exception that 6 μmole of *p*-nitrophenyl-β-D-glucoside was used.

Fraction of organism	Sonic treatment: *M. laidlawii* strain B	Sonic treatment: *M. gallisepticum* strain J	Freeze-thaw *M. laidlawii* strain B
	p-nitrophenol formed (μmole)		
Control (whole organisms)	4·52	4·44	4·86
Residue fraction	3·59	3·72	4·43
Supernatant fraction	0·61	0·42	0·34
Activity in residue fraction (%)	81·9	84·0	91·15

Table 3. *Effect of pH value on β-glucosidase activity in Mycoplasma laidlawii strain B*

Reaction mixture same as Table 1 except 0·048 M-tris-maleate buffer and 6 μmole *p*-nitrophenol-β-D-glucoside were used.

pH value	*p*-Nitrophenol formed (μmole)	pH value	*p*-Nitrophenol formed (μmole)
5·33	0·54	7·02	2·97
5·80	1·59	7·19	2·64
5·90	2·13	7·50	2·64
6·50	2·46	8·00	2·25
6·60	2·64	8·10	2·13
6·80	3·21	8·45	2·04

The specificity of the glucosidases found in various strains of Mycoplasma is shown in Table 4. All strains hydrolysed *p*-nitrophenyl-β-D-glucoside. *Mycoplasma laidlawii* strains A and B, showed very small activity with *o*-nitrophenyl-β-D-galactoside, and no activity was shown by the caprine strain KHS and *M. inocuum*. Amygdalin was partially hydrolysed by the caprine strain KHS and by *M. laidlawii* strain B. All organisms capable of glucose degradation partially hydrolysed salicin. The caprine strain KHS and *M. laidlawii* strain B, hydrolysed 6-bromo-2-naphthyl-β-D-glucoside, for which no activity was shown by *M. laidlawii* strain A and *M. inocuum*. No activity was shown by any strain with 6-bromo-2-naphthyl-α-D-glucoside, α-maltose, cellobiose, gentiobiose, α-methyl D-glucoside, β-methyl-D-glucoside, β-methyl-D-arabinopyranoside, β-methyl-L-arabinopyranoside, β-methyl-D-xylopyranoside, α-methyl-D-xylopyranoside, α-methyl-D-mannopyranoside.

Inhibition of *p*-nitrophenyl-β-D-glucoside hydrolysis by *Mycoplasma laidlawii* strain B occurred when amygdalin was added (Table 5).

Attempts were made to synthesize glycosides with *Mycoplasma laidlawii* strain B. No synthesis of *p*-nitrophenyl-β-D-glucoside, i.e. on decrease in optical extinction

at 400 mμ, was found in the presence of glucose and p-nitrophenol. Likewise, no disappearance of glucose occurred when this was incubated with dialysed sonically treated organisms + cholesterol. Uridine diphosphate glucose (UDPG) did not act as a glucose donor with p-nitrophenol. This inactivity of UDPG was not due to its degradation since no free glucose appeared upon incubation of this compound with the enzyme preparation in the presence or absence of 0·01 M-sodium fluoride.

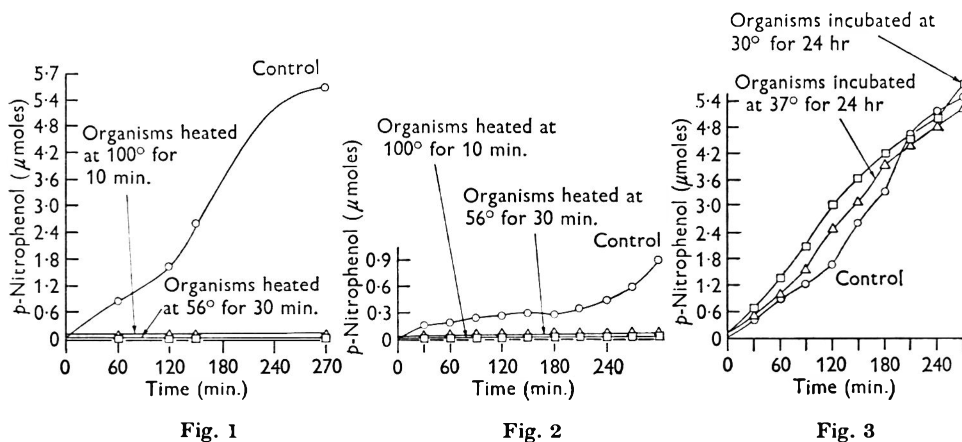

Fig. 1. β-Glucosidase activity in Mycoplasma laidlawii strain B. Reaction mixture: p-nitrophenyl-β-D-glucoside, 6 μmole; glutathione, 0·25 mg.; M/15 phosphate buffer (pH 7·5); organism equivalent to 2 mg. cellular-N; final volume, 3 ml. Reaction time, 3·0 hr at 30°.

Fig. 2. β-Glucosidase activity in Mycoplasma hominis strain 07. Reaction mixture same as in Fig. 1.

Fig. 3. Effect of preincubation on β-glucosidase activity in Mycoplasma laidlawii strain B. Reaction mixture same as in Fig. 1.

Table 4. *Specificity for aryl glucosides by β-glucosidases in saprophytic Mycoplasma strains*

Reaction mixture same as Table 1 except for substrate variation. Each substrate added at 6 μmole.

Substrate	Caprine strain KHS (μmole)	*M. laidlawii* strain B (μmole)	*M. inocuum* (μmole)	*M. laidlawii* strain A (μmole)
p-Nitrophenyl-β-D-glucoside*	4·10	2·10	3·87	3·24
o-Nitrophenyl-β-D-galactoside*	0	0·33	0	0·39
Amygdalin†	0·61	0	0	0·96
Salicin†	0·66	0·15	0·17	0·15
6-Bromo-2-naphthyl-β-D-glucoside†	6·00	0	0	1·89

* Nitrophenol liberation measured spectrophotometrically at 400 mμ.
† Glucose liberation measured by the method of Park & Johnson (1949).

Transglycosidation experiments were made with p-nitrophenyl-β-D-glucoside as donor and methanol, propanol, or butanol as acceptors. Samples were analysed, with known glycosides, by thin-layer chromatography. No glycosides other than p-nitrophenyl-β-D-glucoside were detected.

Table 5. *Inhibition by amygdalin of p-nitrophenyl-β-D-glucoside hydrolysis in Mycoplasma laidlawii Strain B*

Reaction mixture same as Table 1 with the following exceptions: 6 μmole of *p*-nitrophenyl-β-D-glucoside; amounts of amygdalin as shown.

Amygdalin (μmole)	Amygdalin/ *p*-nitrophenyl-β-D-glucoside ratio	Degree of inhibition (%)
0	—	0
1·2	1/5	30·0
6·0	1/1	84·1
12	2/1	96·4
18	3/1	94·6

DISCUSSION

The existence of significant glucosidase activity only in the strains of Mycoplasma which metabolized glucose and its location in the membrane fraction adds supporting evidence to the hypothesis of the role of sterols and carotenols in the membrane transport of glucose (Smith, 1963*b*). Elimination of the lag in hydrolysis of *p*-nitrophenyl-β-D-glucoside by incubation of intact organisms for 24 hr at 30° or 37° may be explained by hydrolysis of endogenous carotenyl or cholesteryl glucoside in the cell membrane (Smith, 1963*b*). It would be expected that the enzyme would preferentially attack the endogenous glucoside not only because it is the natural substrate but also because this substrate is located in the particulate cell-fraction which contains the enzyme. It is unlikely that the lag implicates an induction of the glucosidase since the organisms were grown in the presence of glucose. Bacterial contamination cannot explain the lag, for no increase in bacterial counts were observed, with or without added thallium acetate, during the course of the reaction. Because of the lag two different reaction rates are evident. Therefore, all data presented in this paper were based on the reaction rate which occurred after the initial lag period. The change in rate of reaction after extended incubation, i.e. after 3 hr, was due no doubt to lack of sufficient substrate to saturate the enzyme or to end product-inhibition.

The pH optimum of β-glucosidases is dependent on their source and to a minor degree on the substrate and the buffer (Veibel, 1950). The pH optimum of the β-glucosidases from Mycoplasma is relatively high (pH 6·8) as compared with β-glucosidases from other sources except that from *Saccharomyces cerevisiae* which has an optimum of pH 6·4–6·8 (Duerksen & Halvorson, 1958). The pH optima of β-glucosidases from other sources vary from pH 4 to 5·8 (Veibel, 1950; Jermyn, 1955; Hash & King, 1958; Conchie, 1954).

No added cofactors were required for activity, but this may be a reflexion of the particulate nature of the enzyme. Veibel (1950) demonstrated that the activity of β-glucosidase from almond emulsin doubled when the cations, sodium, potassium, rubidium, calcium, strontium, and barium, and the anions, nitrate, iodide, perchlorate, and chlorate, were added at 0·08 M concentration. However, Duerksen & Halvorson (1958) demonstrated no difference in activity of β-glucosidase from *Saccharomyces cerevisiae* when the cations, sodium, potassium, ammonium, lithium, calcium, magnesium, and manganese, were added at 0·1 M concentration.

The glucosidase in Mycoplasma exhibits relative specificity in contrast to absolute specificity since more than one substrate can be attacked, i.e. *p*-nitrophenyl-*β*-D-glucoside, salicin, amygdalin, and 6-bromo-2-naphthyl-*β*-D-glucoside. The enzyme exhibits high bond specificity since only *β*-glucosides are attacked. High bond specificity also involves the nature of the bonded group such as sugar, alkyl, or aryl aglycons. The only substrates attacked by the enzyme were those with aryl aglycons. Substrates with *α* configuration, i.e. *α*-maltose, *α*-methyl-D-glucoside, 6-bromo-2-naphthyl-*α*-D-glucoside and *α*-methyl-D-mannopyranoside were not attacked by the enzyme which indicated that no *α*-glucosidase was present. Cellobiose, which is of *β* configuration, was not hydrolysed by any of the strains. Such results have been obtained with other organisms. Hash & King (1958) demonstrated *β*-glucosidase in *Myrothecium verrucaria* which hydrolysed aryl *β*-glucosides but not cellobiose. Jermyn (1955) also demonstrated a *β*-glucosidase in *Stachybotrys atra* with similar characteristics. The inactivity of the saprophytic strains toward *β*-methyl-D-glucoside can be compared to the normally slow cleavage of short chained alkyl aglycons by other organisms (Duerksen & Halvorson, 1958). Inability to hydrolyse *β*-methyl-D-xyloside and *β*-methyl-D-arabinopyranoside points to the absolute specificity of the enzyme for carbon atoms 4 and 5. This contrasts with the *β*-glucosidase from almonds which does not exhibit such absolute specificity since *β*-xylosides and *β*-arabinosides are hydrolysed (Veibel, 1950). The *β*-glucosidase from *Saccharomyces cerevisiae* shows absolute specificity for carbon atom number 4 but does not for carbon atom number 5 indicating that *β*-glucosides and *β*-xylosides can be hydrolysed but *β*-galactosides and *β*-arabinosides cannot (Duerksen & Halvorson, 1958). The *β*-glucosidase from *Stachybotrys atra* requires an hydroxyl group at carbon atom number 4 but configuration is not important indicating that *β*-glucosides or *β*-galactosides can be hydrolysed (Jermyn, 1955).

Although all strains of Mycoplasma capable of glucose degradation exhibited activity toward *p*-nitrophenyl-*β*-D-glucoside, no activity was demonstrated toward *o*-nitrophenyl-*β*-D-galactoside even though the aglycon, *o*-nitrophenol, has been shown to promote more activity than *p*-nitrophenol when substituted to glucose (Pigman, 1944). This indicates that the inactivity is due to some characteristic of the glycon, presumably the configuration of the hydroxyl group at carbon atom number 4.

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Lactose Utilization and Hydrolysis in *Saccharomyces fragilis*

BY R. DAVIES

Medical Research Council, Department of Biochemistry, University of Cambridge

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SUMMARY

Sodium azide, 2,4-dinitrophenol and iodoacetate did not inhibit hydrolysis of lactose by cell-free preparations of *Saccharomyces fragilis* β-galactosidase, but with intact organisms fermentation and hydrolysis were inhibited to a similar extent. This suggests that these inhibitors may interfere with the transport of lactose into the cell. Galactose fermentation was inhibited by sodium azide and dinitrophenol to a much greater extent than was glucose or lactose fermentation. Hydrolysis of lactose and *o*-nitrophenyl-β-D-galactoside required potassium ion at an optimal concentration of 0·1 M; ammonium ion also activated, but sodium ion was much less effective and, in presence of optimal concentrations of potassium ion, were inhibitory, as were calcium and zinc ions. Lithium, magnesium and manganese ions were without effect in presence of potassium optimal ion. The β-galactosidase activity of aqueous suspensions of acetone-dried organisms, or of cell-free extracts, decayed rapidly at 25°. This decay was prevented by addition of sodium phosphate or various potassium salts, but not by sodium or potassium chloride or by potassium sulphate. Sodium azide, arsenate or 2,4-dinitrophenol decreased the rate of decay and promoted partial recovery when decayed preparations were subsequently incubated with potassium phosphate. The enzyme was most stable in 0·1 M-potassium phosphate (pH 7·0). A purified (237-fold) preparation of the β-galactosidase was obtained; this too decayed rapidly in water or potassium chloride solution but not in potassium phosphate solution.

INTRODUCTION

Monod & Cohn (1952) compared the properties of β-galactosidases from various species of micro-organisms and found that the enzymes from several strains of *Escherichia coli*, *Aerobacter aerogenes* and *Shigella sonnei* were indistinguishable by substrate or inhibitor specificity or by immunological behaviour, whereas they were clearly differentiated from the β-galactosidases of *Saccharomyces fragilis* and of some Lactobacillus strains. More recently, differences have been reported between the enzymes from strains of *E. coli* in respect to cation activation patterns (Kuby & Lardy, 1953; Wallenfels, Malhotra & Dabich, 1960), turn-over number, absorption spectra (Wallenfels, Zarnitz, Laule, Bender & Keser, 1959) and enzyme stability (Rickenberg, 1959). Little work has been reported on the β-galactosidase of *Saccharomyces fragilis* since the studies by Caputto, Leloir & Trucco (1948). The present paper describes some of the properties of lactose hydrolysis by β-galactosidase preparations from *S. fragilis* and by the intact organisms.

METHODS

Organisms, growth media and suspensions of organisms. The β-galactosidase-producing organism used throughout was a strain of *Saccharomyces fragilis* Jorgenson obtained from the Centraalbureau voor Schimmelcultures, Delft. *Saccharomyces mandshuricus* (Saito, 1916) was obtained from the Carlsberg Laboratory, Copenhagen. The basal yeast-extract medium was that of Davies, Faulkiner, Wilkinson & Peel (1951). For some experiments a basal defined medium was used containing (per litre): $NH_4H_2PO_4$, 1 g.; $(NH_4)_2HPO_4$, 1 g.; KH_2PO_4, 2 g.; 10 ml. trace element solution (Davies *et al.* 1951); biotin, 1 μg.; 200 μg. each of calcium pantothenate, pyridoxin, nicotinic acid and thiamin hydrochloride; 1 mg. *meso*inositol. After sterilization an appropriate source of carbon was added as indicated in the text. The medium was dispensed in Roux Bottles (150 ml./bottle) and inoculated with 0·5 ml. of a 24 hr. culture in basal yeast-extract medium + 2 % (w/v) glucose. For inoculating cultures in defined medium the organisms were harvested, washed twice with sterile water and resuspended in sterile water to approximately the original volume. All cultures were incubated at 25°.

For enzymic tests *Saccharomyces fragilis* cultures were harvested by centrifugation, washed twice with water and suspended in water to a suitable concentration. Optical extinction of suspensions were measured with a Hilger Spekker absorptiometer and converted to mg. dry matter/ml. by means of a calibration curve. All experiments were done at 25°. Where concentrations of solutes are given as % this means % (w/v) in all cases.

Saccharomyces mandshuricus was grown at 25° for 40–48 hr in basal yeast-extract medium + 2 % galactose, the organism harvested, washed twice in water and suspended in 0·2 M-potassium phosphate + citric acid buffer (pH 6·0) containing 8 mM-NaN_3, to give a final concentration of organism equivalent to about 100 mg. dry matter/ml. For experiments in which buffers other than potassium phosphate + citric acid were used, the organism was suspended in 8 mM-NaN_3. The suspension was shaken aerobically for 2 hr at 25° to decrease endogenous CO_2 production to a negligible value. In presence of sodium azide oxidation and assimilation of sugar is inhibited and, under aerobic conditions, glucose and galactose are fermented almost quantitatively (95 %) to ethanol and CO_2.

Cell preparations. Preparations of disrupted *Saccharomyces fragilis* were obtained by shaking with glass beads (ballotini no. 12) in a Mickle shaker (Mickle, 1948) for 15 min. at 4° and filtering through a sintered glass filter (porosity no. 1). Cell-free extracts were obtained by centrifuging the broken organisms at 1000*g* for 15 min. Acetone-dried organisms were prepared by the method of Epps (1944).

Estimation of β-galactosidase activity. This was measured either (*a*) by the increase in reducing sugar, or (*b*) manometrically from the rate of CO_2 evolution when the β-galactosidase preparation was incubated aerobically with 0·075 M-lactose in presence of 2 mM-NaN_3 and galactose-adapted *Saccharomyces mandshuricus*. The latter estimation was done in Warburg manometers containing (in a total volume of 4·0 ml.): 1·0 ml. *S. mandshuricus* suspension; 0·1 M buffer (usually potassium phosphate + citric acid pH 6·0); 0·075 M-lactose; in the side arm, the β-galactosidase preparation. Other additions are described in the text. The gas phase was air except where NaN_3 was omitted, in which case it was 95 % (v/v) N_2 + 5 % (v/v) CO_2. In

presence of 2 mM-NaN_3 the rate of CO_2 evolution was linear up to 15 μl./min. and the yield of CO_2 was 95 % of theoretical for conversion of 1 mole hexose to 2 moles ethanol + 2 moles CO_2. Unless stated otherwise, the rates of CO_2 evolution have been divided by 89·6 and expressed as μmoles lactose hydrolysed/mg. dry matter/hr. One unit of β-galactosidase activity is defined as the amount of enzyme which hydrolyses 1 μmole lactose in 1 hr at 25° in 0·1 M-potassium phosphate + citric acid buffer (pH 6·0).

Hydrolysis of *o*-nitrophenyl-β-D-galactoside (ONPG) was determined by the method of Lederberg (1950) by using 5 mM-ONPG in 0·067 M-potassium phosphate + citric acid buffer (pH 6·5), unless stated otherwise. Activities are expressed as μmoles ONPG hydrolysed/mg. dry matter/hr.

RESULTS

Inducible fermentation of lactose and galactose

The ability of *Saccharomyces fragilis* to ferment lactose and galactose can be induced by growth on basal medium + lactose or galactose. When galactose was the substrate the resulting organisms fermented both lactose ($Q_{CO_2}^{N_2}$ = 240) and galactose ($Q_{CO_2}^{N_2}$ = 233) considerably faster than glucose ($Q_{CO_2}^{N_2}$ = 154). When lactose was the substrate lactose was fermented more rapidly ($Q_{CO_2}^{N_2}$ = 233) than galactose ($Q_{CO_2}^{N_2}$ = 173) or glucose ($Q_{CO_2}^{N_2}$ = 182). After growth in basal medium alone the organism fermented lactose and galactose only very slowly ($Q_{CO_2}^{N_2}$ = 4–8), nevertheless the rates were usually slightly higher than for organisms grown in basal medium + 2 % glucose ($Q_{CO_2}^{N_2}$ = 1–12). A possible cause of the low rate of fermentation of lactose would be a deficiency of β-galactosidase. However, *S. fragilis* grown on basal medium alone, or with the addition of 2 % glucose, possessed considerable β-galactosidase activity after acetone drying, in one instance ninety times the amount required for the observed rate of lactose fermentation and almost twice the amount required for the maximum fermentation rate observed with fully induced organisms (Table 1). *S. fragilis* grown in basal medium alone usually possessed a considerably higher latent β-galactosidase activity than organisms grown in basal medium + 2 % glucose, indicating repression of β-galactosidase synthesis by glucose. Davies (1956), who used *S. fragilis* grown in a chemostat under steady state conditions, observed that synthesis of β-galactosidase was strongly suppressed by glucose concentrations higher than 10^{-3} M.

With *Saccharomyces fragilis* grown in basal defined medium alone fermentation of glucose also was initially slow ($Q_{CO_2}^{N_2}$ = 30–60), suggesting a deficiency in the glycolytic system which would also result in a low rate of galactose and lactose fermentation. Anaerobic incubation of such organisms for 2–4 hr in 0·1 M-potassium phosphate + citric acid buffer (pH 6·0) containing 0·16 M-glucose restored the glucolytic activity to normal ($Q_{CO_2}^{N_2}$ = 181–213), but even after 6 hr in 3% galactose or lactose, fermentation of these sugars remained slow ($Q_{CO_2}^{N_2}$ less than 30). After 21 hr in presence of lactose the rate of fermentation of this sugar increased to $Q_{CO_2}^{N_2}$ = 146–254, but for organisms similarly incubated in galactose $Q_{CO_2}^{N_2}$ (galactose) was only 14–37. Addition of small amounts of glucose (3 mM) and/or vitamin-free casein hydrolysate (0·7 mg./ml.) had no significant effect on the induction of lactose or galactose fermentation. Organisms which had been induced to ferment lactose ($Q_{CO_2}^{N_2}$ = 173) by incubation with lactose for 21 hr were able to ferment galactose at a

much higher rate ($Q_{CO_2}^{N_2}$ = 89) than organisms incubated with galactose for 21 hr ($Q_{CO_2}^{N_2}$ = 9). A possible reason for this difference might be that synthesis of β-galactosidase and a transport system would be sufficient to enable the organisms to derive energy from lactose, whereas for galactose two or three enzymes would be required in addition to a transport system. Robichon-Szulmajster (1958) reported that galactokinase, galactose-1-phosphate-uridyl-transferase and uridyldiphosphate-galactose-4-epimerase are all inducible enzymes in *S. fragilis*, but Mills, Smith & Lockhead (1957) claimed that the epimerase was constitutive.

Table 1. *Lactose fermentation and β-galactosidase activity in Saccharomyces fragilis*

Fermentation rates were measured manometrically at pH 6·0 in an atmosphere of 95% (v/v) nitrogen + 5% (v/v) CO_2. β-Galactosidase activities were measured by the manometric method using acetone-dried organisms or, in one experiment, a crude Mickle extract. To permit direct comparison with β-galactosidase activities, fermentation rates are expressed as μmoles sugar fermented/mg. dry wt. organisms/hr, the values being calculated from the rates of CO_2 evolution by dividing by 44·8 (for glucose) or 89·6 (for lactose).

Growth medium	Period of growth (hr)	Fermentation rate (μmoles/mg./hr) Glucose	Lactose	β-Galactosidase activity (μmoles/mg./hr)
Basal yeast extract (0·02%) only	44	0·94	0·06	2·46
Basal yeast extract (0·02%) only	68	1·50	0·09	8·05
Basal yeast extract (0·02%) + 2% glucose	38	4·12	0·13	1·52 2·10*
Basal yeast extract (0·02%) + 2% glucose	43	5·20	0·05	2·42
Basal yeast extract (0·2%) + 2% lactose	25	5·20	5·42	17·43

* Mickle extract.

Table 2. *Effect of growth in tryptic digest of casein on induction of ability of Saccharomyces fragilis to ferment lactose*

S. fragilis was grown for 21 hr at 25° in basal yeast extract (0·02%) medium supplemented with tryptic digest of casein as indicated. Organisms were harvested, washed and incubated under nitrogen in Warburg manometers with 0·075 M-lactose or glucose in 0·1 M-McIlvaine buffer (pH 4·5) at 25° and the rate of CO_2 production measured.

Incubation period (min.)		Casein added to growth medium as tryptic digest (%) 0	0·02	0·1	0·5	2·0
5	$Q_{CO_2}^{N_2}$ (*glucose*)	63	62	64	66	85
5	$Q_{CO_2}^{N_2}$ (*lactose*)	2	3	16	30	49
240	$Q_{CO_2}^{N_2}$ (*glucose*)	213	208	234	212	260
240	$Q_{CO_2}^{N_2}$ (*lactose*)	7	21	97	115	250

Addition of tryptic digest of casein to a modified basal yeast extract medium (0·02% yeast extract) markedly increased the initial rate of lactose fermentation, and the increase in rate when washed uninduced organisms were subsequently incubated with lactose. The effect increased with increasing concentration of casein digest (Table 2). For all concentrations of casein digest the variation of $Q_{CO_2}^{N_2}$

(lactose) with time had the form shown in Fig. 1 and the value of $Q^{N_2}_{CO_2}$ at the point of inflexion was a function of the casein digest concentration (Fig. 2). The effect was probably due to lactose present in the casein digest since a similar response was obtained by addition of lactose (10^{-4} to 10^{-1} %) to the basal defined growth medium, whereas an acid hydrolysate of vitamin-free casein was ineffective. The value of $Q^{N_2}_{CO_2}$ (lactose) at the point of inflexion probably gives a measure of the initial value of lactose fermenting capacity, the post-inflexion increase in $Q^{N_2}_{CO_2}$ resulting from new synthesis of rate-limiting components of the lactose fermenting system. The β-galactosidase activity of acetone-dried organisms also was increased by addition of small amounts of lactose to the basal defined growth medium, 10^{-4}M giving half maximum production (Fig. 3). Galactose was less effective than lactose at low concentrations but slightly more effective at high concentrations (28 mM).

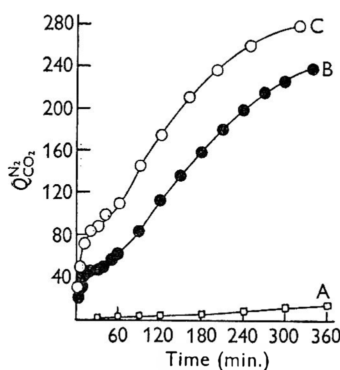

Fig. 1

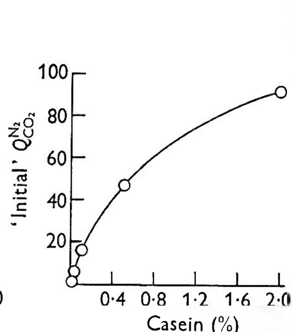

Fig. 2

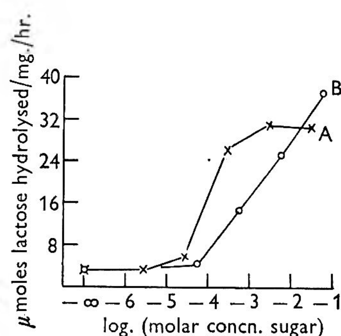

Fig. 3

Fig. 1. Fermentation of lactose by Saccharomyces fragilis. Organisms grown for 24 hr at 25° in basal yeast extract (0·02 %) medium + tryptic digest of casein as indicated. Manometers contained (in 3·5 ml.): 0·05M McIlvaine buffer (pH 4·5), 0·075M-lactose and equiv. 2 mg. dry wt. of S. fragilis. Gas phase N_2. Curve A, no casein; curve B, 0·5 % casein; curve C, 2·0 % casein.

Fig. 2. Effect of addition of casein digest to growth medium on initial rate of lactose fermentation by Saccharomyces fragilis. The organism was grown for 24 hr at 25° in basal yeast extract (0·02 %) medium + tryptic digest of casein as indicated. The initial rate is the rate at the points of inflexion in Fig. 1.

Fig. 3. Effect of growth in presence of lactose or galactose on β-galactosidase activity of acetone-dried Saccharomyces fragilis. Organisms were grown for 48 hr at 25° in basal defined medium + lactose or galactose, as indicated. β-Galactosidase activity was estimated manometrically. Curve A, lactose; curve B, galactose.

Effect of inhibitors on lactose fermentation

The β-galactosidase of *Saccharomyces fragilis*, like that of *Escherichia coli*, is strongly activated by potassium ion (Caputto, Leloir & Trucco, 1948) but intact fully induced organisms have only about one-tenth the β-galactosidase activity of organisms disrupted under suitable conditions (Davies, 1956), although the potassium content of the organisms (0·37 μg. atoms/mg. dry matter, measured by flame photometry (Davies, Folkes, Gale & Bigger, 1953) is about equivalent to the concentration (0·1 M) required for maximum β-galactosidase activity with acetone-dried organisms or cell-free extracts. Because of the potassium requirement of *S. fragilis*

Table 3. *Effect of various treatments and inhibitors on utilization of lactose by Saccharomyces fragilis*

Saccharomyces fragilis was grown for 23 hr at 25° in basal yeast extract medium + 2 % lactose. Fermentations were carried out at 25° under nitrogen in Warburg manometers containing (in 1·0 ml.): 0·1 M-sodium or potassium phosphate + citric acid buffer (pH 6·0), 0·1 M-lactose, equiv. 5·0 mg. dry wt. *S. fragilis* (or equivalent amount of disrupted or acetone-dried organisms) and inhibitors as indicated. Incubation was continued until about one-third of the lactose had been fermented in the uninhibited control system. Organisms and protein were then precipitated with zinc sulphate + barium hydroxide and the supernatant fluids analysed for glucose, galactose and lactose by fermentation with uninduced and galactose-induced *S. mandshuricus*, using acetone-dried *S. fragilis* to hydrolyse residual lactose.

Inhibitor or treatment	Concn.	Buffer: Cation	Buffer: Anions	(*a*) Lactose disappearing (μmole/mg./hr)	(*b*) Lactose fermented (μmole/mg./hr)	(*c*) ½ (Glucose + galactose) accumulating (μmole/mg./hr)	*b*/(*b*+*c*)
None		K^+	Phosphate + citrate	3·05	2·51	0·13	0·95
None		Na^+	Phosphate + citrate	2·80	2·60	0·25	0·91
Iodoacetate	10 mM	K^+	Phosphate + citrate	0·40	0·04	0·46	0·08
		Na^+	Phosphate + citrate	0·53	0·20	0·37	0·35
p-Chloromercuribenzoate	1 mM	K^+	Phosphate + citrate	0·19	0·05	0·04	0·55
		Na^+	Phosphate + citrate	0·15	0	0·1	0
2,4-Dinitrophenol	3 mM	K^+	Phosphate + citrate	0·74	0·66	0·09	0·88
		Na^+	Phosphate + citrate	1·18	1·19	0	1·00
NaN_3	3 mM	K^+	Phosphate + citrate	2·18	1·79	0·08	0·96
		Na^+	Phosphate + citrate	1·36	1·12	0·41	0·73
Toluene	—	K^+	Phosphate + citrate	1·98	0·11	1·85	0·06
		Na^+	Phosphate + citrate	0·24	0	0·20	0
Aerosol OT	2 mg./mg. yeast	K^+	Phosphate + citrate	2·71	0·37	2·10	0·15
		Na^+	Phosphate + citrate	0·16	0·04	0·40	0·09
CTAB	250 μg./mg. yeast	K^+	Phosphate + citrate	4·76	0	5·01	0
		Na^+	Phosphate + citrate	0	0	0	—
Polymyxin	250 μg./mg. yeast	K^+	Phosphate + citrate	5·33	0·29	4·58	0·06
		Na^+	Phosphate + citrate	0·93	0·63	0·11	0·85
Acetone	—	K^+	Phosphate	18·2	0	17·9	0
		Na^+	Phosphate	0·53	0	0·70	0
Mickle extract	—	K^+	Phosphate + methylglyoxaline	23·8	0	24·1	0
		Na^+	Phosphate + methylglyoxaline	0·59	0	0·50	0

β-galactosidase the effect of various treatments and inhibitors on lactose fermentation and hydrolysis by intact organisms was examined in buffer systems in which the cation was either all potassium or all sodium. With intact fully induced uninhibited organisms most of the lactose which disappeared could be accounted for by the CO_2 liberated (assuming a normal alcoholic fermentation) and there was little accumulation of glucose or galactose in the incubation mixtures with either sodium or potassium as the cation (Table 3). Of the inhibitors tested those (iodoacetate, *p*-chloromercuribenzoate, 2,4-dinitrophenol, sodium azide) which, so far as is known

Table 4. *Effect of inhibitors on production of CO_2 from glucose, galactose and lactose by Saccharomyces fragilis*

Saccharomyces fragilis was grown for 23 hr at 25° in basal yeast extract medium + 2 % lactose. Fermentation rates measured manometrically at pH 6·0 with a gas phase of 95 % (v/v) nitrogen + 5 % (v/v) CO_2.

	$Q_{CO_2}^{N_2}$					
	Glucose		Galactose		Lactose	
Inhibitor	K	Na	K	Na	K	Na
None	200	181	159	137	204	155
NaN_3, 0·3 mM	255	215	39	26	167	120
NaN_3, 3 mM	208	167	10	9	87	46
None	319	297	233	210	356	289
2,4-Dinitrophenol, 0·3 mM	360	259	91	94	327	145
2,4-Dinitrophenol, 3 mM	83	67	63	53	72	19
None	216	174	207	183	330	264
Iodoacetate, 1·0 mM	168	126	159	114	330	180
Iodoacetate, 10 mM	15	0	9	0	51	54
None	255	—	252	—	263	—
p-Chloromercuribenzoate, 1·0 mM	120	—	36	—	4	—

are without disruptive effects on the integrity of the cell structures, inhibited CO_2 production and lactose disappearance to about the same extent, irrespective of whether the buffer cation was sodium or potassium; and again there was little accumulation of glucose or galactose. With the exception of *p*-chloromercuribenzoate (100 % inhibition of lactose hydrolysis at 0·63 mM), these substances at the concentrations used did not inhibit β-galactosidase activity of acetone-dried organisms or cell-free extracts. Sodium azide and 2,4-dinitrophenol at 0·3 mM, but none of the other substances tested, inhibited fermentation of galactose to a much greater extent than fermentation of glucose or lactose, irrespective of whether the buffer cation was sodium or potassium (Table 4). Fermentation of both galactose and lactose was more sensitive to inhibition by sodium azide (3 mM) than was fermentation of glucose, despite the fact that cell-free β-galactosidase activity was not inhibited. Inhibition of lactose and galactose fermentation by 2 mM-sodium azide was greater for organisms grown in basal yeast extract medium + 2 % lactose (lactose 68 %; galactose 74 %) than for organisms grown with 2% galactose (lactose 10 %; galactose 57 %).

Treatments or substances which damage cell membrane structures (toluene, Aerosol OT, cetyltrimethylammonium bromide, polymyxin, acetone, disruption in Mickle disintegrator) inhibited CO_2 production from lactose but, provided potassium

ions were present, permitted hydrolysis at a rate which could be many times greater than that required to accommodate the rate of CO_2 production from lactose by intact, fully induced organisms (Table 3).

Effect of pH value on lactose fermentation and β-galactosidase activity

The effect of pH value on the rate of fermentation of lactose by intact *Saccharomyces fragilis* and on the rate of hydrolysis by acetone-dried organisms is shown in Figs. 4*a*, *b*. The fermentation rate at any pH value was similar in sodium or potassium phosphate buffers and was a maximum at pH 5·0–5·5. As with sucrose fermentation (Davies, 1953) there was a pronounced difference between the rates observed

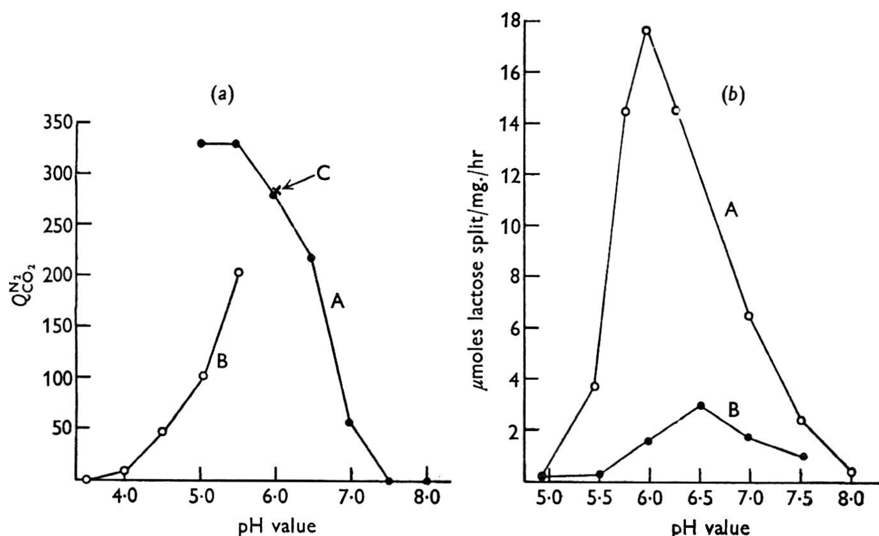

Fig. 4*a*. Effect of pH value on fermentation of lactose by Saccharomyces fragilis. Curve A, potassium phosphate; curve B, sodium acetate; point C, sodium phosphate.

Fig. 4*b*. Effect of pH value on hydrolysis of lactose by acetone-dried Saccharomyces fragilis. Curve A, potassium buffer; curve B, sodium buffer.

Figs. 4*a*, *b*. Organisms were grown for 48 hr at 25° in basal yeast extract medium + 2 % lactose. Fermentation rates were measured in a system containing: 0·075 M-lactose, 0·05 M-phosphate as potassium or sodium phosphate of required pH value, + sufficient $KHCO_3$ or $NaHCO_3$ to give the same pH value in an atmosphere of 95 % (v/v) N_2 + 5 % (v/v) CO_2 and sufficient KCl or NaCl to give a final potassium or sodium ion concentration of 0·1 M. β-Galactosidase activities determined by using similar conditions + galactose-induced S. mandshuricus organisms without sodium azide.

with phosphate and acetate buffers at pH 5·0 and 5·5. In potassium phosphate buffers the rate of lactose hydrolysis by acetone-dried *S. fragilis*, and also by the purified enzyme preparation, showed a sharp maximum at pH 6·0–6·5, and in sodium phosphate buffers a broader maximum at pH 6·5. The rate of hydrolysis of ONPG by acetone-dried organisms showed a sharp maximum at pH 7·0 both in 0·1 M-sodium phosphate and 0·1 M-potassium phosphate. With either substrate and alkali metal ions the enzyme was almost inactive at pH 5·0. Caputto *et al.* (1948) reported maximum lactase activity at pH 6·7–6·8 for a different strain of *S. fragilis*.

Stimulation of β-galactosidase by cations

In these experiments hydrolysis of lactose was not estimated by the continuous *Saccharomyces mandshuricus* technique because of the possibility that thick suspensions of *S. mandshuricus* might remove some of the cations. Consequently hydrolysis was allowed to proceed for 30 min. in the absence of *S. mandshuricus* and the reaction then stopped by addition of zinc sulphate and barium hydroxide (Somogyi, 1945). The precipitate was centrifuged down and the glucose+galactose in the supernatant fluid estimated by fermentation with galactose-induced *S. mandshuricus* organisms in potassium phosphate+citric acid buffer (pH 4·5) containing 2 mM-sodium azide. It was established that, at the concentrations used, none of the

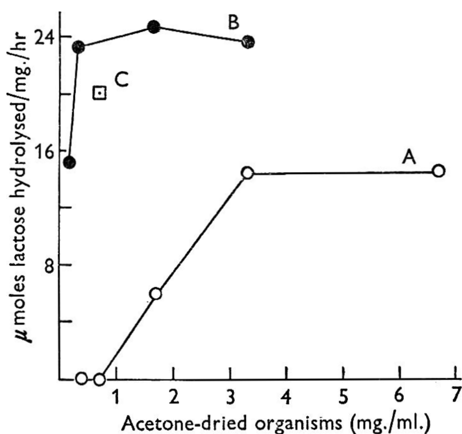

Fig. 5

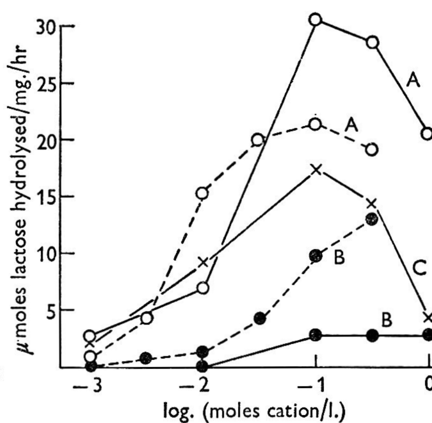

Fig. 6

Fig. 5. Effect of concentration of acetone-dried Saccharomyces fragilis on β-galactosidase activity in absence of added potassium ion. The reactions were done in test-tubes containing 0·097 M-lactose and acetone-dried organisms as indicated, with and without 0·1 M-potassium (as acetate, pH 6·0). After incubation for 30 min. at 25° the free glucose+galactose was estimated by using galactose-induced S. mandshuricus. Curve A, no potassium; curve B, +potassium; point C, +boiled cell suspension at equiv. 6·0 mg./ml.

Fig. 6. Effect of concentration of K^+, Na^+ and NH_4^+ on β-galactosidase activity of acetone-dried Saccharomyces fragilis. The reactions were done in test-tubes containing in 3·0 ml.: 0·097 M-lactose, 2 mg. acetone-dried organisms, and potassium, sodium or ammonium acetate (pH 6·0) at cation concentrations indicated. After 30 min. at 25°, protein was precipitated with $ZnSO_4 + Ba(OH)_2$, and the glucose + galactose in the supernatant fluid estimated with galactose-induced S. mandshuricus. Full lines, lactose; broken lines, ONPG. Curves A, potassium; curves B, sodium; curve C, ammonium.

cations inhibited fermentation of glucose or galactose by *S. mandshuricus*. Suspensions of acetone-dried *S. fragilis* containing more than 1 mg. dry matter/ml. of incubation system had appreciable β-galactosidase activity in the absence of added potassium ion and at 3 mg. dry matter/ml. the enzyme activity (1·45 units/mg. dry matter) was comparable with that observed in presence of 0·1 M-potassium acetate (2·46 units/mg.; Fig. 5). A boiled suspension of acetone-dried organisms at 6 mg./ml. was almost as good an activator as 0·1 M-potassium acetate. These observations can be explained by the fact that the potassium content of acetone-dried organisms was 0·37 μg.atoms/mg. dry matter. The experiments on activation and inhibition of

β-galactosidase by cations were done with a concentration of acetone-dried organisms (0·67 mg./ml.) which was inactive in the absence of added cations.

The β-galactosidase of acetone-dried *Saccharomyces fragilis* was activated by potassium ion and, contrary to the observations of Caputto *et al.* (1948), to a less extent by ammonium ion. Sodium ion activated slightly, but lithium, calcium, magnesium, manganese and zinc (all as acetates, pH 6·0) at concentrations ranging from 10^{-3} to 10^{-1}M did not activate. The lack of activation by magnesium and manganese is at variance with the findings of Caputto *et al.* (1948). β-Galactosidase activity in sodium and potassium phosphate buffers was measured in three ways: (*a*) by continuous fermentation with *S. mandshuricus*; (*b*) by change in optical rotation; (*c*) by increase in reducing sugars. The values obtained were substantially the same (2·7, 1·6 and 0·3 units/mg. dry matter, respectively in 0·1M-sodium phosphate buffer (pH 6·0); 16·9, 16·1 and 18·9 units/mg. in 0·1 M-potassium phosphate (pH 6·0).

The effect of cation concentration on β-galactosidase activation by potassium, ammonium and sodium is shown in Fig. 6. With potassium and ammonium ions maximum activation was achieved at 0·1 M; higher concentrations were less effective. Hydrolysis of ONPG by suspensions of acetone-dried lactose-induced *Saccharomyces fragilis* was stimulated by potassium ion and sodium ion (Fig. 6), the concentrations giving half maximum velocity being about 10^{-1}M for sodium and 10^{-2}M for potassium, although the maximum velocities determined by the method of Lineweaver & Burk (1934) were about the same. Hydrolysis of ONPG was stimulated slightly (50%) by 10^{-2}M- and 10^{-3}M-manganous chloride but not by magnesium chloride at 10^{-2}M or 10^{-1}M.

Table 5. *Inhibition of potassium-activated Saccharomyces fragilis β-galactosidase by other cations*

The reactions were done in test-tubes containing (in 3·0 ml.): 0·097 M-lactose, 0·1 M-potassium as acetate (pH 6·0, except for control without cations), 2 mg. acetone-dried *S. fragilis*, and other cations (as acetates, pH 6·0) as indicated. After 30 min. at 25°, protein was precipitated with $ZnSO_4 + Ba(OH)_2$, and the glucose + galactose in the supernatant fluid estimated with galactose-induced *S. mandshuricus*.

	Cations	β-Galactosidase activity (μmoles lactose hydrolysed/ mg./hr)	Inhibition of K^+ activated enzyme (%)
None	—	1·1	—
K^+ (10^{-1}M)	—	29·0	0
	+ Na^+ (10^{-2}M)	27·0	7
	+ Na^+ (10^{-1}M)	15·6	46
	+ Li^+ (10^{-2}M)	28·0	4
	+ Mg^{++} (10^{-2}M)	29·2	0
	+ Ca^{++} (10^{-2}M)	14·0	42
	+ Zn^{++} (10^{-3}M)	4·8	83
	+ Zn^{++} (10^{-2}M)	0·6	98
	+ Mn^{++} (10^{-2}M)	28·2	3

Inhibition of potassium-activated β-galactosidase by other cations

The effect of various cations on the β-galactosidase activity of acetone-dried *Saccharomyces fragilis* in presence of 0·1 M-potassium ion is shown in Table 5. Zinc ion was strongly inhibitory, as observed by Caputto *et al.* (1948), and sodium and

calcium ions less so. Inhibition by zinc and calcium ions was reported for the β-galactosidase from *Escherichia coli* ML308 (Rickenberg, 1959) and from *E. coli* ML (Cohn & Monod, 1951), but Kuby & Lardy (1953) found calcium ion to be inert with the enzyme from *E. coli* K 12. The degree of inhibition shown by sodium with the enzyme from *S. fragilis* (46% at 0·1 M-sodium) was very much less than was observed for lactose hydrolysis by the enzyme from *E. coli* ML (Cohn & Monod, 1951).

Stability of Saccharomyces fragilis β-galactosidase

The β-galactosidase activity of dry preparations of acetone-dried *Saccharomyces fragilis* decreases only very slowly at room temperature, 50% still remaining after 2 years, but in cell-free extracts or aqueous suspensions of acetone-dried organisms

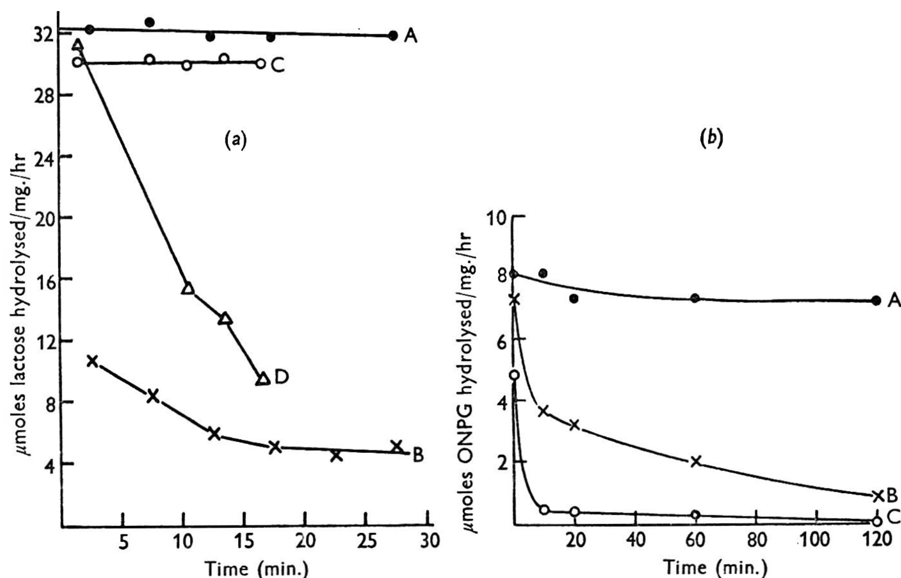

Fig. 7*a*. Decay of β-galactosidase activity of acetone-dried Saccharomyces fragilis and in cell-free extracts at 25°. Enzyme preparations were incubated in various salt solutions for times shown and β-galactosidase activity then estimated by continuous manometric method in a system containing (in 3·0 ml.): 0·1 M-potassium (as acetate or phosphate, pH 6·0, or as chloride), 0·067 M 4(5)-methylglyoxaline-HCl (pH 6·0), 0·075 M-lactose, 2 mM-NaN_3, acetone-dried galactose-induced S. fragilis, or cell-free extract equivalent to 0·5 mg. dry wt. organisms, and 100 mg. dry wt. galactose-induced S. mandshuricus. Curve A, acetone-dried organisms + potassium acetate; curve B, acetone-dried organisms + KCl; curve C, Mickle extract + potassium phosphate; curve D, Mickle extract + KCl.

Fig. 7*b*. Decay of β-galactosidase activity of acetone-dried Saccharomyces fragilis with *o*-nitrophenyl-β-D-galactoside (ONPG) as substrate. Acetone-dried galactose-induced organisms were suspended at 2 mg. dry wt./ml. in: (*a*) 0·1 M (with respect to phosphate) potassium phosphate pH 6·5; (*b*) 0·1 M-KCl; (*c*) water; the suspensions were then incubated at 25°. At the times shown samples (0·5 ml.) were withdrawn for estimation of β-galactosidase by the method of Lederberg (1950). Curve A, potassium phosphate; curve B, KCl; curve C, water.

the enzyme is unstable. Suspensions of acetone-dried organisms in water sometimes lost 80% of their activity in 5 min. at 25° and, frequently, such suspensions were inactive when tested in 0·1 M-KCl or in 0·067 M-4(5)-methylglyoxaline (pH 6·0) with

0·1 M-KCl as sole source of potassium ion. This instability is in contrast to the relative stability of the β-galactosidases from *Bacillus megaterium* KM (Landman, 1957), *Escherichia coli* K12 (Lederberg, 1950) and *E. coli* ML (Cohn & Monod, 1951), although Rickenberg (1959) reported that the β-galactosidases from *E. coli* strains K12 and ML308 lost activity when diluted with water to protein concentrations less than 100 μg./ml., a loss which was prevented by 1–0·2 mM-$MnCl_2$. Landman (1957) observed that the loss of activity which occurred when the *B. megaterium* enzyme was dialysed against ethylenediaminetetra-acetate (EDTA) and triple-distilled water could largely be prevented by the addition of 0·1 M-methionine to the dialysis system.

Decay of *Saccharomyces fragilis* β-galactosidase activity was observed with suspensions of acetone-dried organisms, cell-free extracts and also with the purified enzyme preparation, irrespective of the method used to estimate activity, and it

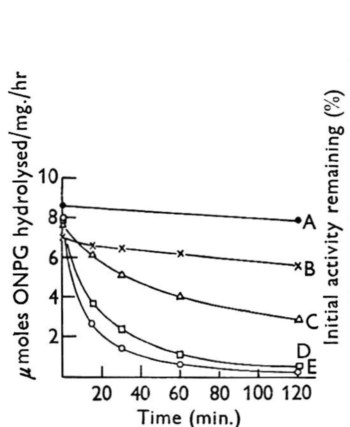

Fig. 8

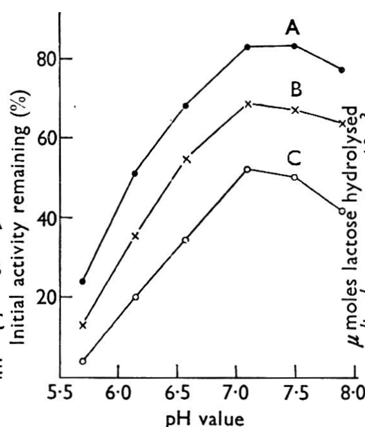

Fig. 9

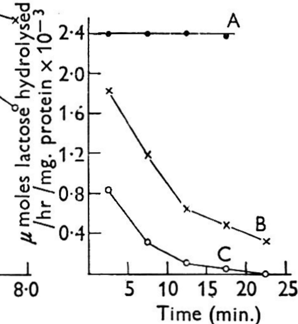

Fig. 10

Fig. 8. Effect of potassium phosphate concentration on decay of β-galactosidase activity. Acetone-dried galactose-induced Saccharomyces fragilis was suspended at 2 mg. dry wt./ml. in potassium phosphate (pH 6·5; 0°) of the concentrations (with respect to phosphate) indicated, and incubated at 25°. Samples (1·0 ml.) were withdrawn at the times shown for estimation of β-galactosidase activity, with ONPG as substrate. Potassium phosphate concentrations: curve A, 0·2 M; curve B, 0·1 M; curve C, 0·033 M; curve D, 0·01 M; curve E, 0·005 M.

Fig. 9. Effect of pH value on decay of β-galactosidase activity at 25°. Acetone-dried galactose-induced Saccharomyces fragilis was suspended at 0° and 2 mg. dry wt./ml. in 33 mM (with respect to phosphate) potassium phosphate of the pH values indicated, and incubated at 25°. At the times shown samples (0·5 ml.) were withdrawn for estimation of β-galactosidase activity, with ONPG as substrate. Curve A, 15 min.; curve B, 30 min.; curve C, 60 min.

Fig. 10. Decay of purified β-galactosidase. Activity was estimated by the continuous manometric method in a system containing (in 3·0 ml.): 0·1 M-potassium as KCl or phosphate (pH 6·0), 5·45 μg. dry wt. β-galactosidase preparation, 0·075 M-lactose, 100 mg. dry wt. galactose-induced S. mandshuricus, 2 mM-NaN_3 and, as indicated, 0·067 M-4(5)-methylglyoxaline-HCl (pH 6·0). Curve A, potassium phosphate only; curve B, potassium phosphate + methylglyoxaline; curve C, KCl + methylglyoxaline.

affected the ability to hydrolyse both lactose and ONPG (Fig. 7*a*, *b*). The extent of decay varied from one enzyme preparation to another and even between suspensions prepared on consecutive days from the same batch of acetone-dried organisms. It

was not obviously related to the conditions of growth, the age of the acetone-dried organisms, or the composition of the incubation system. The activity of crude cell-free extracts or suspensions of acetone-dried organisms did not decay in solutions (0·1 M with respect to anion; pH 6·0) of sodium phosphate, potassium phosphate, acetate, succinate, phthalate, maleate, or ammonium phosphate, but 0·1 M-potassium chloride, potassium sulphate or sodium chloride and also magnesium or manganous chloride at concentrations up to 10^{-2} M afforded no consistent protection, in contrast to the observations of Rickenberg (1959) with *Escherichia coli* β-galactosidase. Although activity of *S. fragilis* β-galactosidase was maintained in potassium succinate it was consistently only about 40 % of that observed with potassium phosphate.

The protective action of potassium phosphate was a function of its concentration (Fig. 8), of pH value (Fig. 9) and of temperature, decay being much slower at 0° (67 % residual activity after 2 hr at 0° in 5 mM-potassium phosphate pH 6·0; 3 % at 25° under similar conditions).

Table 6. *Effect of 2,4-dinitrophenol, sodium azide and sodium arsenate on β-galactosidase activity of acetone-dried Saccharomyces fragilis*

β-Galactosidase activity measured by the continuous manometric method, using double side-arm Warburg manometers containing *in the main compartment:* 1·0 ml. 0·2 M-4(5)-methylglyoxaline-HCl (pH 6·0), 0·5 ml. 20 % lactose, 0·9 ml. galactose-induced S. mandshuricus suspension (equiv. 100 mg. dry wt./ml.) and 0·3 ml. M-potassium as either phosphate (pH 6·0), chloride, or chloride containing 20 mM-2,4-dinitrophenol, sodium azide or sodium arsenate; *in side arm* 1: 0·5 mg. acetone-dried galactose-induced S. fragilis in 0·3 ml. 0·1 M-potassium, as either phosphate (pH 6·0), chloride, or chloride containing 2 mM-2,4-dinitrophenol, sodium azide or sodium arsenate; *in side arm* 2: 0·3 ml. M-KCl or M-potassium phosphate (pH 6·0). The gas phase was 95 % (v/v) nitrogen + 5 % (v/v) CO_2. Before tipping in the acetone-dried S. fragilis the manometers were shaken for 45 min. at 25° to eliminate endogenous CO_2 production. The contents of sidearm 2 was tipped 50 min. after that of side arm 1.

Inhibitor (2 mM):	None	None	2,4-Dinitrophenol	Sodium azide	Sodium arsenate
Potassium salt present at time zero:	Phosphate	Chloride	Chloride	Chloride	Chloride
Potassium salt added at 50 min.:	Chloride	Phosphate	Phosphate	Phosphate	Phosphate
	μmoles lactose hydrolysed/hr/mg. acetone-dried organisms				
Initial activity	23·5	1·3	16·7	12·4	14·8
Activity at 50 min.	16·5	0·7	8·5	4·8	3·5
Activity 5 min. after adding second salt	16·0	1·7	10·4	7·6	4·5
Activity 30 min. after adding second salt	16·0	11·6	14·5	13·7	10·7

The purified enzyme preparation lost 90 % of its activity in 3 weeks at 4° and was inactive after 4 weeks, even when stored in 0·1 M-potassium phosphate (pH 7·0). In presence of 0·067 M-4(5)-methylglyoxaline-HCl (pH 6·0) + 0·1 M-KCl all activity was lost in 20 min. at 25°; in methylglyoxaline + 0·1 M-potassium phosphate (pH 6·0) the activity decreased to 25 % after 20 min., but in potassium phosphate alone there was no loss of activity in 20 min. (Fig. 10). With suspensions of acetone-dried organisms

0·067 M-methylglyoxaline was only slightly inhibitory (10–20 %) when potassium was present as phosphate, acetate or chloride and the rate of decay was not affected.

With suspensions of acetone-dried organisms and with Mickle extracts, sodium azide, sodium arsenate and especially 2,4-dinitrophenol (all at 1·75 mM) decreased the rate of decay. The incubation of partially decayed preparations with 0·1 M-potassium phosphate (pH 6·0) resulted in considerable recovery of activity (Table 6). Rickenberg (1959) reported that 50 mM-phosphate protected *Escherichia coli* β-galactosidase against loss of activity when dilute enzyme solutions were dialysed. With one preparation of acetone-dried *Saccharomyces fragilis*, for which the decay was less pronounced than usual, addition of cysteine (10 mM) in presence of 0·1 M-potassium phosphate (pH 6·0) increased the loss of activity after 90 min. at 25° from 0 to 83 %. Addition of boiled lactose-induced *S. fragilis* at ten times the concentration of acetone-dried organisms neither prevented decay nor stimulated recovery of decayed preparations.

Table 7. *Purification of Saccharomyces fragilis β-galactosidase*

β-Galactosidase activities are expressed as μmoles lactose hydrolysed/hr/unit log (I_0/I) at 278 mμ. The final preparation had log I_0/I (278 mμ) = 1·17 and contained 175 μg. N/ml., corresponding to a β-galactosidase activity of 334 μmoles lactose hydrolysed/mg. N/min. at pH 6·0 and 25°.

Fraction	β-Galactosidase activity	Degree of purification	Recovery (%)
(1) Potassium phosphate extract of acetone-dried organisms	12·6	—	100
(2) Precipitate by 0·9 saturation with ammonium sulphate	75	6 times	92
(3) Adsorbed on $Ca_3(PO_4)_2$ gel, eluted and dialysed against 0·1 M-potassium phosphate (pH 6·0) at 4°	358	28 times	66
(4) Precipitate between 40–50 % acetone, adsorbed to and eluted from $Ca_3(PO_4)_2$ gel	975	77 times	41
(5) Precipitated with 55 % (v/v) acetone and dialysed against 0·1 M-potassium phosphate (pH 6·0) at 4°	3000	237 times	30

Purification of Saccharomyces fragilis β-galactosidase

A purified preparation of *Saccharomyces fragilis* β-galactosidase with an activity of 334 μmoles lactose hydrolysed/mg. N/min. was obtained by the following procedure (Table 7). A suspension of 19·1 g. acetone-dried organisms in 1 l. 0·1 M-potassium phosphate (pH 6·0) was incubated for 20 hr at 25° and centrifuged for 20 min. at 6000g. To the cell-free supernatant fluid was added cysteine (final concentration 10^{-2} M) and solid ammonium sulphate to 0·9 saturation. After standing for 3 hr at 4° the precipitate was centrifuged down (20 min. at 6000g), dissolved in 200 ml. 0·1 M-potassium phosphate (pH 6·0), 60 ml. calcium phosphate gel and 400 ml. water added and the mixture left for 15 min. at 4°. The gel was centrifuged down, suspended in 120 ml. 0·2 M-KH_2PO_4 and the mixture again centrifuged after 20 min. at 4°. The supernatant fluid was dialysed for 11 hr at 4° against 10 l. 0·1 M-potassium phosphate (pH 6·0) and then fractionated by addition of acetone at −10°. The

material which precipitated between 40 and 50% (v/v) acetone was dissolved in 55 ml. 0·1 M-potassium phosphate (pH 6·0), adsorbed on 20 ml. calcium phosphate gel and eluted with 36 ml. 0·2 M-KH_2PO_4. The β-galactosidase was finally precipitated by addition of acetone at $-10°$ to 55% (v/v), the precipitate dissolved in 0·1 M-potassium phosphate (pH 6·0) and dialysed overnight against 0·1 M-potassium phosphate (pH 6·0). The final preparation contained 30% of the initial β-galactosidase activity and had a specific activity of 334 μmoles lactose hydrolysed/mg. N/min. at 25° in 0·1 M-potassium phosphate (pH 6·0). The purification achieved from the crude extract was 237 times with respect to protein. The specific activity of the crystalline β-galactosidase from *Escherichia coli* ML309 is stated to be 127 μmoles ONPG hydrolysed/mg. protein/min. at 20° and that of the electrophoretically homogeneous enzyme from *E. coli* ML308 to be 146 μmoles ONPG hydrolysed/mg. protein/min. at 25° (Wallenfels & Malhotra, 1961). Assuming a Q_{10} of 2·0, these values are approximately equivalent, respectively, to 1200 and 913 μmoles ONPG hydrolysed/mg. N/min. at 25°.

Some properties of the purified β-galactosidase

The purified enzyme required potassium ion for activity, sodium ion being ineffective. Activity was rapidly lost in a system containing 0·1 M-KCl or 0·1 M-potassium phosphate (pH 6·0) + 0·067 M 4(5)-methylglyoxaline (pH 6·0), but not in 0·1 M-potassium phosphate alone (Fig. 10). The pH value for maximum activity was 6·2–6·4, measured by accumulation of glucose and galactose in a fixed time interval, or continuously by liberation of CO_2 in presence of *Saccharomyces mandshuricus*. The preparation did not react with rabbit antibodies prepared using purified *Escherichia coli* β-galactosidase as antigen.

DISCUSSION

It has been clearly established by the work of Monod, Cohn, Lederberg, Halvorson and others that β-galactosides are hydrolysed by *Escherichia coli* only after transport into the cell. Lactose utilization by *Saccharomyces fragilis* has not been studied so extensively, but the available data support the conclusion that a similar situation exists. The 10- to 20-fold increase in β-galactosidase activity observed when *S. fragilis* is disrupted in various ways (first reported by Caputto *et al.* 1948), coupled with the observations reported in the present paper and those of Sanches & Sols (1962) that only very small amounts of free glucose and galactose can be detected when lactose is metabolized under conditions which involve no obvious damage to the cell membranes, all suggest that transport into the *S. fragilis* cell may be the rate-limiting factor in lactose hydrolysis by this organism. Simultaneous inhibition of metabolism and hydrolysis of lactose by substances (sodium azide, 2,4-dinitrophenol, iodoacetate) which do not inhibit activity of the cell-free β-galaetosidase suggests that these substances interfere with transport of lactose into the cell. Sodium azide (3 mM) does not inhibit glucose fermentation, but galactose fermentation is strongly inhibited even by 0·3 mM-azide and is also much more sensitive to inhibition by 2,4-dinitrophenol than is fermentation of glucose or lactose. This also may be due to an inhibition of transport of lactose into the cell.

The properties of the β-galactosidases of *Escherichia coli* and *Saccharomyces fragilis* are, in general, similar. Wallenfels, Malhotra & Dabich (1960) found that the

rate of hydrolysis of *o*-nitrophenyl-β-D-galactoside (ONPG) by the enzyme from *E. coli* ML309 was maximal at pH 6·6 for both the sodium-activated and the potassium-activated enzyme, whereas hydrolysis of lactose showed two maxima (pH 6·5 and 8·1) with the potassium-activated enzyme but only one (at pH 7·0) with the sodium-activated enzyme. In the case of *S. fragilis* β-galactosidase ONPG hydrolysis was maximal at pH 7·0 with both sodium- and potassium-activated enzyme, whereas lactose hydrolysis was maximal at pH 6·5 with the sodium-activated enzyme but at pH 6·0–6·5 with potassium activation. There was no evidence of a second maximum at pH 8 for hydrolysis of either lactose or ONPG by the potassium-activated enzyme. Other workers have reported pH optima for *S. fragilis* β-galactosidase which vary from pH 6·6 (van Dam, Revallier-Warffemius & van Dam-Schermerhorn, 1950) to pH 7·8 (Sanches & Sols, 1962). These differences may be due to strain variations since the pH optimum for ONPG hydrolysis by β-galactosidases from different strains of *Escherichia coli* varies from pH 6·6 to 7·3 (Wallenfels & Malhotra, 1961).

The preferential activation of *Saccharomyces fragilis* β-galactosidase by potassium ion when lactose is the substrate is similar to the behaviour of the enzyme from *Escherichia coli* ML strains, but hydrolysis of ONPG by the *S. fragilis* enzyme is also preferentially activated by potassium ion, in contrast to the behaviour of the *E. coli* enzymes. In presence of the optimal concentration of sodium ion, manganese and magnesium have been reported to stimulate hydrolysis of ONPG by the β-galactosidases of *E. coli* ML308 (Reithel & Kim, 1960) and *E. coli* K12 (Kuby & Lardy, 1953). A similar situation exists for the *S. fragilis* enzyme, but it is possible that the effect is not an activation of the enzyme but rather a protection from decay (Szabó & Davies, 1964). Rickenberg (1959) reported that 0·2–1·0 mM-$MnCl_2$ prevented the loss of activity which occurred when aqueous solutions of the β-galactosidase from *E. coli* ML308 were diluted to less than 100 μg. protein/ml.

An absolute requirement for magnesium ion for the hydrolysis of ONPG, but not lactose, by *Escherichia coli* ML308 β-galactosidase was reported by Reithel & Kim (1960), based on the observation that ONPG hydrolysis was inhibited by 10^{-4}M-EDTA, the inhibition being annulled by magnesium ion. A similar inhibition of ONPG hydrolysis by 10^{-4}M-EDTA which is annulled by various metal cations, notably 10^{-4}M-manganese and 10^{-4}M-ferrous ions, has been observed with the *Saccharomyces fragilis* enzyme (Szabó & Davies, 1964). The annulment of inhibition might be due to removal of EDTA by chelation with the added cation, rather than a re-activation by some essential metal ion. As with the enzyme from *E. coli* ML308 (Reithel & Kim, 1960), hydrolysis of lactose by *S. fragilis* β-galactosidase was not significantly affected by magnesium or manganous ions, contrary to the findings of Caputto *et al.* (1948).

The reasons for the rapid loss of activity when the *Saccharomyces fragilis* β-galactosidase preparations, crude or purified, were incubated in KCl or NaCl solutions or in water, and for the protective effect of orthophosphate, arsenate, 2,4-dinitrophenol and sodium azide, are not clear. With crude preparations, degradation of β-galactosidase by proteolytic enzymes is a possibility, but this seems less likely for the purified enzyme preparation. Rapid decay of the purified enzyme preparation might be related to the presence of 4(5)-methylglyoxaline, since potassium phosphate protected in the absence but not in the presence of this compound. Decay

of the crude preparations, however, was not affected by the methylglyoxaline. Histidine, which has the same imidazole ring as 4(5)-methylglyoxaline, has been suggested as one group involved in β-galactosidase activity (Wallenfels & Malhotra, 1961).

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Studies on the β-Galactosidase Activity of *Saccharomyces fragilis*

BY G. SZABO* AND R. DAVIES

Medical Research Council, Department of Biochemistry, University of Cambridge

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SUMMARY

The decay of β-galactosidase activity which occurs when acetone-dried *Saccharomyces fragilis* organisms are suspended in water at 25° is prevented by various potassium salts, sodium sulphide, azide, fluoride and citrate, manganous chloride and sulphate, cobaltous chloride, oxine, diethyldithiocarbamate, dimethylglyoxime and 2,4-dinitrophenol. Ethylenediaminetetra-acetate (EDTA) has no effect on the decay but inhibits the enzyme, the inhibition being annulled by various metal cations, notably manganous and ferrous ions. Sodium pyrophosphate increased β-galactosidase activity of intact organisms. This effect is to some extent reversible and is prevented by glucose. It is not accompanied by uptake of sodium pyrophosphate into the organisms. The possible mode of action of pyrophosphate is discussed.

INTRODUCTION

β-Galactosidase was isolated from a strain of *Saccharomyces fragilis* by Caputto, Leloir & Trucco (1948), who examined some of its properties and found the enzyme to be optimally active at pH 6·8 and activated by potassium, manganous or magnesium ions. Davies (1964) reported that both crude and partially purified preparations of the enzyme were unstable in water but fairly stable in potassium phosphate solutions. Davies (1956) found a smaller β-galactosidase activity in intact organisms than in disrupted organisms, irrespective of the conditions under which they had been grown. The present paper describes investigations concerning the activity and decay of β-galactosidase preparations and factors affecting activity of this enzyme in intact organisms.

METHODS

Organism and media. The organism used throughout was a strain of *Saccharomyces fragilis* Jorgenson obtained from the Centraalbureau voor Schimmelcultures, Delft. The basal growth medium was that of Davies, Faulkiner, Wilkinson & Peel (1951), but without the trace elements solution. To this basal medium were added trace metals and an appropriate source of carbon as indicated in the text. The basal medium and glucose or galactose solutions were separately sterilized by autoclaving. Lactose was sterilized by Seitz filtration. For growth the medium was dispensed into Roux bottles (150 ml./bottle), inoculated with 0·5 ml. of

* Present address: D.O.T.E., Biological Institute, Debrecen 12, Hungary.

a 24 hr culture grown in basal medium + 2% (w/v) glucose and incubated at 30°. For enzymic tests the organism was harvested by centrifugation, washed with glass-distilled water and resuspended as stated in the text. Log I_0/I values of suspensions were measured with a Hilger 'Spekker' absorptiometer and converted to mg. dry matter/ml. by means of a calibration curve. All experiments were done at 25° and glass-distilled water was used throughout.

Preparation of broken organisms and of suspensions of acetone-dried organisms. Broken organisms were prepared by shaking with glass beads (Ballotini no. 12) in the Mickle disintegrator (Mickle, 1948). Acetone-dried organisms were prepared by the method of Epps (1944) and suspended at a suitable concentration (usually about 1·0 mg./ml.) in glass-distilled water at 0° and maintained at 0° until used. For studies of the decay of β-galactosidase activity, samples (4·5 ml.) of this suspension were mixed at 0° with 0·5 ml. of a solution containing the substances under study. The system was then incubated at 25° and after an appropriate time 0·5 ml. samples were withdrawn for estimation of β-galactosidase activity. Zero-time samples were taken before raising the temperature to 25°.

Experiments with whole organisms. The general technique was that used with acetone-dried organisms, except that in some experiments the organisms were recovered by centrifugation and washed before estimating the β-galactosidase activity. No significant changes in activity were observed as a result of washing.

Estimation of β-galactosidase activity. This was measured essentially by the method of Lederberg (1950). The organisms were incubated for 30 min. at 25° in a system consisting of (in 3·0 ml.): 0·067 M (with respect to phosphate) potassium phosphate (pH 6·8) and 3·3 mM-*o*-nitrophenyl-β-D-galactoside (ONPG). The reaction was stopped by adding 0·5 ml. M-Na_2CO_3, the organisms removed by centrifugation at 3000g and the extinction at 420 mμ measured with a Beckman model DU spectrophotometer. Activities are expressed as μmoles ONPG hydrolysed/mg. dry wt./hr.

Radioactivity measurements. The amount of radioactive phosphorus taken up from ^{32}P-labelled sodium pyrophosphate by intact organisms was measured in the following way. The organisms were harvested by centrifugation at 3000g, washed twice with water and a suitable sample (0·1 ml.), containing equiv. 0·1–0·5 mg. dry wt. organisms, was plated on to a planchet (2 cm. diam.) which also held a piece of lens tissue moistened with 0·03 ml. 1% (w/v) cetyltrimethylammonium bromide to facilitate spreading of the sample. The samples were dried under an infrared lamp and counted by using a thin mica end-window Geiger-Müller tube in association with a Panax Type 100C scaler. To determine uptake of radioactivity from ^{14}C-methyl-β-D-thiogalactoside (TMG) the organism was filtered off on Oxoid membrane filters (2 cm. diam.), washed thoroughly with water, the membranes dried and counted as described above. Filtration through membrane filters could not be used for experiments with ^{32}P because of the very strong adsorption of pyrophosphate by the filters.

Fermentation and respiration rates. These were measured at 25° using Warburg manometers and following the procedures described by Umbreit, Burris & Stauffer (1949).

Chemicals. *o*-Nitrophenyl-β-D-galactoside was obtained from L. Light and Co. Synthetic ^{14}C-methyl-β-D-thiogalactoside was a gift from Professor J. Monod, to whom we express our thanks. ^{32}P-Labelled sodium pyrophosphate was obtained

from the Radiochemical Centre, Amersham, England. Gut juice from *Helix pomatia* was kindly given by Dr D. H. Northcote. All other chemicals were of analytical grade when available.

RESULTS

Factors affecting the decay of β-galactosidase activity in aqueous suspensions of acetone-dried Saccharomyces fragilis

The use of water distilled from metal stills may have influenced the results of the experiments on *Saccharomyces fragilis* β-galactosidase described by Davies (1964). It was therefore considered advisable to re-investigate certain aspects of this work.

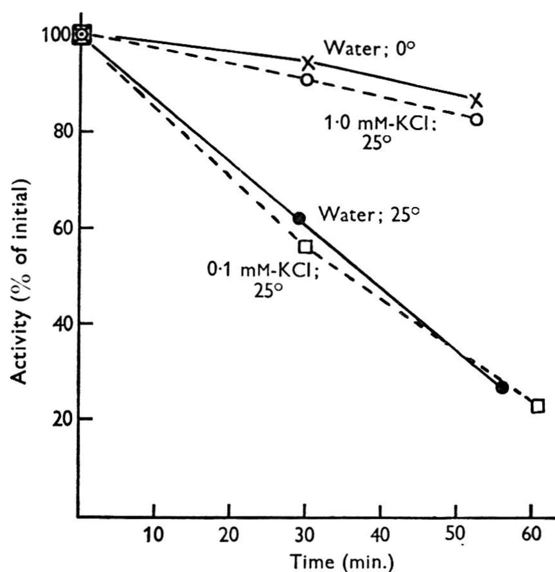

Fig. 1. Effect of temperature and potassium chloride on decay of β-galactosidase activity of suspensions of acetone-dried Saccharomyces fragilis. Acetone-dried organisms were suspended at 0° in water (solid lines) or potassium chloride solution (broken lines), incubated at 0° or 25° and the β-galactosidase activity measured at the times shown.

Effect of sodium and potassium salts. Using glass-distilled water the optimum pH value for hydrolysis of ONPG by the acetone-dried organisms in 0·067 M-potassium phosphate buffers was pH 6·7. It was confirmed that the enzymic activity decayed rapidly when acetone-dried organisms were suspended in water at 25°, but only slowly at 0°, and that the activity could be preserved by suspension in potassium phosphate buffer (pH 7·0). Contrary to the findings of Davies (1964), concentrations of potassium chloride greater than 5 mM were found to preserve β-galactosidase activity (Fig. 1). This discrepancy has not been resolved. Various potassium salts were effective at 5 mM concentration, but sodium salts were generally much less effective except for sodium citrate (Table 1).

Effect of concentration of acetone-dried organisms. This had a marked effect on the rate of decay when acetone-dried organisms were suspended in water. After 60 min.

at 25° the decay was very marked with 1·0 mg. dried organisms/ml., but zero with 10 mg./ml. A hot water extract of the dried organisms inhibited decay.

Effect of various metal ions. Cupric, zinc and mercuric ions (100μM) inhibited β-galactosidase activity; at 100μM other metal ions tested were without appreciable effect (Table 2). Manganous chloride and, to a lesser extent, cobaltous chloride

Table 1. *Effect of various salts on decay of β-galactosidase activity of acetone-dried Saccharomyces fragilis*

A suspension (4·5 ml.) of acetone-dried organisms in glass-distilled water at 0° was mixed at 0° with 0·5 ml. of the appropriate salt solution at pH 6·8. β-Galactosidase activity was estimated before and after incubation for 1 hr at 25°. Results are expressed as μmoles o-nitrophenyl-β-D-galactoside (ONPG) hydrolysed/mg. dry wt./hr.

		μmole ONPG split/mg./hr after incubation for			
		K+		Na+	
Anion	Concn. (mM)	0	1 hr	0	1 hr
Water control	—	8·5	3·6	10·8	0·8
Acetate	5	8·6	8·7	8·9	2·0
	50	16·2	14·0	10·3	.
	200	12·8	13·7	10·0	14·3
Phosphate	5	8·5	12·1	8·5	2·3
	50	8·7	16·0	9·5	7·1
	200	8·9	16·4	12·8	.
Succinate	5	11·6	11·1	.	7·9
	50	12·2	15·8	9·8	13·1
	200	11·7	14·2	8·5	3·2
Lactate	5	13·0	8·0	10·1	1·1
	50	11·4	10·4	10·2	1·2
	200	11·2	15·9	9·5	0·1
Chloride	5	9·2	9·8	11·9	2·0
	50	11·7	18·0	12·0	.
	200	9·2	16·9	.	9·1
Oxalate	5	11·8	9·8	8·9	2·0
	50	8·2	12·5	10·3	.
	200	13·6	16·0	10·0	14·3
Phthalate	5	9·6	6·9	10·3	1·7
	50	8·7	11·9	7·6	0·0
	200	8·1	18·7	3·0	0·0
Citrate	5	13·3	20·6	9·9	10·2
	50	13·6	14·6	10·2	10·5
	200	7·7	9·9	10·3	7·6

partially protected the enzyme, but at low concentrations the effect was variable. At higher concentrations manganous ion increased the initial zero-time activity and protected the enzyme; manganous chloride was more effective than manganous sulphate (Table 3). Manganous chloride prepared from 'Analar' potassium permanganate was as effective as 'Analar' manganous chloride, indicating that the effect was a property of manganese and not of impurities in the 'Analar' manganous chloride. At 100μM other metal ions tested had no effect on the rate of decay.

Effect of some enzyme inhibitors. EDTA, semicarbazide, p-chloromercuribenzoate, and to a smaller extent hydroxylamine, inhibited β-galactosidase activity (Table 4).

Oxine, dimethylglyoxime and especially hydrazine and 2,4-dinitrophenol increased the initial activity. 2,4-Dinitrophenol, sodium sulphide, sodium azide, potassium cyanide, diethyldithiocarbamate and, to a lesser extent, oxine, dimethylglyoxime,

Table 2. *Effect of various metal ions on decay of β-galactosidase activity of acetone-dried Saccharomyces fragilis*

Acetone-dried *S. fragilis* organisms were suspended at equiv. 1·0 mg. dry wt./ml. and 0° in solutions of substances shown in the Table. β-Galactosidase activity estimated before and after incubation for 1 hr at 25°.

Substance added	Concn. (μM)	μmole ONPG split/mg./hr after incubation for		Initial activity remaining (%)
		0	1 hr	
None	.	14·3	2·3	16·0
None	.	17·0	2·1	12·3
$MnCl_2$	100	18·4	11·3	61·5
$MgSO_4$	100	15·4	2·2	14·3
$FeCl_3$	100	10·1	1·7	16·8
$FeSO_4$	100	10·1	1·1	10·9
$ZnSO_4$	100	3·5	0·1	2·9
$CoCl_2$	100	15·5	6·5	41·8
$CuCl_2$	100	0·0	0·0	.
$CuCl_2$	10	0·3	0·1	.
$CaCl_2$	100	14·9	2·5	16·7
$HgCl_2$	100	0·0	0·0	.
Basic lead acetate	100	13·1	2·1	16·0
H_2MoO_4	100	14·1	1·9	13·5
H_3BO_3	100	9·2	2·0	21·7

Table 3. *Effect of manganese concentration on decay of β-galactosidase activity of acetone-dried Saccharomyces fragilis*

Acetone-dried *S. fragilis* organisms were suspended at equiv. 1·0 mg. dry wt./ml. and 0° in solutions of substances shown in the Table. β-Galactosidase activity estimated before and after incubation for 1 hr at 25°.

Salt	Concn. (M)	μmole ONPG split/mg./hr after incubation for		Initial activity remaining (%)
		0	1 hr	
None	(Water control)	8·4	0·7	8·0
$MnCl_2$	10^{-7}	6·6	0·1	1·5
	10^{-6}	8·0	0·1	1·3
	10^{-5}	8·5	0·5	5·9
	10^{-4}	10·7	0·5	4·7
	10^{-3}	10·2	1·9	18·6
	10^{-2}	12·3	3·6	29·2
	10^{-1}	21·6	11·8	54·5
$MnSO_4$	10^{-7}	7·3	0·1	1·4
	10^{-6}	9·0	0·4	4·4
	10^{-5}	8·6	0·5	5·8
	10^{-4}	10·0	0·4	4·0
	10^{-3}	11·2	1·2	10·7
	10^{-2}	12·5	3·6	28·8
	10^{-1}	15·0	4·2	28·0

Table 4. *Effect of various enzyme inhibitors on β-galactosidase activity of whole and of acetone-dried Saccharomyces fragilis*

For acetone-dried organisms conditions were the same as in Table 3. Experiments with whole organisms were done in a similar manner, suspensions of the organisms in water replacing the acetone-dried organisms.

		Acetone-dried organisms			Whole organisms		
		µmole ONPG split/mg./hr after incubation for		Initial activity remaining	µmole ONPG split/mg./hr after incubation for		Initial activity remaining
Substance added	Concn. (mM)	0	1 hr	(%)	0	1 hr	(%)
None	—	5·7	0·5	10	0·09	0·06	67
None	—	6·0	1·6	26	.	.	.
EDTA	10	0·2	0·0	.	0·06	0·05	83
Oxine	1	.	.	.	0·25	0·14	56
Oxine	10	9·3	5·3	57	.	.	.
Hydrazine	10	10·2	1·7	17	0·11	0·13	121
Hydroxylamine	10	3·9	0·0	0	0·12	0·06	50
Semicarbazide	10	0·3	0·0	.	0·09	0·05	56
Na sulphide	10	6·8	8·9	130	0·12	0·07	58
Na fluoride	5	6·4	4·1	64	0·12	0·07	58
Na azide	1	6·6	6·7	101	.	.	.
Na azide	5	7·3	7·4	101	0·09	0·05	56
Na azide	10	.	.	.	0·11	0·05	45
Na cyanide	1	6·3	7·0	103	0·12	0·08	67
Na cyanide	10	6·2	5·5	89	0·19	0·17	89
Na pyrophosphate	10	5·4	0·0	0	0·23	1·00	435
Na citrate	0·1	5·5	3·6	65	0·14	0·08	57
Na diethyldithiocarbamate	1	0·2	9·2	126	0·11	0·09	82
Dimethylglyoxime	1	9·6	4·0	41	.	.	.
Dimethylglyoxime	5	.	.	.	0·11	0·11	100
Salicylaldoxime	0·1	7·0	2·1	31	0·10	0·07	70
2,4-Dinitrophenol	0·1	10·7	11·8	110	.	.	.
2,4-Dinitrophenol	0·01	.	.	.	0·11	0·05	45
p-Chloromercuribenzoate	0·1	0·3	0·2	.	0·09	0·02	26

sodium fluoride and sodium citrate all prevented decay, whereas hydroxylamine and sodium pyrophosphate accelerated decay (Table 4).

Inhibition of β-galactosidase activity by the chelating agent ethylenediamine-tetra-acetate (EDTA) and its annulment by manganese and magnesium was shown with the *Escherichia coli* enzyme by Reithel & Kim (1960). β-Galactosidase activity in *Saccharomyces fragilis* is similarly inhibited by EDTA, the inhibition being annulled

Table 5. *Effect of EDTA and various metal ions on β-galactosidase activity of acetone-dried Saccharomyces fragilis*

Acetone-dried organisms were suspended at 0° in water or in 0·1 mM-EDTA with and without 0·1 mM solutions of the substances listed ($CuCl_2$, 1·0 μM). β-Galactosidase activity estimated before and after incubation for 1 hr at 25°.

Incubation system	μmole ONPG split/mg./hr after incubation for 0	1 hr	Initial activity remaining (%)
Water control	13·1	2·1	15
Water control	13·5	6·1	45
EDTA	3·8	2·7	69
EDTA + $MnCl_2$	17·2	12·4	72
EDTA + $MgSO_4$	7·1	4·7	66
EDTA + $FeCl_3$	9·7	1·6	16
EDTA + $FeSO_4$	11·7	5·5	46
EDTA + $ZnSO_4$	1·6	0·1	6
EDTA + $CoCl_2$	9·0	1·8	20
EDTA + $CuCl_2$	4·9	0·5	10
EDTA + $CaCl_2$	4·4	2·3	59
EDTA + basic lead acetate	3·9	2·1	55
EDTA + H_2MoO_4	4·9	2·0	43
EDTA + H_3BO_3	6·3	2·0	32

Table 6. *Effect of sodium pyrophosphate plus various metal ions on β-galactosidase activity of acetone-dried organisms*

Conditions as for Table 5 with 0·01 M-sodium pyrophosphate in place of EDTA.

Incubation system	μmole ONPG split/mg./hr after incubation for 0	1 hr	Initial activity remaining (%)
Water control	13·1	2·1	15
Water control	13·5	6·1	45
Sodium pyrophosphate	7·1	0·2	3
Na pyrophosphate + $MnCl_2$	14·6	7·7	53
Na pyrophosphate + $MgSO_4$	7·8	1·2	15
Na pyrophosphate + $FeCl_3$	7·3	0·7	10
Na pyrophosphate + $FeSO_4$	6·8	0·9	13
Na pyrophosphate + $ZnSO_4$	12·3	1·3	10
Na pyrophosphate + $CoCl_2$	11·4	6·9	60
Na pyrophosphate + $CuCl_2$	5·9	0·9	15
Na pyrophosphate + $CaCl_2$	5·1	1·2	24
Na pyrophosphate + basic lead acetate	3·5	0·3	9
Na pyrophosphate + $HgCl_2$	0·2	0·0	—
Na pyrophosphate + H_2MoO_4	6·2	0·8	13
Na pyrophosphate + H_3BO_3	6·3	1·8	29

by various metal cations (Table 5). Manganese was most effective, followed by ferrous and ferric iron, cobalt and then magnesium. Sodium pyrophosphate is a chelating agent and the effect of various metal ions on the accelerated decay promoted by pyrophosphate is shown in Table 6. Manganese and cobalt were effective inhibitors of pyrophosphate action. Zinc, which by itself at 0·1 mM is a strong inhibitor of the activity of cell-free β-galactosidase preparations, did not inhibit in presence of 10 mM-pyrophosphate. Potassium phosphate (10 mM, pH 6·7), which by itself prevented decay of β-galactosidase activity, was completely ineffective in this respect in presence of 0·01M- sodium pyrophosphate (Table 7).

Table 7. *Effect of pyrophosphate and potassium ion on β-galactosidase activity of acetone-dried Saccharomyces fragilis*

Acetone-dried organisms were suspended at 0° in water, or in potassium phosphate (pH 6·8) of the concentrations shown, with and without 0·01 M-sodium pyrophosphate. β-Galactosidase activities estimated before and after incubation for 1 hr at 25°.

Potassium phosphate pH 6·8 (mM)	Incubation for 0, 0·01 M sodium pyrophosphate −	Incubation for 0, 0·01 M sodium pyrophosphate +	Incubation for 1 hr, 0·01 M sodium pyrophosphate −	Incubation for 1 hr, 0·01 M sodium pyrophosphate +	Initial activity remaining (%) −	Initial activity remaining (%) +
	μmole ONPG split/mg./hr					
0	10·0	3·8	2·5	0·6	25	16
0·1	8·8	3·6	1·8	0·0	21	0
1·0	8·9	3·9	7·5	0·0	84	0
10·0	.	4·0	12·8	0·0	.	0

β-Galactosidase activity of intact Saccharomyces fragilis

The rate of hydrolysis of β-galactosides by intact *Saccharomyces fragilis* organisms may be controlled by permeability factors as well as by the β-galactosidase content. The response to inhibitory or stimulatory substances may therefore differ from that of cell-free preparations of β-galactosidase. With the exceptions of oxine (1 mM), cyanide (10 mM), pyrophosphate (10 mM) and possibly sodium citrate (0·1 mM), the substances listed in Table 4 had no significant effect on the β-galactosidase activity of intact *S. fragilis* organisms.

The most interesting effect was that of sodium pyrophosphate, which gave a twofold stimulation of initial activity and a further fourfold stimulation after 1 hr. This effect was only evident at an alkaline pH value, but alkalinity was probably not responsible for the increase in activity since other buffers at the same concentration and pH value had no comparable effect. Nor could the effect of pyrophosphate be reproduced by other phosphorus-containing compounds (Table 8). The effect of pyrophosphate was not influenced significantly by 0·1–10 mM-orthophosphate or by 1 to 100μM-uranyl chloride, but simultaneous addition of glucose prevented it, the inhibition being greater at higher glucose concentrations (Table 9).

With the exceptions of manganese and zinc, the increased activity resulting from pyrophosphate treatment was not significantly affected by the simultaneous presence of various cations at 0·1 mM (Table 10). Zinc and manganese significantly

increased the effect of pyrophosphate, maximum stimulation occurring with 1·0 mM (Table 11). Mixtures of zinc or manganese with EDTA did not show these effects. The β-galactosidase activity of organisms which had been pretreated with sodium pyrophosphate, washed and resuspended in water was not inhibited by the cations (0·1 mM) listed in Table 10. This is in marked contrast to the inhibitory effect of

Table 8. *Effect of pyrophosphate, pH value and some other phosphorus-containing compounds on the β-galactosidase activity of intact Saccharomyces fragilis*

Incubation system	Concn. (mM)	pH value	Initial activity remaining after 1 hr (%)
Water	.	.	72
Water	.	.	80
Na pyrophosphate	1	8·65	70
	2	8·65	75
	10	8·65	307
	10	7·0	100
	10	7·5	109
	10	7·8	120
	10	8·2	226
	10	8·65	306
	10	8·9	338
	10	9·0	184
Na orthophosphate	10	8·65	100
	10	10·0	58
K orthophosphate	20	8·65	112
Na hexametaphosphate	10	8·65	75
Adenosine diphosphate	7	8·65	82
Adenosine triphosphate	7	8·65	61
Deoxyribonucleic acid	0·5 mg./ml.	8·65	90
Tris + HCl buffer	20	8·7	91
	20	9·0	70
$NaHCO_3 + Na_2CO_3$	20	9·15	100
$NaHCO_3 + Na_2CO_3$	20	9·65	75

Washed, intact organisms were suspended at 0° in solutions shown above. β-Galactosidase activity was measured before and after incubation for 1 hr at 25°.

Table 9. *Effect of pyrophosphate plus glucose on the β-galactosidase activity of whole Saccharomyces fragilis organisms*

Conditions as for Table 8.

Incubation system	Glucose concn. (mM)	Incubation for 0 (μmole ONPG split/mg./hr)	Incubation for 1 hr (μmole ONPG split/mg./hr)	Initial activity remaining (%)
Water control	.	0·10	0·05	50
0·01 M-Na pyrophosphate	Nil	0·16	0·42	262
Na pyrophosphate + glucose	0·55	0·17	0·21	123
	5·5	0·16	0·14	88
	55·0	0·17	0·12	71

many of these ions on the activity of the enzyme in cell-free extracts or suspensions of acetone-dried organisms.

Organisms treated with sodium pyrophosphate released material absorbing light at 260 mμ, but suspensions of the treated organisms showed no change in viable count and only a 10 % decrease in rate of respiration or fermentation; nor was any

Table 10. *Effect of various metal ions on pyrophosphate stimulation of β-galactosidase activity of whole organisms of Saccharomyces frcgilis*

Conditions as for Table 8.

Incubation system		Metal ion concn. (mM)	Incubation for 0: μmole ONPG split/mg./hr	Incubation for 1 hr: μmole ONPG split/mg./hr	Initial activity remaining (%)
Water		.	0·22	0·16	73
Na pyrophosphate (0·01 M)		.	0·15	0·56	374
Na pyrophosphate (0·01 M)	+ KCl	20	0·23	0·76	330
	+ $MnCl_2$	0·1	0·25	1·19	477
	+ $MgSO_4$	0·1	0·15	0·72	480
	+ $FeCl_3$	0·1	0·20	0·56	280
	+ $FeSO_4$	0·1	0·19	0·60	316
	+ $ZnSO_4$	0·1	0·29	1·00	345
	+ $CaCl_2$	0·1	0·13	0·74	569
	+ $HgCl_2$	0·1	0·02	0·10	500
	+ basic lead acetate	0·1	0·10	0·66	660
	+ H_2MoO_4	0·1	0·15	0·69	460
	+ H_3BO_3	0·1	0·24	0·68	283
	+ $CuCl_2$	1·0 μM	0·19	0·80	421

Table 11. *Effect of concentration of manganese, magnesium and zinc ions on pyrophosphate stimulation of β-galactosidase activity of whole Saccharomyces fragilis organisms*

Conditions as for Table 8.

		μmole ONPG split/mg./hr after incubation for 0		1 hr		Initial activity remaining (%)	
		0·01 M-Na pyrophosphate					
Cation	Concn. (mM)	−	+	−	+	−	+
None	.	0·44	1·07	0·44	4·87	100	451
Mn^{2+}	0·1	0·46	1·58	0·62	7·40	135	468
	1·0	0·60	1·97	0·38	11·80	63	600
	10	1·25	1·00	3·20	1·42	256	142
	100	1·01	.	1·03	.	102	.
Mg^{2+}	0·1	0·52	0·90	0·40	3·93	77	437
	1·0	0·48	1·00	0·43	4·60	90	460
	10	0·44	0·39	0·53	0·40	120	102
	100	0·31	.	0·28	.	90	.
Zn^{2+}	0·1	0·30	1·10	0·46	4·30	153	391
	1·0	0·40	1·35	0·32	7·30	80	540
	10	0·38	0·34	0·39	0·27	103	79
	100	0·50	.	0·30	.	60	.

change in cell morphology evident on examination by phase-contrast microscopy. Examination of the suspension fluid after sodium pyrophosphate treatment showed that only very low β-galactosidase activity was present. However, it is possible that enzyme released into the suspension may have been inactivated by the pyrophosphate present (see Table 4).

The increased β-galactosidase activity of pyrophosphate-treated *Saccharomyces fragilis* was mostly lost when the organisms were incubated for 1 hr at 30° in tris + HCl buffer (5 mM, pH 6·8); but a second incubation with pyrophosphate again

Table 12. *Reversibility of pyrophosphate stimulation of β-galactosidase activity of whole Saccharomyces fragilis organisms*

Whole *S. fragilis* organisms were incubated (*a*) in water at 25° for 1 hr; (*b*) in 0·01 M-sodium pyrophosphate at 25° for 1 hr; (*c*) treated as (*b*), then organisms centrifuged down, suspended in 5 mM-tris + HCl buffer (pH 7·0) and incubated for 1 hr at 30°; (*d*) treated as (*c*), then organisms centrifuged down, resuspended in 0·01 M-pyrophosphate and incubated for 45 min. at 25°. β-Galactosidase activity was then estimated after incubation.

Incubation conditions	μmole ONPG split/mg./hr	Water control value (%)
(*a*) Water control	0·115	100
(*b*) 0·01 M-sodium pyrophosphate	0·352	306
(*c*) as (*b*), followed by incubation in tris + HCl buffer (pH 7·0)	0·195	169
(*d*) as (*c*), followed by incubation in 0·01 M-pyrophosphate	0·240	208

Table 13. *Uptake of* ^{14}C *from* ^{14}C*-methyl-β-D-thiogalactoside by whole organisms of Saccharomyces fragilis*

Intact organisms were incubated in a system containing (per ml.): washed organisms (glucose-grown equiv. 10·8 mg. dry wt.; galactose-grown, 4·3 mg.), 0·06 M-potassium phosphate (pH 6·8), 0·5 mM-^{14}C-methyl-β-D-thiogalactoside (2×10^4 counts/min./μmole) and, where added, 2 mM-$MnCl_2$. Organisms pretreated with pyrophosphate were incubated for 1 hr at 25° in 0·01 M-sodium pyrophosphate, centrifuged down and washed with water before incubation with ^{14}C-methyl-β-D-thiogalactoside as described above. Samples (0·5 ml.) were filtered through Oxoid membrane filters, washed with water, dried and counted. Counting rates have been corrected for background (15 counts/min.).

		Counts/min./mg. dry wt. organisms after			
		0		1 hr	
Organisms grown on	Incubation system	0°	25°	0°	25°
Glucose	Glucose + phosphate	19	15	6	17
Galactose	Glucose + phosphate	17	30	33	161
Galactose	Glucose + phosphate + $MnCl_2$	.	21	.	124
Galactose	Glucose + phosphate (organisms pretreated with sodium pyrophosphate)	.	26	.	78

resulted in an increase in activity (Table 12). Loss of the increased activity was not observed when pyrophosphate-treated organisms were incubated with potassium orthophosphate (0·05 M, pH 6·8).

Uptake of radioactive sodium pyrophosphate by intact organisms. There was no significant uptake of ^{32}P when *Saccharomyces fragilis* organisms were incubated in

0·01 M-sodium pyrophosphate labelled with ^{32}P. In presence of manganous chloride (1 mM) there was a small but significant uptake (about 3 μm-moles/mg. dry wt. organisms) within the first few seconds and little further increase in 1 hr.

Uptake of radioactive methyl-β-D-thiogalactoside (TMG) by intact organisms. The uptake of ^{14}C from ^{14}C-TMG was examined at 0° and 25° in presence of 0·01 % glucose as energy source. Organisms grown with glucose as sole carbon source did not accumulate TMG to an appreciable extent, whereas organisms grown on galactose accumulated TMG at 25° to a degree which represented a slight concentration, probably not more than twofold, over that present in the suspension medium. Galactose-grown organisms accumulated a small amount of TMG at 0°. Manganese (2 mM) decreased slightly the amount of TMG taken up at 25°, as did treatment of galactose-grown organisms with sodium pyrophosphate (Table 13). TMG was not fermented by *Saccharomyces fragilis* organisms grown on glucose or on galactose.

DISCUSSION

Caputto *et al.* (1948) reported that manganese and magnesium ions stimulated hydrolysis of lactose by cell-free preparations from *Saccharomyces fragilis*. Such stimulation of lactose hydrolysis was not observed by Davies (1964), although hydrolysis of *o*-nitrophenyl-β-D-galactoside was stimulated about 50 % in 0·1 M-potassium phosphate (pH 6·5) + 0·01 M-manganous chloride. In the present work, with glass-distilled water used for all reagents, manganous ion was found to protect the β-galaetosidase activity of acetone-dried *S. fragilis* against decay. The extent of this protection was such that the activities of freshly prepared suspensions in 0·1 M-manganous chloride were two to three times greater than similar suspensions in water alone. A similar protective effect of manganous ion (0·2–1·0 mM) on *Escherichia coli* ML 308 β-galactosidase was reported by Rickenberg (1959). In the present work manganous ion, and to a lesser extent cobaltous ion, also prevented the marked acceleration of decay brought about by incubation with sodium pyrophosphate. This effect of pyrophosphate might be due to chelation of essential metal ions. In this connexion aureomycin, which chelates manganese ions, has been found to antagonize activation and protection of *Saccharomyces fragilis* β-galactosidase by manganese (Szabo, unpublished results). Some chelating agents (oxine, sodium diethyldithiocarbamate) protected the enzyme against decay, but at the pH value used (6·8) chelation of manganese by these reagents would be rather weak. These effects might be interpreted on the basis that metal ions, possibly manganous ions, hold the β-galactosidase molecules in an active configuration and that addition of substances which strongly chelate the metal ions may result in loss of the active configuration and even in denaturation of the enzyme. Calcium ion seems to have this function in microbial α-amylases and in the case of *Bacillus amyloliquefaciens* α-amylase they can be replaced by strontium or, less effectively, by magnesium (Oikawa, 1959). Wallenfels & Malhotra (1961) analysed preparations of several times recrystallized *Escherichia coli* ML 309 β-galactosidase and found calcium to be consistently present at about 270 μg./g. protein; magnesium was not detected. It has been reported that manganous ion can substitute for calcium ion for the activation of *Vibrio cholerae* neuraminidase (Mohr & Schramm, 1960).

Stimulation of the β-galactosidase activity of intact *Saccharomyces fragilis*

organisms by pyrophosphate seems to have no obvious explanation. Physical disruption of the organisms might be expected to result in increased activity since most treatments which have a disruptive action on cell membranes have this effect (Davies, 1964). The necessity for relatively high concentrations of pyrophosphate, the high pH value requirement, and the release of material absorbing at 260 mμ, are facts which favour this explanation. However, physical disruption would not seem to explain all the effects which are observed to follow pyrophosphate treatment. For example, the retention of an increased β-galactosidase activity when pyrophosphate-treated organisms were washed with water, the lack of inhibition by zinc ion and the observation that the effect of pyrophosphate could be annulled by incubation in tris buffer, when the organisms again became susceptible to pyrophosphate, do not fit with an explanation based on simple physical disruption of the organisms. The chelating properties of sodium pyrophosphate may be important, but chelation is probably not the only factor involved in this phenomenon since other chelating agents (oxine, EDTA) do not produce this effect. The sodium pyrophosphate effect seems to be specific; no other phosphorus compound tested had a similar activity.

It is possible that pyrophosphate treatment of whole organisms increases the permeability to *o*-nitrophenyl-β-D-galactoside, which might result in increased β-galactosidase activity. It has been found that the β-galactosidase activity of *Saccharomyces fragilis* incubated with gut juice from *Helix pomatia*, or with papain, increases before material absorbing at 260 mμ is released from the organisms (Szabo, unpublished results). The uptake of methylthiogalactoside by untreated galactose-grown organisms probably exceeded what would be expected from a simple diffusion equilibrium; this uptake was slightly decreased by treatment with pyrophosphate. It is possible that pyrophosphate interferes with the mechanism by which the galactoside is transported into the cell, perhaps by inhibiting synthesis of some component of the cell membrane. This idea is supported by the observation that the effect of pyrophosphate was prevented in presence of glucose (but not by glucose + sodium azide; Szabo, unpublished results). Although uptake of methylthiogalactoside by *S. fragilis* is very much less than that observed with *Escherichia coli*, the considerable difference between the amounts accumulated by glucose-grown and galactose-grown organisms suggests the presence of an inducible transport system for β-galactosides. Experiments to determine whether thiomethylgalactoside can induce formation of β-galactosidase or a β-galactoside permease in *S. fragilis* have so far given inconclusive results.

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Differences in the Resistance of Sulphate-reducing Bacteria to Inhibitors

By A. M. SALEH*

National Chemical Laboratory, Teddington, Middlesex

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SUMMARY

Forty-five strains of sulphate-reducing bacteria showed marked differences in their resistance to Hibitane (I.C.I. Ltd.) and, to a lesser degree, to cetyltrimethylammonium bromide. Panacide (British Drug Houses Ltd.) was effective against all the strains. The relevance of these findings to modern views on the taxonomy of the sulphate-reducers and to the problem of inhibiting these organisms in the field is discussed.

INTRODUCTION

The anaerobic sulphate-reducing bacteria are of interest both on account of their great economic importance (Postgate, 1960) and of their unique type of oxidative metabolism, which involves the utilization of sulphate or other sulphur compounds as terminal electron acceptors in place of oxygen. Numerous strains of these bacteria have now been isolated, and although few studies on their carbon sources or other aspects of their nutrition have yet been made on which a classification might be based (the feasibility of such a classification was discussed by Macpherson & Miller, 1963), two genera and four species are now recognized on morphological and biochemical grounds: *Clostridium nigrificans*, *Desulfovibrio desulfuricans*, *D. gigas* (Le Gall, 1963) and *D. orientis* (Adams & Postgate, 1959). The majority of strains isolated hitherto have been assigned to *D. desulfuricans*, a species apparently characterized by the presence of cytochrome c_3 and desulphoviridin (Postgate, 1956) though variable in NaCl requirement (Littlewood & Postgate, 1957; Ochynski & Postgate, 1963), ability to dismute pyruvate (Postgate, 1952) and to metabolize choline (Baker, Papiska & Campbell, 1962).

In a survey of the effects of inhibitors on sulphate-reducing bacteria, Saleh, Macpherson & Miller (1964) drew attention to some marked differences in the resistance of different strains to certain inhibitors, which, though probably attributable in some cases to unstandardized experimental techniques, must in others reflect genuine physiological differences between strains. For example, in these authors' own comparative studies on the mesophile *Desulfovibrio desulfuricans* strain Hildenborough and the thermophilic spore-former *Clostridium nigrificans* strain Delft 74T, the thermophile proved much more susceptible to most of the inhibitors tested, growth often being suppressed by one-tenth of the concentration required to inhibit the Hildenborough strain. In the present report, the effects of four inhibitors

* Present address: Sanitary Engineering Department, Faculty of Engineering, Alexandria University, Alexandria, Egypt.

on forty-five strains of sulphate-reducing bacteria, representative of all the four species mentioned above, were examined to discover whether there is a correlation between resistance and taxonomic position.

METHODS

Organisms. Forty-five strains of sulphate-reducing bacteria were obtained as freeze-dried ampoules from the National Collection of Industrial Bacteria (NCIB). These strains were assumed to be in pure culture; however, it is now known (Professor L. L. Campbell, personal communication) that strains Avonmouth NCIB 8398 and probably Norway 4 NCIB 8310 (Miller & Saleh, in preparation) were mixed populations of two different types of sulphate-reducers.

The strains used, with their NCIB numbers, were as follows:

(1) Thermophiles, growth temperature 55°. Strains of *Clostridium nigrificans*; all spore-formers; all freshwater organisms: Teddington Garden 8351; Staines G 8353; Holland CT 8356; Delft 3T 8359; Delft 15T 8361; Delft 74T 8395; unnamed strain 8706; unnamed strain 8788.

(2) Mesophiles, growth temperature 30°; strains 8364 and 8365 are stated (unpublished work at the National Chemical Laboratory) to grow optimally at 28° .

(*a*) *Clostridium nigrificans*, Coleman's strain 42 (8452). This sporulating freshwater strain, originally assigned to *Desulfovibrio orientis*, has now been identified as a mesophilic *C. nigrificans* (Postgate & Campbell, 1963).

(*b*) *Desulfovibrio desulfuricans*—freshwater strains: Teddington M 8302, Hildenborough 8303, Wandle 8305, Essex 6 8307, Holland D-6 8311, Teddington R 8312, Beckton 8319, Holland C-6 8372, Marseille (Gaz 54) 8386, Berre Eau 8387, Berre Sol 8388, Llanelly 8446, Woolwich 8457, Byron 8458, Monticello 2 9442, 'Vibrio cholinicus' 9467; salt-water strains: El Agheila C 8308, Norway 4 8310, New Jersey SW-8 8315, Venice 1 8322, Australia 8329, Canet 40 8363, California 43:63 8364, Louisiana 43:11 8365, El Agheila Z 8380, Canet 20 8391, Canet 32 8392, Canet 41 8393, Walvis Bay 8397, Avonmouth 8398, British Guiana 8403, Sylt 3 9335, Aberdovey (a strain recently isolated by the author) 9492.

(*c*) *Desulfovibrio gigas* (freshwater) 9332.

(*d*) *Desulfovibrio orientis* (spore-former; freshwater) strains Singapore 1 8382, Singapore 2 8445.

Cultivation. Cultures were raised from the freeze-dried condition at 30° or 55°, as appropriate, in the medium of Baars (1930) containing 1·0 g. Difco yeast extract/l. and 5 mM-cysteine hydrochloride. NaCl 25 g./l. was added for salt-water organisms. Details of the preparation, sterilization and pH adjustment of this medium are given elsewhere (Saleh *et al.* 1964). Stock cultures were maintained, for reasons unconnected with the present work, in the medium C of Butlin, Adams & Thomas (1949) with additional lactate and sulphate (Dr J. R. Postgate, personal communication) and NaCl where necessary. All the strains grew satisfactorily in this medium and in Baars's medium; it was not found necessary to incorporate thiolacetate to ensure growth of the *Desulfovibrio orientis* strains (Adams & Postgate, 1959). Subcultures were made weekly. Stock and experimental cultures were grown in Pyrex test tubes plugged with cotton-wool and incubated in McIntosh & Fildes's anaerobic jars under an atmosphere of 99 % H_2 + 1 % CO_2 (v/v).

Tests of inhibitors. A bacteriostatic screening method was used. Tests were carried out in Baars's medium with yeast extract and cysteine, in conformity with the practice at the National Chemical Laboratory over a number of years. Four substances known to have strong bacteriostatic or bactericidal effects on sulphate-reducers (Saleh *et al.* 1964) were tested against the 45 strains. These were bis-(*p*-chlorophenyl-diguanido-)hexane diacetate (Hibitane, Imperial Chemical Industries Ltd.), cetyltrimethylammonium bromide (CTAB), 5,5′-dichloro-2,2′-dihydroxy-diphenyl methane (Panacide; British Drug Houses Ltd.), and 2,4-dinitrophenol. Solutions as obtained were assumed to be sterile; CTAB and 2,4-dinitrophenol were made into concentrated solutions, allowed to stand for a few hours and assumed to be sterile. Serial dilutions were made in sterile distilled water, 2 ml. of the appropriate dilution added to 18 ml. sterile Baars's medium, and the medium adjusted when necessary to pH 7·0–7·2 (bromothymol blue) with sterile NaOH or HCl. Each experimental medium was dispensed into three test tubes and inoculated with enough stock culture of the appropriate organism to give an initial concentration of between 10^5 and 5×10^5 bacteria/ml. After anaerobic incubation the tubes were examined for blackening (formation of FeS); cultures which showed doubtful growth were examined microscopically.

RESULTS

Effects of Hibitane and CTAB

Hibitane and CTAB were separately tested against the 45 strains at 0·1, 0·25, 0·5, 1·0, 2·5, 5·0, 10, 25, 50, 100, 250, 500 and 1000 μg./ml. (parts per million). The minimum inhibitory concentration for each strain was noted after incubation for 10 days; the results were the same after a further 20 days of incubation. The difference in the bacteriostatic concentration of Hibitane towards different strains proved to be greater than ten-thousandfold (see Table 1), and was almost as great in the case of CTAB (Table 2). In both cases the spore formers (strains of *Clostridium nigrificans* and *Desulfovibrio orientis*) were more sensitive than the non-sporulators. Hibitane was the more effective inhibitor, a greater number of strains being inhibited by a given concentration than by the same concentration of CTAB.

Effects of Panacide and dinitrophenol

These two inhibitors were tested over the range of concentrations used for Hibitane and CTAB. Tubes were examined for growth after 10 days. Panacide at 50 μg./ml. was effective against all the strains tested (Table 3), 2,4-dinitrophenol at 100 μg./ml. (Table 4), that is, a poorer separation of the strains was obtained with these two compounds. The representatives of *Clostridium nigrificans* and *Desulfovibrio orientis* were, again, more susceptible than the other strains.

Viability tests

Viability tests were made on bacteria recovered from tubes containing the minimum inhibitory concentrations of three of the inhibitors. The contents of such tubes were centrifuged aseptically for 5 min. at about 11,000 *g* and the precipitated organisms suspended in Baars's medium, transferred to test tubes and incubated. All strains except *Clostridium nigrificans* 8356 and 8706 and *Desulfovibrio desulfuricans* 8302, 8307, 8311, 8312, 8319, 8398, 8446 and 9442 had been killed by incubation for

Table 1. *The minimum concentration of Hibitane inhibitory to various strains of sulphate-reducing bacteria*

Concentration of Hibitane (μg./ml.)					
0·1	1	2·5	10	25	>1000
Organism affected					
*C.n.** 8351† T‡	*C.n.* 8452 M	*D.d.* 8302 M 1	*D.d.* 8307 M 2	*D.d.* 8315 MH	*D.d.* 8303 MH 3
C.n. 8353 T		*D.d.* 8303 M 1	*D.d.* 8310 MH 2¶	*D.d.* 8363 MH 2	*D.d.* 8364 MH 3
C.n. 8356 T		*D.d.* 8305 M 1	*D.d.* 8312 M 2	*D.d.* 8391 MH 2	*D.d.* 8365 MH 3
C.n. 8359 T		*D.d.* 8311 M 1	*D.d.* 8322 MH 2	*D.d.* 8392 MH 2	*D.d.* 8403 MH 3
C.n. 8361 T		*D.d.* 8319 M 1	*D.d.* 8329 MH	*D.d.* 8393 MH 2	
C.n. 8395 T		*D.d.* 8386 M 1	*D.d.* 8372 M 2		
C.n. 8706 T		*D.d.* 8397 MH 1	*D.d.* 8380 MH 2		
C.n. 8788 T		*D.d.* 9442 M 1	*D.d.* 8387 M 2		
D.o. 8382§ M		*D.d.* 9446 M 1	*D.d.* 8388 M 2		
D.o. 8445‖ M		*D.g.* 9332 M	*D.d.* 8398 MH 3¶		
			D.d. 8457 M		
			D.d. 8458 M		
			D.d. 9335 MH 2		
			D.d. 9467 M 2		
			D.d. 9492 MH		

* *C.n.* = *Clostridium nigrificans*; *D.d.* = *Desulfovibrio desulfuricans*; *D.g.* = *D. gigas*; *D.o.* = *D. orientis*.

† NCIB number of strain.

‡ T = thermophile; M – mesophile; H – marine strain (sometimes termed 'halophilic'); 1,2,3 – groups of *D. desulfuricans* based on DNA composition (Saunders *et al.* 1964).

§ Inhibited by 0·25 μg./ml.

‖ Inhibited by 0·05 μg./ml.

¶ Mixed population: see text.

Table 2. *The minimum inhibitory concentration of CTAB towards sulphate-reducers*

For explanation of symbols see Table 1.

Concentration of CTAB (μg./ml.)					
0·25	10	25	50	250	>1000
Organism affected					
C.n. 8353* T	*C.n.* 8452† M	*D.d.* 8307 M 2	*D.d.* 8322 MH 2	*D.d.* 8329 MH	*D.d.* 8308 MH 3
C.n. 8356 T	*D.d.* 8302 M 1	*D.d.* 8310 MH 2	*D.d.* 8380 MH 2	*D.d.* 8363 MH 2	*D.d.* 8315 MH
C.n. 8359 T	*D.d.* 8303 M 1	*D.d.* 8312 M 2		*D.d.* 8391 MH 2	*D.d.* 8364 MH 3
C.n. 8361 T	*D.d.* 8305 M 1	*D.d.* 8372 M 2		*D.d.* 8392 MH 2	*D.d.* 8365 MH 3
C.n. 8395 T	*D.d.* 8311 M 1	*D.d.* 8387 M 2		*D.d.* 8393 MH 2	*D.d.* 8403 MH 3
C.n. 8706 T	*D.d.* 8319 M 1	*D.d.* 8388 M 2			*D.d.* 9492 MH
C.n. 8788 T	*D.d.* 8386 M 1	*D.d.* 8398 MH 3			
D.o. 8382 M	*D.d.* 8397 MH 1	*D.d.* 8457 M			
D.o. 8445 M	*D.d.* 8446 M 1	*D.d.* 8458 M			
	D.d. 9442 M 1	*D.d.* 9335 MH 2			
	D.g. 9332 M	*D.d.* 9467 M 2			

* Inhibited by 0·5 μg./ml.

† Inhibited by 5 μg./ml.

Table 3. *The minimum inhibitory concentration of Panacide towards sulphate-reducers*

For explanation of symbols see Table 1.

Concentration of Panacide (μg./ml.)

0·5	10		25	50
Organism affected				
C.n. 8351 T	*C.n.* 8452 M	*D.d.* 8387 M 2	*D.d.* 8315 MH	*D.d.* 8308 MH 3
C.n. 8353 T	*D.d.* 8302 M 1	*D.d.* 8388 M 2	*D.d.* 8329 MH	
C.n. 8356 T	*D.d.* 8303 M 1	*D.d.* 8391 MH 2		
C.n. 8359 T	*D.d.* 8305 M 1	*D.d.* 8392 MH 2		
C.n. 8361 T	*D.d.* 8307 M 2	*D.d.* 8393 MH 2		
C.n. 8395 T	*D.d.* 8310 MH 2	*D.d.* 8397 MH 1		
C.n. 8706 T	*D.d.* 8311 M 1	*D.d.* 8398 MH 3		
C.n. 8788 T	*D.d.* 8312 M 2	*D.d.* 8403 MH 3		
D.o. 8382 M	*D.d.* 8319 M 1	*D.d.* 8446 M 1		
D.o. 8445 M	*D.d.* 8322 MH 2	*D.d.* 8457 M		
	D.d. 8363 MH 2	*D.d.* 8458 M		
	D.d. 8364 MH 3	*D.d.* 9335 MH 2		
	D.d. 8365 MH 3	*D.d.* 9442 M 1		
	D.d. 8372 M 2	*D.d.* 9467 M 2		
	D.d. 8380 MH 2	*D.d.* 9492 MH		
	D.d. 8386 M 1	*D.g.* 9332 M		

Table 4. *The minimum inhibitory concentration of 2,4-dinitrophenol towards sulphate-reducers*

For explanation of symbols see Table 1.

Concentration of 2,4-dinitrophenol				
1·0	5	25	50	100
Organism affected				
C.n. 8351 T	*C.n.* 8452 M	*D.d.* 8302 M 1	*D.d.* 8307 M 2	*D.d.* 8446 M 1
C.n. 8353 T		*D.d.* 8303 M 1	*D.d.* 8308 MH 3	*D.d.* 8457 M
C.n. 8356 T		*D.d.* 8305 M 1	*D.d.* 8310 MH 2	*D.d.* 9467 M 2
C.n. 8359 T		*D.d.* 8311 M 1	*D.d.* 8315 MH	
C.n. 8361 T		*D.d.* 8312 M 2	*D.d.* 8363 MH 2	
C.n. 8395 T		*D.d.* 8319 M 1	*D.d.* 8365 MH 3	
C.n. 8706 T		*D.d.* 8322 MH 2	*D.d.* 8380 MH 2	
C.n. 8788 T		*D.d.* 8329 MH	*D.d.* 8387 M 2	
D.o. 8382 M		*D.d.* 8364 MH 3	*D.d.* 8388 M 2	
D.o. 8445 M		*D.d.* 8372 M 2	*D.d.* 8391 MH 2	
		D.d. 8386 M 1	*D.d.* 8392 MH 2	
		D.d. 8397 MH 1	*D.d.* 8393 MH 2	
		D.d. 8458 M	*D.d.* 8398 MH 3	
		D.d. 9335 MH 2	*D.d.* 8403 MH 3	
		D.d. 9442 M 1	*D.d.* 9492 MH	
		D.g. 9332 M		

10 days with the minimum inhibitory concentration of Hibitane. After similar treatment with CTAB only *C. nigrificans* 8353 and *D. orientis* 8445 were viable; with Panacide no strain was viable. In all cases the minimum inhibitory concentration was bactericidal after 30 days of incubation; and in all cases twice (or 2·5 times) the minimum inhibitory concentration was bactericidal after 15 days.

DISCUSSION

Sigal, Senez, Le Gall & Sebald (1963) determined the base composition of the DNA of seven strains of sulphate-reducing bacteria, representative of all four known species, and found significant differences which correlated with taxonomic position. Saunders, Campbell & Postgate (1964), in a much wider survey of the species *Desulfovibrio desulfuricans*, recognize three groups. Group 1, with a guanine + cytosine content of 60–62 % of the total DNA base, is composed mainly of freshwater organisms, unable to dismute pyruvate or to metabolize choline. Group 2 organisms (54–56 % guanine + cytosine) are predominantly salt-water forms, though adaptable in all cases but one to NaCl-free medium, and are able to dismute pyruvate and metabolize choline. Members of group 3 (45·6–46·6 % guanine + cytosine) are strict salt-water strains. In the present work, the group numbers assigned by Saunders *et al.* to strains of *D. desulfuricans* are shown in Tables 1–4. The most striking feature was the very high resistance of group 3 organisms to Hibitane and CTAB; the only exception, strain Avonmouth (8398), is now known to be a mixture of two types, one corresponding to group 2 but the dominant organism belonging to group 3. Members of group 2 were somewhat more resistant to these inhibitors than were group 1 strains, a partial separation of freshwater from salt-water members of group 2 being noticeable. Curiously, though in keeping with the findings of Saleh *et al.* (1964), the strains of *C. nigrificans* (both thermophilic and mesophilic) and of *D. orientis*, all spore-formers, were less resistant to any of these inhibitors than were strains of *D. desulfuricans* or *D. gigas*.

Shewan, Hodgkiss & Liston (1954) and Shewan, Hobbs & Hodgkiss (1960) described a simple method of differentiating common non-pathogenic pseudomonads and vibrios on the basis of their resistance to penicillin, terramycin and a vibriostatic pteridine; Callao & Montoya (1960) differentiated four *Azotobacter* species on their resistance to certain dyes. The present work shows that *Desulfovibrio desulfuricans* group 3 strains and the spore-forming types may be unequivocally distinguished from other sulphate-reducing bacteria by their high and low resistances to Hibitane, respectively, and that within a narrower concentration range a separation of group 1 and 2 organisms may be observed. Hence resistance to inhibitors may be a useful taxonomic character within this group of bacteria.

The remarkable range of susceptibility of the sulphate-reducers to Hibitane and CTAB illustrates the importance of conducting laboratory screening tests on strains isolated from the site in which the inhibitor is to be used (Allred, Mills & Fisher, 1954), especially when the site is a salt-water one in which *Desulfovibrio desulfuricans* group 3 may be present. Alternatively, strains of known group should be included in screening tests to determine the spectrum width of the inhibitor. Panacide was found in the present work to be a broad-spectrum inhibitor of sulphate-reducing bacteria, preventing the growth of all strains at 50 μg./ml. and all but three at 10 μg./ml.

The observed bacteriostatic concentration of an inhibitor was in all cases the same on the 30th day as the 10th; thus a 10-day incubation period is probably sufficient in this type of experiment. Viability tests showed that Hibitane, CTAB and Panacide had considerable bactericidal power at their bacteriostatic concentration; the bactericidal effect of 2,4-dinitrophenol was studied by Bennett & Bauerle (1960).

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Lipase Activity of Mycoplasma

By S. ROTTEM and S. RAZIN

Department of Clinical Microbiology, Hebrew University-Hadassah Medical School, Jerusalem, Israel

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SUMMARY

All of eight Mycoplasma strains tested were capable of hydrolysing tributyrin. The saprophytic *Mycoplasma laidlawii* strains showed the lowest lipolytic activity, and the parasitic *Mycoplasma gallisepticum* the highest. The properties of *M. gallisepticum* lipase were studied in some detail. The lipolytic activity of this organism was highest at the logarithmic phase of growth and declined steeply afterwards. The enzyme was not bound to the cell membrane and appeared in the soluble fraction of disrupted cells. The cell extract hydrolysed tributyrin much faster than trilaurin or triolein. Methyl oleate and 'Tween 80' were only slowly hydrolysed. Cholesteryl acetate and stearate were not hydrolysed by cell extract or by intact *M. gallisepticum*. Partial purification of the lipase was accomplished by ammonium sulphate fractionation of cell-extract proteins, followed by anion-exchange chromatography. The partially purified enzyme did not require inorganic ions for activity and its optimal pH value varied between 7·5 and 8·0, depending on the substrate tested.

INTRODUCTION

In a study of the nutritional requirements of Mycoplasma for long-chain fatty acids, *Mycoplasma laidlawii* was found to utilize methyl oleate or 'Tween 80' as a source for oleic acid. There was evidence pointing to the enzymic hydrolysis of Tween 80 by this organism (Razin & Rottem, 1963). The presence of lipases in Mycoplasma had long been suspected. During growth on horse-serum agar several Mycoplasma strains form a pearly film with little spots, found to consist of calcium and magnesium soaps; the film contained cholesterol and phospholipids (Edward, 1954). On media enriched with egg yolk, spots were formed by some additional strains which did not form them with horse serum. A clearing of egg-yolk medium by mycoplasmas was also noted. These observations prompted Edward (1954) to suggest that Mycoplasma strains contain lipases which break down phospholipids. These indications for an active lipid metabolism in Mycoplasma notwithstanding, very little was done to study the enzymes involved. Smith (1959) described cholesterol esterase activity in several Mycoplasma strains. One human strain was also found to contain a lipase which hydrolysed triglycerides. In view of the importance of lipids as essential nutrients and building blocks of the Mycoplasma cell membrane (Rodwell & Abbott, 1961; Smith, 1963; Tourtellotte, Jensen, Gander & Morowitz, 1963; Razin, Argaman & Avigan, 1963) it seemed worthwhile to examine the lipid metabolism of these organisms.

METHODS

Organisms. Mycoplasma laidlawii strain A (PG8), *M. laidlawii* strain B (PG9), *M. mycoides* var. *mycoides* (PG1) and *M. bovigenitalium* (PG11) were obtained from Dr D. G. ff. Edward (The Wellcome Research Laboratories, Beckenham, Kent, England). *M. mycoides* var. *capri* was provided by Dr E. Klieneberger-Nobel (The Lister Institute of Preventive Medicine, London). *M. gallisepticum* named (R) was obtained from Mrs Ruth Bernstein (Faculty of Agriculture, The Hebrew University, Rehovot, Israel). Strain S6 of *M. gallisepticum* was provided by Dr H. E. Adler (School of Veterinary Medicine, University of California, Davis, U.S.A.). *M. laidlawii* (oral strain) was isolated in our laboratory from the human oral cavity. Most of the work to be described was done with *M. gallisepticum* (R).

Washed suspensions. The organisms were grown in 1 to 3 l. volumes of a modified Edward medium (Razin, 1963) for 24–72 hr at 37°. The organisms were collected by centrifugation in a Sharples Supercentrifuge at 4°. The paste of organisms so obtained was resuspended in 0·25M-NaCl solution and centrifuged at 16,000*g* for 5 min. at 4°. The sedimented organisms were then washed in cold 0·02M-phosphate buffer (pH 7·5) and resuspended in similar buffer. The amount of organisms in suspension was expressed as mg. cell protein/ml. suspension. In suspensions of the main test organism, *M. gallisepticum* (R), cellular protein was determined by comparing the extinction at 500 mμ to a previously prepared standard curve, which related extinction and cellular protein. Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951).

Cell-free extracts. Mycoplasma gallisepticum organisms were disrupted in an M.S.E. ultrasonic disintegrator (60 W.). Seven ml. of a heavy suspension of the washed organisms in 0·02M-phosphate buffer (pH 7·5) were treated at 1·5 amp. for 2·5 min. Cell debris was removed by centrifugation at 34,000*g* for 30 min. and the supernatant fluid separated and kept at −20° until used.

Chemicals. Tri-*n*-butyrin, di-*n*-butyrin, 1-monobutyrin and mono-olein were obtained from Eastman Organic Chemicals (Rochester 3, New York, U.S.A.). Triolein and trilaurin were the products of Mann Research Laboratories (New York 6, N.Y., U.S.A.). Methyl oleate, cholesteryl acetate and cholesteryl stearate were obtained from Nutritional Biochemicals Corporation (Cleveland, Ohio, U.S.A.). Aqueous emulsions of lipids were prepared by ultrasonic treatment. The lipid (about 200μmoles) was dissolved in diethyl ether (1–2 ml.) and the solution injected with a syringe into 15 ml. of 0·02M-phosphate buffer containing 0·02M-2-hydroxymethylpropane-1:3-diol (tris) buffer (pH 7·7; subsequently this will be referred to as phosphate tris buffer). The buffer solution contained in addition 200μmole sodium taurocholate. The suspension was immediately treated in an M.S.E. ultrasonic disintegrator at 1·5 amp. for 4 min. without cooling. During this treatment the temperature of the emulsion reached 40°, causing the ether to evaporate. These emulsions obtained remained stable for at least several days.

Determination of lipolytic activity

Qualitative assays. Edward medium plates containing 0·4% (w/v) tributyrin were prepared. Tributyrin dissolved in a small volume of acetone was added to basal

Edward medium containing Bacto-heart infusion broth, Bacto-peptone, NaCl and Bacto-agar (Razin & Oliver, 1961; Razin, 1963). The medium was autoclaved at 121° for 20 min., cooled to 50° and all the other ingredients (Razin, 1963) added. The medium, still liquid, was transferred to a sterile 50 ml. beaker and treated in the ultrasonic disintegrator at 1·5 amp. for 3 min. Twelve ml. volumes of this medium were then poured into sterile Petri dishes and allowed to solidify. In some experiments the tributyrin plates were covered with a thin film of polyvinyl formal ('Formvar', Shawinigan Ltd., London, E.C. 3). A solution of 0·5 % (w/v) Formvar in chloroform was quickly poured on the surface of the agar, and allowed to drain off by inclining the plates. After evaporation of the chloroform a fine film of Formvar was produced on the agar surface. This film was freely permeable to medium nutrients, but prevented direct contact of Mycoplasma organisms placed on top of it, with the medium underneath. The organisms to be tested were grown in 5 ml. volumes of liquid Edward medium for 24–48 hr and washed as described above. The washed organisms obtained from a 5 ml. culture were suspended in 0·3 ml. of 0·02 M-phosphate buffer (pH 7·5) and 0·02 ml. drops of these suspensions were placed on the tributyrin plates. The plates were incubated at 37° for 24 hr; clear zones which appeared in and around the drop areas indicated lipolytic activity.

A simple semi-quantitative method for estimating lipase activity in chromatographic fractions of Mycoplasma proteins was devised. Tributyrin, dissolved in a small volume of acetone, was added in a final concentration of 0·4 % (w/v) to 0·02 M-phosphate buffer (pH 7·5) containing 0·9 % (w/v) Bacto-agar. The medium was autoclaved at 121° for 20 min., cooled to 50° and treated in the ultrasonic disintegrator at 1·5 amp. for 3 min. Twelve ml. volumes of the resulting suspensions were poured into sterile Petri dishes and allowed to solidify. Drops (0·02 ml.) of each of the chromatographic fractions were placed on the plates, which were then incubated at 37° for 24 hr. The presence and degree of lipolytic activity in these fractions could be then estimated by the size of the clear zones which appeared in the drop areas (Pl. 1, fig. 1).

Quantitative assays. The reaction mixtures were usually composed of washed organisms, cell extracts or chromatographic fractions in 0·02 M-phosphate buffer (pH 7·5). The lipid was added to the reaction mixture as a finely dispersed suspension prepared by ultrasonic treatment as described above. Thallium acetate, which did not affect lipolytic activity, was added to a final concentration of 0·04 % (w/v) to prevent growth of contaminating organisms during long incubation periods. The total volume of the reaction mixture was 5–10 ml. Experiments with washed organisms or cell-free extracts were done in 100 ml. Erlenmeyer flasks shaken in a 37° water bath. Lipase activity in chromatographic fractions was assayed in test tubes incubated statically at 37°. Hydrolysis of tri-, di- and monobutyrin was determined by titration of freed butyric acid with 0·01 N-NaOH, measured by a glass electrode. Hydrolysis of triglycerides and other esters of long-chain fatty acids was determined according to Dole (1956). The liberated long-chain fatty acids were eluted from the reaction mixture with heptane + water + isopropanol (36 + 28 + 36, by vol.) and titrated with 0·02 N-NaOH, with Nile blue as indicator. Corrections were always made for titritable acidity of reaction mixtures containing boiled organisms, cell extracts or chromatographic fractions. One enzyme unit was defined as that amount which under the conditions of the test liberated 1 μequiv.

of acid/6 hr. Specific activity of the lipase was defined as the number of enzyme units/mg. protein in the volume of reaction mixture titrated.

For measurement of tributyrin hydrolysis by cell extracts or chromatographic fractions, a rapid turbidimetric method was devised. A suspension containing 32 μmoles tributyrin/ml. in 0·02 M-phosphate buffer (pH 7·5) was prepared by ultrasonic treatment at 1·5 amp. for 3 min. Three ml. samples of cell extracts or chromatographic fractions in 0·02 M-phosphate buffer (pH 7·5) were incubated in a 37° water bath. Each sample received 0·2 ml. tributyrin suspension and the extinction was measured immediately in a 'Unicam SP 500' spectrophotometer at 625 mμ. Decrease in extinction of suspensions was determined after 10 and 20 min. of incubation at 37°. Corrections were made for the decrease of extinction in a control tube containing tributyrin suspended in 0·02 M-phosphate buffer (pH 7·5). The extinction in the control tube during the incubation period decreased only (slightly by not more than 0·03 unit scale reading at 625 mμ). One enzyme unit, according to the turbidimetric method, was defined as that amount which under the conditions of the test caused a decrease of 0·01 unit scale reading at 625 mμ after 20 min. incubation. Specific activity of the lipase was defined as the number of enzyme units/mg. protein in 1 ml. of reaction mixture.

Hydrolysis of cholesteryl esters was measured by titration of the free acid liberated according to Dole (1956) and by colorimetric determination of the free cholesterol formed. Free cholesterol was separated from its esters by thin-layer chromatography (Razin *et al.* 1963; Argaman & Razin, to be published) and estimated by the $FeCl_3$ reaction (Wycoff & Parsons, 1957).

Partial purification of lipase. Ammonium sulphate was added to cell extract of *Mycoplasma gallisepticum* (R) to 50 % saturation (31·3 g./100 ml.). The solution was left overnight at 4°; the resulting precipitate was centrifuged down at 25,000 *g* for 1 hr in the cold, and discarded. More ammonium sulphate was then added to the supernatant fluid to 80 % saturation (24·8 g./100 ml.) and the solution kept overnight at 4°. This second precipitate was collected by centrifugation at 25,000 *g* for 1 hr at 4° and dissolved in 0·02 M-phosphate buffer (pH 7·5). The solution was then dialysed at 4° for 15 hr against the same buffer with constant stirring. The buffer was changed several times during this dialysis. A granular precipitate which appeared in the dialysis bag was separated by centrifugation and discarded. The clear protein solution was then chromatographed on a DEAE-Sephadex A-50 (medium) column. Fractions were collected by a fraction collector and tested for lipase activity. The fractions containing lipase activity were combined, dialysed against de-ionized water overnight at 4° and freeze-dried.

Analytical methods. Butyric acid liberated during hydrolysis of tributyrin was identified by paper chromatography (Block, Durrum & Zweig, 1958). The developing solvent was *n*-propanol + concentrated ammonia, sp.gr. 0·880 (70 + 30, by vol.). The paper was sprayed with an aqueous solution of bromophenol blue (0·05 %, w/v) acidified with citric acid (0·2 %, w/v). The ammonium salt of butyric acid appeared on the paper as a blue spot on a yellow background.

Degradation products of triglycerides containing long-chain fatty acids were identified by thin-layer chromatography. The chromatoplates were covered with silica gel G containing Rhodamine (Razin *et al.* 1963). The lipid to be chromatographed was dissolved in a small volume of benzene and applied to the plates as a

short band. The developing solvent was light petroleum (B.P. 40–60°)+diethyl ether (50+50, by vol.) and the running time was about 1 hr. After chromatography, the tri-, di- and monoglycerides were visible under ultraviolet radiation as pink-yellow fluorescent areas on a pale-green background.

RESULTS

Occurrence of lipolytic activity in various Mycoplasma strains

All the Mycoplasma strains tested were found to hydrolyse tributyrin when grown on Edward medium plates containing this substrate (Pl. 1, fig. 2). The saprophytic *Mycoplasma laidlawii* strains showed the lowest lipolytic activity and the parasitic *M. gallisepticum* strains the highest (Table 1). Most of the work was therefore carried out with *M. gallisepticum* strains.

Table 1. *Hydrolysis of tributyrin by various Mycoplasma strains*

Reaction mixtures consisted of washed organisms, 40 μmoles tributyrin and 20 μmoles sodium taurocholate in 5 ml. 0·01 M-phosphate buffer (pH 7·5). The results represent the mean values obtained in 3 to 6 experiments with different batches of organisms.

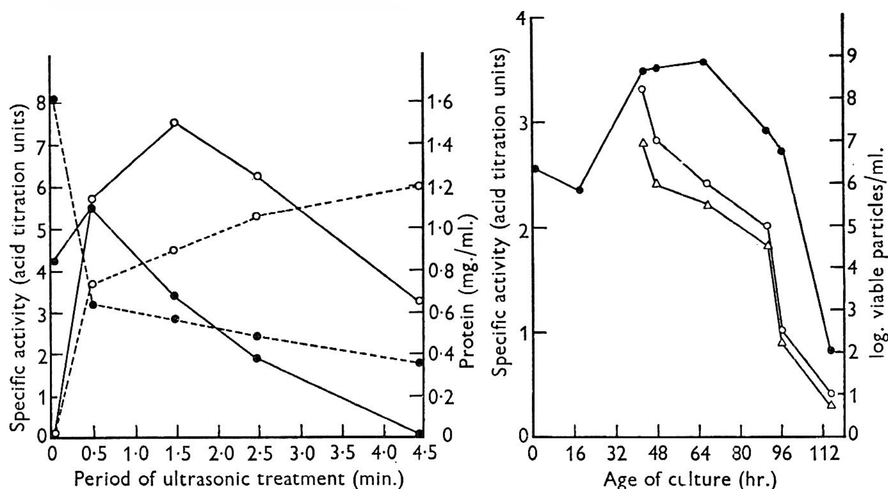
Organism	Specific lipase activity (μequiv. acid liberated/mg. protein/6 hr)
Mycoplasma gallisepticum (S6)	3·20
M. gallisepticum (R)	2·60
M. mycoides var. *mycoides*	2·10
M. mycoides var. *capri*	1·60
M. bovigenitalium	1·60
M. laidlawii strain A	0·66
M. laidlawii strain B	0·54
M. laidlawii oral strain	0·42

Localization of lipase activity in the Mycoplasma organism

The hydrolysis of tributyrin in and around the growth areas on tributyrin plates (Pl. 1, fig. 2) suggested that lipases of Mycoplasma are soluble and not cell-bound. Tributyrin was also hydrolysed by organisms grown on a Formvar film which prevented direct contact between the organisms and the substrate in the growth medium. When *Mycoplasma gallisepticum* organisms were disrupted by ultrasonic treatment, the lipase activity was found in the fraction not sedimented by centrifugation at 34,000*g* for 30 min. (Fig. 1). Lipolytic activity in this 'soluble' fraction was maximal after ultrasonic treatment for 1·5–2·5 min. Further treatment diminished enzymic activity, probably because of denaturation of proteins by the ultrasonic treatment (Hughes, 1961). Lipase activity in the cell-debris fraction was much lower than in the 'soluble' fraction. On centrifugation of the 'soluble' fraction at 100,000*g* for 30 min. the lipolytic activity remained in the supernatant fluid; the sediment was practically lipase-free. Lipase activity of cell-free extracts of *M. gallisepticum* did not significantly decrease during 6 months storage at 4°.

Relation of lipase activity to age of culture

The lipolytic activity of *Mycoplasma gallisepticum* whole organisms and cell extracts was highest at the logarithmic phase of growth, declining steeply afterwards. This decline paralleled the rapid death of *M. gallisepticum* in the glucose-containing Edward medium (Fig. 2).



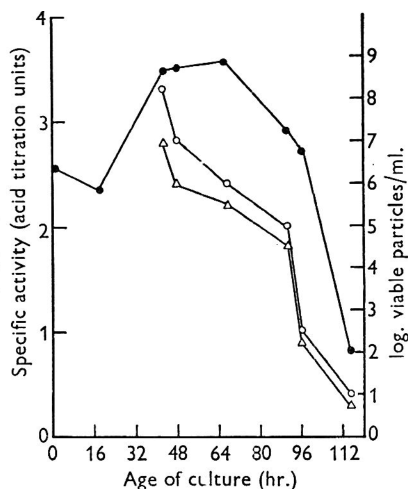


Fig. 1. Lipase activity of extract and debris of Mycoplasma gallisepticum (R) organisms treated ultrasonically for various periods of time. Cell debris was separated from cell extract by centrifugation at 34,000g for 30 min. Lipase activity was determined in the fractions obtained, with tributyrin as substrate. Cell extract (○); cell debris (●). ——, lipase activity; - - -, protein content of cell fraction.

Fig. 2. Lipase activity of Mycoplasma gallisepticum (R) harvested at different phases of growth. Lipase activity was determined as described in Table 1 with tributyrin as substrate. Lipase activity of washed organisms (△) and of cell-extracts (○). Number of viable particles (●) determined according to Butler & Knight (1960).

Specificity of Mycoplasma gallisepticum lipase towards various substrates

The cell extract of *Mycoplasma gallisepticum* hydrolysed glycerides of short- and long-chain fatty acids. Tributyrin was hydrolysed at a much higher rate than was trilaurin or triolein. The oleic acid esters, methyl oleate and 'Tween 80', were also hydrolysed, but only slowly (Table 2). The optimal pH value for hydrolysis varied with the nature of the substrate; thus mono-, di- and tributyrin were best hydrolysed at pH 7·5, trilaurin and triolein at pH 8·0. The rate of hydrolysis of methyl oleate and 'Tween 80' was about the same at pH 7·5 and 8·0 (Table 2). During the hydrolysis of triolein and trilaurin the corresponding di- and monoglycerides appeared in the reaction mixture, as shown by thin-layer chromatography. Cholesteryl acetate and stearate were not hydrolysed by washed organisms or extracts of *M. gallisepticum* (R) or by washed organisms of *M. gallisepticum* (S6). During the incubation no free fatty acids were liberated from the cholesteryl esters (Table 2), and the amount of free cholesterol did not increase, as shown by thin-layer chromatography. Hydrolysis of cholesteryl esters was tested at various pH values ranging from 6·5 to 9·0.

Partial purification of Mycoplasma gallisepticum lipase

The lipase of *Mycoplasma gallisepticum* was precipitated between 50 and 80% saturation with ammonium sulphate. The precipitated proteins were chromatographed on a DEAE–Sephadex A-50 column. Fractions 6 to 10 eluted from the column showed lipase activity, rising to a sharp peak in fractions 7 and 8 (Fig. 3). Table 3 summarizes the steps involved in the purification of the lipase, the degree of purification and the yield of enzyme.

Table 2. *Substrate specificity of Mycoplasma gallisepticum lipase*

The reaction mixture consisted of 5 ml. *M. gallisepticum* cell extract in 0·02M-phosphate tris buffer, containing 20 μmoles sodium taurocholate and substrate in the amounts indicated in the table.

Substrate	pH value of reaction mixture	μequiv. acid liberated/mg. protein in 6 hr	24 hr
Tributyrin (20 μmoles)	7·5	6·52	9·20
	8·0	4·54	6·90
Dibutyrin (30 μmoles)	7·5	4·23	—
	8·0	2·82	—
1-Monobutyrin (60 μmoles)	7·5	3·04	—
	8·0	2·39	—
Trilaurin (20 μmoles)	7·5	0·07	0·21
	8·0	0·42	0·96
Triolein (20 μmoles)	7·5	0·06	0·16
	8·0	0·32	0·62
Methyl oleate (60 μmoles)	7·5	0·10	0·23
	8·0	0·12	0·24
Tween 80 (1%, w/v)	7·5	0·22	0·52
	8·0	0·26	0·52
Cholesteryl stearate (20 μmoles)	7·5	0	0
	8·0	0	0

Table 3. *Partial purification of Mycoplasma gallisepticum lipase*

Step	Preparation	Volume (ml.)	Protein (mg./ml.)	Specific activity*	Degree of purification	Yield (%)
1	Crude cell extract	20·0	3·45	20·4	1·0	100·0
2	Supernatant fluid obtained after 50% saturation with $(NH_4)_2SO_4$	29·0	1·04	33·0	1·6	69·4
3	Precipitate formed at 80% saturation with $(NH_4)_2SO_4$	2·2	8·70	45·0	2·2	61·8
4	Chromatography on DEAE-Sephadex A-50					
	Fraction 7	4·0	0·052	594·0	27·6	25·6
	Fraction 8	4·0	0·050	495·0	24·1	
	Fraction 9	4·0	0·036	450·0	22·0	

* Determined by the turbidimetric method.

Properties of the partially purified lipase

The hydrolysis of the various triglycerides increased at about the same rate during the purification procedure (Table 4). Tributyrin was used as substrate in testing the properties of the partially purified lipase. Optimal temperature for activity was 37° and pH 7·5 was optimal (Fig. 4). The partially purified lipase was completely inactivated by heating at 65° for 10 min. (Fig. 5), and was rapidly inactivated when

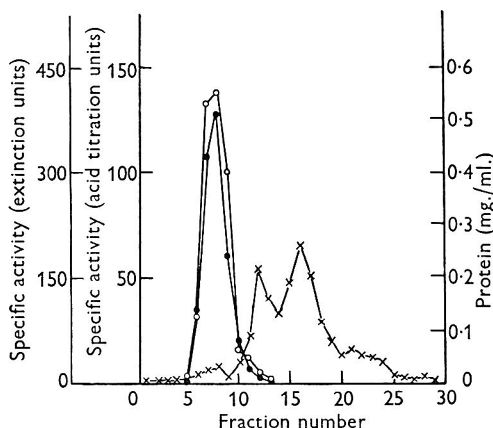


Fig. 3

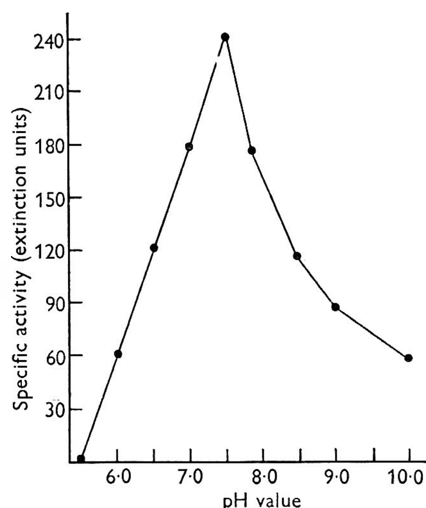


Fig. 4

Fig. 3. Partial purification of Mycoplasma gallisepticum lipase by anion-exchange chromatography of cell-extract proteins. Two ml., containing 12 mg. protein obtained by ammonium sulphate precipitation, were placed on a DEAE-Sephadex A-50 (medium) column (1·4 × 14 cm.) equilibrated with 0·02 M-phosphate buffer (pH 7·5). Elution was performed by successive addition of 20 ml. volumes of NaCl solution of increasing molarity (0·1, 0·2, 0·3, 0·4, 0·5 and 0·6 M-NaCl in 0·02 M-phosphate buffer, pH 7·5). Flow rate was about 20 ml./hr and fraction volume 4 ml. Hydrolysis of tributyrin by all fractions was tested by the acid titration (●) or by the turbidimetric (○) method. Protein concentration in fractions (×).

Fig. 4. Effect of pH value on the hydrolysis of tributyrin by the partially purified lipase of Mycoplasma gallisepticum. Lipase activity was determined by the turbidimetric method. The reaction mixtures contained 0·02 M-phosphate tris buffer.

Table 4. *Comparison of the hydrolysis rate of various triglycerides by the partially purified lipase and cell extract of Mycoplasma gallisepticum* (*R*)

Reaction mixtures as in Table 2. Hydrolysis of tributyrin was tested at pH 7·5 and that of trilaurin and triolein at pH 8·0.

Substrate	Specific lipase activity (acid titration units)	
	Partially purified lipase	Cell extract
Tributyrin	55·20	2·30
Trilaurin	4·21	0·24
Triolein	3·90	0·15

incubated in highly acid or alkaline solutions (Fig. 6). The divalent cations magnesium, calcium and manganese did not stimulate lipase activity. Prolonged dialysis of the enzyme solution, or addition of ethylenediaminetetra-acetic acid (EDTA) in a final concentration of 0·025 M did not affect enzymic activity.

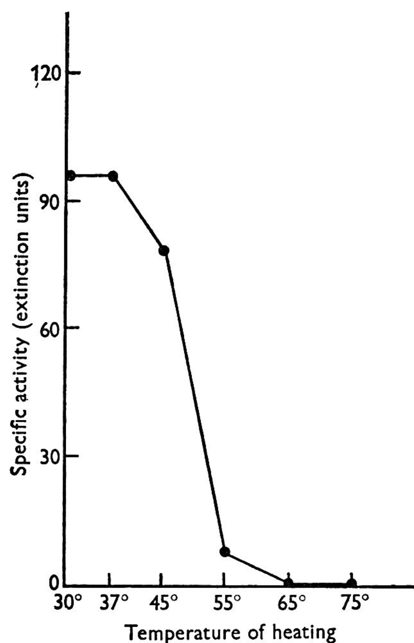


Fig. 5

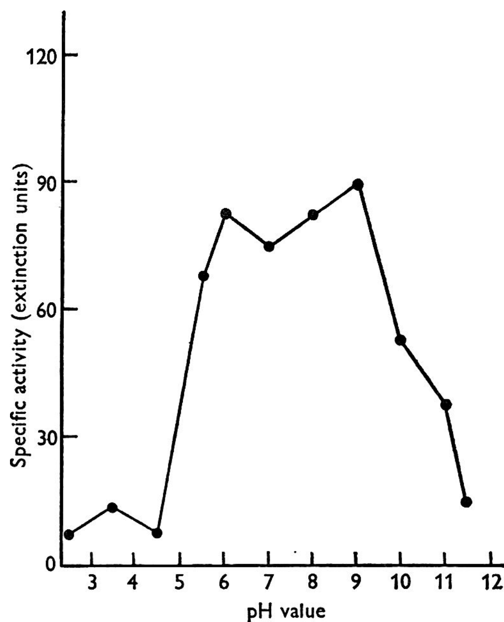


Fig. 6

Fig. 5. Heat inactivation of Mycoplasma gallisepticum lipase. The partially purified enzyme solution was heated for 10 min. at the temperatures indicated in the figure. Hydrolysis of tributyrin by the heated solutions was tested turbidimetrically.

Fig. 6. Inactivation of Mycoplasma gallisepticum lipase in acid or alkaline solutions. The pH value of the partially purified enzyme solution in 0·02 M-phosphate buffer was adjusted with 0·1 N-HCl or 0·1 N-NaOH to the values indicated in the figure. The enzyme solutions were incubated at 37° for 30 min. The ability of these solutions to hydrolyse tributyrin was tested after re-adjustment to pH 7·5.

DISCUSSION

The hydrolysis of tributyrin by all eight of the Mycoplasma strains tested indicated the widespread occurrence of lipolytic activity in this group of organisms. The saprophytic *Mycoplasma laidlawii* strains showed a much lower lipolytic activity than the parasitic strains. The saprophytic strains have already been found to differ from the parasitic strains in their nutritional requirements (Razin, 1962), lipid composition (Rothblat & Smith, 1961; Razin, Argaman & Avigan, 1963) and osmotic fragility (Razin, 1963). The lipases of mycoplasmas seem to resemble lipases of other micro-organisms in being soluble and not cell-bound (Davies, 1954). This was clearly shown for the lipase of *M. gallisepticum*. However, lipase activity of a human Mycoplasma strain was found to be associated with the cell membrane by Smith (1959).

The steep decline in the lipolytic activity of *Mycoplasma gallisepticum* at the end

of the logarithmic phase of growth may be explained in several ways. Activity may decline owing to oxidation or digestion of the lipase by autolytic enzymes (Sierra, 1957). The enzyme may be denatured by the high acidity developed in the growth medium; the pH value of Edward medium containing glucose (0·5 %, w/v) decreases to pH 5·2–5·4 during the decline phase of growth. At this low pH value the partially purified lipase of *M. gallisepticum* lost most of its activity (Fig. 6). The soluble lipase might also diffuse out of dying organisms. We detected no lipase activity in the growth medium after removal of the organisms. This may, however, be due to the relatively low activity of the lipase and its dilution in the growth medium.

The cell extract and the partially purified lipase of *Mycoplasma gallisepticum* hydrolysed triglycerides of short- and long-chain fatty acids, as well as methyl oleate and Tween 80. The rate of hydrolysis of trilaurin or triolein was only 1–10 % of that of tributyrin. The optimal pH value for hydrolysis also varied with the nature of the substrate. Dependence of the rate of hydrolysis and the optimal pH value on the nature of the substrate is a well-known characteristic of many lipases (Goldman & Rayman, 1952; Nashif & Nelson, 1953; Bier, 1955). Hence one enzyme only may be responsible for the hydrolysis of the various substrates by *M. gallisepticum*. The unchanged substrate specificity following purification of the lipase supports this assumption. The inability of *M. gallisepticum* organisms and cell extracts to hydrolyse cholesteryl esters has also been observed when radioactive cholesteryl oleate was used. *M. gallisepticum* incorporated this ester from the growth medium, but did not hydrolyse it (Argaman & Razin, to be published).

Formation of a film and spots in the growth medium due to degradation of lipids serves as a criterion for the classification of Mycoplasma (Edward, 1954; Freundt, 1958). In view of our results strictly defined conditions should be prescribed for this test. *Mycoplasma laidlawii*, for example, which is classified as a 'non-film and spots' producer (Freundt, 1958), will form a film and spots when Tween 80 and $CaCl_2$ supplement the Edward medium (Razin & Rottem, 1963).

The role of lipases in the physiology of Mycoplasma is yet to be elucidated. The lipases may hydrolyse lipids in the growth medium and liberate long-chain fatty acids required for growth of Mycoplasma (Rodwell & Abbott, 1961; Tourtellotte *et al.* 1963; Razin & Rottem, 1963). On the other hand, the liberated long-chain fatty acids may have a deleterious effect on the organisms when they accumulate above a certain concentration in the growth medium. The mycoplasmas, having only a thin lipoprotein membrane, are very susceptible to lysis by surface-active substances, including long-chain fatty acids (Edward & Fitzgerald, 1951; Rodwell, 1956; Smith & Boughton, 1960; Razin & Rottem, 1963).

This work forms part of an investigation made by S. Rottem in partial fulfilment of the requirements for the Ph.D. degree at the Hebrew University, Jerusalem. The study was supported in part by a grant from the Joint Research Fund of the Hebrew University-Hadassah Medical School.

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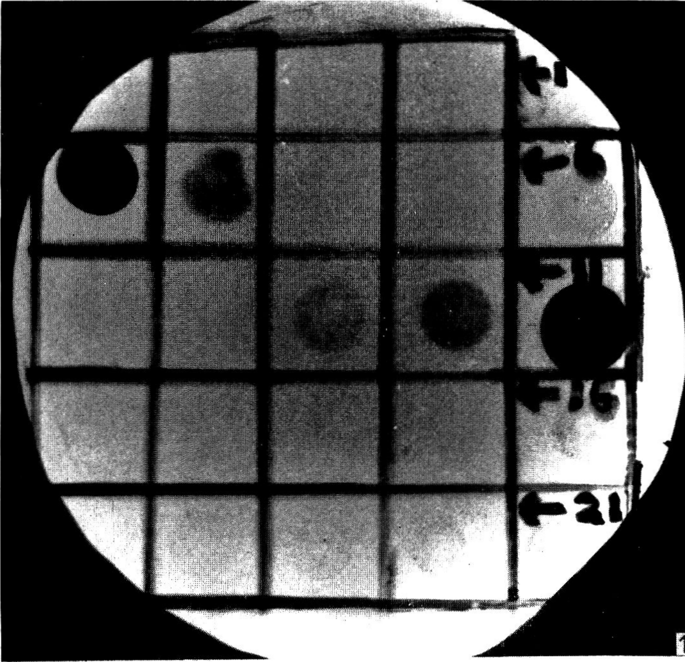
EXPLANATION OF PLATE

Fig. 1. Detection of lipolytic activity in fractions collected during anion-exchange chromatography of Mycoplasma gallisepticum proteins. Plates contained 0·4 % (w/v) tributyrin dispersed in 0·02 M-phosphate buffer (pH 7·5) and 0·9 % (w/v) Bacto-agar (purified). Drops (0·02 ml.) of the chromatographic fractions were placed on the plate, and lipolytic activity determined after incubation at 37° for 24 hr.

Fig. 2. Hydrolysis of tributyrin by various Mycoplasma strains. Drops (0·02 ml.) of washed suspensions of organism were placed on Edward medium plates containing 0·4 % (w/v) tributyrin; incubation at 37° for 48 hr. 1 = Mycoplasma laidlawii strain A; 2 = M. laidlawii strain B; 3 = M. mycoides var. capri; 4 = M. gallisepticum (R); 5 = boiled organisms of M. gallisepticum (R); 6 = 0·02 ml. crude pancreatic lipase solution (1 mg./ml.).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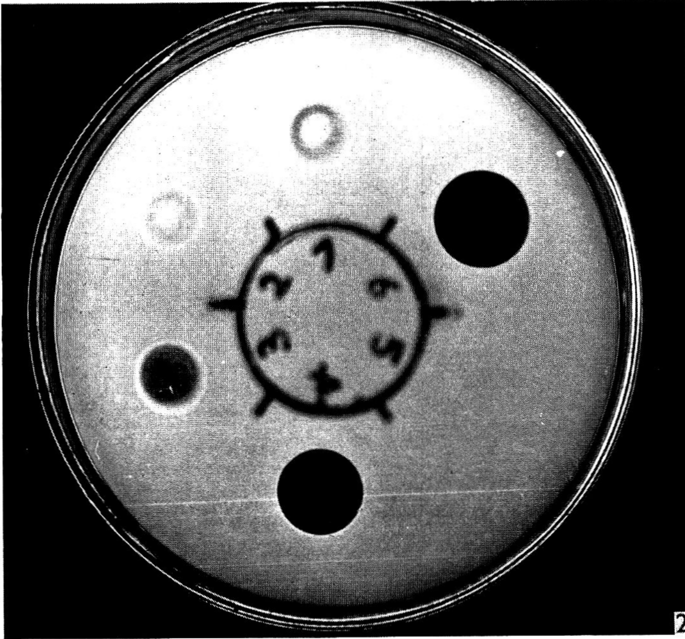
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The Type A Phages of *Salmonella typhimurium*: the Significance of Mixed Clones Arising from Singly-Infected Bacteria

By J. S. K. BOYD and D. E. BIDWELL

The Wellcome Laboratories of Tropical Medicine, The Wellcome Building, Euston Road, London, N.W. 1

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SUMMARY

Salmonella typhimurium Q1, when grown in standard medium at a temperature of 37°, is a uninuclear organism in which nuclear division precedes cell division, so that some 25 to 30% of the bacteria show various stages of nuclear division. When incubated at a lower temperature (e.g. 25°) nuclear division outruns cell division, and binucleate and quadrinucleate forms appear. In experiments at 37°, infection of Q1 or superinfection of Q1 (A1a) with temperate phage A1b temporarily arrests bacterial division, producing a lag period of 30–45 min. Decision to lyse is taken from 1 to 9 min. after exposure to infection, and the ensuing productive development leading to the release of a brood of temperate phage particles is an immediate sequel of this decision. Decision to lysogenize, as evidenced by immunity to a heavy challenge by homologous virulent phage, is taken within one minute of infection. 60 to 70% of bacteria infected or superinfected with a single phage particle produce pure clones of lysogenized, prophage-substituted, or doubly lysogenized bacteria. It is suggested that the mixed clones of uninfected and converted bacteria derived from the remaining 30 to 40% may result from the lysogenization of one-half of a nucleus already embarked on the process of division; evidence favouring this theory is discussed. It is concluded that, contrary to current belief, and under the conditions of our experiments, the infective material of a phage particle which has penetrated a sensitive bacterium proceeds with little delay either to become integrated with the chromosome as prophage or to undergo productive development and produce lysis, and does not remain for one or more bacterial generations an unattached cytoplasmic inclusion.

INTRODUCTION

The events which intervene between the penetration of a sensitive bacterium by the infective material of a temperate phage particle, and the ultimate attachment of this material to the bacterial chromosome as prophage, present problems of considerable interest. Several facts suggest that this process of lysogenization may be completed without delay and certainly before the infected bacterium divides. Thus, while the decision to lyse is usually taken within the first 10 min. both by virulent phage and by the virulent particles of temperate phage, the decision to lysogenize is made even more quickly, is immediately dominant over lysis, and confers an immunity which resists indefinitely the aggression of heavy infections of homologous virulent phage. Accompanying the development of this immunity, there is a

well-marked prolongation of the division time of the first generation of phage-infected bacteria which is not seen in subsequent generations, and which suggests a considerable but transient disturbance of the bacterial metabolism at nuclear level. This disturbance might well be related to the integration of the phage DNA into the bacterial chromosome. However, it is currently held (references will be given in the discussion) that the phage genetic material remains as a free cytoplasmic inclusion for one or more bacterial generations before it becomes prophage; accordingly during this period each division of the bacterium which carries the inclusion produces one potentially lysogenic and one sensitive daughter cell, thus giving rise to a mixed clone of sensitive and infected organisms. We found difficulty in reconciling this conclusion with certain observations made when working with *Salmonella typhimurium* Q1 and the A phages which lysogenize it, and decided to investigate the problem in more detail.

METHODS

The materials and methods used were mainly those described in previous papers of this series (Boyd, 1950; Boyd & Bidwell, 1957, 1959, 1961, 1962). The bacterial strains were *Salmonella typhimurium* Q1 and *S. typhimurium* Q1 (A1a). The phages, both temperate and their virulent mutants, were members of the A series. Phage A1b is believed to be identical with phage P22, since both were isolated from the same bacterial strain.

The smears subsequently stained by Giemsa's method were prepared on coverslips, dried in the incubator, fixed in absolute ethanol for 5 min., washed, hydrolysed in N-HCl for 9 min., very thoroughly washed in distilled water, stained overnight in dilute Giemsa stain (1 drop of Giemsa stain to 1 ml. of buffer in distilled water, pH 7·0), washed in the same buffer, dried and mounted. For convenient reference, details of certain of the methods used are given in the text.

Terminology. Clones of unchanged *Salmonella typhimurium* Q1 or Q1 (A1a) are designated 'uninfected'; clones showing lysogenization, prophage substitution or double lysogenization are designated 'converted'; clones containing both uninfected and converted bacteria are referred to as 'mixed'.

The nuclei of Salmonella typhimurium Q1

It is not infrequently stated that in the exponential state of growth rod-shaped bacteria are multinucleate, containing two or more nuclei (Bertani, 1953, 1958; Jacob & Wollman, 1953). This is a matter of some importance in investigating the progeny of newly lysogenized cells, as is also the occurrence of 'doublets', i.e. bacteria nearing the stage of division (Bertani, 1960). In lysogenization experiments it would obviously be ideal to use cultures in which division of the bacteria had been synchronized. However, in our hands attempts to produce synchronization by the method described by Lark & Maaløe (1954) were unsuccessful, and we had recourse to the use of cultures in which multiplication had been decreased to a minimum, though even in such preparations the percentage of bacteria in process of division was considerable. The number and developmental state of the nuclei was determined by examining the bacteria either by phase-contrast illumination or in stained smears. Samples were taken from standard cultures at the stage of preparation corresponding to zero-point in the experiments, i.e. immediately before the addition of

infecting or superinfecting phage. For phase-contrast examinations, 1 ml. of 40% (w/v) formaldehyde was added to 9 ml. of the culture, and a measured volume of the formalin-treated culture was added to an equal quantity of melted agar in a test tube, which was then thoroughly shaken. A small drop of this mixture was placed on a heated slide, a coverslip applied and pressed firmly down to give as thin a layer of agar as possible, and the edges of the preparation were sealed with melted Vaseline. The bacteria, being trapped in the now solidified agar, were quite motionless and easy to examine. The Giemsa-stained smears were examined with a $\frac{1}{16}$ in. oil immersion lens and a ×25 ocular. With strong illumination through a compensating condenser, the purple-stained nuclear masses were clearly defined. The two methods gave very similar results. In the smallest bacteria, which were short, almost oval in

Table 1. *Percentage of bacteria showing nuclear division in a standard broth culture of Salmonella typhimurium*

Samples were taken from standard broth cultures at the stage of preparation corresponding to zero-point in the experiments.

Expt. no.	Strain of *S. typhimurium*	No. of bacteria examined	% showing undivided nuclei	% showing incompletely divided nuclei	% showing completely divided nuclei	Total % showing division	
1	Q1	100	73	21	6	27	Examined by phase contrast
2	Q1	400	72	22	6	28	
3	Q1	1000	70	24	6	30	
4	Q1	500	73	22	5	27	
5	Q1 (A1a)	500	72	23	5	28	
6	Q1 (A1a)	1000	71	23·5	5·5	29	
7	Q1 (A1a)	500	72	23	5	28	
8	Q1 (A1a)	200	74	20	6	26	
9	Q1 (A1a)	1000	75·5	20	4·5	24·5	
10	Q1 (A1a)	1000	74·5	.	.	25·5	Examined as stained smears
11	Q1 (A1a)	300	76	.	.	24	
12	Q1 (A1a)	500	75	.	.	25	

shape, and obviously the product of a recent division, there was a single small nuclear mass. At the other end of the scale there were 'doublets' (Bertani, 1960) with two distinct nuclei separated by an indentation in the bacterial wall. Between these two extremes were intermediate forms in all stages of development towards division. As the bacterium elongated, the nucleus also became more rod-shaped and ultimately showed, at the middle of its long axis, an indentation which appeared before any corresponding indentation of the cell wall was seen. As far as *Salmonella typhimurium* Q1 is concerned, therefore, the doublet conception is correct. When in the logarithmic phase of growth at 37°, the bacterium is uninucleate, and two separate nuclei can be seen only when its division is imminent. Counts made in a number of preparations, both by phase-contrast illumination and by the direct examination of stained smears, are recorded in Table 1.

In some experiments an attempt was made to differentiate three grades, uninucleate, binucleate (on the point of division) and intermediate forms with elongated nuclei showing a greater or lesser degree of indentation. In others only two types were recorded, those with unindented ovoid nuclei and those with elongated nuclei showing indentations of all degrees up to complete division. In the cultures used in

these experiments, examined at the time when, in the lysogenization experiments, phage was added, approximately 5 % of the bacteria had double nuclear masses, and a further 20–25 % had elongated nuclei showing various degrees of indentation. None of the many thousands of bacteria examined was quadrinucleate.

Since these results were at variance with the findings of Lark, Maaløe & Rostock (1955), we repeated the experiment following more or less the technique used by these workers. A broth culture of *Salmonella typhimurium* Q1 which had been kept at a temperature of 25° for 5 hr was raised to 37° by adding broth warmed to 90°, and was maintained at 37° in a water bath. At intervals after the temperature was raised to 37°, smears were prepared and stained with Giemsa. Binucleate and quadrinucleate bacteria as described by Lark *et al.* (1955) were seen in the early smears, but after 60 min. at 37°, when active multiplication was established, the picture had reverted to normal, and the organisms in the culture had the characters we found in our preparations. It would therefore seem that, when growth takes place at a temperature of 25°, nuclear division runs ahead of cellular division, producing multinucleate forms. When, however, the culture is restored to the optimum temperature for multiplication, cellular division takes place immediately after nuclear division, so that the bacteria are uninucleate except when they are about to divide.

The lag period following bacteriophage infection

Preliminary experiments showed that the smooth progress of the growth curve of a culture of *Salmonella typhimurium* in broth was upset by minor and transient variations of temperature and by the addition of relatively small quantities (10 %) of the culture medium in which the organism was growing. To overcome these difficulties, all culture media, phage preparations, pipettes, etc., were maintained and used at a temperature of 37°. Broth, inoculated with the organism under test taken from an overnight culture on nutrient agar, was incubated for 2 hr at 37°. Two ml. of this culture were added to 15 ml. broth and incubated at 37° for a further 45 min., when it contained approximately 10^8 organisms/ml. This is the preparation referred to as a 'standard' culture. Of this culture 4·95 ml. were placed in each of two test tubes. To one, the control, 0·05 ml. of broth was added: to the other 0·05 ml. of a phage suspension adjusted to give, in the final concentration, 10^9 particles/ml. (or more or less, according to the phage : bacteria ratio required). The tubes were returned to the water bath at a temperature of 37° and, except in experiments in which it was necessary to time from the moment phage was added, a period of 5 min. was allowed for adsorption to occur. Thereafter samples were removed at predetermined intervals, diluted as required, and plated in 0·5 ml. quantities either on nutrient agar in the case of the controls, or on nutrient agar impregnated with virulent phage when counts of lysogenized bacteria were required.

Curves of the rate of multiplication of newly lysogenized bacteria in five members of the series are given in Fig. 1. The lag periods varied from 30 to 45 min. In the case of newly lysogenized *Salmonella typhimurium* Q1 (A1b), Q1 (A1c) and Q1 (A3) the rate of multiplication, once the lag period was passed, was similar to that of the control. In the case of *S. typhimurium* Q1 (A2d) and particularly Q1 (A2e), the rate of division was slower than normal.

Lag curves were also plotted (Fig. 2) in certain superinfections known to give a high rate of prophage substitution or double lysogenization (Boyd & Bidwell, 1962).

Superinfection of *Salmonella typhimurium* Q1 (A1a) with phage A1b and phage A1c provided good examples of the progress of bacterial multiplication following prophage substitution. As might be expected, the lag period in these superinfections was similar to that found in heavy infections of *S. typhimurium* Q1 (see Fig. 1). Subsequent multiplication was at a slightly slower rate than in a control culture of

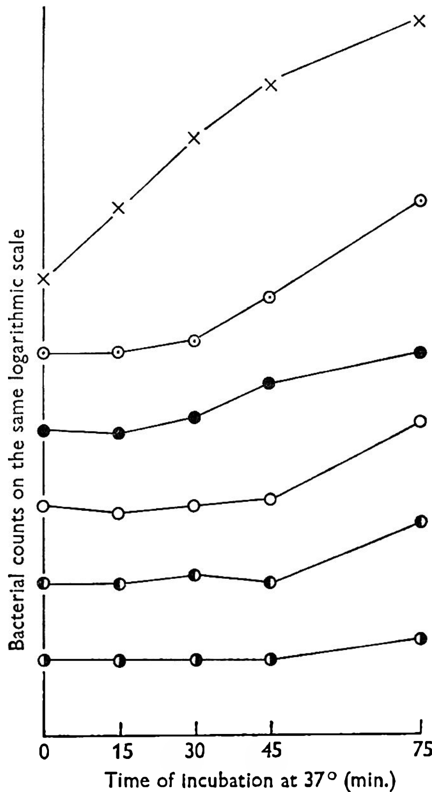

Fig. 1

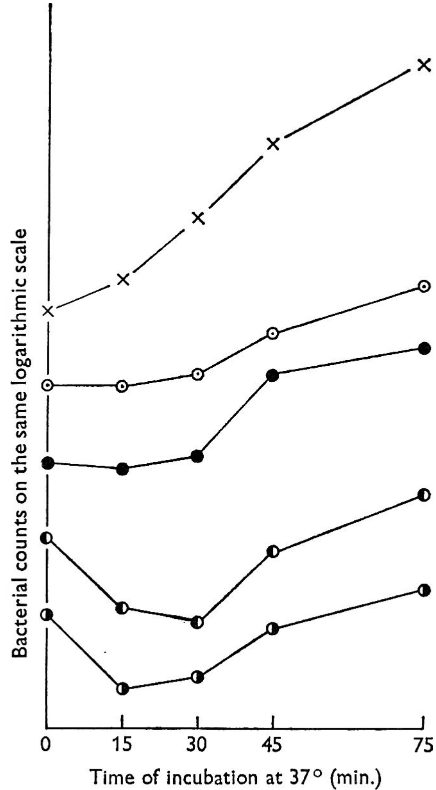

Fig. 2

Fig. 1. The lag period in simple lysogenization. The points on the curves are counts of viable bacteria made from cultures maintained at 37° in a water bath. The control is uninfected Salmonella typhimurium Q1, and the remaining five curves record the lysogenic bacteria in identical cultures exposed to the various phages in a phage:bacteria ratio of 10:1. The bacterial viable counts at zero were approximately 10^8/ml. The subsequent counts are plotted on the same logarithmic scale as the control, and therefore show comparable slopes. ×, is S. typhimurium Q1; ⊙, Q1 exposed to phage A1b; ●, exposed to phage A1c; ○, exposed to phage A3; ◐, exposed to phage A2d; ◑, exposed to phage A2e.

Fig. 2. The lag period in superinfection. Legend as in Fig. 1, except that the culture used was Salmonella typhimurium Q1 (A1a). In superinfection with phages A2d and A2e, note the decrease in viable bacteria during the first 30 min. of incubation. ×, is S. typhimurium Q1 (A1a); ⊙, is Q1 (A1a) exposed to phage A1b; ●, exposed to phage A1c; ◐, exposed to phage A2d; ◑, exposed to phage A2e.

S. typhimurium Q1 (A1a). Superinfection of *S. typhimurium* Q1 (A1a) with phages A2c, A2d and A2e (giving double lysogenization) produced an unexpected result. After incubation for 15 min. the number of viable doubly lysogenic organisms

decreased to about one-half the number present in the sample plated immediately after exposure. This finding was readily repeated. To confirm that this decrease in the number of lysogenized bacteria was not caused by lysis resulting from productive development, total counts (by microscope) and viable counts (by culture) of *S. typhimurium* Q1 (A1a) superinfected with phage A2d, and of a control culture of *S. typhimurium* Q1 (A1a), were carried out in parallel (Table 2). The total count showed no decrease corresponding to the decrease in number of viable doubly-lysogenized bacteria.

Table 2. *Correlation of total and viable counts of Salmonella typhimurium Q1 (A1a) and Q1 (A1a) superinfected with A2d*

A standard culture of *S. typhimurium* Q1 (A1a) was divided into two portions and phage A2d was added to one of them to give a phage:bacteria ratio of 10:1.

Total counts of bacteria were made by microscopical methods, and include both viable and non-viable organisms. Viable counts were made by plating measured quantities on nutrient agar, incubating overnight, and examining for its prophage content each colony which developed from the superinfected sample. The decrease by over 50% of the doubly-lysogenized organisms which took place during the first 15 min. of incubation had no counterpart in the corresponding total count nor in the counts of the control *S. typhimurium* Q1 (A1a).

Time of incubation (min.)	*S. typhimurium* Q1 (A1a)		*S. typhimurium* Q1 (A1a) superinfected with phage A2d		
	Total count	Viable count	Total count	Viable count Q1 (A1a)	Viable count Q1 (A1a, A2d)
0	$1\cdot45\times10^{8}$	$8\cdot1\times10^{7}$	$1\cdot4\times10^{8}$	$4\cdot56\times10^{7}$	$2\cdot04\times10^{7}$
15	$1\cdot72\times10^{8}$	$9\cdot26\times10^{7}$	$1\cdot45\times10^{8}$	$4\cdot4\times10^{7}$	$9\cdot66\times10^{6}$
30	$1\cdot99\times10^{8}$	$1\cdot5\times10^{8}$	$1\cdot48\times10^{8}$	$5\cdot0\times10^{7}$	$1\cdot1\times10^{7}$

Development, subsequent to penetration, of infecting or superinfecting phage

Most of the reported observations on the processes of lysis and lysogenization have been made on bacteria exposed to concentrations of phage sufficiently high to give a preponderance of multiple infections. We have preferred to experiment with a low phage:bacteria ratio, giving single infections of most of the bacteria. Such a technique is laborious because of the large proportion of blanks (uninfected bacteria) in every experiment, but gives results which avoid any fallacy which may result from multiple infection. Earlier work (Boyd & Bidwell, 1961) showed that when a culture of *Salmonella typhimurium* Q1 was exposed to a concentration of temperate phage sufficiently low to give single infections, some of the bacteria were lysogenized and others were lysed after productive development of the phage. Under standard conditions all the type A phages produce relatively constant proportions of lysogenesis and lysis, but each member of the series has its own lysogenesis:lysis ratio. We explain this phenomenon by postulating: that the particle which becomes prophage is adequately endowed with some property essential for establishing lysogenesis; that the temperate particle which produces lysis is deficient but not wholly lacking in this property; that the virulent mutant, which by the use of a suitable technique can be recovered from most temperate phage suspensions (Boyd, 1951) and which is incapable of producing lysogenesis, is completely devoid of it. These concepts are recapitulated here because they have an important bearing on the interpretation of the experiments which follow.

Decision to lyse; virulent phage. Lwoff, Kaplan & Ritz (1954) found that when *Salmonella typhimurium* 1404 (an indicator strain used in earlier experiments) was exposed simultaneously to high concentrations of a temperate A1a phage and a virulent mutant of this same phage (temperate phage ratio 11:1, virulent phage ratio 9:1) the temperate phage proved to be dominant, and produced the same percentage of surviving (lysogenized) bacteria as a control in which the bacteria were exposed only to the temperate phage. These workers also did lysogenization experiments: (*a*) in media to which 5-methyltryptophan had been added, (*b*) with incubation at a temperature of 42°, and (*c*) in medium containing citrate which was subsequently neutralized by the addition of magnesium sulphate. The results were claimed to show that the critical time at which the decision to lyse or lysogenize was reached lay between the 6th and 9th minute after infection, and that at this time the genetic material of the phage became irrevocably committed to one or other of these lines of development.

Table 3. *Domination of temperate phage over virulent phage*

Standard preparations were used. The % of *Salmonella typhimurium* survivors was ascertained by plating suitably diluted quantities on nutrient agar. All survivors in (2) were found to be lysogenic.

	Time of incubation (min.)				
	0	3	6	9	12
	% of surviving *S. typhimurium* organisms in samples taken after incubation for times indicated				
(1) Virulent phage A1b added to *S. typhimurium* Q1 to give a 10:1 ratio	56·2	4·8	0·33	0·7	0
(2) Same as (1), but with temperate phage A1b added in a 10:1 ratio at the time of sampling, and allowed to act for 10 min. before plating	75·2	38·8	8·6	0·73	0

We have repeated the first of the experiments of Lwoff *et al.* with *Salmonella typhimurium* Q1 and phage A1b (Table 3); the culture of *S. typhimurium* Q1 was infected with a 10:1 ratio of the virulent mutant of phage A1b, and to this mixture temperate phage A1b was added at intervals to give a 10:1 ratio. As in the experiment of Lwoff *et al.*, the protective power of the temperate phage was well marked when it was added shortly after the bacteria had been exposed to the virulent phage, but this protective power was absent when the temperate phage was added 9 min. after this exposure. However, it is noteworthy that, in terms of the time-interval, the decline in the protective power of the temperate phage was graded, and that, after as short an interval as 3 min., approximately only one-half of the bacteria which were protected at zero time were 'saved' by the temperate phage.

Decision to lyse; temperate phage. Salmonella typhimurium Q1 was exposed to a concentration of temperate phage A1b calculated to give 15–20% of lytic reactions (Boyd & Bidwell, 1961), and an excess of the same temperate phage was added to portions of this infected culture at 3-min. intervals (Table 4). The time interval until the decision to lyse became irreversible was rather longer than in the case of sensitive

bacteria exposed to virulent phage. In some of the bacteria the point of no return was reached in 6 min.: after 12 min. the addition of excess temperate phage did not produce any protective action.

Table 4. *Time of decision to lyse (i.e. undergo productive development). Temperate phage A1b and Salmonella typhimurium Q1*

Temperate phage A1b to give a low phage:bacteria ratio was added to a standard culture of *S. typhimurium* Q1 and the resulting mixture distributed into a number of test tubes which were then incubated at 37°. Excess of temperate phage A1b to give a high phage:bacteria ratio was added to successive tubes at 3 min. intervals. The % of 'bursts' was estimated by the method described in a previous communication (Boyd & Bidwell, 1961).

Mixture of temperate phage A1b and *S. typhimurium* Q1 to give a phage:bacteria ratio of 0·5:1				
No further temperate A1b added	Temperate phage A1b to give a phage:bacteria ratio of 10:1 added after incubation for time (min.) indicated			
	3	6	9	12
% of 'bursts'				
14	0	2·1	7	18·4

Table 5. *Time of decision to lysogenize; temperate phage A1b and Salmonella typhimurium Q1*

Temperate phage A1b was added to 50 ml. of a standard culture of *S. typhimurium* Q1 to give a phage:bacteria ratio of 0·5:1, and the mixture distributed in 0·5 ml. quantities into a series of tubes and incubated at 37°. From one series of tubes samples were taken at 3 min. intervals and the % of lysogenized bacteria determined by plating 0·5 ml. of 10^{-5} dilution on a lawn of *Salmonella typhimurium* T gal^{-} on galactose agar (Boyd & Bidwell, 1961), this technique being adopted in preference to the 'impregnated agar' method to avoid exposing the bacteria to excess of virulent phage A1b. To a second series virulent phage A1b to give a 10:1 ratio was added at corresponding intervals, and after incubation for a further 5 min. the % of bacteria lysogenized was determined by plating 0·5 ml. of 10^{-5} dilutions on nutrient agar and counting the lysogenic colonies which developed.

	Temperate phage A1b added to *S. typhimurium* Q1 in a 0·5:1 ratio		
	% of bacteria lysogenized		
Time of sampling (min.)	In control	Virulent phage A1b added to give a 10:1 ratio	Time of adding virulent A1b (min.)
1	3	5·6	1
4	10·6	14·3	4
7	17·6	19·6	7
10	15·5	16·3	10
13	12	16·3	13

Decision to lysogenize; temperate phage. A standard culture of *Salmonella typhimurium* Q1 was infected with a low concentration of phage A1b, giving a phage: bacterium ratio of 0·5:1, and then divided into two portions. From the first portion (the control) samples were removed at intervals and the percentage of lysogenized bacteria therein calculated. At the same intervals virulent phage A1b was added to

measured volumes from the second portion to give a 10:1 ratio. After incubation for 5 min. to allow the virulent phage to be adsorbed, the percentage of lysogenized bacteria in this test series was also calculated. The results (Table 5) show that the percentage of lysogenic responses was slightly greater in the test series than in the control, a variation which can be attributed to differences in the techniques followed. The apparent delay in lysogenization seen in both control and test can be accounted for by the low phage concentration and the interval of time which consequently elapsed before all the phage particles came in contact with bacteria, since a standard culture of *S. typhimurium* Q1 exposed to a high concentration of temperate phage A1b produced maximum lysogenization much more quickly (Table 6). The salient fact which emerges from the experiment recorded in Table 5 is that the temperate phage had established its decision to lysogenize in the earliest stages of the test, and maintained this decision in the face of a heavy challenge by virulent phage.

Table 6. *Establishment of immunity*

Temperate phage A1b was added to *Salmonella typhimurium* Q1 in a 10:1 ratio. Samples were taken at the times recorded, diluted to 10^{-4} in antiphage serum to neutralize free phage, and plated on agar impregnated with virulent phage A1b. The percentages of bacteria lysogenized (immune to virulent phage) were calculated in terms of a control culture not exposed to either temperate or virulent phage.

Time of sampling (min.):	0	0·5	1	3	5	10
% of bacteria lysogenized	37	45	82	80	81	81·9

Onset of productive development. Productive development is an immediate sequel to the decision to lyse. This is readily seen when the phage: bacteria ratio is low, say 1:10 (Fig. 3). Adsorption led to a decrease in the count of free phage particles for about 15 min. (More detailed experiments, not recorded here, showed that adsorption was in fact overtaken by phage production in 18–20 min.) Thereafter phage production continued actively for the next 45 min., reaching in about 40 min. a concentration sufficient to produce, quite abruptly, lysogenization in the majority of the bacteria which had escaped infection at the beginning of the experiment. There was no detectable clearing of the culture until about the 80th min., and little change in the total count of viable bacteria in 120 min.

Establishment of lysogenesis. In doing experiments to determine the immediate fate of the infective material of a lysogenizing temperate phage which has just penetrated a sensitive bacterium, it was deemed advisable to eliminate certain possible sources of error. The first precaution is one which we believe to be of great importance. Only stable 'wild' phages were used: they were from lines which have never been subjected to artificial mutagenic influences, and which, over the many years they have been in use, have never produced any mutants other than the virulent type previously described (Boyd, 1951) which can be recovered without difficulty from all the temperate A phages except phage A4. No semi-virulent forms have ever been encountered, nor when culturing together different members of the group have we, with one possible exception, found any evidence of genetic recombination, although this possibility was constantly in mind. Secondly, we have confined our observations to experiments with low multiplicities calculated to produce a majority of single infections, and we have not used more than one type of infecting phage in any one experiment.

Two slightly different techniques were used to obtain clones from single organisms. In one a standard culture of *Salmonella typhimurium* Q1 (or Q1 (A1a) for superinfections) containing about 10^8 bacteria/ml. was infected with phage A1b at a 0·5:1 ratio. After 5 min., or in some experiments 10 min., at 37° to allow adsorption to take place, the culture was diluted $10^{-7} \times 1/16$ in broth, and a 0·4 ml. sample of this dilution placed in each of 100 to 150 small sterile tubes, giving a distribution of one bacterium per four tubes. After further incubation for a standard time (as

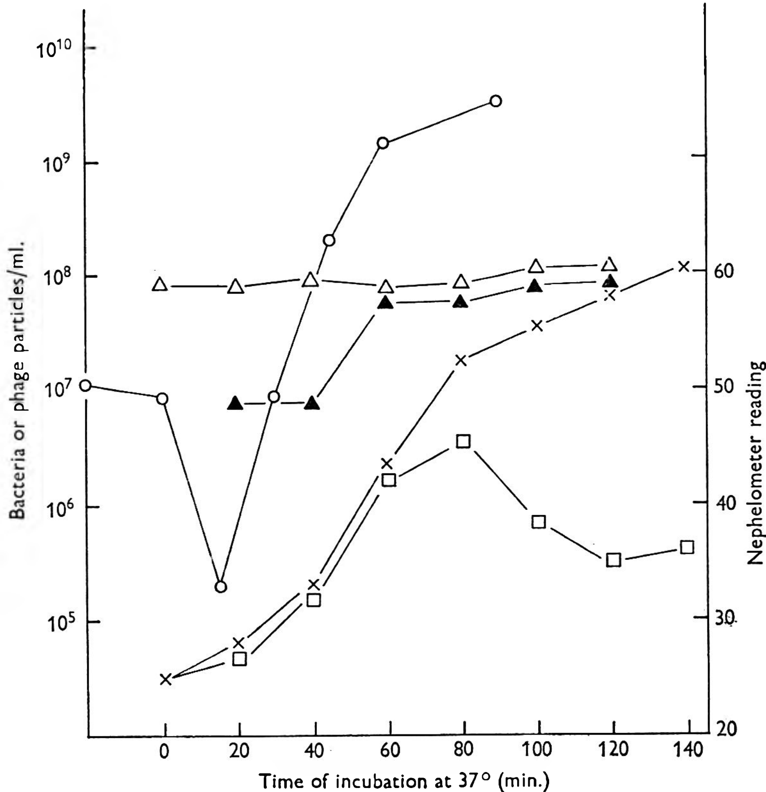

Fig. 3. Onset of lysis, primary and secondary lysogenization, and clearing of the culture. A standard culture of Salmonella typhimurium Q1 was exposed to phage A1b in the low phage:bacteria ratio of 1:10. Lysis, revealed by the production of free phage, began around 15 min. Primary lysogenization was at its peak by 20 min. Secondary lysogenization of residual sensitive bacteria took place between 40 and 60 min., when there was a high concentration of free phage. Clearing of the culture did not occur until around 80 min. ○, free phage; △, count of all viable bacteria, lysogenized and non-lysogenized; ▲, count of lysogenic bacteria; ×, turbidity curve of control; □, turbidity curve of infected culture.

detailed later) the contents of each tube were flooded over a plate of nutrient agar and incubated overnight. Colonies developed on about 25% of the plates, and each colony on every plate was examined for lysogenicity. Where the second technique was followed, the infected culture was distributed, not into small tubes, but into 5 ml. screw-cap bottles. After the standard period of incubation, each bottle was half filled with melted nutrient agar at 45° and culture and agar thoroughly mixed.

The bottles were then placed in the hot-room (37°) and left there overnight. Colonies which developed in the depth of the medium were easily reached with a platinum needle and were subjected to the usual examination for lysogenicity. The di-advantage of this second method was that, in a variable number of bottles in each batch, one or more of the bacteria found its way into the water of condensation and

Table 7. *The average number of Salmonella typhimurium bacteria arising from the division of a single organism after standard incubation*

Temperate phage A1b was added to standard cultures of *S. typhimurium* Q1 and Q1 (A1a) in a ratio of 0·5:1 and, after incubation for 10 min. at 37°, diluted and distributed in tubes or 5 ml. screw-cap bottles in quantities calculated to give not more than 1 organism per tube or bottle. After further incubation sufficient to allow two or three bacterial divisions to occur (*a*) the contents of each tube were poured over a plate of nutrient agar, or (*b*) a quantity of nutrient agar was added to each bottle and thoroughly mixed with the culture. Each colony which developed after incubation was examined for lysogenicity. The figures are compiled from the results of 27 experiments.

S. typhimurium Q1 exposed to phage A1b				*S. typhimurium* Q1 (A1a) exposed to phage A1b			
Type of colony	No. of tubes or bottles	No. of colonies	Average	Type of colony	No. of tubes or bottles	No. of colonies	Average
Q1 (unchanged)	110	511	4·6	Q1 (A1a) unchanged	107	487	4·55
Q1 (A1b)	30	89	3	Q1 (A1b)	20	58	2·9
Mixed	20	41	2·05	Mixed	17	59	3·5
Q1		57	2·85	Q1 (A1a)		48	2·8
Q1 (A1b)				Q1 (A1b)			

Table 8. *Progeny of single organisms of Salmonella typhimurium Q1 exposed to a low concentration of phage A1b*

For technique see Table 7.

			Converted			
	Uninfected clones		Pure clones Q1 (A1b)		Mixed clones Q1 and Q1 (A1b)	
Expt. no.	Total	Clones of 10 or more organisms	Total	Clones of 10 or more organisms	Total	Clones of 10 or more organisms
1	15	1	5	—	—	—
2	22	1	7	—	3	1
3	14	1	2	—	3	—
4	6	1	4	—	3	1
5	9	2	2	—	2	—
6	15	—	1	—	—	—
7	8	—	2	—	—	—
8	11	1	2	—	1	—
9	2	—	3	—	1	—
10	8	—	3	—	5	—
11	5	—	3	—	1	—
12	10	—	3	—	1	—
13	11	—	5	—	—	—
14	12	—	6	—	2	—
Grand total	148	7	48	—	22	2

in multiplying overgrew the surface of the medium; bottles thus affected were discarded. The advantage of this method was economy of medium, and the certainty that all viable bacteria were examined, since none was lost by transfer from one container to another.

To ascertain the optimum time of incubation needed for adequate but not excessive multiplication of the bacteria, calculations were made from the lag-curve experiments already described. They showed that, in these experiments which began at a

Table 9. *Progeny of single organisms of Salmonella typhimurium Q1 (A1a) exposed to a low concentration of phage A1b*

For techniques see Table 7.

	Uninfected clones		Converted: Pure clones of Q1 (A1b)		Converted: Mixed clones of Q1 (A1a) and Q1 (A1b)	
Expt. no.	Total	Clones of 10 or more organisms	Total	Clones of 10 or more organisms	Total	Clones of 10 or more organisms
1	16	1	1	—	2	—
2	13	—	1	—	—	—
3	10	—	2	—	5	—
4	17	—	5	—	1	1
5	8	—	3	—	—	—
6	11	—	1	—	5	1
7	3	—	1	—	2	—
8	12	—	5	—	1	—
9	15	2	—	—	3	—
10	8	—	2	—	1	—
11	7	1	1	—	4	—
12	5	—	4	—	—	—
13	10	—	4	—	1	—
14	10	1	4	—	2	—
Grand total	145	5	34	—	27	2

Table 10. *Percentages of pure clones and mixed clones of Salmonella typhimurium resulting from infection or superinfection with single particles of phage A1b*

The figures are from Tables 8 and 9. Bottles or tubes producing ten or more colonies have been excluded on the assumption that they were seeded with more than one bacterium.

S. typhimurium Q1 exposed to phage A1b				*S. typhimurium* Q1 (A1a) exposed to phage A1b			
		Converted				Converted	
Total	Uninfected	Pure clones of Q1 (A1b)	Mixed clones of Q1 and Q1 (A1b)	Total	Uninfected	Pure clones of Q1 (A1b)	Mixed clones of Q1 (A1a) and Q1 (A1b)
Number of clones							
209	141	48	20	198	140	34	25
Relative % of pure and mixed clones							
.	.	70·6	29·4	.	.	57·6	42·4

point when exponential growth was well advanced, the number of viable bacteria increased on an average four times in 105 min. The plates or bottles were accordingly incubated from 105 to 120 min. and were expected to give up to a maximum of ten colonies. The presence of more than ten colonies—a rare occurrence—was taken as evidence that the tube or bottle had been charged with more than one bacterium, and this tube or bottle was therefore excluded from further calculations.

In all, thirty such experiments were performed. In fifteen of these, *Salmonella typhimurium* Q1 was infected with phage A1b, and in the other fifteen *S. typhimurium* Q1 (A1a) was superinfected with this same phage. The results are recorded in Tables 7, 8, 9 and 10. In calculating the average number of bacteria per tube (Table 7) the results of three experiments in which the standard incubation period was exceeded have been excluded. Tables 8 and 9 give the findings in detail, showing the number of tubes or bottles in which only unaffected bacteria were found, the number of tubes or bottles containing only bacteria which had undergone conversion (lysogenization or prophage substitution), and the number of tubes or bottles with mixed clones. These figures, after certain corrections had been made, are consolidated in Table 10. The findings are analysed in the discussion. They show that the majority of the bacteria which were infected by phage produced pure converted clones.

DISCUSSION

By direct observation it has been shown that, contrary to the experience of other workers who used different conditions of culture (Lark *et al.* 1955), *Salmonella typhimurium* Q1, as we have prepared and used it, has a single nucleus. This, before the division of the bacterium as a whole, elongates, becomes indented or dumb-bell shaped, and finally divides. *S. typhimurium* Q1 is therefore not a binucleate organism in the accepted sense of the term, and is comparable to any other cell which periodically divides into daughter cells, differing from the cells of higher orders only in the short duration of the generation cycle. However, some 25% of the cells in our standard cultures showed nuclei in varying stages of division, and it is possible that earlier changes, not detectable by microscopic examination, had taken place in an even larger percentage of the organisms. It is to be noted that the percentages of organisms which showed signs of nuclear division (Table 1) were calculated in terms of the *total* count, which includes viable and non-viable bacteria. We have shown elsewhere (Boyd & Bidwell, 1961) that about 1/3 of the bacteria in the cultures we use are non-viable. If non-viable organisms are stillborn, or become non-viable at an early stage in their growth cycle, then the percentage of dividing bacteria, calculated in terms of *viable* bacteria, would be considerably higher than the figures given in Table 1. However, we have no experimental evidence to show the stage at which non-viability supervenes. Bertani (1960) drew attention to the significance of 'doublets' (bacteria which have gone through the internal processes of division, but in which the potential daughters still remain attached to each other, so that for purposes of counting or distribution they remain a single organism) and the somewhat anomalous results which may follow the infection of one-half of such a doublet by a phage particle. Presumably a bacterium may be infected by a phage particle at any stage in its cycle of development, and therefore in 25% or more the nucleus will have embarked on the process of division when penetration by the phage

occurs. The bearing which this may have on the occurrence of mixed clones will be discussed later.

The lag-period curves in uncomplicated lysogenization of *Salmonella typhimurium* Q1 (Fig. 1) shows clearly that the inhibition of bacterial division is largely confined to the first generation, and that subsequently the lysogenized bacteria multiply at approximately the same rate as do the unlysogenized control organisms, though there are exceptions to this rule. Superinfection of *S. typhimurium* Q1 (A1a) with phages A1b and A1c (Fig. 2) follows much the same pattern as simple infection of *S. typhimurium* Q1, with the maximum delay affecting the first generation, but showing a slightly decreased division rate in subsequent generations. The curves given by superinfection with phages A2d and A2e, which produce double lysogenization (Boyd & Bidwell, 1962), are of a peculiar and distinctive pattern. The remarkable decrease in the count of doubly-lysogenized organisms which occurs in the first 15 min. of the experiment is not paralleled by a similar decrease in the number of organisms as counted by the microscope and therefore cannot be the result of bacterial destruction caused by productive development or 'lysis-from-without'. Since adsorption of phage which has been presented in high concentration continues during this period, it is possible that multiple infection of these doubly-lysogenized organisms may render them non-viable without bringing about immediate lysis. Some similar effect may be responsible for the flat curve following infection of *S. typhimurium* Q1 with phage A2e. Leaving aside these exceptions, the balance of evidence indicates that the major disturbance of bacterial metabolism, as shown by delayed division, occurs in the newly infected or superinfected organism. This is compatible with the conception that the phage establishes its union with the bacterial chromosome before bacterial division occurs, and in doing so upsets the timing of the routine mechanism of division. Conversely, it is unlikely that a cytoplasmic inclusion which persists as such through several generations would disturb the division of the first bacterial division but not of subsequent generations. As can be seen in Table 7, the numbers of uninfected and converted organisms in mixed clones were approximately the same; i.e. there was no runaway multiplication of the uninfected bacteria to suggest that they divided more rapidly than did the converted bacteria.

That productive development is the immediate sequel to the decision to lyse is clearly demonstrated by the results set out in Fig. 3. In so far as the decision to lysogenize is revealed by the establishment of immunity (i.e. protection against the lethal productive development of virulent phage) it can be seen that, given an equal start, this decision precedes and dominates the decision to lyse taken by virulent phage (Tables 3, 4 and 5). Even though immunity stems from the action of only a single lysogenizing particle, it cannot be overborne by a heavy multiple infection with virulent phage (Table 5). Can such immunity be conferred by the lysogenizing particle as a freelance cytoplasmic inclusion, or does it indicate that the phage has linked up with the bacterial chromosome as prophage and, in its role of supplementary gene, is playing its part in regulating the metabolism of the organism by causing a specific repressor to be elaborated?

It was to confirm or refute the suggestion that the phage DNA remains for one or more generations as a cytoplasmic inclusion or pre-prophage that the experiments recorded in Tables 7, 8, 9 and 10 were performed. The total number of viable bacteria

cultured from each tube in the various experiments provides information of some significance (Table 6). Where converted clones developed, the average number of bacteria was significantly lower than in the clones of non-infected bacteria. This is of course, a result of the lag period. The total number of bacteria in the mixed clones was slightly higher than in the non-infected clones, but the number of converted bacteria was practically the same in both the mixed and converted series, namely 3, 2·85, 2·9 and 2·8, respectively. This strongly suggests that in both series the process of division had been in progress for the same length of time or, in other words, that in pure converted clones and in mixed clones the parent lysogenic bacteria took origin about the same time, and that in the mixed clones lysogenesis was not delayed until the pre-prophage had passed through one or more generations as an unattached cytoplasmic inclusion. Further evidence to this effect is to be found in the relative proportions of uninfected and converted organisms in the mixed clones. If the pre-prophage does in fact remain unattached for one or more bacterial generations, then the first bacterial division will produce an uninfected organism and one containing pre-prophage. If during this generation the pre-prophage becomes prophage, then after the next bacterial division the clone will contain two uninfected and two converted bacteria, and in subsequent divisions the numbers of the two types will remain approximately equal. If, however, the pre-prophage is again carried over unattached, the score would be three uninfected and one infected bacteria. Thus it is to be expected that the uninfected bacteria would always outnumber the infected or converted ones. However, this does not happen. Results, not recorded in the tables, show that in the phage A1b versus *Salmonella typhimurium* Q1 experiments, 13 of the 20 mixed clones gave a distribution of this pattern, but in the remaining 7 clones the position was reversed, as one or two uninfected organisms occurred in association with from three to seven converted ones. In the superinfection experiments 9 of 24 mixed clones showed similar reversed proportions of non-infected and converted organisms.

The consolidated figures in Table 9 show that when *Salmonella typhimurium* Q1 was exposed to a low multiplicity of infection with phage A1b (a single infection in the majority) some 70% of the infected bacteria were converted before division occurred, and so produced pure clones: the remaining 30% gave mixed clones. Corresponding figures for the superinfection of *S. typhimurium* Q1 (A1a) with phage A1b are roughly 59 and 41%. Clearly lysogenization—the integration of the phage genetic material as prophage into the chromosome of the bacterium—occurred before the first division in the majority of cases. What then is the explanation of the mixed clones? With the distribution technique which was used and the corrections which were made, it cannot be attributed to the introduction of two separate bacteria into one tube. On the other hand, it may be related to infection of a bacterium in which the nucleus has already embarked on the process of division. The conception of unilateral infection of a doublet—a cell in which nuclear division is complete although separation of the daughters has not yet taken place—presents no difficulty. But, if infection occurs during the early stages of nuclear division, what happens? It seems improbable that an undivided 'pre-prophage' could attach itself to a partially-divided nucleus and accelerate its own rate of division so as to come into step with the dividing chromosome and thus produce ultimately two prophage-bearing daughter chromosomes. The alternative would be to remain unattached until

division of the chromosome had reached the stage when two separate sites of attachment, one on each of the daughter chromosomes, were available, and then to become attached as prophage to one of these sites. This would result in a unilaterally lysogenic doublet. Followed to its logical conclusion, this theory implies that every organism in which nuclear division has started when it becomes infected with a single particle of temperate phage is destined to become a unilaterally infected doublet and the potential parent of a mixed clone. In terms of this theory the conception that, after penetration of the bacterium, the phage DNA becomes pre-prophage and remains for some time a free cytoplasmic inclusion would be correct in a limited sense. In an organism in which nuclear division had started, the pre-prophage would remain 'free' until such division was completed. There is no reason, however, to think that it would remain unattached for one or more bacterial generations.

In summary, it may be said that the results of the present culture experiments, together with various items of indirect evidence (lag period, early establishment of immunity, analogy of decision to lyse and immediate onset of productive development), suggest that in single infections and superinfections with stable temperate phage, in this particular *Salmonella typhimurium* system, lysogenization is completed before the infected bacterium divides. When the phage penetrates the bacterium before the start of nuclear division the result in culture is to produce a pure clone of lysogenic bacteria. When infection occurs after nuclear division has started, the phage DNA remains as pre-prophage until the nucleus divides, and then becomes attached as prophage to one of the halves, forming a unilaterally lysogenic doublet. If such a unilaterally lysogenic doublet is separated and cultured individually before it divides, it gives rise to a mixed clone of uninfected and converted progeny. This theory is compatible with the numbers of bacteria with dividing nuclei present in our cultures, remembering that the percentages given in Table 3 relate to the total count, and might be considerably higher if it were possible to calculate them in terms of viable bacteria.

How are these conclusions to be reconciled with the currently accepted theory that pre-prophage remains as an unattached cytoplasmic inclusion for one or more generations? This was first suggested by Lieb (1953), who infected *Escherichia coli* K12 with phage λ at a multiplicity of 3 to 20, and found mixed clones arising from the progeny of single bacteria treated in this way. She calculated that the period intervening between penetration of the bacterium by the phage and the establishment of lysogenesis was about one hour. Bertani (1954) found a similar state of affairs in superinfections of lysogenic strains of *Shigella dysenteriae* with multiple infections of phage P2 (originally derived from *Escherichia coli*) and certain of its mutants. He proposed the name 'pre-prophage' for the unattached phage genetic material and suggested that in the pre-prophage state some multiplication of the phage components may occur. This theory gains weight from an observation made by Levine (1957), who found that in mixed infections of *Salmonella typhimurium* with mutants of phage P2 a good proportion of the lysogenized bacteria carried prophages with markers from both phages, which suggested that multiplication and genetic recombination had occurred. Luria (1959) recorded experiments in which *S. typhimurium* was exposed to phage P22, resulting in extensive segregation of sensitive non-carrier bacteria in practically every clone. In some cases carrier

bacteria continued to harbour more than one type of phage for many generations. He regarded the early establishment of a prophage in one cell nucleus as an unlikely event. Further experiments were done in which single clones of *S. typhimurium* infected with phage P22 were analysed by using micromanipulation by a modified De Fonbrunne technique. Each of these clones was the progeny of a single bacterium that had been infected with phage particles of one or more genetic types. It was found that, even with multiple infections, a phage element could be maintained for several generations by single unilinear transmission within a clone that continued to produce non-carrier sensitive bacteria. Such clones occasionally gave a typical lysogenic subclone. Clearly these results, and particularly those reported by Luria (1959), are completely at variance with ours.

Why should this be? Two possible explanations may be suggested. (1) Our experiments were made with single infections, the other experiments with multiple infections. In the case of Lieb's original observation, this appears to be the only difference. (2) The phages used were not stable wild strains, but were mutants. In some of the experiments lysogenic bacteria were superinfected with a related phage mutant, in others non-lysogenic sensitive bacteria were exposed simultaneously to two different phage mutants. Without a detailed study of the overall characters of these mutants, particularly of their stability and lysogenizing properties when used singly, no reliable conclusion can be reached. Nevertheless, it seems possible that related and perhaps unstable mutants when present as multiple infections and when competing with each other for a particular site on the chromosome, might for a time mutually obstruct lysogenization. This is a theory which could be confirmed or disproved by suitable experiment. A third possibility to be considered in relation to the micromanipulator experiments (Luria, 1959) is that the parent bacterium of the clone, picked out in this way, may have been a unilaterally infected doublet. If similar results were obtained consistently and repeatedly, as no doubt they were, this explanation can be disregarded.

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Sedimentation Coefficients of Yeast Ribosomes

By J. De Ley

Laboratory for Microbiology, Faculty of Sciences, State University, Ghent, Belgium

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SUMMARY

The corrected sedimentation coefficients, expressed as $(S_{20,w})_0$, of ribosomes from nine different yeasts, representing a wide taxonomic range, were found to be $51{\cdot}5 \pm 2{\cdot}4$, $68{\cdot}6 \pm 3{\cdot}3$ and $86{\cdot}7 \pm 2{\cdot}9 \times 10^{-13}$ sec. Very small quantities of a faster (approximately 128×10^{-13} sec.) and a slower particle were sometimes detected. Yeast ribosomes have thus sedimentation coefficients which are distinctly different from bacterial ribosomes.

INTRODUCTION

It appears that ribosomes are not the same in all groups of organisms; it is generally assumed that ribosomes from bacteria are smaller or lighter than those from other organisms. It was recently shown (De Ley, 1964) that the corrected sedimentation coefficients of ribosomes from a wide variety of bacteria are nearly identical and had the values of 29·5, 37·5, 56·3, 76·7 and 110·5 S (Svedberg units). It seemed desirable to determine these constants for other micro-organisms, e.g. yeasts, under the same experimental conditions and with the same equipment. Since information on yeast ribosomes is limited to *Saccharomyces cerevisiae*, it was decided to extend observations to other yeasts, representing a wide taxonomic spectrum. Ribosomal particles in *S. cerevisiae* were discovered by Schachman, Pardee & Stanier (1952). The sedimentation coefficient at infinite dilution $(s_{20,w})_0$ was found to be 80×10^{-13} sec. (Chao & Schachman, 1956); a similar value was also reported by Bowen, Dagley, Sykes & Wild (1961). In solutions poor in Mg and Ca, the 80 S ribosome dissociated into 60 and 40 S particles (Chao, 1957); papain action led to a similar breakdown (Morgan, Greenspan & Cunningham, 1963). Morgan (1962) reported values of 71, 75, 80 and 82 for the 80 S ribosomes, 54, 56, 57 and 60 for the 60 S ribosomes and 33, 36 and 39 for the 40 S ribosomes. A new particle, originating from the 60 S ribosome, and with a sedimentation coefficient of about 50 S was detected. Koehler (1962) reported the corrected values of about 120 S, 80–95 S, 58–66 S, 42–53 S and 20–32 S. Furthermore, Koehler's results allowed the conclusion that yeast ribosomes were smaller than bacterial ones, and not bigger as was generally assumed. Differences in the relative amounts of the ribosomes during changes in the physiological state of the yeast cells have been reported by Wolfe (1956), Ashikawa (1958) and Koehler (1962).

METHODS

Organisms and culture conditions. The strains used are mentioned in Table 1. With the exception of *Saccharomyces cerevisiae* Hansen which we isolated in pure culture from commercial baker's yeast, all the strains used were obtained through the

courtesy of the Centraalbureau voor Schimmelkultures, Baarn, the Netherlands. Unless otherwise stated, these yeasts were grown in liquid unhopped malt-extract (8° Balling; pH 6). *S. cerevisiae* grew somewhat better in (%, w/v): 1·5, malt extract broth (Difco); 0·078, Bacto-peptone (Difco); 1·2, maltose; 0·2, glycerol; 0·1, K_2HPO_4; 0·1, NH_4Cl; final pH 6·5. The organisms were grown in 200 ml. volumes of the medium in 1 l. Erlenmeyer flasks on a reciprocal shaker at 30°, except for *Lipomyces lipoferus* which was grown at room temperature (18–23°) and *Nadsonia fulvescens* which was grown at 18°. After 1–2 days, young cultures in the exponential phase were harvested by centrifugation for 15 min. at 12,000*g*. Another set of flasks, with the yeasts in the stationary phase, were harvested after 4–7 days. Before harvesting, all cultures were examined microscopically for contamination.

Preparation of cell-free extracts. The organisms were washed by centrifugation two to three times from TMS buffer (pH 7·1; 0·01M-tris, 0·004M-succinic acid, 0·005M-$MgSO_4$); this buffer was used in all the work. To improve subsequent mechanical breakage, the paste of organisms was frozen overnight at −20°, and then disrupted in the Hughes block (Shandon Scientific Co., London) by a few severe blows with the Denbigh Fly Press no. 4 (Th. Ward Ltd., Albion Works, Sheffield). The resulting mass was suspended in an equal volume of TMS buffer. After addition of a minute crystal of crystalline DNAase (Worthington, Freehold, N.J., U.S.A.), the suspension was left for 1 hr at 22° and then centrifuged at 12,000g for 15 min. at 4° in a Servall RC2 Centrifuge (Ivan Servall Inc., Norwalk, Conn., U.S.A.) to eliminate unbroken organisms and large pieces of debris. The supernatant fluid was used at once for ultracentrifugal analysis.

Analytical ultracentrifugation was carried out exactly as described before (De Ley, 1964).

RESULTS

Corrections to be applied to experimental S values. The same corrections were applied as described in the previous paper (De Ley, 1964). The effect of pressure and dilution was again found to be negligible. The effect of concentration was the most important one. From each crude extract dilutions of 3/4, 1/2, 1/4, 1/8 and sometimes 1/16 in TMS buffer were made and the sedimentation coefficients determined. These values were extrapolated to infinite dilution and called $(s_{20,\mathrm{TMS}})_0$. The difference between the experimental sedimentation coefficient s_c in the crude extract and the extrapolated value was in the range 12–42%. As in the case of bacteria, where similar differences were observed, quite considerable errors can be introduced in the S nomenclature of ribosomes when this correction is not taken into account.

The corrected sedimentation coefficients of yeast ribosomes. The results are collected in Table 1. Five types of ribosomes were seen; only three of them were present in sufficient amounts for measurement of their sedimentation coefficient with certainty. The average $(s_{20,\mathrm{TMS}})_0$ values, expressed as Svedberg units (S), were calculated to be $49·8 \pm 2·3$, $66·4 \pm 3·2$ and $83·9 \pm 2·8$. Faster particles, with an average S of 124, occurred only in small amounts in some strains. Small particles of the order of 30 S were seen only rarely and in minute amount. According to our previous paper, the reduction to standard conditions was calculated with the formula $(s_{20,w})_0 = 1·035\ (s_{20,\mathrm{TMS}})_0$. The reduced values are $51·5 \pm 2·4$, $68·6 \pm 3·3$, $86·7 \pm 2·9$ and about 128.

Influence of the medium and its Mg^{2+} concentration. It is known that the Mg^{2+}

concentration of the medium affects the relative amounts of bacterial ribosomes. In a previous paper (De Ley, 1964) it was reported that their sedimentation coefficients were not noticeably influenced. A similar result was obtained with yeast ribosomes. The sedimentation coefficients of Saccharomyces ribosomes were determined both from organisms grown on the complex malt medium in the absence of added Mg^{2+} and from organisms grown on a defined glucose + salts + vitamin medium (Lodder & Kreger-van Rij, 1952), containing 0·004M-$MgSO_4$. There was no noticeable difference between the sedimentation coefficients observed.

Table 1. *Corrected sedimentation coefficients, expressed as* $(s_{20,\mathrm{TMS}})_0 \times 10^{13}$ *sec., of ribosomal particles from several yeasts*

Values in parentheses are from particles present in very small amount only in the highest concentration of crude extract examined. They could not be determined by extrapolation, but s_C *versus* $(s_{20,\mathrm{TMS}})_0$ plots according to De Ley (1964) were used. These values are only close approximations.

Organism	CBS* no.	Age (days)	Value of $(s_{20,\mathrm{TMS}})_0 \times 10^{13}$ sec.			
Saccharomyces cerevisiae Hansen	(Own isolate)	1	None	65·1	83·6	None
		4	48·9	66·3	83·2	(125)
Nematospora coryli Peglion	2608	2	51·8	68·5	85·0	(143)
		5	54·2	None	86·7	(119)
Sporobolomyces salmonicolor (Fischer et Brebeck) Kluyver et van Niel	490	2	(51·3)	64·3	84·1	(130)
		5	None	65·9	83·7	None
Hansenula anomala (Hansen) H. et P. Sydow	110	2	(47·3)	65·9	83·3	None
		4	50·6	None	84·2	None
Cryptococcus laurentii (Kufferath) Skinner	139	2	None	67·5	83·4	(117)
		4	None	(67·3)	84·0	None
Nadsonia fulvescens (Nadson et Konokotina) Sydow	2596	2	48·8	69·6	83·3	(127)
Lipomyces lipoferus (den Dooren de Jong) Lodder et van Rij	914	2	None	None	83·5	None
Schizosaccharomyces pombe Lindner	356	3	None	(70·2)	(87·5)	(125)
Endomycopsis capsularis (Schiönning) Dekker	2519	2	(51·5)	None	83·8	None
		7	None	None	83·6	None

* CBS = Centraalbureau voor Schimmelkultures, Baarn.

Influence of age of organisms. It is known that the physiological age of the organisms changes the relative amounts of ribosomal types (see Introduction). This was confirmed in the present work but not further examined. Table 1 shows that the sedimentation coefficients of ribosomes from young and old organisms were not noticeably different.

Dissociation of yeast ribosomes. Chao (1957) reported that the 80 S ribosomes of Saccharomyces dissociated into 60 and 40 S particles in conditions of Mg^{2+} or Ca^{2+} deficiency. We confirmed this with several types of yeasts. Decreasing the Mg^{2+}

concentration in the tris+succinate buffer from 0·015 M to 0·005 and 0·0005 M, resulted in a decrease of our 86 S particles and a concomitant increase in the 51 S and 68 S particles. Increasing the Mg^{2+} concentration to 0·05 M resulted in a decided decrease in the amount of the particles, possibly due to aggregation and very quick sedimentation.

DISCUSSION

Our corrected sedimentation coefficients are in good agreement with the values given by Koehler (1962). The ribosomes of the nine different yeasts examined appear to be identical or nearly identical. These organisms were selected as fairly representative yeasts in general. It thus seems likely that the ribosomes will be similar for all yeasts; if there are differences between different organisms, they fall within the experimental error. Yeast ribosomes have sedimentation coefficients which are distinctly different from bacterial ones.

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