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Contents

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No. 3 *issued September* 1965

THE JOURNAL OF GENERAL MICROBIOLOGY

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

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

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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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Extracellular Products of Blue-Green Algae

By B. A. WHITTON
Department of Botany, University of Durham

(*Received* 4 *June* 1964)

SUMMARY

An examination was made of soluble extracellular materials produced by *Anabaena cylindrica* and some other species of blue-green algae, to examine whether they have any functional importance. Organisms of all the 15 species (representing 10 genera) examined produced extracellular pigment; with at least 10 of these species part of this pigment was not diffusible on dialysis, though the actual amount varied from 14 to 50%. The dialysis residue fractions of the extracellular products of *A. cylindrica* and 4 other species had similar amino acid compositions, rather different from those of extracellular polypeptides produced by other micro-organisms. The algal polypeptides had large proportions of serine and glycine, but no basic amino acids. The dialysis residue fraction of *A. cylindrica* extracellular product had no effect on spore formation and did not cause an increase in uptake of phosphate. A biological effect of this fraction was the decrease in the toxic effect of polymyxin B against *A. cylindrica* and *Anacystis nidulans*. For the latter organism there was a linear relationship between concentration of polymyxin B and amount of algal dialysis residue fraction which just permitted the alga to survive. This effect of extracellular products on polymyxin B toxicity might operate in nature since *Bacillus polymyxa* often occurs in association with blue-green algae.

INTRODUCTION

There is much evidence that the production of extracellular substances by blue-green algae is widespread and sometimes quantitatively important; mucilaginous sheaths are frequently seen, and these are often coloured, usually in shades of yellow or brown. Fogg (1952) surveyed the literature about the production of soluble extracellular nitrogenous substances by blue-green algae, and concluded that this is a general feature of these organisms; later papers have supported this view (see review by Fogg, 1962; Fay, 1962; Stewart, 1963; Taha & Elrefai, 1962; Venkataraman, 1961). However, little attempt has been made to assess the possible functional significance of this soluble extracellular material.

Jakob (1954, 1957) showed that the extracellular products of a strain of *Nostoc muscorum* contained a dialysis diffusate component which was biologically active on various other micro-organisms. Filtrates of old cultures with many heterocysts were especially active; when heterocysts were absent, the filtrate was not active. Fogg (1952) showed that the extracellular polypeptides of *Anabaena cylindrica* were able to complex 0·324 mg. copper with each mg. total peptide-nitrogen. Fogg & Westlake (1955) gave further evidence for an interaction between algal polypeptide and cations, and showed that colour changes occurred with pH value in the case of

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Fe (III) and polypeptide mixtures. Algal polypeptide binding of copper decreased its toxicity towards *A. cylindrica*; similar binding was also found with other elements which might be important for the growth of the alga. Superficially at least, it seems strange that the chelation of one metal decreases its toxicity, whilst the chelation of others may be expected to increase their availability.

Fogg (1952) found that in later stages of growth of *Anabaena cylindrica*, the production of extracellular nitrogen always occurred. However, comparison at equivalent stages of growth showed that such production was increased considerably by deficiency of certain elements (e.g. iron) and was decreased slightly by a deficiency of molybdenum. The extracellular combined nitrogen was mainly polypeptide, with lesser amounts of an amide, the proportions of which decreased as the cultures aged. Fogg concluded that the polypeptides were liberated during metabolism at the outer surface of the organism. Hughes, Gorham & Zehnder (1958) and Gorham (1960) showed that a substance, toxic to various mammals and termed 'fast death factor' (FDF), was present in the organisms and in the culture fluid of an uni-algal culture of *Microcystis aeruginosa*. Bishop, Anet & Gorham (1959) showed that FDF extracted from the organisms was one of a mixture of 5 distinct polypeptides (see Table 2), and concluded that FDF was probably cyclic and of molecular weight between 1300 and 2600.

Soluble extracellular products of blue-green algae may thus have several quite different kinds of biological effect, some of which may be of ecological interest. The present work was done mainly with *Anabaena cylindrica*. Some comparative observations on other blue-green algae are also included in this paper.

METHODS

Anabaena cylindrica was obtained from the culture collection of Westfield College, London. There are at present two strains (at least) of this organism in culture; one produces spores in older cultures (Cambridge collection no. 1403/2), and the other does not. The strain used in all experiments described here produced spores. The alga was grown in the medium described by Fogg (1949) with the modification that iron was added as Fe (III). EDTA chelate and the trace element mixture used was that of Allen & Arnon (1955). The alga was inoculated into 250 ml. volumes of medium contained in penicillin flasks, and incubated at 25° and a light intensity of 7000 mc. During incubation the flasks were shaken at 1–2-day intervals. The cultures were harvested at a late exponential stage of growth and filtered through kieselguhr and then through an Oxoid membrane filter. When sterile samples were required membrane filters were used, filtration being done with a sterile filter flask, the lower surface of the filter having been sterilized by ultraviolet irradiation.

Growth studies were done with 25 ml. medium in 100 ml. conical flasks incubated in a thermostat-controlled water bath provided with a shaking mechanism (Miller & Fogg, 1957). Extracellular material of *Anacystis nidulans* was obtained by growing this organism in the same way, but in the medium of Kratz & Myers (1955).

Other organisms were grown in media and under conditions in which they are known to grow well; these cultures were harvested at late stages in growth.

Some field materials were also used. These included a dense bloom of *Oscillatoria planctonica* taken from St James's Park lake, London, in August 1962, a sample of

London reservoir water containing *Microcystis aeruginosa*, and colonies of *Nostoc commune* brought from The Burren, Co. Clare, Ireland. The samples of water containing blooms were filtered through kieselguhr and then membrane filters. The Nostoc colonies were grown in media in the laboratory for a few weeks and then treated like the pure cultures. With field collections it was obviously not possible to be sure that any extracellular material found had been released by the algae. However, with the *O. planctonica* in particular the alga had been overwhelmingly dominant in the lake for some weeks before collection, so that material discussed below may have come from this alga.

Table 1. *List of cultures used in various studies*

Organism	Westfield no.	Cambridge no.	Pigment	Absorption spectra	Dialysis	Amino acids
Pure culture						
Anabaena cylindrica Lemm.	10	1403/2	+	+	+	+
A. variabilis Kütz. (Allen's strain)	24	.	+	.	.	.
A. variabilis Kütz. (N-fixer)	32	.	+	.	.	.
Anabaena sp.	28	.	+	+	+	.
Anabaena sp.	31	.	+	+	+	.
Anacystis nidulans Drouet	33	.	+	+	+	+
Calothrix sp.	20	.	+	+	.	.
Chlorogloea fritschii Mitra	50	1411/1	+	+	+	+
Cylindrospermum sp.	19	1415/1	+	.	.	.
Mastigocladus laminosus Cohn	2	1447/1	+	+	.	.
Microcoleus vaginatus (Vaucher) Gom.	5	1459/6	+	+	.	.
Nostoc sp.	53	.	+	.	.	.
Pseudanabaena sp.	49	.	+	.	.	.
Synechocystis salina Wislouch	55	.	+	+	.	.
Field samples						
Microcystis aeruginosa Kütz.	.	.	+	+	+	.
Nostoc commune Vaucher	.	.	+	+	+	+
Oscillatoria planctonica Wolszyńska	.	.	+	+	+	+

A list of the algae used and a summary of investigations made is given in Table 1. Polymyxin B was supplied by Burroughs Wellcome and Co. in 10,000 unit packs.

Amino acids were separated by two-way descending chromatography on Whatman 3 MM paper. The first solvent was phenol saturated with water, with a trace of ammonia in the chromatography tank. The second solvent was butanol + glacial acetic acid + water (90 + 10 + 29, by vol.). Amino acids were located by ninhydrin spray.

RESULTS

Physical and chemical observations on extracellular material

Pigments. The filtrate from old cultures of *Anabaena cylindrica* was reddish brown by reflected light, brown by transmitted light. Filtrates from cultures of 14 algae were also observed to be coloured during the later stages of growth (Table 1). After concentration of those samples of culture filtrate where colour was not very intense, the colours were: *Mastigocladus laminosus*, purple; *Anacystis nidulans*, lime-yellow; the remainder, shades of brown to brown-yellow. Similar colours have been noted frequently in the sheaths of natural populations of blue-green algae. In some cases (e.g. *Nostoc verrucosum*) these pigments are probably intimately associated with the mucilaginous sheaths and are insoluble. In the field

samples investigated here, all the colours showed evidence of being associated with soluble pigments. Filtrates of the lake water containing Oscillatoria were coloured brown, whilst filtrates of the water containing *Microcystis aeruginosa* showed a brown-yellow colour after concentration.

Absorption spectra of filtrates. Absorption spectra of 10 filtrates and the two natural water samples (see Table 1) were examined between 240 and 600 mμ. All the filtrates absorbed throughout this range, generally with high absorption at 240 mμ, gradually decreasing at longer wavelengths. High ultraviolet absorption values were not necessarily associated with those media which were strongly coloured. For instance the two highest sets of absorption readings over most of the ultraviolet range were from *Anacystis nidulans* and *Chlorogloea fritschii*, the filtrates of which were only slightly coloured. *Anabaena cylindrica* showed a marked peak at 280–290 mμ, *Mastigocladus laminosus* at 270–280 mμ. All the others except *Synechocystis salina* showed inflexions implying peaks in their spectra in this region. With the exception of *A. nidulans*, there was no tendency for peak formation or detectable inflection elsewhere. The filtrate of an old *A. nidulans* culture showed marked inflexions at 300 and 360 mμ; pteridines found in old cultures of this species (Forrest, van Baalen & Myers, 1959) have a main absorption peak at about 320 mμ. However, the particular compound later isolated from *A. nidulans* organisms showed neither of its peaks in this region.

Preparation of Anabaena cylindrica polypeptide. Several methods (ion-exchange, activated charcoal, gel filtration) were tested as means for partially purifying extracellular polypeptide of *Anabaena cylindrica*. Part of the culture filtrate was non-dialysable; some polypeptide was present in this fraction which was chosen for experiments, being salt-free material obtained by a method unlikely to have caused much breakdown of polypeptide. The absorption spectra of a sample of filtrate before and after prolonged dialysis ('Visking' tubing) against distilled water, after making allowance for slight changes in the spectrum with changes in pH value, were different. Relatively more material absorbing in the ultraviolet region passed through the membrane than material which absorbed in the visible region. The molecule causing a peak at 290 mμ passed preferentially through the membrane as compared with other absorbing material. Possibly the peak was caused by the aromatic groups known to be present in *A. cylindrica* filtrates (tyrosine, phenylalanine; Fogg, 1952). A comparison between retention of chromophore and retention of nitrogen showed as follows:

	Extinction E_{400}	mg. N/l.
Culture filtrate	0·397	37·5 ± 1·4
Dialysis residue	0·145	10·2 ± 0·7
Proportion in dialysis residue (%)	36·5	27·2

Dialysis of culture filtrates of five other blue-green algae and of the two natural water samples (see Table 1) gave similar results. The most obvious differences between the materials from different organisms were considerable differences in the proportion of material in the dialysis residue, and the fact that prolonged dialysis of *Anacystis nidulans* culture filtrate against water led to a precipitation, which did not occur with filtrates of the other species.

Amino acid composition of dialysis residue fraction of filtrates. The dialysis residue fraction of *Anabaena cylindrica* filtrate contained nitrogen; its amino acid composition was examined together with samples representing 4 other genera (Table 1). Samples of the dialysis residue fractions of filtrates were evaporated to about 5% their original volume, and then heated with an equal volume of concentrated HCl for 24 hr. at 105°. After this hydrolysis there was much charred material in all the samples except that from *Anacystis nidulans*.

Table 2. *Amino-acid composition of blue-green algal non-dialysable extracellular materials, together with various results in the literature (for details, see text)*

	Anabaena (Fogg, 1952)				Non-dialysable fractions					
	12-day culture		28-day culture						FDF (Hughes *et al.* 1958)	Phormidium wall (Frank *et al.* 1962)
Amino acids and amines	Pre-h.	Post-h.	Pre-h.	Post-h.	Anabaena	Anacystis	Nostoc	Oscillatoria		
Alanine	+	+	+	+	3	3	4	4	+	+
β-Alanine	.	.	.	.	1	0	1	0		
Aspartic	.	+	.	.	1	1	1	1	+	+
Ethanolamine	.	.	.	.	0	0	0	1	.	.
Glutamic	+	+	+	+	2	2	2	2	+	+
Glutamine	+	?+	.	.	0	0	0	0	.	.
Glycine	?+	+	.	+	4	4	4	4	.	+
Leucine	+	+	.	+	2	3	2	2	+	.
Phenylalanine	.	+	.	.	1	0	1	0	.	.
Proline	.	.	.	.	1	0	1	0	.	.
Serine	?+	+	.	+	4	4	4	4	+	+
Threonine	.	.	.	+	3	2	3	2	.	+
Tyrosine	.	+	.	+	?tr	1	?tr	2	.	.
Valine	+	+	.	+	2	4	2	2	+	.
Unknown	.	.	.	.	+	2	+	1	.	.
Misc. non-basic	.	.	.	.	.	.	1	.	.	+
Basic acid(s)	.	+	.	.	0	0	0	0	+	.
Total/poly-peptide	.	.	.	.	11	10	12	11	7	(7)

The use of numbers for individual amino-acids indicates the relative proportion of the various acids in a particular polypeptide. '? tr' = possibly a slight trace present. Pre-h., Post-h. = pre- and post-hydrolysis.

Duplicate chromatograms were run with Anabaena and Oscillatoria material and single ones with the other samples. The amino acids found are shown in Table 2, together with the list for an undialysed sample of filtrate (Fogg, 1952) and the fast death factor (FDF) of *Microcystis aeruginosa* (Hughes *et al.* 1958). Several unidentified spots are omitted from Table 2. The most prominent of these was one separating from and moving slightly ahead of leucine in the butanol + acetic acid + water solvent. This was present in Anacystis and Oscillatoria material and possibly also in others. This unrecognized compound is almost certainly not an artifact due to the breakdown of leucine. The several other faint spots did not include one which seemed likely to be diaminopimelic acid.

Good separation was not obtained with the ninhydrin-reactive material on the Chlorogloea chromatogram. From the other 4 chromatograms it was concluded

that the samples of filtrate contained polypeptides of rather similar amino acid composition. They agreed in the prominence of alanine, glycine and serine, and the presence of aspartic acid, glutamic acid, leucine, threonine and valine. There were several differences among the other substances, though the Anabaena and Nostoc products were similar, qualitatively and quantitatively. Two observations are worth noting: (i) the presence of β-alanine in Anabaena and Nostoc material; (ii) the probable absence of tyrosine as compared with its marked presence with the other two algae. Tyrosine was found in both the desalted hydrolysed filtrate samples studied by Fogg (1952). Other observations and tests made on the non-dialysable fraction of the filtrate of *Anabaena cylindrica* were reported by Whitton (1963).

Some biological effects of algal polypeptides

Samples of the dialysis residue fraction of *Anabaena cylindrica* culture filtrate were used in all the following experiments. Several samples of culture filtrate were used, but all the experiments with polymyxin B were made with the same sample of filtrate. The filtrates were evaporated to about one-twentieth of their original volume, the temperature not being allowed to rise above 65°. The concentrate was membrane-filtered, dialysed against water, re-filtered, and stored at a concentration corresponding to an extinction of 10 units at 400 mμ. This material will be called polypeptide below, although it may have contained other extracellular non-dialysable components.

Effect of polypeptide on spore formation. *Anabaena cylindrica* can be subcultured, for at least a few times, in a condition almost free from mucilage and spores, yet when growth in shaken cultures of limited volume the organism always changes from a freely suspended planktonic state to a mucilaginous and spore-producing state. This occurred quite early in growth, and thus might not be due to a deficiency of a nutrient, but rather to the secretion of extracellular material above a given concentration. Replicate sets of cultures grown with and without polypeptide added to the medium showed no difference in spore counts after incubation for 7 days.

Effect of polypeptide on phosphate uptake. Experiments were done to see whether the presence of polypeptide in the medium affected the short-term uptake of phosphate by the alga. There was no significant effect.

Effect of polypeptide on the toxicity of polymyxin B. The results of Hunter & McVeigh (1961) indicated that *Anabaena cylindrica* was very resistant to polymyxin B; there was no inhibition of growth at a concentration of polymyxin B 200 μg./ml. Other results in the literature indicate that blue-green algae are much more sensitive to polymyxin B than is *A. cylindrica*; this was confirmed by tests made in the present work. Some explanation was therefore sought for Hunter & McVeigh's result. Experiments were made to see whether *A. cylindrica* polypeptide might decrease the toxic affect of polymyxin B on the alga. (The term polypeptide is used in this section as synonymous with the algal dialysis residue. It is however possible that the effects observed are due to a component other than polypeptides.)

Certain procedures were used in all the following experiments with *Anabaena cylindrica*. Medium (20 ml.) was added to fourteen 100-ml. conical flasks, sterilized and K_2HPO_4 solution was added, to four-fifths the concentration normally used, to minimize the formation of precipitate. Polypeptide was added to the experi-

mental set of flasks, and then the polymyxin B solution to a series of concentrations in control and experimental flasks. Water was added where necessary to bring the volume up to 25 ml. After inoculation with spore-free filaments to give 28–40 cells/mm.^{3}, the cultures were incubated at 25° with continuous shaking.

Toxic effect of polymyxin B on Anabaena cylindrica. In the first experiment of this series, a concentration of the stock polypeptide sample was used corresponding to extinction E_{400} 0·020, and polymyxin B concentrations of 0, 2, 4, 6, 8, 10, 12 units/ml. Some cultures grew only after a lag, and a rough estimate of this lag was made visually. The results are in Table 3. It was concluded that addition of algal polypeptide caused more polymyxin B to be needed before it was toxic to young algal filaments. Microscopic observation of those cultures which lagged in growth showed that many filaments were killed and the cells fragmented. Another experiment with a polypeptide concentration equivalent to E_{400} 0·040 showed a similar picture. There was no visible inhibition of growth at polymyxin B 18 μg./ml. (the highest dose tested) with polypeptide present; without polypeptide there was a lag of 1 day with polymyxin B 6 μg./ml. and no survival above this concentration.

Table 3. *Effect of Anabaena cylindrica dialysis residue on toxicity of polymyxin to Anabaena cylindrica*

Polymyxin concentration tested (μg./ml.)	− Algal dialysis residue	+ Algal dialysis residue
0	+	+
2	+	+
4	Lag (1 day)	+
6	−	Lag (1 day)
8	−	Lag (3 days)
10	−	Lag (2 days)
12	−	Lag (2 days)

+, No inhibition of growth. −, No growth after 3 weeks' incubation.

A further experiment with a polypeptide concentration equivalent to E_{400} 0·100, gave rather different results. Without polypeptide, growth occurred with polymyxin B 4 units/ml., but not 6 units/ml. With added polypeptide and a range of polymyxin concentrations from 30–150 units/ml. the filaments in each case remained intact and apparently healthy for several days (in contrast to those without polypeptide and with polymyxin B at 6 units/ml. and above). But microscopic observation on day 5, showed that the filaments had become moribund, and no growth subsequently occurred. Unlike the cultures without polypeptide the cells did not disintegrate.

Toxicity of polymyxin B to Anacystis nidulans. Experiments like the previous ones were done with *Anacystis nidulans* to test whether the effect of *Anabaena cylindrica* polypeptide was specific for its own cells, or whether it could decrease polymyxin B toxicity for other organisms. The experiments were like the first experiments with *A. cylindrica* except that the *A. nidulans* inoculum gave an initial cell count of 84 cells/mm.^{3}, and Kratz & Myers's (1955) medium at 35° was used. The *A. cylindrica* polypeptide concentration tested was equivalent to E_{400} 0·020. The results obtained after incubation for 7 days are shown in Table 4. From these

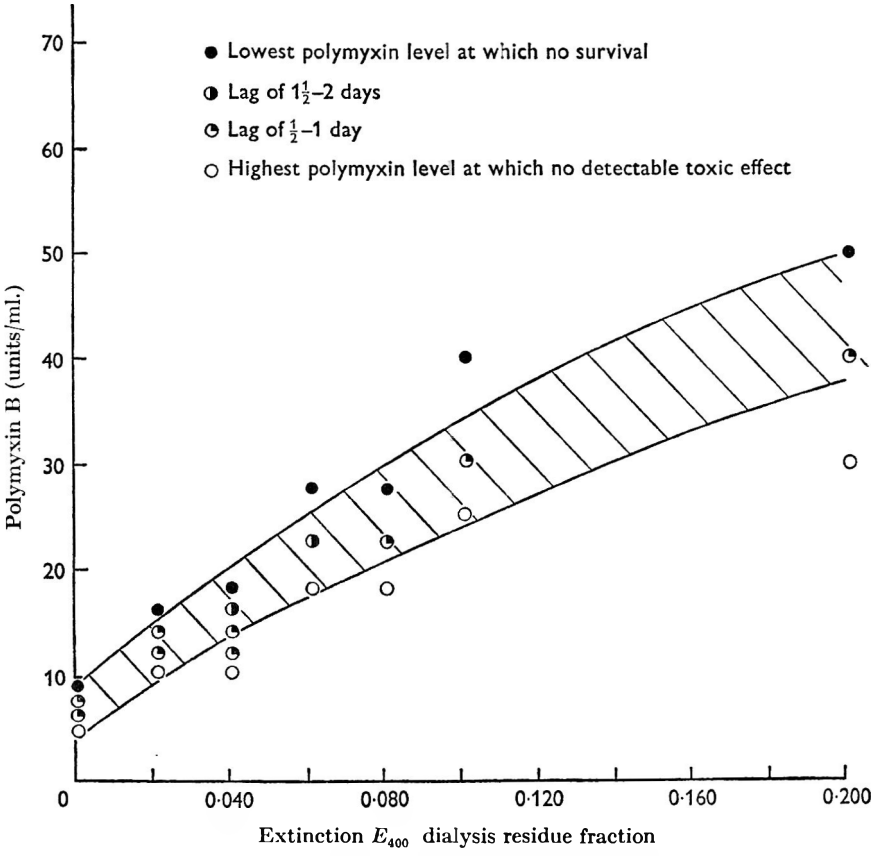

Fig. 1. Minimum lethal dose of polymyxin to *Anacystis nidulans* plotted against concentration of algal (*Anabaena cylindrica*) extracellular material added to the growth medium.

Table 4. *Effect of Anabaena cylindrica dialysis residue fraction on toxicity of polymyxin to Anacystis nidulans*

Polymyxin concentration tested (μg./ml)	− Algal dialysis residue	+ Algal dialysis residue
0	+	+
2	+	+
4	−	+
6	−	Lag (1½ day)
8	−	Lag (2 days)
10	−	−
12	−	−

+, No inhibition of growth. −, No growth after 1 week's incubation.

it was concluded that the lowering of the toxicity of polymyxin B by *A. cylindrica* polypeptide was not specific for *A. cylindrica* only. Because there was variation in the behaviour of the controls with regard to sensitivity to polymyxin B, the effects of a range of algal polypeptide and polymyxin concentrations simultaneously were compared. This experiment was done with *A. cylindrica* polypeptide and *A. nidulans*.

The experiment was done in a hundred 4 ml. tubes, with a 2·5 ml. medium in each. The growth medium was that of Kratz & Myers (1955) at half strength, and with added $NaHCO_3$ (0·2 g./l.). The tubes were incubated without shaking at 35°. The results are illustrated in Fig. 1. It was concluded that there was an almost linear relationship between concentration of polymyxin B toxic to *A. nidulans* and the concentration of *Anabaena cylindrica* polypeptide, over the range tested. The algal polypeptide caused no precipitation addition to a solution of polymyxin B 1000 μg./ml. Several cultures in which the polymixin B added had had no toxic effect were filtered; these showed no difference in absorption spectrum in the visible range as compared with that of the polypeptide originally present. The retention of extracellular material by a dialysis membrane was decreased only slightly by polymyxin B 100 μg./ml. It seems likely that the algal polypeptide acted in these experiments by competing with the polymyxin B for some site on or in the organism rather than by combining with it in the medium.

DISCUSSION

The results reported suggest that the extracellular substances from the pure cultures tested, and also from the field materials, have many features in common.

Whitton (1963) compared the production and amino acid composition of extracellular polypeptides of blue-green algae and bacteria. All the non-dialysable blue-green algal polypeptides are more closely related to each other than to any bacterial ones yet described. There is much evidence suggesting that a situation in many ways similar to that occurring with the blue-green algae studied is also widespread among bacteria. This is perhaps especially true of the groups including some nitrogen-fixing forms, and amongst these, the Bacillaceae. For instance, Benedict & Stodola (1949) noted the production of a brown pigment in the culture medium of *Bacillus polymyxa* during growth. They stated that, while there is some relationship in colour between the supernatant fluid and polymyxin yields, the correlation between the two was not sufficiently significant to allow use of colour measurement as an analytical procedure for polymyxin assay.

Two examples from the literature which look particularly close to those of blue-green algae are from the genus *Clostridium* (Rosenblum & Wilson, 1950; Izumi, 1962). In the mucopolysaccharides isolated from culture medium of *C. perfringens* by Izumi, the main constituents were neutral sugars, amino acids, uronic acids and oligopeptides. This material is particularly close to the blue-green algal products studied here in that a range of amino acids is present (all of them occurring with *Anabaena cylindrica*), and that there are no basic ones present.

Finally the work of Lilly (1962) on a lipomucoprotein released into the medium by a lysine-requiring mutant of *Escherichia coli* may be mentioned. Lilly pointed out that the amino acid composition of this lipomucoprotein differed from that of *E. coli* cell walls mainly in the absence of diaminopimelic acid, and suggested that it represented an outer layer of the cell wall which was sloughed off under certain nutrient deficiency conditions.

It seems possible that such a situation may also hold with blue-green algae. Frank, Lefort & Martin (1962) studied the composition of the cell wall of *Phormidium uncinatum*. The amino acids present in addition to the mucopeptide

fraction are shown in Table 2. It can be seen that the amino acid composition has many similarities to the extracellular polypeptide of *Oscillatoria planctonica*.

Function of algal polypeptide. The lowering of polymyxin toxicity by the algal extracellular polypeptide is of interest for several reasons. *Bacillus polymyxa* is a very widespread organism, and grows under conditions suitable for many blue-green algae (see Benedict & Stodola, 1949). Bacillus rods are frequently visible on staining the sheaths of blue-green algae, and Miss V. G. Collins of the Freshwater Biological Association (personal communication) states that these are often *B. polymyxa*, and that with some species at least the rods are generally present and often very abundant. Bisset (1962) stated that *B. polymyxa* was a well-known plant pathogen, and that it produced an (extracellular) pectinase. This pectinase might presumably be expected to attack the mucilages of blue-green algae. Inspection of the data in Benedict & Stodola (1949) shows that the formation of extracellular polypeptide might determine frequently whether or not an alga might be killed by the polymyxin formed by the bacteria. It thus seems likely that the ability to withstand the polypeptide antibiotics produced by *B. polymyxa* is likely to be of considerable biological significance for the alga.

A further feature of interest in this phenomenon is that it provides a possible clue to another important biological function of the polypeptide. Newton (1953) showed that there was some inhibition of polymyxin toxicity by metal cations, and he concluded that the polymyxin and cations competed directly for sites on the cells. Since blue-green algal polypeptide is known to complex various metals, and since the results indicate that the algal extracellular material containing polypeptide probably competes directly with polymyxin for sites on or in the cell, it is suggested that it may function by presenting certain polyvalent cations in a chelated form to loci on or in the cell, and there exchange them for univalent cations, probably H^+.

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The Interaction of Hydrogen Ion, Carbon Dioxide and Potassium Ion in Controlling the Formation of Resistant Sporangia in *Blastocladiella emersonii*

By D. H. GRIFFIN*

Botany Department, University of California, Berkeley, California, U.S.A.

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SUMMARY

Continuous culture and batch culture experiments on the control of morphogenesis of the aquatic phycomycete *Blastocladiella emersonii* led to the following conclusions: (1) The metabolism of Blastocladiella under the conditions of these experiments was highly oxidative, all of the substrate being accounted for as unused substrate, fungus-carbon and CO_2. (2) Morphogenesis was dependent on potassium-ion concentration, ephemeral sporangiate organisms developed in low K^+ concentration and resistant sporangiate organisms developed in higher K^+ concentration. (3) Morphogenesis in these experiments was independent of CO_2 partial pressure and pH value. (4) Morphogenesis was also dependent on an ionized species of carbonic acid (bicarbonate, carbonate) depending on the medium used. Ephemeral sporangia developed at low concentrations of these ions, K^+, HCO_3^- and CO_3^{2-}, and resistant sporangia developed at higher concentrations. (5) The potassium and magnesium content and pH value of the organisms were independent of the pH value and potassium concentration of the medium over the ranges studied, and of the sporangium type produced by the organisms. The continuous culture apparatus used is described and continuous culture theory applicable to experiments in morphogenesis has been developed and tested.

INTRODUCTION

Blastocladiella emersonii Cant. & Hyatt is an aquatic phycomycete in the order Blastocladiales. The organisms consist of two portions at maturity, a vegetative portion and a sporangium. The basal vegetative portion which bears rhizoids is separated by a septum from the terminal sporangium. The organisms are unbranched and produce only one sporangium. Two types of sporangia occur: (1) thin-walled hyaline ephemeral sporangia which discharge zoospores soon after formation; (2) thick-walled brown resistant sporangia which must age for several weeks before discharging zoospores. The symbols OC, ordinary colourless, for ephemeral sporangiate organisms and RS for resistant sporangiate, were used by Cantino (1961) and are used in the present paper in the same sense. The life-cycle of *B. emersonii* is of the brachyallomyces type; there is no evidence of syngamy or meiosis (Cantino, 1961). Thus the zoospores from either type of sporangium can develop into organisms which bear either type of sporangium. With this ambivalent behaviour it is clear that some factor or factors in the environment play a role in determining which

* Present address: Botany Department, University of Iowa, Iowa City, Iowa 52240.

type of sporangium an individual zoospore will produce. Cantino (1961) studied the development of ephemeral and resistant sporangia by *B. emersonii*. He observed (Cantino, 1951) that zoospores widely spread on peptone agar grew into organisms bearing ephemeral sporangia. These organisms discharged zoospores that germinated in the surface water around the parent organism and grew to form a cluster of organisms. Within these clusters, and only there, did organisms with resistant sporangia develop. With sodium bicarbonate or calcium carbonate included in the medium, widely spread zoospores developed into resistant sporangiate (RS) organisms. From these observations Cantino (1951, p. 331) concluded that 'CO_2 was indeed of fundamental importance in determining whether or not RS plants...were formed', and that the appearance of resistant sporangia in clusters of organisms was the result of local accumulation of carbon dioxide. Cantino (1951, p. 333) reported that 'a relatively small increase in available CO_2...appeared to be necessary for the formation of individual RS plants', but (Cantino, 1951, p. 332) that 'inconsistent results were obtained with plates placed in gas chambers containing different quantities of CO_2', and that (1951, pp. 333–335) 'extreme reduction of the atmospheric CO_2 concentration often induced the formation of large quantities of RS...'. In these experiments no record was made of the pH values of the media.

Cantino (1956) presented further data about the relationship between pH value and bicarbonate effects on the formation of resistant sporangia. These experiments are difficult to interpret because bicarbonate concentration and hydrogen-ion concentration were varied simultaneously, and the experiments were made on agar media where the pH value and CO_2 environment of the plants is difficult to determine accurately.

The differences in development of ephemeral sporangiate plants and resistant sporangiate plants are complex (Cantino, 1961) as one would expect for such a drastic difference in function. The short-lived ephemeral sporangiate plants are the rapidly reproducing portion of the life cycle and make their appearance when the environment has the proper conditions of nutrition, temperature, aeration, moisture, etc., for such growth. The resistant sporangium with its thick relatively impervious wall, lipid reserves and inactive metabolism allows Blastocladiella to survive adverse conditions. As the environment shifts from growth conditions to adverse conditions some factors characteristic of this change act on the developmental control machinery of the organism causing changes which result in the growth of a resistant sporangium. Since there are many ways in which an environment can become adverse, it is likely that there are many ways to affect the control mechanisms of the organism. One question is, what form of carbon dioxide is important? The carbon dioxide equilibrium with water is the crux of this question. The three variables, pH value, carbon dioxide concentration and bicarbonate concentration, at equilibrium are described by the Henderson-Hasselbalch equation. To determine which factor is important, two of the factors, e.g. carbon dioxide and bicarbonate must be varied over a wide range at each of several values of the third variable, the pH value. The environmental control necessary to do these experiments is difficult to attain in batch culture because the growing organisms make continuing changes in the culture as they grow: nutrients are used and metabolic products, including carbon dioxide, are formed, leading to changes in the hydrogen ion, bicarbonate ion and carbon dioxide concentrations. In continuous culture these variables can be

precisely controlled because new medium is continuously fed into the culture and spent medium and organisms are continuously washed out. The growth rate and wash-out rate of the culture are so balanced in continuous culture that a steady state is achieved in which the concentrations of organisms, nutrients and metabolic products are all constant. Thus there need be no opportunity for alterations of pH value, bicarbonate or carbon dioxide concentrations because of the activities of the organism.

Continuous culture theory. Continuous culture theory has been developed (Monod, 1950; Novick & Szilard, 1950) and tested experimentally (Herbert, Elsworth & Telling, 1956). The theoretical treatment of continuous culture is dependent on the assumption that there is complete and instantaneous mixing of the contents of the culture such that the concentration of the components in the effluent is equal to their concentration in the vessel. The residence times of the components of the culture are determined by the ratio of the flow rate, f, to the volume of the vessel, V. This ratio is called the dilution rate, D. There are two sources of change in the concentration of organisms, x, the wash-out rate of the organisms by dilution of the culture,

$$-dx/dt = Dx, \tag{1}$$

and the growth of the organisms,

$$dx/dt = \mu x. \tag{2}$$

Equation (2) is the familiar logarithmic growth equation where μ is the specific growth constant.

During continuous culture, changes in the concentration of organisms are dependent on the balance between the growth rate and the wash-out rate,

$$dx/dt = \mu x - Dx. \tag{3}$$

During a steady state dx/dt is zero and therefore D equals μ.

One can calculate the length of time it takes for a zoospore to develop into a mature organism during steady-state growth with equation (4), where P_M is the number of mature organisms in the culture, P_T is the total number of organisms in the culture, D is the dilution rate, and G is the development time of the organisms:

$$P_M/P_T = e^{-DG}. \tag{4}$$

This equation is derived from the kinetics of continuous culture theory. It is directly useful when the culture contains only ephemeral sporangiate Blastocladiella organisms.

To interpret the results of continuous culture experiments with Blastocladiella one would like to know the proportion of organisms in a culture which are developing resistant sporangia. Since there is a large difference in the development times of ephemeral- and resistant-sporangiate organisms, many which develop along the resistant sporangium pathway will be washed out of the culture before they reach a recognizable state of maturity, whereas most of the rapidly developing ephemeral sporangiate organisms will reach maturity in the culture tube. When the numbers of mature ephemeral and resistant sporangiate organisms are counted, the culture will have a disproportionate number of ephemeral sporangiate forms. By using equation (4) to solve simultaneously for the fraction of the total number of ephemeral and resistant sporangiate organisms that will reach maturity during their residence in the culture tube, one obtains equation (5), where P_{RS} and P_{OC} are,

respectively, the numbers of mature resistant sporangiate organisms and mature ephemeral sporangiate organisms, and G_{RS} and G_{OC} are, respectively, the development times in hours of the resistant and ephemeral sporangiate organisms, and ρ_{RS} is the porportion of organisms which produce resistant sporangia:

$$\rho_{RS} = \frac{(P_{RS}/P_{OC}) \exp\{-D(G_{OC}-G_{RS})\}}{1+(P_{RS}/P_{OC}) \exp\{-D(G_{OC}-G_{RS})\}}. \quad (5)$$

Equation (5) takes into account the variation in dilution rates between cultures and the disparity in development times of the two kinds of sporangia when estimating the proportion of organisms which will develop resistant sporangia.

The results of the present paper support the hypothesis that a variety of factors can stimulate the formation of resistant sporangia. Resistant sporangium formation is stimulated by bicarbonate or carbonate ion, but these interact in some way with the yeast-extract peptone medium used. Potassium ion also stimulates the formation of resistant sporangia and this effect is independent of the bicarbonate effect and of differences in the yeast-extract peptone media.

METHODS

The strain of *Blastocladiella emersonii* used in these experiments was obtained from a subculture of Cantino's original isolate which has been maintained in the Berkeley Culture Collection since 1949.

Table 1. *Compositions of culture media used*

PYG medium			
Yeast extract (Difco)	12·5 g.	Tris (2-amino-2-hydroxymethyl-	
Peptone (Difco)	12·5 g.	1,3-propanediol) buffer	6·0 g.
Glucose	30 g.	De-ionized water	10 l.
PG/6 medium			
Peptone (Difco)	2·0 g.	Tris	6·0 g.
Glucose	5·0 g.	De-ionized water	10 l.
Defined medium			
Glucose	10 g.	Mn	0·5 p.p.m.
L-glutamic acid	10 g.	Zn	0·1 p.p.m.
DL-methionine	0·4 g.	Mo	0·2 p.p.m.
Thiamine.HCl	200 μg.	Co	0·2 p.p.m.
KH_2PO_4	10·6 g.	Cu	0·1 p.p.m.
$K_2HPO_4.3H_2O$	9·6 g.	Ca	0·8 p.p.m.
$MgSO_4.7H_2O$	2·0 g.	B	0·5 p.p.m.
Fe (versenol chelate)*	0·3 p.p.m.	De-ionized water	10 l.

* Na_3-N-hydroxyethylethelenediaminetriacetate (Dow Chemical Co., Midland, Mich., U.S.A.)

The ingredients of the media used (defined medium, Barner & Cantino, 1952; PYG; PG/6) are listed in Table 1. For all the media the glucose, the nitrogenous organic materials, the minerals, and the bulk of the water (about 9·5 l. after autoclaving) were autoclaved separately from each other. The pH values of the media were adjusted by adding KOH or H_2SO_4; the K^+ concentration was adjusted with K_2SO_4 except where noted in the results.

The culture apparatus (Fig. 1) (Griffin, 1962) was a modified version of that

developed in the Bio-organic Chemistry Laboratory at Berkeley for the continuous culture of algae. The vessel consists of an outer tube 9 cm. in diameter by about 70 cm. long and an inner tube 7 cm. in diameter by about 65 cm. long projecting concentrically into the outer tube. The two tubes are sealed together at the top so there is a cylindrical space of about 1·5 cm. between them. Both tubes taper conically at the bottom. The volume of the culture is about 600 ml. The inner tube,

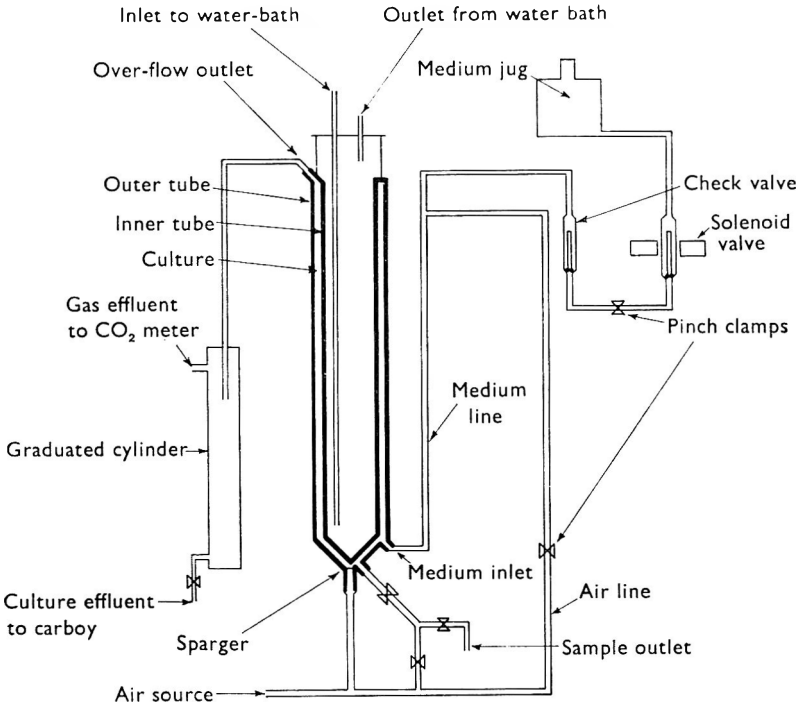

Fig. 1. Diagram of continuous culture apparatus.

closed at the bottom and open at the top, is a water bath through which water is circulated from a thermo-regulated water bath. The outer tube has an opening at the bottom through a ground glass joint wherein a sintered glass sparger mounted in the tip of the male portion of the ground glass joint is inserted. There are two openings in the side of the outer tube near the bottom, one for the medium inlet and one for withdrawing samples and inoculating the apparatus. The effluent opening is located at the top of the outer tube just below the ring-seal joining the inner and outer tubes at the top. The culture is contained in the space between the inner and outer tubes.

The flow rate of medium into the culture is controlled by a clock-operated solenoid valve. The back-up valve in the medium line prevents the culture from accidentally flowing into the medium jug. Air is slowly bled into the lower portion of the medium line to sweep it free from medium and to prevent zoospores from swimming up the medium line.

All experiments in the continuous culture apparatus were made at 25° in light

from G.E., cool white fluorescent bulbs. The intensity at the surface of the culture tube, measured by a Weston Foot Candle Meter was 600 ft.-c.

Inocula were prepared by growing cultures on medium PYG + 2 % agar at room temperature (about 23°) for 15–24 hr, then flooding them for 5 min. to 3 hr with sterile dilute pond water (1 vol. filtered pond water + 2 vol. distilled water; Emerson, 1958). The resulting suspension of zoospores was used to inoculate the cultures. At intervals after inoculation samples were withdrawn for observation. The pH value of the medium was determined with samples freshly aerated with effluent gas of the parent culture. The numbers of mature organisms were determined in samples containing 200 or more.

Glucose was measured by the method of Bernfeld (1955) and glutamic acid by the micro-Kjeldahl method. For mineral analyses the dry ashing procedure of Johnson & Ulrich (1959) was used. After taking the ash up in 3 N-HCl and filtering off unburned carbon, K^+ analyses were made with a Beckman DU Spectrophotometer equipped for flame photometry. A portion of the HCl solution was made up to 0·1 N-$HClO_4$ and 5·7 mM-$SrCl_2$ for Mg^{2+} analyses in an atomic absorption spectrophotometer. Carbon dioxide analyses were done in a Liston–Becker infra-red analyser.

RESULTS

Carbon balance on a steady-state continuous culture

According to Cantino (1961, p. 246) 'The evidence suggests that a predominantly fermentative type of metabolism is involved. For example, a homolactic fermentation is demonstrable under certain conditions during growth.' The observations of Cantino & Hyatt (1953) which showed the presence of the tricarboxylic acid cycle enzymes, and of Cantino & Lovett (1960) which showed the high rate of endogenous O_2 uptake by ephemeral sporangiate Blastocladiella organisms, are not compatible with this view. To help resolve this inconsistency a carbon balance on a steady-state continuous culture under aerobic conditions was made to determine whether the dissimilatory metabolism of *Blastocladiella emersonii* growing in defined medium had a fermentative or oxidative character. The rates of consumption of the carbon sources, glucose and glutamic acid, were correlated with the rates of production of fungus material and CO_2. If the catabolism of the fungus was primarily fermentative, one would expect that only a small portion of the carbon consumed would be converted to fungus material; and in the case of a homolactic fermentation no CO_2 would be produced. On the other hand, if the catabolism was principally oxidative, one would expect that up to 50 % of the carbon consumed would be converted to fungus material (Foster, 1949) and the remainder to CO_2.

Cultures were grown in defined medium (pH 7·5) in the continuous culture apparatus at 25°. The aeration rate was 0·67 l./min. The inflowing gas was air containing 0·0005 atmosphere of CO_2, and the effluent gas contained 0·0010 atmosphere of CO_2. Glucose and glutamic acid analyses were made as described in Methods.

The data showing the rates of consumption of the carbon sources, glucose and glutamic acid, and the fate of this carbon as fungus material and CO_2 are given in Table 2. Assuming the carbon content of the fungus to be 45 % of the dry weight (Cochrane, 1958), the rate of incorporation of carbon into the fungus was calculated from the dry-weight data. As can be seen from Table 2, all the carbon taken from

the medium was accounted for by fungus material + CO_2. This is compatible with the hypothesis that under these particular conditions of nutrition and aeration, the metabolism of the *Blastocladiella emersonii* was largely oxidative and no lactic acid was produced. The conclusion that lactic acid was not produced is supported by the carbon balance and by there being no decrease in pH value. In several batch cultures with defined medium in the continuous culture apparatus, the pH value became more alkaline as the culture grew, also indicating that acid was not being produced. The data of Cantino & Lovett (1960) which showed a high degree of lactic acid production may have been due to a lower degree of aeration than was maintained in the present experiments.

Table 2. *Steady-state carbon balance of Blastocladiella growing in defined medium*

	Growth rate (mg. dry wt./hr/ mg. dry wt.)	Carbon consumed/100 ml. culture: Glucose (μmole/hr)	Glutamic acid (μmole/hr)	Total carbon (μatom/hr)
*A	0·061	33·9	40·9	408 ± 10
B	0·063	35·0	43·0	425 ± 10

	Carbon accounted for/100 ml. culture: CO_2 (μmole/hr)	Fungus (mg. dry wt/hr)	Fungus (μatom c/hr)	Total carbon (μatom/hr)
*A 0·061	180	6·1	229	409 ± 35
B 0·063	187	6·8	255	442 ± 35

* Lines A refer to one determination; lines B refer to a second determination on the same steady state 10 hr after the first.

Growth of resistant sporangiate and ephemeral sporangiate Blastocladiella organisms in continuous culture

The conditions under which resistant sporangiate, and ephemeral sporangiate, organisms developed were studied in continuous culture with medium PYG, and in batch culture with medium PG/6. The reasons for using different methods of cultivation for these two media are explained below. Other conditions and methods were as previously described.

Continuous culture experiments. Equation (5) was used to help interpret the results of these experiments by accounting for the differences in dilution rates between cultures and the differences in development times of ephemeral and resistant sporangiate organisms. The development time of an ephemeral sporangiate organism is the time for a zoospore to grow into an organism bearing a thin-walled colourless sporangium with cleaved-out zoospores. The development time of a resistant sporangiate organism is the time for a zoospore to grow into an organism bearing a pigmented thick-walled resistant sporangium.

The development times of ephemeral sporangiate blastocladiellas, G_{oc}, were calculated from continuous culture data by using equation (4); the G_{oc} values varied from 13 to 19 hr (Table 3). A burst of mature ephemeral sporangiate blastocladiellas was evident in all of the cultures within the first 24 hr. These were the first ephemeral

sporangiate forms to mature from the inoculum and also gave a measure of the development time. These G_{OC} values are in general agreement with the development times reported by Cantino and co-workers: 12 hr (McCurdy & Cantino, 1960), 17–21 hr (Cantino & Horenstein, 1959), 34 hr (Cantino, 1961).

In those cultures where a significant number of resistant sporangiate organisms developed, a burst of mature ephemeral sporangiate organisms occurred within the first 24 hr after inoculation, indicating that the G_{OC} value was also of the order of 20 hr in these cultures. Mature resistant sporangiate organisms began to appear in these cultures from 40 to 60 hr after inoculation and gradually increased thereafter, indicating that the G_{RS} value for individual organisms was highly variable; but the average value was certainly longer than 60 hr. Cantino (1961) reported that the time for 5% of the RS organisms to mature averaged 108 hr.

Table 3. *Development times of ephemeral sporangiate blastocladiellas in medium PYG: continuous culture*

pH value	CO_2 partial pressure (atm. $\times 10^2$)	K^+ concentration (m-equiv./l.)	Development time (hr)	D (hr^{-1})
8·1	0·05	8·0	13	0·10
7·2	4·0	8·0	16	0·18
7·2	4·0	8·0	19	0·15
7·35	0·05	8·0	19	0·12

Table 4. *The probability of a blastocladiella producing a resistant sporangium under various conditions of H^+, K^+ and CO_2 concentration* in continuous cultures, assuming G_{OC} = 20 hr and G_{RS} = 100 hr*

pH value	CO_2 partial pressure (atm. $\times 10^2$)	K^+ concentration (m-equiv./l.)	K^+ added as	D (hr^{-1})	$\frac{P_{RS}}{P_{RS}+P_{OC}}$	ρ_{RS}
8·8	0·05	8·0	KOH	0·048	0·41	0·97
8·1	0·4	8·0	KOH	0·052	0·46	0·98
7·6	4·0	27	KOH	0·047	0·98	1·0
8·0	0·05	27	K_2SO_4	0·015	0·83	0·94
7·7	0·05	28·6	KCl	0·046	0·92	1·0
6·7	0·05	27	K_2SO_4	0·038	0·83	0·94

* Two methods of stimulating resistant sporangium formation were used in these experiments, high CO_3^{2-} and high K^+ concentration. These factors stimulate RS formation independently of each other.

The proportion of blastocladiellas producing resistant sporangia, ρ_{RS}, in cultures containing resistant sporangiate organisms, was calculated by using equation (5) and G_{OC} values of 20 hr and G_{RS} values of 100 hr. For comparison the ratio $P_{RS}/(P_{RS}+P_{OC})$ (the value of ρ_{RS} if one were to consider only the ratio of the number of favourable events to the total number of events without taking into account the variation in dilution rates between cultures and the disparity in the development times between the two types of blastocladiella) is shown in Table 4.

The effect of potassium ion on the development of resistant sporangia

The effect of K^+ concentration on development of Blastocladiella in continuous culture is summarized in Table 5. Potassium at 27 m-equiv./l. induced resistant sporangia. This effect was independent of pH value between pH 6·7 and 8·0, and also independent of the anion; K_2SO_4 and KCl at equivalent concentrations had the same effect. Batch culture experiments in medium PG/6 also showed that potassium ion induced the development of resistant sporangia. These experiments were performed as batch culture experiments because the growth rate of those cultures in which resistant sporangiate organisms developed was very slow and even the slowest dilution rate maintainable in continuous cultivation washed the culture out of the tube. This slow growth rate appeared to be the result of the small number of ephemeral sporangiate organisms in the culture rather than a change in the rate of development of ephemeral sporangiate organisms. Changes in pH value in batch cultures in medium PG/6 were generally less than 0·1 pH unit and often less than 0·05.

Table 5. *The effects of K^+ on the formation of resistant sporangia in medium PYG; continuous culture*

pH value	CO_2 pressure (atm. × 10^2)	K^+ concentration (m-equiv./l.)	K^+ added as	HCO_3^- (m-equiv./l.)	CO_3^{2-} (m-equiv./l.)	Kind of sporangia produced by *c.* 100 % of the organisms
7·2	0·05	8·0	K_2SO_4	0·11	0·162	Ephemeral
8·1	0·05	8·0	K_2SO_4	0·90	10·4	Ephemeral
7·6	4·0	27	KOH	23	80	Resistant
8·0	0·05	27	K_2SO_4	0·72	6·6	Resistant
7·6	0·05	27	KCl	0·29	1·0	Resistant
6·7	0·05	32·5	K_2SO_4	0·36	0·0138	Resistant

Table 6. *The effect of K_2SO_4 on the formation of resistant sporangia in medium PG/6; batch cultures*

pH value	CO_2 partial pressure (atm. × 10^2)	HCO_3^- (m-equiv./l.)	K^+ (m-equiv./l.)	Kind of sporangia produced by *c.* 100 % of the organisms
7·9	0·05	0·58	3·4	Ephemeral
7·95	0·05	0·6	22	*c.* 50 % ephemeral and 50 % resistant
7·9	0·05	0·58	28	Resistant
7·95	0·05	0·6	34	No growth
8·85	0·05	5·0	20	No growth

In the batch culture experiments there were three types of results: (1) those cultures in which 40–70 % of the organisms developed ephemeral sporangia in about 20 hr (designated OC cultures); (2) those cultures in which 1–10 % of the organisms developed ephemeral sporangia in 20 hr and 10–30 % of the organisms developed resistant sporangia in 80–100 hr (designated RS cultures); (3) those cultures in which less than 10 of the organisms developed ephemeral sporangia and less than two developed resistant sporangia in 100 hr (designated as no growth).

The effect of varying K_2SO_4 concentration is summarized in Table 6. At a low K^+ concentration (3·4 m-equiv./l.) ephemeral sporangiate organisms, and at a higher concentration (28 m-equiv./l.) resistant sporangiate organisms developed; above this latter concentration growth was inhibited. These experiments suggest that resistant sporangiate organisms were formed under particular stress conditions. They developed in a narrow zone between where ephemeral sporangiate organisms ceased to develop and where no growth occurred.

The effects of pH, CO_2 and HCO_3^- on the development of resistant sporangia

Experiments to determine the effect of H^+, CO_2 and HCO_3^- concentration on development were made in medium PYG. It was necessary to do these experiments in continuous culture in order to maintain a constant pH value, since the pH value of resistant sporangiate cultures changed rapidly in batch culture (see Table 9). These experiments, summarized in Table 7, showed, under the conditions used, the following results:

(1) Control of development was independent of CO_2 partial pressure since resistant sporangiate organisms and ephemeral sporangiate organisms both developed over the range of 0·0005 to 0·004 atmosphere CO_2. (2) Control of development was independent of pH value since resistant sporangiate organisms and ephemeral sporangiate organisms both developed at the same pH value, pH 8·1. (3) Control of development was independent of HCO_3^- concentration since resistant sporangiate cultures developed from 4·5 to 7·2 m-equiv./l. HCO_3^-, and ephemeral sporangiate cultures developed from 0·11 to 9·2 m-equiv./l. HCO_3^-. (4) Control of development was dependent on CO_3^{2-} concentration since resistant sporangiate cultures developed above 80 m-equiv./l. CO_3^{2-} and ephemeral sporangiate cultures developed below 12·8 μ-equiv./l. CO_3^{2-}.

Table 7. *The effects of pH, CO_2, HCO_3^- and CO_3^{2-} on the formation of resistant sporangia in medium PYG; continuous culture*

pH value	CO_2 partial pressure (atm. × 10^2)	K^+ concentration (m-equiv./l.)	K^+ added as	HCO_3^- (m-equiv./l.)	CO_3^{2-} (μ-equiv./l.)	Kind of sporangia produced by *c.* 100 % of the organisms
7·2	0·05	8·0	K_2SO_4	0·11	0·162	Ephemeral
8·1	0·05	8·0	K_2SO_4	0·90	10·4	Ephemeral
7·2	4·0	8·0	KOH	9·2	12·8	Ephemeral
8·8	0·05	8·0	KOH	4·5	260	Resistant
8·1	0·4	8·0	KOH	7·2	82	Resistant

A similar series of experiments performed with medium PG/6 gave slightly different results. These were batch culture experiments, as previously described, and pH changes during a run were less than 0·1 pH unit. The results are summarized in Table 8 and indicate the following conclusions. (1) Control of development was independent of pH value since resistant sporangiate organisms developed from pH 6·7 to 8·9 and ephemeral sporangiate organisms developed from pH 7·1 to 7·9. (2) Control of development was independent of CO_2 partial pressure, although some CO_2 was required for growth. Resistant sporangiate organisms developed in 0·0005–0·10 atmosphere CO_2 and ephemeral sporangiate organisms developed in less than

0·0001–0·01 atmosphere CO_2. (3) Control of development was dependent on bicarbonate concentration, since resistant sporangiate plants developed between 5 and 8 m-equiv./l. bicarbonate and ephemeral sporangiate plants developed at or below 2 m-equiv./. bicarbonate.

Table 8. *The effects of pH, HCO_3^- and CO_2 on the formation of resistant sporangia in medium PG/6; batch cultures*

pH value	CO_2 partial pressure (atm. × 10^2)	HCO_3^- concentration (m-equiv./l.)	CO_3^{2-} concentration (μ-equiv./l.)	K^+ (m-equiv./l.)	Kind of sporangia produced by *c.* 100 % of the organisms
7·9	0·05	0·58	4·0	3·4	Ephemeral
7·1	1·0	1·8	2·0	3·4	Ephemeral
7·1	0·05	0·09	1·10	3·4	Ephemeral
8·9	0·05	5·8	400	3·4	Resistant
7·95	0·42	4·9	32	3·4	Resistant
7·2	4·2	8·1	13	3·4	Resistant
6·7	10	7·2	0·44	3·4	Resistant
8·85	0·00	0·00	0·00	3·4	No growth
7·9	0·00	0·00	0·00	3·4	No growth
7·9	< 0·01	< 0·01	< 0·80	2·9	Ephemeral

The internal pH value, K^+ content and Mg^{2+} content of ephemeral sporangiate and resistant sporangiate blastocladiellas grown in single-generation batch cultures

The foregoing experiments showed that the external K^+ concentration and pH value, since concentration of ionized CO_2 species are dependent on pH value, were important parameters in the morphogenesis of *Blastocladiella emersonii*. The following experiments were done to determine whether the internal pH value and K^+ concentrations were correlated with resistant sporangium formation. Since K^+ affects the uptake of the other major cation of the cell, Mg^{2+} (Rothstein, 1955, 1961), the cell content of Mg^{2+} was also measured. Sufficient material for analysis of resistant sporangiate cultures could not be obtained from continuous cultures. Therefore, single-generation cultures were grown in 10 l. of medium PYG in 20 l. carboys. The culture was inoculated with a suspension of zoospores prepared as previously described but standardized with a Klett–Summerson colorimeter so that $1·7 \times 10^7$ zoospores were inoculated into each 10 l. culture. The cultures were vigorously aerated with air blown through a bubbler tube. They were grown at 25° in dim light from G.E. white fluorescent bulbs at less than 1 ft.-c. at the surface of the carboys as measured by a Weston Foot Candle Meter. The results are given in Table 9. The results from OC cultures are the average of three replicates and the results from RS cultures are the average of two replicates. The oldest of the OC cultures contained about 5 of the organisms with cleaved or discharged zoospores. The RS cultures contained very few ephemeral sporangiate organisms; about half the organisms had reached maturity by 72 hr.

Table 9 shows no significant differences with respect to internal pH value, potassium content or magnesium content as between ephemeral sporangiate organisms (OC) and resistant sporangiate organisms (RS). There were differences between organisms grown under different conditions, but these differences were not

correlated with the type of organism produced in the culture. The pH value of ephemeral sporangiate organisms varied from pH 6·4 to 7·3, and of resistant sporangiate organisms from pH 6·5 to 7·0. The K^+ content of ephemeral sporangiate organisms varied from 21 m-equiv./100 g. dry wt. organism to 41 m-equiv./100 g. dry wt. organism; that of resistant sporangiate organisms varied from 14 m-equiv./100 g. dry wt. organism to 57 m-equiv./100 g. dry wt. The Mg^{2+} content of ephemeral sporangiate organisms varied from 6·4 m-equiv./100 g. dry wt. organism to 15 m-equiv./100 g. dry wt.; that of resistant sporangiate organisms from 3·2 m-equiv./100 g. dry wt. to 10 m-equiv./100 g. dry wt.

Table 9. *The internal pH, K^+ and Mg^{2+} content of blastocladiellas grown in single generation batch cultures in medium PYG*

	Medium			Analysis of organisms			
					Minerals (m-equiv./100 g. dry wt.)		
Time of growth (hr)	Initial pH value	Final pH value	K^+ as K_2SO_4 m-equiv./l.	Internal pH value	K^+	Mg^{2+}	Kind of sporangia produced by *c.* 100 % of the plants
13	7·3	7·4	8·0	6·4	21	15	Ephemeral
17·5	7·3	7·3	8·0	6·6	22	6·4	Ephemeral
12	6·75	6·7	8·0	7·3	35	8·9	Ephemeral
16	6·75	6·7	8·0	7·1	41	10·6	Ephemeral
24	7·8	7·4	27	7·0	51	10	Resistant
48	7·8	7·4	27	7·0	20	4·8	Resistant
72	7·8	7·5	27	7·0	14	5·1	Resistant
24	7·3	7·3	27	6·6	57	10	Resistant
48	7·3	6·5	27	6·6	28	3·8	Resistant
72	7·3	6·5	27	6·6	31	5·5	Resistant
24	8·4	7·9	8·0	6·9	28	7·2	Resistant
48	8·4	7·7	8·0	6·9	17	3·4	Resistant
72	8·4	6·6	8·0	6·5	23	3·2	Resistant

DISCUSSION

The results show that several factors can influence resistant sporangium formation in *Blastocladiella emersonii*, namely, potassium ion, bicarbonate ion and carbonate ion. The latter two also appear to interact with a factor in yeast extract or peptone; however the potassium effect appears to be independent of this. One would like to know by what mechanisms these factors operate and whether they are affecting the same or closely related processes. The end product appears morphologically identical regardless of the set of conditions in which the resistant sporangiate organisms were grown. There are, doubtless, biochemical features common to resistant sporangiate organisms developing under different conditions. The known biochemical differences between ephemeral sporangiate organisms grown at a high H^+ and low HCO_3^- concentration, and resistant sporangiate organisms at low H^+ and high HCO_3^-, were summarized by Cantino (1961). There may also be other differences. Some, if not all, of these characteristics must be common to the particular developmental pathway regardless of the environmental factors which direct it. Thus, whether the concentration of potassium, bicarbonate or carbonate directs development, there is a common result, and these variables must act on the same initial components of the cell, those found in the young plants.

Rothstein (1955) mentioned a number of interesting phenomena in his review on the effects of electrolytes on the metabolism of yeast. Potassium stimulates the fermentation of glucose without affecting respiration. This stimulation is dependent on the external K^+ concentration and is independent of internal concentration. The effect is not dependent on the simultaneous uptake of potassium. The effect is only on sugar metabolism and not on the metabolism of alcohol or endogenous substrates. Potassium not only affects the rate of fermentation but also changes the ratios of the products, glycerol, ethanol and carbon dixoide, and inhibits the production of polysaccharide, a by-product of metabolism. These effects of K^+ concentration are pH dependent. In the absence of potassium the rate of sugar fermentation shows a bimodal dependence on pH with optima at pH 4·5 and 8·5. In high K^+ concentration the curve becomes flat over the range of pH 2 to 10. The internal pH as measured by the freeze-thaw technique of Conway & Downey (1950) is constant over this external pH range. Rothstein (1955) interpreted these data as an effect of potassium at the cell surface on the metabolism of the cell but the mechanism of action has not yet been explained.

There are certain interesting parallels between the yeast story and the effects observed with *Blastocladiella emersonii*, namely, the internal pH of the blastocladiellas is not correlated with the external pH and the internal K^+ concentration is not correlated with the external concentration over the ranges studied. Perhaps the effect of K^+ in directing the development of *B. emersonii* is also at the cell surface. It is possible that the effects of HCO_3^- and CO_3^{2-} have a similar explanation. Cantino (1961) stated that bicarbonate acts as a CO_2 source, reversing the decarboxylation steps centring around α-ketoglutarate in a weakly functional TCA cycle. One would expect that an increase in free CO_2 would be more effective than increasing the bicarbonate concentration in equilibrium with the CO_2. The data from the batch culture experiments in medium PG/6 and the continuous culture experiments in medium PYG are against the hypothesis that bicarbonate acts as a source of free CO_2, since it was shown that the partial pressure of CO_2 itself was unimportant in determining the development of the blastocladiellas. The carbon balance and the difficulties in sweeping out the metabolic CO_2 in the 'CO_2-free' cultures also suggest that *B. emersonii* has a strong CO_2-forming metabolism rather than a homolactic fermentation. Furthermore, if it can be assumed that unhydrated CO_2 molecules are freely diffusable through a cell membrane (as supported by Conway & Downey, 1950), then the internal pH value of a cell under known partial pressures of CO_2 determines the internal bicarbonate concentration and thus the total CO_2 content. The internal pH of ephemeral sporangiate blastocladiellas grown at pH 6·7 was higher than that of resistant sporangiate organisms grown at pH 8·4. Thus the internal pH value and presumably the internal bicarbonate concentration are not correlated with the external values, and they are just the opposite of what one would expect for bicarbonate to act directly inside the cell in the manner postulated by Cantino. Thus the effects of HCO_3^- and CO_3^{2-} may also be at the cell surface. However, if the primary effect is at the cell surface, it is also transmitted to the internal machinery of the cell to act on such mitochondrial constituents as succinic dehydrogenase and cytochrome oxidase (Cantino, 1961).

One of the principal difficulties yet to be overcome in the study of development of resistant sporangia and ephemeral sporangia of *Blastocladiella emersonii* is the

inability to obtain resistant sporangia in a defined medium. This, coupled with the difference between cultures grown in media PYG and PG/6, suggests that some unknown factor in the peptone and yeast extract is playing a central role in the formation of resistant sporangia. Understanding the nature of this factor will lead to further insight into the machinery of the control of morphogenesis in *B. emersonii*.

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APPENDIX

Continuous culture theory. This treatment depends on the assumptions that dilution is continuous and that there is complete and instantaneous mixing of the inflowing medium with the culture contents.

A given concentration of blastocladiellas, P_T, at any time, t, under a constant dilution rate will be washed out at the rate

$$-dP_T/dt = DP_T. \tag{6}$$

Integration of equation (6) yields

$$P_T = P_{\text{zoosp}}e^{-Dt}, \tag{7}$$

where P_{zoosp} is the initial concentration of zoospores inoculated into the apparatus and P_T is the concentration of growing blastocladiellas in the apparatus after dilution for t hours since inoculation. When these blastocladiellas are releasing zoospores, $t = G$, the development time, and there will be P_0 blastocladiellas in the culture according to equation (8),

$$P_0 = P_{\text{zoosp}}e^{-DG}. \tag{8}$$

Under conditions of steady-state growth with a multi-generation culture it is not possible to follow a single set of zoospores. Since the growth rate of the culture is constant, the number of zoospores, P_z, is constant. All mature blastocladiellas $= G$ hr old. Let Q = the number of hours older than G any particular mature blastocladiella is. Then the number of mature blastocladiellas, P_{mature}, $G+Q$ hours old is

$$P_{\text{mature}} = P_z e^{D-(G+Q)} \tag{9}$$

The total number of mature blastocladiellas, P_M, is given by

$$P_M = \int_0^\infty P_z e^{-D(G+Q)} dQ, \tag{10}$$

and

$$P_M = (P_z/D)e^{-DG}. \tag{11}$$

All immature plants are $< G$ hours old. Let R = the age of any immature plant. R may take values from 0 to G. The number of immature blastocladiellas, P_{immature}, at some age, R, is

$$P_{\text{immature}} = P_z e^{-DR}. \tag{12}$$

The total number of immature blastocladiellas, P_i, is given by

$$P_i = \int_0^G P_z e^{-DR} dR, \tag{13}$$

and

$$P_i = (P_z/D)(1-e^{-DG}) \tag{14}$$

Dividing equation (14) by equation (11) and rearranging gives

$$P_M/(P_M+P_i) = e^{-DG}. \tag{15}$$

Since $P_i+P_M = P_T$, equation (15) can be written

$$P_M/P_T = e^{-DG}, \tag{16}$$

which is identical to equation (4). The logarithmic form (equation (17)) of equation (16) is used for ease of calculation,

$$G = D^{-1}\ln(P_T/P_M). \tag{17}$$

If one knows the development times of ephemeral sporangiate blastocladiellas and resistant sporangiate blastocladiellas, the probability of a zoospore developing into a resistant sporangiate glastocladiella can be calculated from the ratio of mature resistant sporangiate blastocladiellas to mature ephemeral sporangiate blastocladiellas.

For the case where resistant sporangiate blastocladiellas and ephemeral sporangiate blastocladiellas both occur in the same culture, equation (16) can be rewritten into two simultaneous equations (equations (18) and (19)), where P_{RS} is the number of mature resistant sporangiate blastocladiellas, T_{RS} is the total number of blastocladiellas developing along the resistant sporangial pathway, and G_{RS} is the development time of a resistant sporangiate blastocladiella. The subscript OC refers to the corresponding values for ephemeral sporangiate blastocladiellas,

$$P_{RS}/T_{RS} = \exp\{-DG_{RS}\}, \tag{18}$$

$$P_{OC}/T_{OC} = \exp\{-DG_{OC}\}. \tag{19}$$

The total number of blastocladiellas, $TP1$, is given by

$$T_{p1} = T_{RS}+T_{OC}. \tag{20}$$

Therefore the probability of a zoospore developing into a resistant sporangiate blastocladiella, ρ_{RS}, is

$$T_{RS}/T_{p1} = \rho_{RS} \tag{21}$$

By combining equations (18), (19) and (21) and making the proper rearrangements one gets equation (22),

$$\rho_{RS} = \frac{(P_{RS}/P_{OC})_{exp}\{-D(G_{OC}-G_{RS})\}}{1+(P_{RS}/P_{OC})_{exp}\{-D(G_{OC}-G_{RS})\}}. \tag{22}$$

If $G_{OC} = G_{RS}$, then equation (22) reduces to

$$\rho_{RS} = P_{RS}/(P_{OC}+P_{RS}). \tag{23}$$

Inspection of equation (22) shows that for a given ρ_{RS}, when $G_{OC} = G_{RS}$, the observed ratio of mature resistant sporangia to ephemeral sporangia is highly dependent on the dilution rate. Therefore, comparison of results between cultures based on the ratio P_{RS}/P_{OC} is only valid when the dilution rates of the cultures are all identical. Use of equation (22) eliminates the necessity of precisely duplicating dilution rates in different cultures.

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Development of Flagella by *Proteus mirabilis*

By JUDITH F. M. HOENIGER

Department of Microbiology, School of Hygiene, University of Toronto, Toronto, Canada

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SUMMARY

The sequence of flagellar development accompanying differentiation during multiplication in a plate culture of *Proteus mirabilis* was investigated with the electron microscope and the negative-staining technique; the sequence of development can best be seen from the electron micrographs. The first flagella were produced towards the end of the first hour, and increased to a peak at about 6 hr and then decreased. The bacteria changed from coccoid to rod-shaped to elongated forms; the latter measured up to 80 μ in length and were equipped with several thousand flagella. On the basis of measurements of flagellar complement, the elongated forms (or swarmers) can be regarded as 'flagellin-factories'. The fine structure of both flagella and fimbriae was examined and several new features were seen, in particular certain structures at the bases of both appendages. The diameter of Proteus fimbriae was found to be about 40 Å.

INTRODUCTION

This paper deals with the various stages of flagellar development in a plate culture of *Proteus mirabilis* inoculated sparsely all over, a sequence not previously photographed. This paper is one of a series, earlier papers having examined by phase-contrast microscopy the changes in living organisms (Hoeniger, 1964), and by ordinary microscopy the changes manifest in stained preparations (Hoeniger, 1965).

Flagella are now generally regarded to be the locomotor organelles of most eubacteria. In life these organelles are shaped like a helix, but when dried before viewing in the ordinary light microscope or in the electron microscope, their helical structure becomes flattened on the supporting surface and they then assume a sinusoidal shape in the plane, with a wavelength that is fairly constant from one species to another. The chemical and physical properties of bacterial flagella have largely been worked out with Proteus flagella (particularly *Proteus vulgaris*). We know that Proteus flagella consist almost entirely of protein, called flagellin (Astbury, Beighton & Weibull, 1955), which has a chracteristic amino acid composition (Weibull, 1949; Kobayashi, Rinker & Koffler, 1959) and a molecular weight of about 20,000 (Kobayashi *et al.* 1959). It is believed that these protein molecules constitute the subunits of the flagellum proper. According to all recent models, strands or rows of flagellin molecules are arranged helically or lengthwise in the intact flagellum (Kerridge, Horne & Glauert, 1962; Abram, Vatter & Koffler, 1964*a*; Lowy & Hanson, 1964). In the case of the Proteus flagellum, the subunits have a diameter of 45 Å (Rogers & Filshie, 1963) or 50 Å (Lowy & Hanson, 1964), and the flagellum proper a diameter of 120 Å.

More relevant to the present study is the past work on the flagellated Proteus bacteria themselves. Leifson and his associates (Leifson, Carhart & Fulton, 1955; Leifson, 1960) studied Proteus flagellation under various physiological conditions by staining for light microscopy; Pijper, Neser & Abraham (1956) made comparable experiments, viewing living bacteria by sunlight dark-ground illumination. Electron micrographs have been published from time to time illustrating flagellated Proteus organisms (Houwink & van Iterson, 1950; van Iterson, 1953; 1954; Preusser, 1958; Robinow, 1960; Thornley & Horne, 1962), but these did not follow an orderly sequence of differentiation or flagellar development. The only study thus far of flagellar development in Proteus is that by Bisset and his co-worker (Bisset, 1951; Bisset & Hale, 1951).

In the present work, the bacteria were inoculated uniformly but sparsely on a solid medium, and stages of flagellar development followed in the electron microscope by using the negative staining technique. Flagellation was correlated with type of motility in living cultures. Microscopic observations were combined with the determination of the growth-curve in an attempt to correlate morphological with physiological events. This study also afforded an opportunity for re-examining the fimbriae of Proteus since the inoculum contained many fimbriate organisms; and for examining the basal appendages of both flagella and fimbriae.

METHODS

Organism, media and incubation. The strain of *Proteus mirabilis* used in this work was isolated from a stool specimen and maintained on egg slopes (Mackie & McCartney, 1960) at 4°. The inoculum was prepared from cultures grown on slopes of heart infusion agar (Difco) or passed through a Craigie-type motility tube (Tulloch, 1939) of semi-solid agar (heart infusion broth + 0·3 % agar). The organisms were suspended in physiological saline to a concentration of about 10^9 bacteria/ml.; 1 ml. of this suspension was spread uniformly over the surface of heart infusion agar in a 15 cm. diameter Petri dish, thus giving an inoculum of approximately one bacterium/20 μ^2 of surface. Incubation was at 30°, this temperature having been found previously (Hoeniger, 1964) to be most suitable for following the morphological changes in living organisms by phase-contrast microscopy.

Measurement of growth. Growth was measured by dry weight and colony count. Bacteria harvested in saline from three plates were pooled and made up to 100 ml. with saline. For colony counts, a 1 ml. sample was diluted, and 0·1 ml. plated on heart infusion medium containing 4 % agar to inhibit swarming (Mackie & McCartney, 1960). The remaining 99 ml. of bacterial suspension were centrifuged, and the deposit washed and dried to constant weight at 105°.

Electron microscopy. Negative staining was done with living or fixed bacteria. With living organisms, the bacteria were floated off the agar by adding saline to the plate and rocking to and fro (to avoid breaking off flagella by shearing); the bacteria were then decanted into a centrifuge tube, spun down, and washed with distilled water. With fixed organisms, the bacteria were taken up in 0·5 % (w/v) formaldehyde (neutral) and allowed to fix for several hours, then centrifuged down and washed as before. The bacteria were suspended in distilled water at a concentration of about 10^8 organisms/ml. and mixed with an equal volume of 2 % (w/v)

potassium phosphotungstate (PTA; Thornley & Horne, 1962) + 0·02–0·04 % bovine serum albumin (BSA; Fraction V from bovine plasma; Armour Pharmaceutical Company, Kanakee, Illinois, U.S.A.); the BSA, which promotes the spreading of the PTA solution by decreasing the surface tension, was added to the PTA solution immediately before staining. The stained bacteria were placed on an electron microscope grid coated with Formvar and carbon by breaking a thin film in a 5 mm. platinum loop on to the surface of the supporting membrane (Murray, 1963). The grid itself rested on a pad of filter paper so that excess fluid drained away as the film of bacteria dried down rapidly. Electron micrographs were taken with a Philips 100 B electron microscope, operating at 80 kV with a 25 μ objective aperture, at instrumental magnifications of $\times$ 2000–10,000.

In most experiments with the electron microscope, correlated observations were made on living bacteria. The stage of differentiation in a plate culture could be determined quickly by focusing on the surface of growth derived from the sparsely inoculated plate. Motility, whether rotational, translational or absent, was checked with the phase-contrast microscope in hanging-drop mounts of organisms from the plates suspended in broth (see Stocker & Campbell, 1959).

Measurements of bacteria and flagella. The width and length of bacteria were measured directly from electron micrographs; estimates of volume were based on the assumption that the organisms were cylindrical. The number of flagella/bacterium was likewise determined from micrographs, while the length of each flagellum was measured with a piece of string laid along the curves from free end to point of insertion, whenever possible. In the case of the elongated bacteria seen during later stages of differentiation (which can have hundreds, even thousands of flagella), a limited number (about 50–100) was counted or measured over a bacterial length of several μ.

RESULTS

The choice of inoculum

Previous studies (Hoeniger, 1964, 1965) were made with an inoculum of organisms which had passed through a tube of semi-solid agar, i.e. motility-tube cultures incubated for about 40 hr. When such cultures were now examined in the electron microscope, they were found to consist largely of bacteria which were fully fimbriate (with usually 100–300 fimbriae/bacterium—as in Pl. 1, fig. 1) or had both fimbriae (50–100) and flagella (usually 1–3, but occasionally more as in Pl. 1, fig. 2); the remainder of the bacteria were flagellate but non-fimbriate, or had no appendages. All these organisms were small in size and coccoid to rod-shaped (0·6–0·7 μ wide by 0·7–2·0 μ long).

The finding of large numbers of fimbriate bacteria from the 40-hr motility tubes was surprising, and it seemed advisable to investigate the types of appendages on organisms from such cultures incubated for various times up to 48 hr, and to compare them with cultures of similar ages grown on solid medium, i.e. agar slopes. The results are given in Table 1; since only a hundred organisms were screened per specimen the data are intended to show trends rather than finite values. The table shows that: in motility-tube cultures the proportion of fimbriate bacteria (i.e. total of bacteria with fimbriae only and those with fimbriae + flagella) increased with incubation-time; the proportion of flagellate bacteria (i.e. total of bacteria with

flagella only and with flagella + fimbriae) decreased; the proportion of non-flagellate non-fimbriate bacteria remained more or less constant. With bacteria grown on solid medium: the slope cultures always contained a large proportion of flagellate bacteria with usually 1–4 flagella/bacterium, less often 5–10. These flagellate organisms closely resembled the 12 hr bacteria whose morphology will be described in detail below (see Pl. 5, fig. 10). The slope cultures contained far fewer fimbriate organisms than the motility-tube cultures, and there were always some without any appendages.

Since 30–36 hr motility tubes and 18 hr slopes had approximately the same proportion of flagellate bacteria, these cultures were used for inoculating plates in the growth experiments which follow.

Table 1. *Types of appendages on bacteria of Proteus mirabilis passed through semi-solid medium (motility-tube culture) or grown out on agar slopes over a period of 18–48 hr*

	Percentage of bacteria with			
Type of culture	Flagella only	Flagella + fimbriae	Fimbriae only	No appendages
Motility-tube, 18 hr*	—	—	—	—
Motility-tube, 24 hr	93	0	0	7
Motility-tube, 30 hr	58	10	20	12
Motility-tube, 36 hr	32	26	28	14
Motility-tube, 42 hr	22	22	46	10
Motility-tube, 48 hr	24	10	56	10
Slope, 18 hr	67	3	8	22
Slope, 24 hr	66	4	4	26
Slope, 30 hr	50	4	24	22
Slope, 36 hr	43	2	19	36
Slope, 42 hr	44	6	26	24
Slope, 48 hr	46	9	15	33

* Sufficient bacteria for electron microscopy had not yet passed through medium.

Sequence of growth in plate cultures

Figure 1 gives the results of an experiment in which growth was measured in terms of viable count (upper curve, open circles) and dry weight (lower curve, closed circles). It will be seen that the dry weight curve has a short lag phase (*c.* 1 hr), an exponential phase of approximately 5 hr, a retardation phase of 4 hr, and a stationary phase beginning at 12 hr. The colony count curve shows an almost imperceptible lag phase, an exponential phase of *c.* 6 hr, a retardation phase of 4 hr, and the stationary phase again begins at 12 hr. There is no true phase of decline during the 48 hr of the experiment. The generation time of *Proteus mirabilis* in this experiment was 40 min., a value which agrees fairly well with phase-contrast observations of division in living organisms (Hoeniger, 1964).

When one correlates the growth sequence in Fig. 1 with morphological changes on the plates and in the organisms themselves, several points seem to emerge: the short lag phase encompasses the time during which the bacteria are known from phase-contrast studies (Hoeniger, 1964) to be enlarging prior to their first division; the latter half of the exponential phase corresponds to the period when most organisms differentiate into long motile filaments commonly called swarmers (see

Discussion); and the retardation and stationary phases to the time during which the elongated organisms break down into progressively shorter rods that eventually become quite small resting organisms.

As for growth starting from a slope culture, i.e. an inoculum of mainly flagellate bacteria, the two curves are similar to those in Fig. 1, and so are the morphological changes. Evidently, resting organisms from slope cultures, with few fimbriate bacteria, behave very much like those from motillty-tube cultures, with *c.* 50 % fimbriate bacteria.

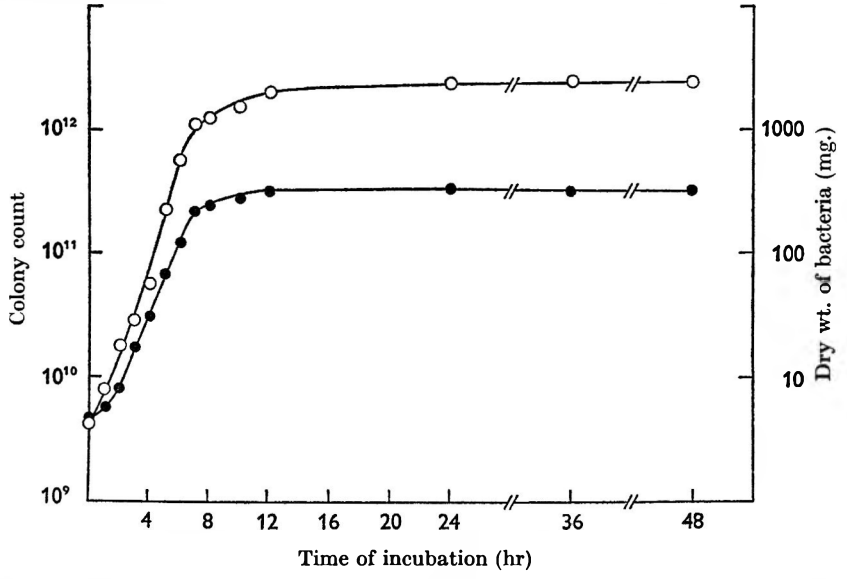

Fig. 1. Growth of *Proteus mirabilis* on heart infusion agar starting from an inoculum of partly fimbriate, partly flagellate bacteria (motility-tube culture). Calculated as colony count (open circles) and dry weight (closed circles), both parameters being derived per crop from three 15 cm. plates.

Electron microscopy of negatively stained bacteria

To turn now to the major part of this paper: the morphological sequence, particularly as regards flagellar development, which takes place during 48 hr of incubation. The first observable change occurs during the first hour: the bacteria enlarge significantly, their diameter increasing from 0·6–0·7 μ to between 0·9 and 1·2 μ; new flagella grow out and appear as short curved spikes (see Pl. 2, figs. 3, 4). The bare bacterium in Pl. 2, fig. 3 has 6 apparently new flagella, and the fimbriate bacterium in fig. 4 has 9. (Of course, in this type of experiment it is impossible to distinguish new from old flagella with certainty.) The mean flagellar lengths on these two bacteria are 1·03μ and 1·09 μ, respectively; the distribution of lengths is plotted as a histogram in Fig. 2 parts (*a*) and (*b*). When organisms from 1 hr plates were observed in the living condition using hanging-drop mounts, many showed a rotating motility. In this and other observations of motility, it was necessary to allow for that proportion of the inoculum, i.e. approximately 50 %, which already possessed long flagella and so moved in quite a rapid, translatory fashion (see Table 1).

That the bacteria continue to grow and synthesize flagella during the next hour can be seen from Pl. 2, fig. 5. This rod has 36 flagella with a mean length of 1·98 μ;

the distribution of the lengths of its flagella is shown in Fig. 2 (*c*). Hanging-drops of such 2 hr cultures showed that the proportion of bacteria with rotating motility had decreased, many now moving in a translational manner.

The end of another bacterium from a 2 hr culture is to be seen in Pl. 6, fig. 11: this bacterium bore a cluster of some hundred fimbriae at either end. In fact, fimbriae which were present on about 50 % of the inoculated organisms were

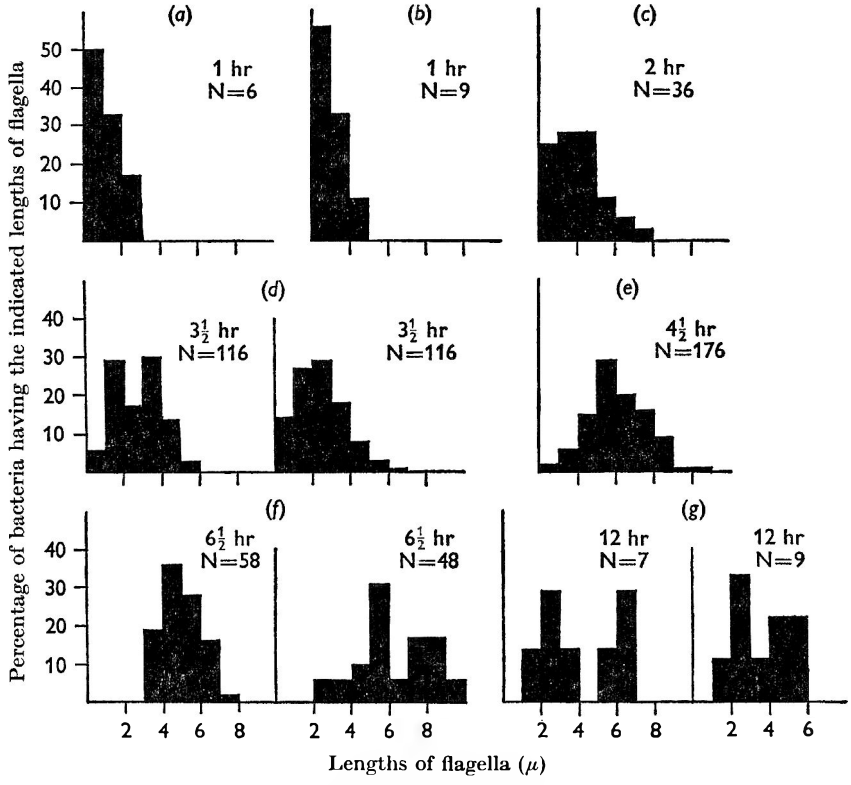

Fig. 2. Distribution of lengths of flagella on *Proteus mirabilis* bacteria shown in electron micrographs. Age of culture is indicated in hr; N = no. of flagella/bacterium.

progressively segregated to the tips of the bacteria in succeeding generations and were not seen beyond 2½ hr of incubation. We shall have more to say about Pl. 6, fig. 11, later.

By the time 3½ hr have elapsed, elongated forms are to be found: a pair of these which had not yet separated is shown in Pl. 3, fig. 6. Both organisms have 116 flagella and many of these have not yet attained their full length, the mean lengths being 2·4 and 2·7 μ. The range of flagellar lengths has been plotted in the histograms of Fig. 2 (*d*). It was further observed that by this time most bacteria in living preparations moved in a translational manner.

During the next 2–3 hr progressively longer and significantly thinner bacteria are to be seen, the well-known Proteus swarmers. These can range anywhere from 6 to 80 μ long, are 0·65–0·75 μ wide, and are equipped with hundreds, even thousands of flagella. A relatively short swarmer from a 4½ hr culture is shown in Pl. 4, fig. 7;

its complement of 176 flagella had a mean length of 4·0 μ; the actual distribution of lengths is plotted in Fig. 2 (*e*). Plate 4, fig. 8, presents the tip of a longer (i.e. 22 μ) swarmer from a 5½ hr culture; by this time more than 60% of the bacteria on the agar plate had differentiated into such long motile filaments. Approximately 300 flagella could be counted on a 5 μ length at the tip of the bacterium in Pl. 4, fig. 8; thus, by inference, the whole organism had over a thousand (*c.* 1300). The flagella range from 2 to 10 μ long, the mean from a measurable sampling of 50 being 4·9 μ; as it obviously is not possible to plot all the lengths of flagella on this organism, no histogram has been prepared. Further it is to be noted that these flagella are produced all over the surface of the organism, a feature which could not be distinguished in most shadow-cast specimens (Houwink & van Iterson, 1950; Preusser, 1958; Robinow, 1960). When one examines such bacteria in the living condition they are seen to move in a sinuous manner which is doubtless responsible for their synonym of 'snakes'.

After 6 hr of incubation the elongated forms start breaking down into progressively shorter units. Plate 5, fig. 9, shows a fairly short swarmer which had divided quite symmetrically to give two rods still attached together at the time of fixation. The average length of flagella on these rods is 4·9 and 6·2 μ respectively; the range of lengths in each case is to be seen in Fig. 2 (*f*). Living bacteria at this stage (i.e in a 6½ hr culture) were all still moving in a translatory fashion.

The process of subdivision continues until the culture consists of coccoid bacteria or short rods like those (from a 12 hr plate) in Pl. 5, fig. 10, which move either rotationally or translationally, depending on the length of the flagella. It will be noted that some of the flagella on the bacteria in Pl. 5, fig. 10, are shorter than during previous stages (their average lengths are 3·7 and 4·1 μ); the distribution of length is plotted in Fig. 2 (*g*).

The sequence of cellular differentiation and flagellar development described in the preceding paragraphs is summarized in Table 2. The data for each age of culture in regard to shape and size-range of organisms, range of number of flagella/bacterium, and average flagellar length (mean $\pm$ S.D.) have been determined from at least 10 micrographs; the one exception is the 5½ hr measurement of average flagellar length which comes from a single specimen (that in Pl. 4, fig. 8). The heading 'Developing flagella' is based on the assumption that the shorter flagella seen during early stages of differentiation are newly developed ones (see above, p. 33). Further, in determining the type of motility in hanging-drop mounts, allowance was always made for the inoculated bacteria (about 50%) known to possess long flagella and hence moving in a translatory fashion.

As Table 2 shows, the average flagellar length increases from 1 to 5½ hr, then falls. An even more informative measure of flagellar synthesis is obtained by calculating the number of flagella/unit volume of bacterium and the total flagellar length/unit volume, as presented in Table 3. It will be seen that there is a gradual increase in both number and total length of flagella to a maximum of about 5½ hr, and then a decline. That the average flagellar length increases with time to a peak, and then declines can also be seen from the histograms of Fig. 2. The rise naturally reflects an increase in the production of flagellin, so that we can really regard the swarmers as 'flagellin-factories'. The decline suggests that in later stages the bacteria are synthesizing flagella at a lower rate.

Table 2. *Characteristics of bacteria and flagella of Proteus mirabilis growing out on agar over 48 hr at 30°, inoculated from motility-tube culture*

	Bacteria			Developing flagella		
Age of culture	Shape or type	Range of diameter (μ)	Range of length (μ)	Range of no./bacterium	Average length (μ)*	Type of Motility
0 hr (inoculum)	Coccoid to rod-shaped	0·6–0·7	0·7–2·0	—	—	—
1 hr	Coccoid to rod-shaped	0·9–1·2	1·1–2·4	1–10	$0·75 \pm 0·29$	Rotating
1½ hr	Rods	0·9–1·2	1·7–2·6	5–30	$1·54 \pm 0·22$	Rotating
2 hr	Rods	0·8–1·0	2·0–3·7	15–60	$2·02 \pm 0·32$	Rotating and translational
2½ hr	Rods	0·8–0·9	3·0–4·2	65–80	$2·38 \pm 0·04$	Translational
3½ hr	Rods and short swarmers	0·7–0·8	3·8–6·0	90–140	$2·61 \pm 0·11$	Translational
4½ hr	Swarmers	0·65–0·75	6·0–*c.* 20·0	*c.* 150–450	$3·84 \pm 0·32$	Translational
5½ hr	Swarmers	0·65–0·75	*c.* 16–80	*c.* 500–5000	5·25	Translational
6½ hr	Rods and short swarmers	0·65–0·75	3·0–8·0	40–120	$4·85 \pm 1·0$	Translational
8 and 12 hr	Coccoid to rod-shaped	0·65–0·8	1·0–2·0	5–30	$3·69 \pm 0·41$	Translational
24 and 48 hr	Coccoid to rod-shaped	0·55–0·65	0·6–1·4	1–10	$2·46 \pm 1·70$	Rotating and translational

* Mean ± S.D.

Characteristics of Proteus flagella and fimbriae

Apparently fully grown flagella, 6–11 μ in length, are to be seen on a proportion of the inoculated bacteria (as in Pl. 1, fig. 2), on the swarmers (Pl. 4, figs. 7, 8) and on short rods derived by the breakdown of the latter (see Pl. 5, fig. 9). These organelles have a mean wavelength or pitch (Leifson, 1960) of $2{\cdot}13 \pm 0{\cdot}14$ μ (S.D.), and are 120 Å wide. In a number of preparations where the flagella had been pulled off as the PTA dried down, hook-like endings were observed. Plate 6, fig. 11, shows the end of a rod from a 2 hr culture with one free flagellum just above the cell and another, indicated by arrows, on the lower surface, evidently still attached and penetrating the wall. Both flagella terminate in hooks. These basal hooks measure 300–400 Å in length, and have the same diameter as the rest of the flagellum. Sometimes the hooks were found attached to rounded cytoplasmic structures about

Table 3. *Development of flagella by Proteus mirabilis in plate culture at* 30°; *data calculated from electron micrographs*

	No. flagella/unit vol. bacterium (μ^3)		Total flagellar length (μ)/ unit vol. bacterium (μ^3)	
Age of culture	Mean	S.D.	Mean	S.D.
1 hr	3·15	1·82	2·64	1·60
1½ hr	9·91	5·64	16·36	9·65
2 hr	18·27	7·63	37·38	14·08
2½ hr	37·43	11·51	74·07	15·79
3½ hr	45·58	6·17	116·55	14·54
4½ hr	80·90	7·67	305·33	32·56
5½ hr	150·00	—	603·00	—
6½ hr	44·36	8·55	214·05	28·96
8 and 12 hr	16·84	5·23	60·37	14·86
24 and 48 hr	18·10	9·61	61·02	44·82

200 Å wide (i.e. between 150 and 250 Å); a cluster of flagella ending in such structures can be observed in the partially lysed swarmer of Pl. 6, Fig. 12. The possible significance of these rounded structures as 'basal bodies' for the flagella will be discussed below. The free ends of the flagella always appear squared off. The presence of an axial core of phosphotungstate in a number of short, developing flagella suggested that the flagella themselves are hollow.

The fimbriae of *Proteus mirabilis* are to be found chiefly on inoculum bacteria taken from motility-tube cultures (see Pl. 1, figs. 1, 2) and in plate cultures derived from them (see Pl. 2, fig. 4 and Pl. 6, fig. 11). On the inoculated bacteria they numbered between 50 and 300 and were peritrichously arranged. In plate cultures, the fimbriae were gradually 'diluted out' as the population multiplied, being segregated towards the poles of the bacteria. A particularly good example of polar fimbriae is to be found in Pl. 6, fig. 11. Proteus fimbriae are straight filaments, in contrast to the wavy flagella, and usually project singly from the periphery of the organism though occasionally they form small bundles with the individual fibres twisted around one another. The fimbriae are 0·2–1·3 μ in length and have a diameter close to 40 Å; i.e. they are one-third as wide as the flagella. In a few instances, evidence was obtained of an attachment organelle on Proteus fimbriae, as can be seen in Pl. 6, fig. 13. These polyhedral structures are *c.* 250 Å wide, and closely

resemble the protusions from which fimbriae have been found projecting on the surface of lysed organisms of a Pseudomonas species by Dr T. Yamamoto (personal communication).

DISCUSSION

The sequence of bacterial differentiation described, which started from a uniformly distributed inoculum, corresponds precisely to that observed by phase-contrast microscopy in plate cultures of *Proteus mirabilis* inoculated at a single point (Hoeniger, 1964—see Figs. 10–19). It seemed, therefore, justified to apply the term 'swarmer' to the elongated forms observed at about 4–6 hr in the type of experiment described in this paper. Swarmers removed from either of these two types of cultures all move in the same sinuous translatory manner when viewed in hanging-drop mounts. Further, the sequence here described of the breakdown of swarmers into progressively shorter organisms was exactly the same as that observed in my earlier work (Hoeniger, 1964; Figs. 25–27). However, one is probably not justified in using the term 'swarming' to refer to the period of churning, swirling activity that takes place on the uniformly inoculated plate between 4 and 6 hr. 'Swarming' should be restricted to the spreading of growth over the uninoculated area of the medium. It is indeed to this latter type of migration by Proteus swarmers that the term was applied in the past (Russ-Münzer, 1935; Lominski & Lendrum, 1947; Kvittingen, 1949; Hughes, 1957).

Several aspects of the sequence by which the small Proteus bacteria of the inoculum are differentiated, via rod-shaped organisms, into elongated swarmers merit comment. The fact that the peritrichous fimbriae present on about 50 % of the inoculum are progressively segregated during the first 2½ hr to the poles of derivative bacteria suggests that new cell-wall material is being synthesized in the equatorial region rather than by diffuse intercalation of new material with old. Such an equatorial manner of cell-wall replication has already been observed in streptococci (Cole & Hahn, 1962; Chung, Hawirko & Isaac, 1964); *Salmonella typhi*, on the other hand, synthesizes new wall by intercalation (Cole, 1964). The application of fluorescent antibody staining to cell-wall replication during the differentiation of Proteus swarmers will throw further light on this problem. The synthesis of flagella during bacterial differentiation appears to be a random process. The longest, and presumably the first, flagella (which develop during the 1–2 hr after inoculation) are located either at the equator (as in Pl. 2, fig. 3), or towards a pole (Pl. 2, fig. 4), or in both regions (Pl. 2, fig. 5). There is no evidence to support the view that the first flagellum is invariably produced by Proteus at a single pole (Bisset & Hale, 1951). Nor were the flagella shed during later stages as earlier observed by Bisset & Hale.

During the developmental sequence leading to swarmers, the whole physiological organization of the bacterium changes. There is a switch from the production of vegetative rods to that of elongated forms. This switch is presumably mediated through an inhibition of the normal mechanism of cell division. The bacteria thus produced are quite different from their progenitors: not only are they much longer and significantly thinner, but they have many more flagella/unit volume (see Table 3). In fact, the swarmers have already been called 'flagellin-factories'. This suggests that there is a partial curtailment in the production of cellular protein which results in the greatly increased production of flagellar protein.

To discuss now the characteristics of Proteus flagella and fimbriae described in this paper. The flagellar width of 120 Å agrees well with that reported for shadowed preparations of *Proteus vulgaris* by Astbury & Weibull (1949); the wavelength of $2{\cdot}13 \pm 0{\cdot}14\ \mu$ is likewise close to previous determinations (Reichert, 1909; Weibull, 1950; Leifson *et al.* 1955; Pijper *et al.* 1956). The hook-shaped endings seen in Pl. 6, figs. 11 and 12, are reminiscent of the little hooks or 'rootlets' observed on isolated flagella of *Agrobacterium radiobacter* (Houwink & van Iterson, 1950), *P. vulgaris* (Rogers & Filshie, 1963) and *Vibrio metchnikovii* (Glauert, Kerridge & Horne, 1963), and of the hockey-stick shaped endings seen on intact flagella of *Spirillum* spp. (Houwink, 1953; Murray & Birch-Andersen, 1963) and *V. metchnikovii* (Glauert *et al.* 1963). The observation that the flagellar hooks are attached within the cell to rounded structures about 200 Å wide (see Pl. 6, Fig. 12) is indeed interesting. Similar basal discs were recently observed in lysed bacteria of *V. metchnikovii* (Glauert *et al.* 1963), *P. vulgaris* (Abram, Vatter & Koffler, 1964*b*) and some Bacillus species (Abram *et al.* 1964*b*). Van Iterson & Leene (1964) have found the basal regions of the flagella in *P. vulgaris* to be sites for binding reduced tellurite, which suggests the presence there of reductive enzymes. It is the opinion of the present writer that these 'basal bodies' are the regions in which the flagellin molecules are aggregated to form flagella, there being anywhere between 3 and 10 strands or rows of such flagellin molecules/flagellum according to the various models proposed to date (Kerridge *et al.* 1962; Abram *et al.* 1964*a*; Lowy & Hanson, 1964).

The fimbriae of *Proteus mirabilis* are thinner than those of other enterobacteria (Duguid & Gillies, 1958; Duguid, 1959; Brinton, 1959; Thornley & Horne, 1962). In fact, the dimension given here of *c.* 40 Å is significantly lower than previously reported from shadowed preparations of intact fimbriae (Coetzee, Pernet & Theron, 1962; Shedden, 1962), and slightly lower than that from negatively stained preparations of isolated fimbriae (Rogers & Filshie, 1963). Of particular note is the presence of a 'basal body' also on these organelles, as can be seen in Pl. 6, fig. 13. This body may arise either within the cytoplasm proper or in the cytoplasmic membrane. Thus fimbriae and flagella in general appear to originate inside of the bacterial cell wall. Indeed, both structures are retained when appropriate strains of *Escherichia coli* or *P. mirabilis* are converted to sphaeroplasts by treatment with penicillin (Lederberg & St. Clair, 1958; Maccacaro & Turri, 1959; Martin, 1963). The basal structures on the fimbriae, like those on the flagella, are probably the sites at which the protein subunits (Brinton & Stone, 1961; Brinton, 1965) are assembled into the fimbriae proper.

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EXPLANATION OF PLATES

In figs. 1–10, the marker represents 1 μ; in figs. 11–13, it represents 0·1 μ.

PLATE 1

Electron micrographs of inoculum organisms which had passed through a tube of semi-solid agar, i.e. from a motility-tube culture.

Fig. 1. Pair of fimbriate coccobacilli.

Fig. 2. Fimbriate rod with 6 long flagella.

PLATE 2

Electron micrographs of bacteria showing early stages of flagellar development.

Fig. 3. Bare rod with 6 young flagella; from 1 hr plate.

Fig. 4. Fimbriate rod with 9 young flagella; from 1 hr plate.

Fig. 5. Rod with 36 flagella of various lengths: from 2 hr plate.

PLATE 3

Fig. 6. Electron micrograph of a pair of young swarmers which have not yet separated; from a $3\frac{1}{2}$ hr plate.

PLATE 4

Electron micrographs of Proteus swarmers.

Fig. 7. Short swarmer from a $4\frac{1}{2}$ hr plate.

Fig. 8. 6 μ tip of a much longer (i.e. 22 μ) swarmer taken from a $5\frac{1}{2}$ hr plate.

PLATE 5

Electron micrographs of bacteria derived by the breakdown of swarmers.

Fig. 9. Pair of rods from $6\frac{1}{2}$ hr plate culture, with many long flagella.

Fig. 10. Pair of rods from 12 hr plate, with flagella of various lengths.

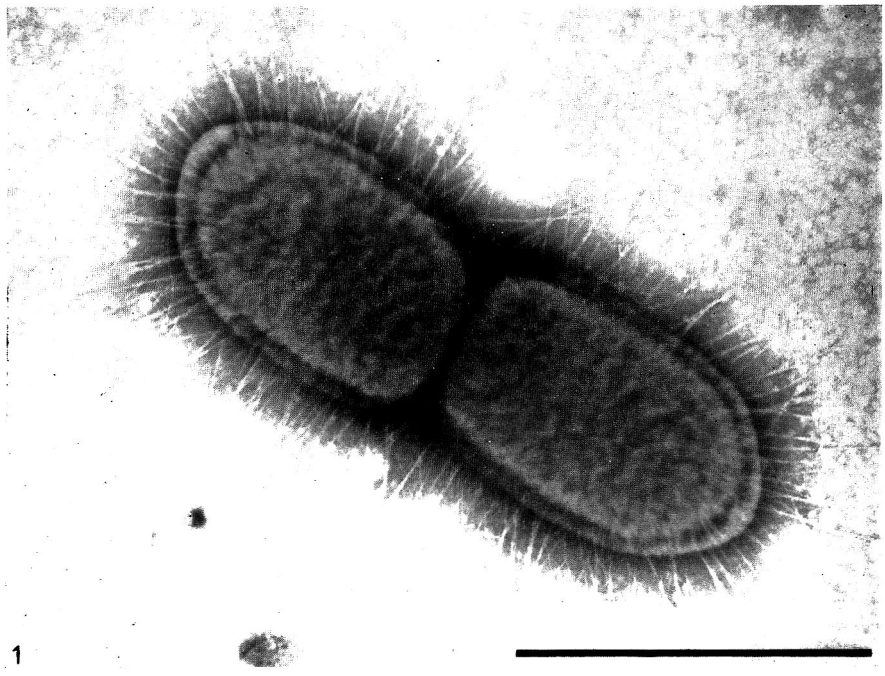
PLATE 6

Electron micrographs of Proteus flagella and fimbriae showing details of basal structure.

Fig. 11. Tip of rod from a 2 hr plate bearing flagella and fimbriae; terminal hooks on 2 flagella are indicated by arrows.

Fig. 12. Flagella penetrating the cellular envelope of a partially lysed swarmer; each flagellum ends in a hook which is attached to a spherical mass of cytoplasm.

Fig. 13. Three fimbriae with polyhedral appendages presumed to be cytoplasmic in origin.



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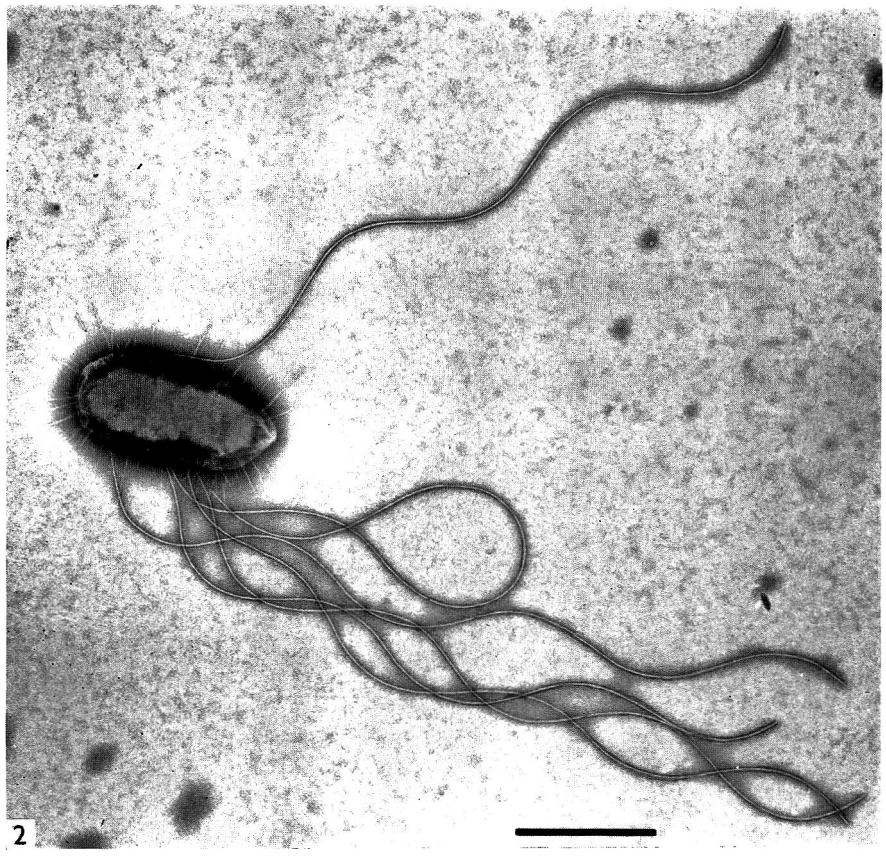

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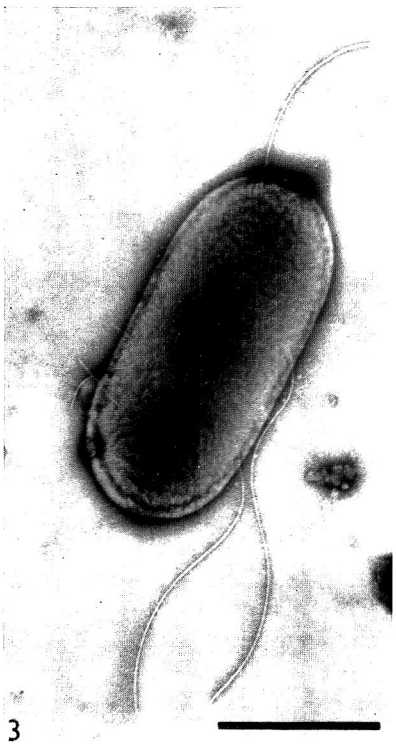

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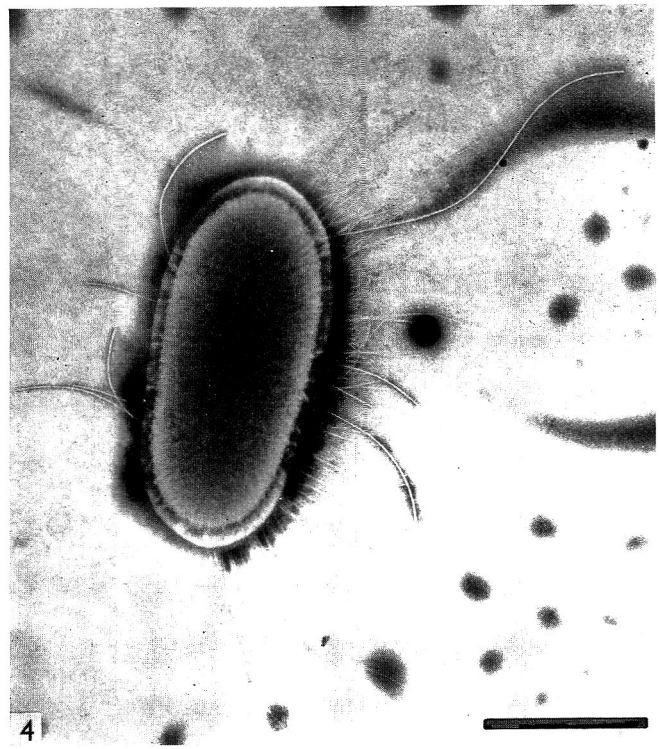

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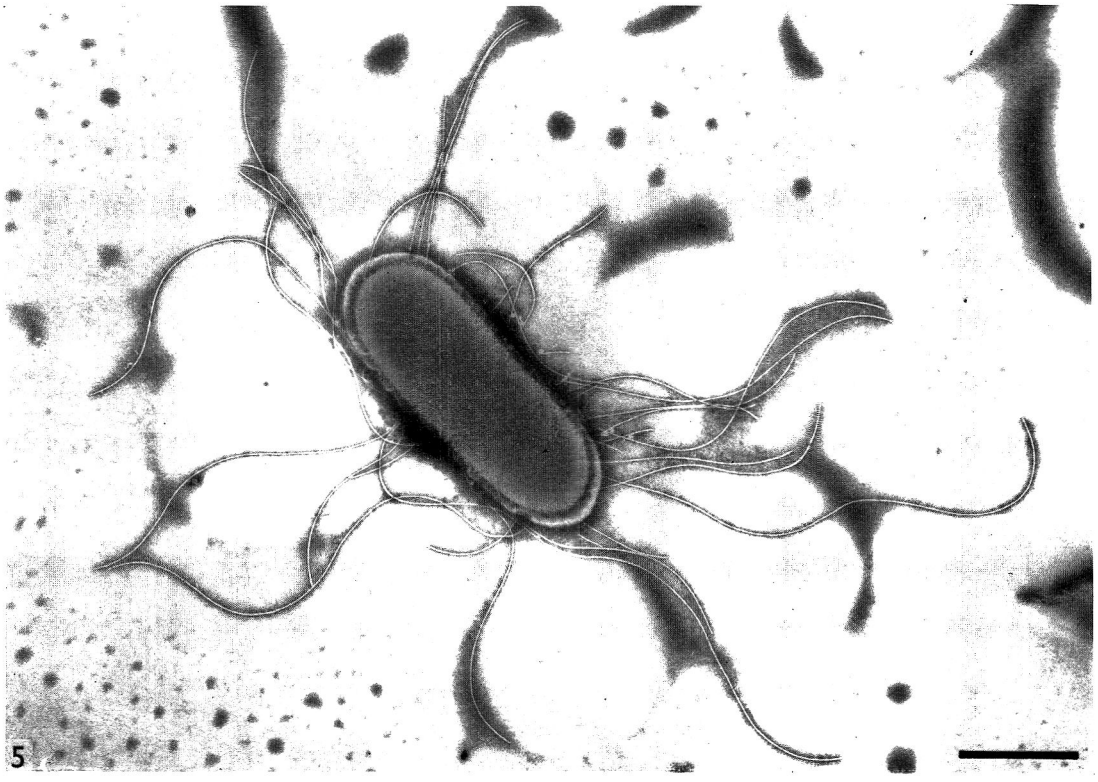

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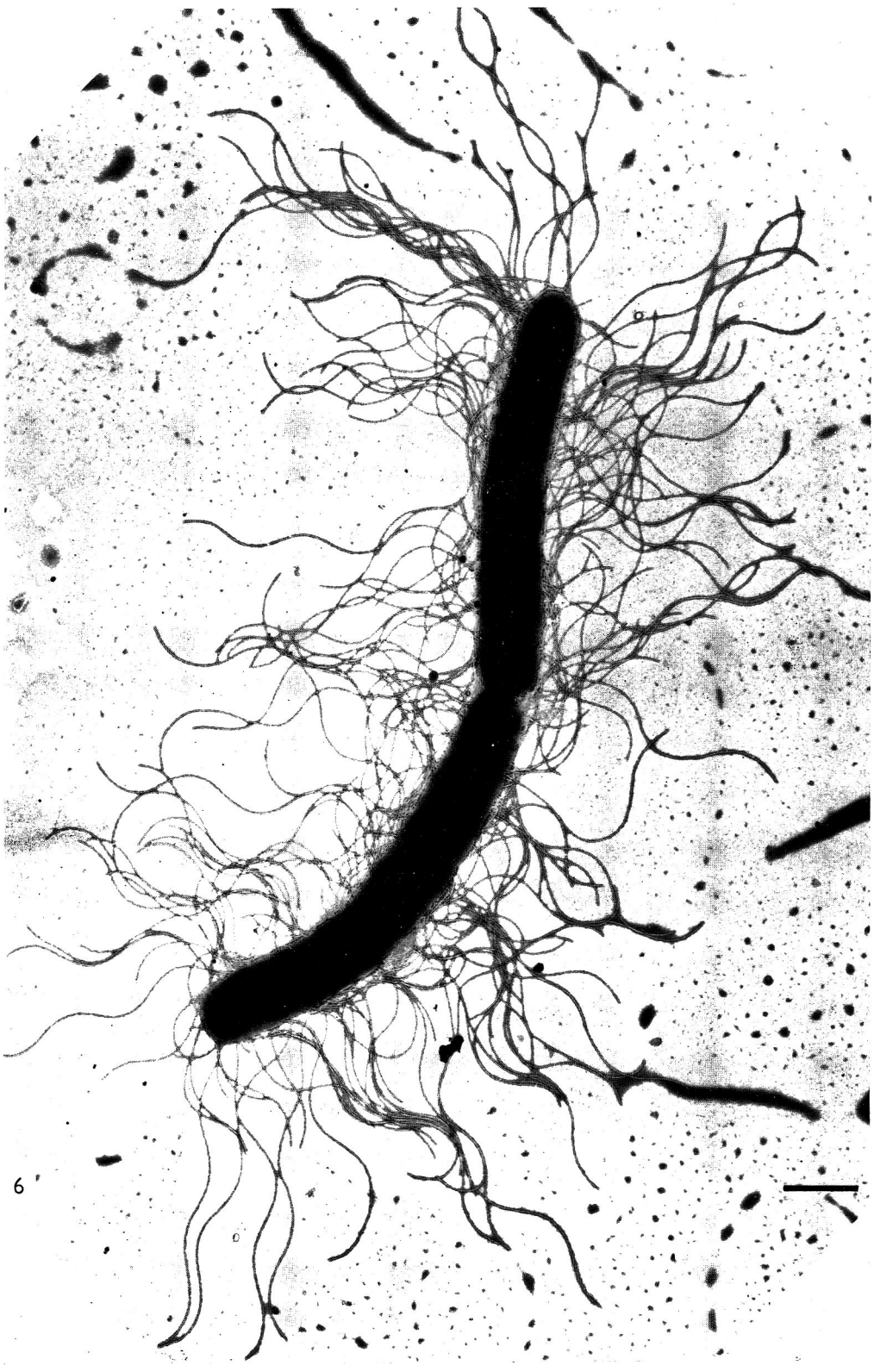

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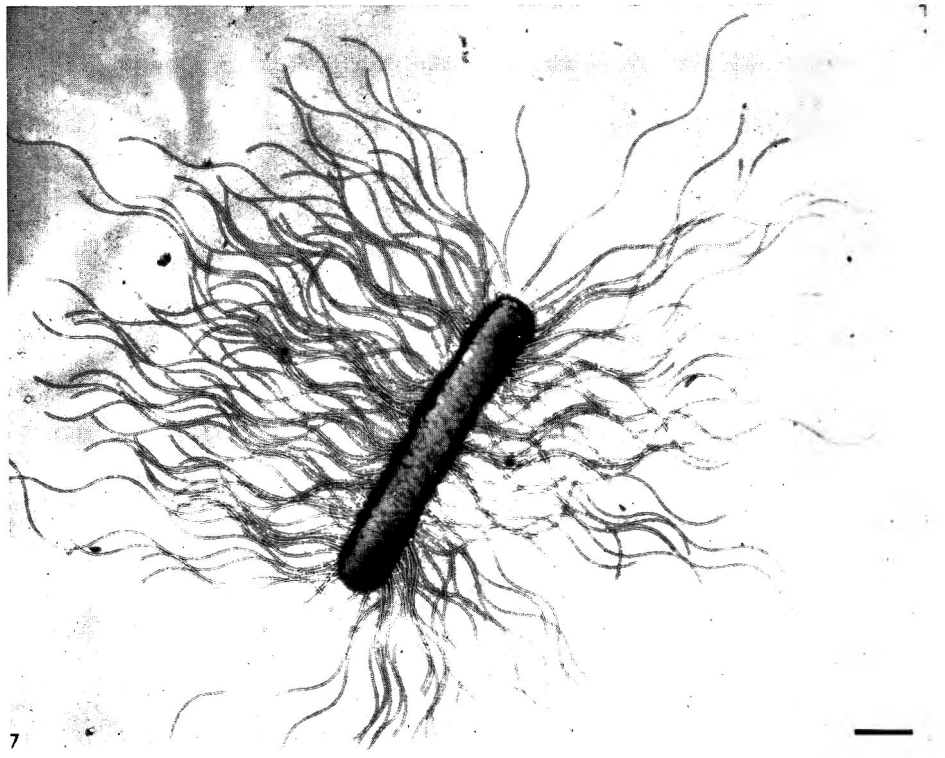

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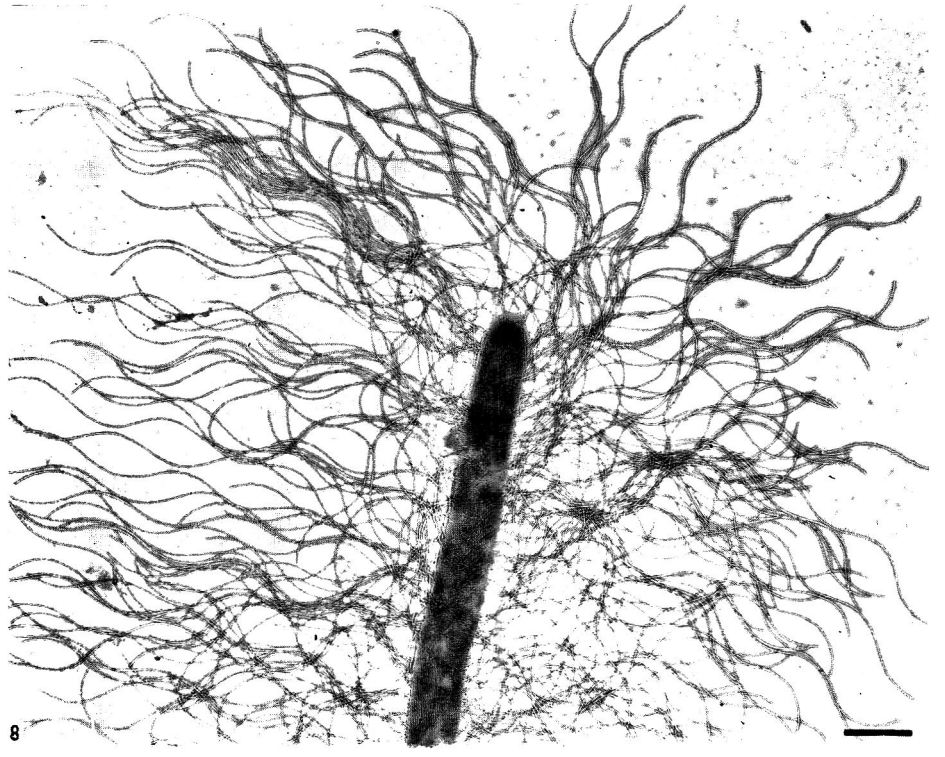

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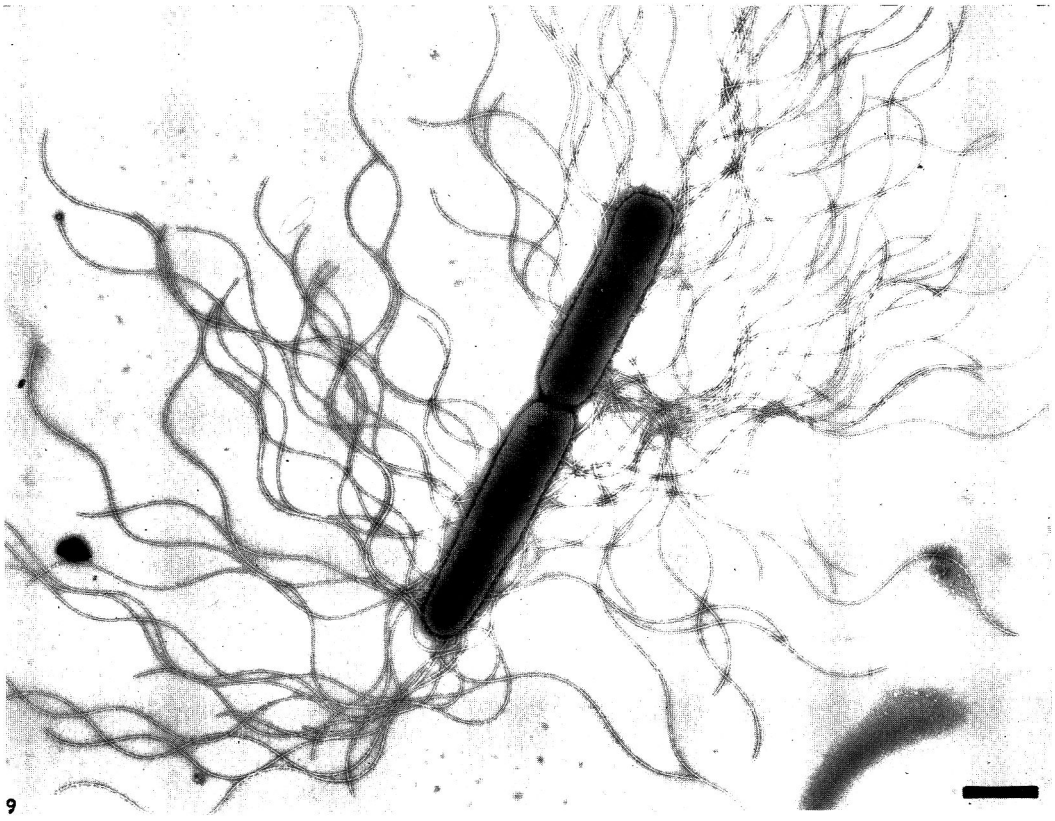

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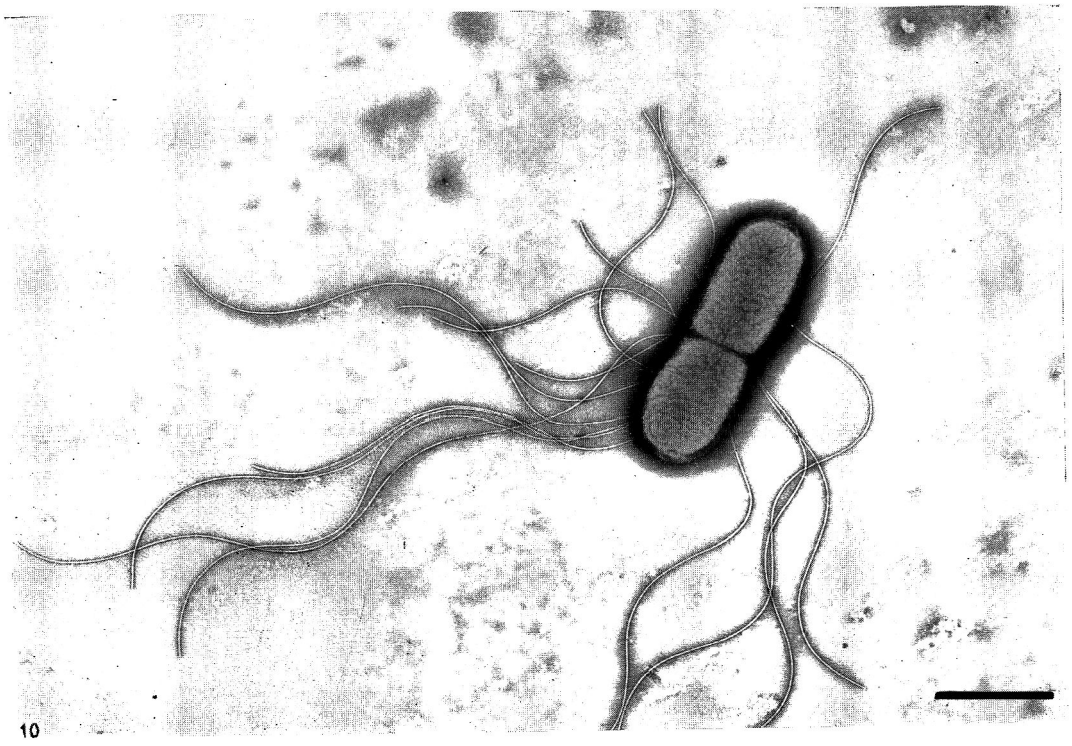

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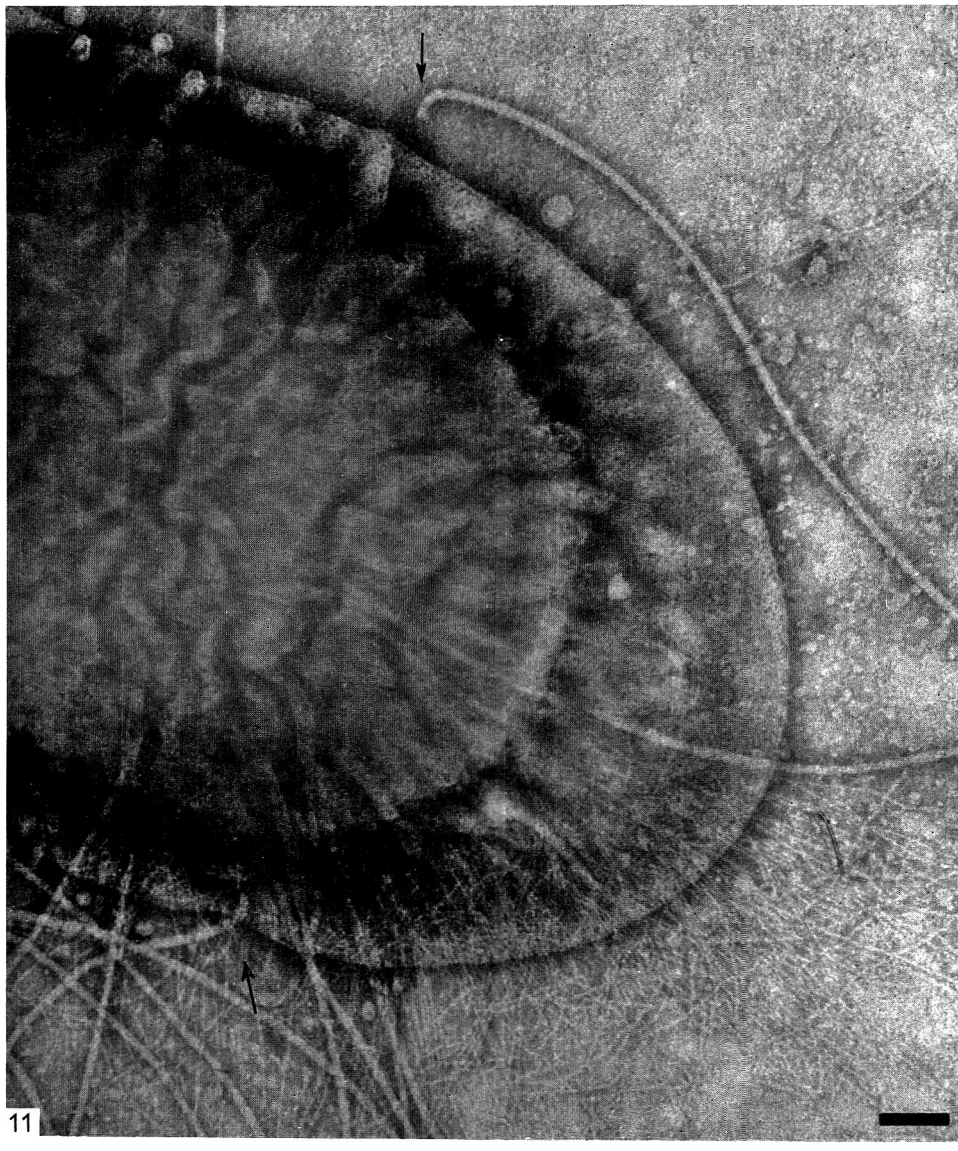

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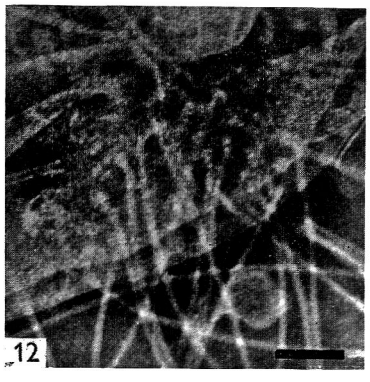

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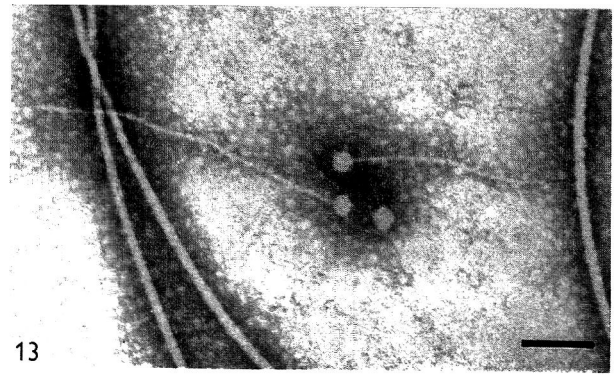

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Resistance to the Bactericidal Effect of Ultraviolet Radiation Conferred on Enterobacteria by the Colicine Factor *colI*

By SHEILA HOWARTH*

Guinness-Lister Research Unit, Lister Institute of Preventive Medicine, Chelsea Bridge Road, London, S.W. 1

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SUMMARY

Strains of *Salmonella typhimurium* and *Escherichia coli* which have acquired the colicine factor, *colI*, are less sensitive to the lethal effects of ultraviolet (u.v.) radiation than the non-colicinogenic parent strain. The dose of u.v. radiation required to kill 50 % of the population of *S. typhimurium* strain LT2 colicinogenic for colicine I was greater by a factor of three than that required to kill 50 % of the non-colicinogenic parent. The number of survivors of the non-colicinogenic strain decreased more or less exponentially with dose; the survival curve of the colicinogenic strain had a pronounced 'shoulder'. Experiments with a strain of *E. coli* K12 carrying lambda prophage indicated that the presence of *colI* decreased the incidence of phage induction following u.v. irradiation.

INTRODUCTION

Some strains of the Enterobacteriaceae produce colicines which are antibiotics active against some other members of this family, and for this reason are termed 'colicinogenic' (Gratia, 1925; Fredericq, 1957). The ability to produce colicine is attributed to the presence in the colicinogenic strain of a transmissible genetic determinant called a 'colicine factor' regarded as belonging to a group of genetic elements termed 'plasmids' (Lederberg, 1952). Many different colicines are known, the ability to form each one being determined by a different factor.

Non-colicinogenic strains may be made colicinogenic for certain colicines by growth in mixed culture with an appropriate colicinogenic strain (Fredericq, 1954). During culture together, cell conjugation occurs, initiated by the presence of the colicine factor, which is thereby transmitted to the non-colicinogenic strain (Ozeki, Stocker & Smith 1962). Amongst the factors transferred in this way is that responsible for the production of colicine I, that is colicine I factor (henceforth referred to as *colI*). In *Salmonella typhimurium* strain LT2 and *Escherichia coli* strain K12, *colI* behaves as a sex factor, analogous to the mating factor, F, of *E. coli* K12, so that under certain conditions not only are colicine factors transferred but also chromosomal genes (Ozeki & Howarth, 1961; Clowes, 1961; Smith & Stocker, 1962).

An attempt was made to increase the number of recombinants arising from colicine-factor-mediated recombination in *Salmonella typhimurium*, as outlined above, by u.v. irradiation of one or other of the parent strains (see Hayes, 1953); it was then

* Present address: Department of Microbiology, University of Otago Medical School, Dunedin, New Zealand.

found that the parent lines differed considerably in u.v. sensitivity; the strain carrying *colI* was more resistant than the non-colicinogenic parent.

Colicines are produced spontaneously by only a small fraction of a colicinogenic population and these bacteria are, in consequence, non-viable (Ozeki, Stocker & de Margerie, 1959). Some colicine factors are inducible by u.v. radiation (Fredericq, 1957) but inducibility has not been demonstrated for *colI* when present in *Salmonella typhimurium* (Ozeki *et al.* 1959). Amati (1963) and Monk & Clowes (1964) have, however, reported u.v. induction of colicine I production in *Escherichia coli* K12. Therefore, one would expect, *a priori*, the presence of *colI* to have no effect on u.v. survival or that possibly it might increase sensitivity, if induction did occur

METHODS

Bacterial strains. Wild-type *Salmonella typhimurium* LT2 and auxotrophic mutants came from the Department of Genetics, Carnegie Institution, Cold Spring Harbour, New York. Mutant *cysD-36* (Clowes, 1958) requires cysteine for growth; *athC-5* (Yura, 1956) requires adenine and thiamine for growth. Colicinogenic derivatives of these and other strains are indicated by the addition of the symbol for the colicine factor concerned; for example *cys*D-*36* (*colI*) is a derivative producing colicine I. The following colicinogenic derivatives, *cysD-36* (*colI*), *cysD-36* (*ColE1*), *cysD-36* (*colI*) (*colE2*), *cysD-36* (*col11–17*), *cysD-36* (*colB1*) *and athC-5* (*coll*), were from stocks maintained at the Guinness-Lister Unit and have been described by Ozeki, *et al.* (1962). The source of the *colI* factor was *Shigella sonnei* strain P9. *Salmonella typhimurium* SL427, wild-type LT2 'cured' of a B prophage (Boyd, 1950), maintained at the Guinness-Lister Unit, had been supplied originally by Dr N. Zinder, Rockefeller Institute for Medical Research, New York.

A non-colicinogenic, non-lysogenic subline of *Escherichia coli* K12 HfrC was obtained from Dr K. W. Fisher, Microbial Genetics Unit, Medical Research Council, Hammersmith Hospital, London. It is an Hfr recombinant of Hfr Hayes cured of lambda (λ) prophage, requiring thiamine for growth, resistant to azide and sensitive to λ phage. Three colicinogenic and/or lysogenic derivatives of HfrC were prepared, (λ) *col*$^-$, λ^- (*colI*) and (λ) (*colI*). Lysogeny for lambda phage is indicated by (λ). *Salmonella typhimurium* strain *cysD-36* (*colI*) was used as the source of *colI*. Lambda phage for lysogenization was kindly supplied by Dr G. G. Meynell of the Guinness–Lister Unit.

Media. Nutrient broth was made from a tryptic digest of beef. For growth studies involving the use of a nephelometer, cultures were grown in buffered glucose peptone water containing: glucose, 1 g.; peptone, 10 g.; NaCl, 5 g.; sodium glycerophosphate 31 g.; distilled water, 1000 ml.; brought to pH 7·2 by adding N-HCl. Peptone agar, used as a routine medium for viable counts consisted of: peptone (Evans), 20 g.; NaCl, 5 g.; distilled water, 1000 ml.; agar, 13 g.

Culture conditions. Broth cultures were grown in loosely capped 25 ml. bottles ('Universal containers') containing 10 ml. nutrient broth and incubated at 37°, without aeration or shaking. Plate cultures were also incubated at 37°.

Estimation of bacterial growth. Growth curves were plotted from liquid cultures, samples being estimated in a nephelometer (Evans Electroselenium Ltd., Bishop's Stortford, Herts.).

Preparation of colicinogenic derivatives. The methods used for obtaining colicinogenic strains of *Salmonella typhimurium* have been described in detail by Ozeki *et al.* (1962). In the case of *colI*, which is readily transmitted by mixed culture, it consisted of growing together a suitable colicinogenic donor strain and the non-colicinogenic acceptor strain. For preparation of colicinogenic derivatives of wild-type LT2 and SL427, strain *cysD-36* (*colI*) was the source of *colI*. A strain of *Escherichia coli*, CL104, was used as the colicine-sensitive indicator strain (Ozeki *et al.* 1962).

Escherichia coli is sensitive to colicine I, unlike *Salmonella typhimurium*. Resistant mutants of both the non-lysogenic parental strain HfrC and its derivative lysogenic for λ (see below) were isolated from colonies appearing in colicine-inhibition zones (Fredericq, 1957). Resistant strains were made colicinogenic for colicine I by culture in common with *cysD-36* (*colI*).

Preparation of lysogenic strains of Escherichia coli HfrC. Bacteria were infected with phage λ at high multiplicity and survivors tested for lysogeny by u.v. induction.

Ultraviolet irradiation. Overnight unaerated broth cultures were centrifuged, washed and resuspended in saline, then usually agitated in the M.S.E. blendor for 2 min. at a speed of 12,000 rev./min. to break up clumps and pairs of bacteria. This suspension contained 10^8–10^9 bacteria/ml., depending on the organism. It was usually diluted 10^{-2} or 10^{-3} in saline for irradiation. A 3 ml. volume of the final suspension was u.v. irradiated in an open Petri dish, on a mechanical rocker, to ensure uniform irradiation. Dose was measured as time of exposure, at a standard distance from the u.v. source. Treated bacteria were plated for viable counts on peptone agar.

Two lamps were used for u.v. irradiation: one was a high-pressure mercury arc giving an ouput in many regions of the u.v. spectrum; the other was a low-pressure Westinghouse Sterilamp, type no. 782L-30, emitting more than 80 % of its radiation in the region of 2537 Å.

Ultraviolet induction of prophage λ (Lwoff, Siminovitch & Kjeldgaard, 1950). A saline suspension of λ-lysogenic bacteria containing about 10^7 bacteria/ml. was plated for viable count and plaque count (Adams, 1959) before and after irradiation. The λ-sensitive strain HfrC was used as an indicator for plaque counts. After overnight incubation bacteria which formed plaques at the time of plating in overlays produced large distinct plaques, distinguishable from the small clearings surrounding most colonies of the lysogenic strain.

To measure the effect of different doses of u.v. radiation on induction, separate 3 ml. samples of the saline suspension were irradiated for increasing periods. To ensure a high percentage of induction in irradiated bacteria, they were incubated in broth for 30 min. before plating for plaque counts (Weigle & Delbrück, 1951).

RESULTS

Effect of colI on the u.v. sensitivity of Salmonella typhimurium LT2

Typical dose-survival curves, obtained for the pair of strains *cysD-36 col*$^-$ and *cysD-36* (*colI*), are given in Fig. 1. The non-colicinogenic strain, *cysD-36 col*$^-$, was killed more or less exponentially, with a slight increase in death-rate at doses greater than 2 min.; the curve of the colicinogenic strain, on the other hand, showed

a pronounced shoulder. The final slopes of the two curves were about the same. From examination of survival curves the dose of u.v. radiation required to give 50 % survival of the non-colicinogenic strain was about 30 sec. and that for the colicinogenic strain about 100 sec. Repeated testing of the two strains, *cysD-36 col*$^-$ and *cysD-36* (*colI*), gave reproducible results when the high-pressure mercury arc lamp was used for u.v. irradiation (see Methods). This lamp was used for all experiments to be described, but results were later checked by using a low-pressure Westinghouse Sterilamp, when the same difference in survival of colicinogenic and non-colicinogenic strains was apparent.

In the experiment represented in Fig. 1 possible photo-reactivation was prevented by doing the experiment in a darkened room with only subdued yellow light. However, daylight, when not direct sunlight, had no noticeable effect upon survival and so all subsequent experiments were done under normal laboratory conditions of lighting.

Several possible explanations of the apparent increase in resistance to u.v. radiation, brought about by *colI* agent, were tested experimentally.

(1) That the resistance of the colicinogenic strain was due to clumping of bacteria in the irradiated suspension. As a precaution against clumping, suspensions were routinely agitated in a blendor before irradiation, at a speed sufficient to break up all clumps and pairs of bacteria.

(2) That a substance or substances which affected survival after u.v. treatment might be secreted into the medium during growth. In one experiment, an overnight culture of the colicinogenic strain was centrifuged and samples of bacteria resuspended in supernatant fluids of overnight cultures of the colicinogenic and the non-colicinogenic strains where they were left for 1 hr at 37°, before dilution in saline for irradiation. Bacteria of the non-colicinogenic strain were treated in the same way. In a second test, a saline suspension of the colicinogenic strain was u.v. irradiated in the usual way, but before plating for viable counts, the bacteria were diluted in supernatant fluids of overnight cultures of the colicinogenic and of the non-colicinogenic strains. In neither test was the survival of a strain noticeably changed by exposure to the two different supernatants.

(3) That the difference in the sensitivity of the two strains might have been due to differences in sensitivity to the indirect effects of u.v. radiation, for example, to peroxide ions formed in the irradiated medium. This was tested by irradiating bacteria in saline containing 0·1 % sodium sulphite, to eliminate free peroxide ions. The difference in the survival curves was the same as obtained by u.v. irradiation in plain saline. Furthermore, when bacteria were u.v. irradiated after being gently spread on the surface of peptone agar plates, when indirect effects of irradiation would be minimized, the difference between the two curves was unchanged, though the rate of killing of both strains was slightly greater than when the same doses of radiation were given to the bacteria in suspension.

(4) The results of Alper & Gillies (1958, 1960) suggest that a slow-growing strain should have a greater chance of survival after u.v. irradiation than a faster growing strain. The growth of cultures of several pairs of strains, non-colicinogenic parent and colicinogenic derivative ,were compared by turbidity measurements. The strains tested were *cysD-36 col*$^-$ and *cysD-36* (*colI*); *athC-5 col*$^-$ and *athC-5* (*colI*); LT2 (wild-type) *col*$^-$ and LT2 (wild-type) (*colI*); SL427 *col*$^-$ and SL427 (*colI*). In no case

did the growth rate of a pair of strains differ once growth was in the exponential phase. However, in each case the irradiated non-colicinogenic strain showed a longer lag period (about 3 hr after 1 min. dose) than the colicinogenic strain (about 1 hr after 1 min. dose), presumably because there were fewer survivors in the former suspension. A dose of 1 min. was expected to give about 70 % survival of the colicinogenic strain and about 15 % survival of the non-colicinogenic strain. With increase in dose there was increase in the length of the lag period, more apparent in the case of the non-colicinogenic strain in which the lag period increased to about 5 hr with a 2 min. dose, whereas the lag period of the colicinogenic strain increased only slightly to about 1 hr 30 min. with a 2 min. dose.

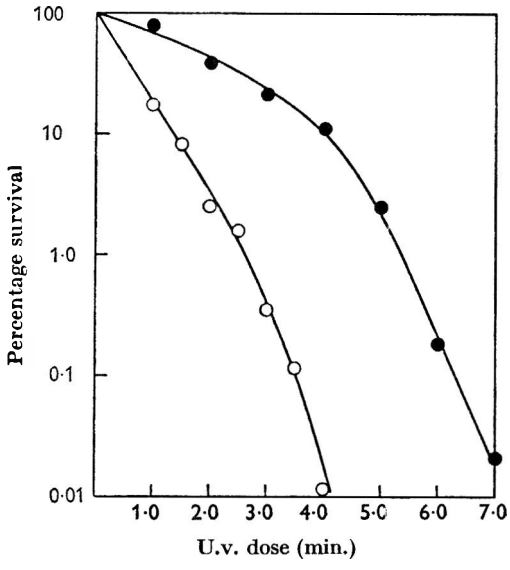

Fig. 1

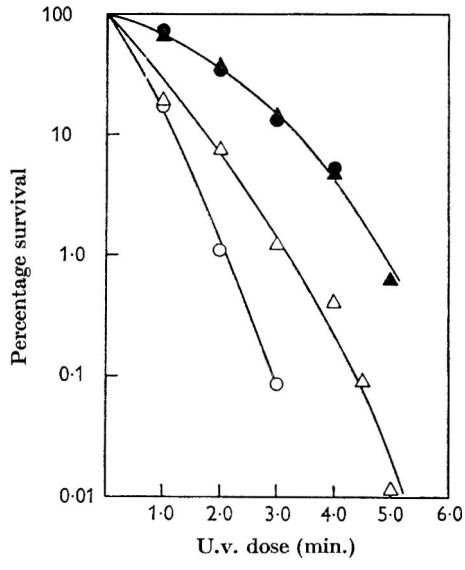

Fig. 2

Fig. 1. Ultraviolet survival curves of *Salmonella typhimurium* LT2: ○, strain *cysD-36 col*⁻; ●, the colicinogenic derivative *cysD-36* (*colI*). Bacteria grown overnight in unaerated broth culture, were agitated in an M.S.E. blendor for 2 min. at a speed of 12,000 rev./min. to break up clumps and pairs of bacteria, then resuspended in saline at a concentration of about 2×10^7 bacteria/ml. Separate 3 ml. volumes of this suspension were irradiated at each dose level. Irradiation was carried out in an open Petri dish on a mechanical rocker, using a high pressure mercury arc as u.v. source (see Methods). For viable counts samples were diluted in saline and plated on peptone agar.

Fig. 2. Ultraviolet survival curves. ○, *Salmonella typhimurium* LT2 wild-type; ●, the colicinogenic derivative LT2 wild type (*colI*); △, SL427 (*S. typhimurium* wild-type, cured of a B-phage); ▲, the colicinogenic derivative SL 427 (*colI*). Saline suspensions containing about 2×10^6 bacteria/ml. were irradiated. For other details see legend to Fig. 1.

None of the results outlined gave an explanation for the observed difference in sensitivity to u.v. radiation of a non-colicinogenic strain and a derivative carrying the *colI* factor. The generality of the effect of *colI* in *Salmonella typhimurium* LT2 was established by transmitting *colI* to wild-type LT2 and to SL427 (wild-type *S. typhimurium*, cured of a B phage). These strains and an additional auxotrophic strain, *athC-5* (*colI*), were u.v. irradiated, together with the non-colicinogenic strains from which they were derived. Survival curves of the pairs of strains LT2 (wild-

type) *col*⁻ and LT2 (wild-type) (*colI*) and *athC-5 col*⁻ and *athC-5* (*colI*) were similar to those of the corresponding pair *cysD-36 col*⁻ and *cysD-36* (*colI*). Strain SL427 was less sensitive to u.v. radiation than wild-type LT2 (Fig. 2), possibly because the B phage carried by wild-type LT2 is to some extent induced by u.v. radiation. However, the u.v. sensitivity of SL427 was significantly decreased by the presence of *colI* factor (Fig. 2).

The effect of colicine factors, other than colI, on u.v. sensitivity of Salmonella typhimurium, strain LT2

The u.v. sensitivity of strains *cysD-36* (*col 11–17*) and *cysD-36* (*colB1*) were compared with the sensitivity of strain *cysD-36* (*colI*). *colB1* resembles *colI* in being readily transmitted by mixed culture (Ozeki *et al.* 1962; Stocker, Smith &

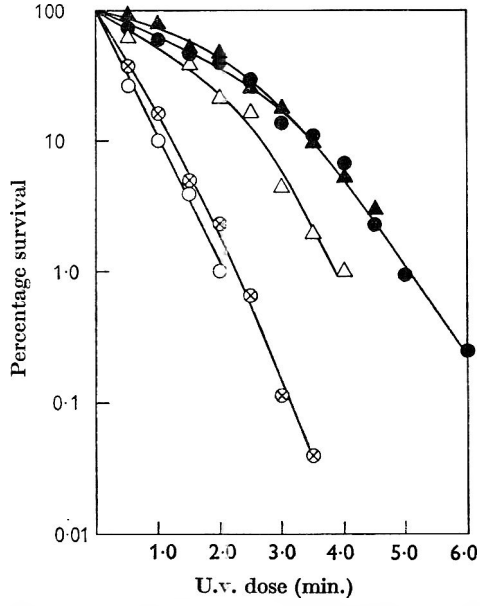

Fig. 3. Ultraviolet survival curves of *Salmonella typhimurium* LT2 carrying various colicine factors: ○, *colE2*; ●, *colI*; △, *colB1*; ▲, *col 11–17*; ⊗, *colE1*. Each was present in a separate subline of the auxotroph *cysD-36*. Saline suspensions containing 2×10^9 bacteria/ml. were irradiated. Survival curves of strains carrying *colE1* and *colI* were obtained in a separate experiment from those of strains carrying *col 11–17*, *colB1* and *colE2*. For other details see legend to Fig. 1.

Ozeki, 1963; Smith, Ozeki & Stocker, 1963); *col 11–17*, a factor determining production of a colicine different from the 'standard' types and common in *Salmonella typhimurium* strains from Australia (Prof. B. A. D. Stocker, personal communication) is likewise readily transmissible. These factors also conferred relative resistance to u.v. radiation but *colB1* to a lesser degree than *colI*. These results are shown in Fig. 3, together with the results of a further experiment in which strain *cysD-36* (*colI*) was u.v. irradiated, as was a strain colicinogenic for colicine E1, *cysD-36* (*colE1*). The survival curve of the strain *cysD-36* (*colE1*) was similar to that of the non-colicinogenic parent (Figs. 1, 3). As production of colicine E1 is known to be induced by

u.v. irradiation in *S. typhimurium* LT2 (Ozeki *et al.* 1959) one might have expected *cysD-36* (*colE1*) to be more sensitive to u.v. than the non-colicinogenic parent. The absence of such enhanced sensitivity has been observed before (H. Ozeki personal communication). A strain colicinogenic for E2, also u.v. inducible, similarly gave a survival curve resembling that of the non-colicinogenic parent (Fig. 3). The presence of *colI* in bacteria carrying *colE1* increased their survival after u.v. irradiation to about the same extent as when present in non-colicinogenic bacteria. Survival of bacteria carrying both *colE2* and *colI* was slightly lower. Moreover, newly acquired *colI* is also effective, as was demonstrated by the increased survival of a strain carrying *colE1* and *colI*, of which about half the bacteria had acquired *colI* during overnight growth in mixed culture with *cysD-36* (*colI*), and the remainder had acquired *colI* during growth in broth for 2 hr before irradiation.

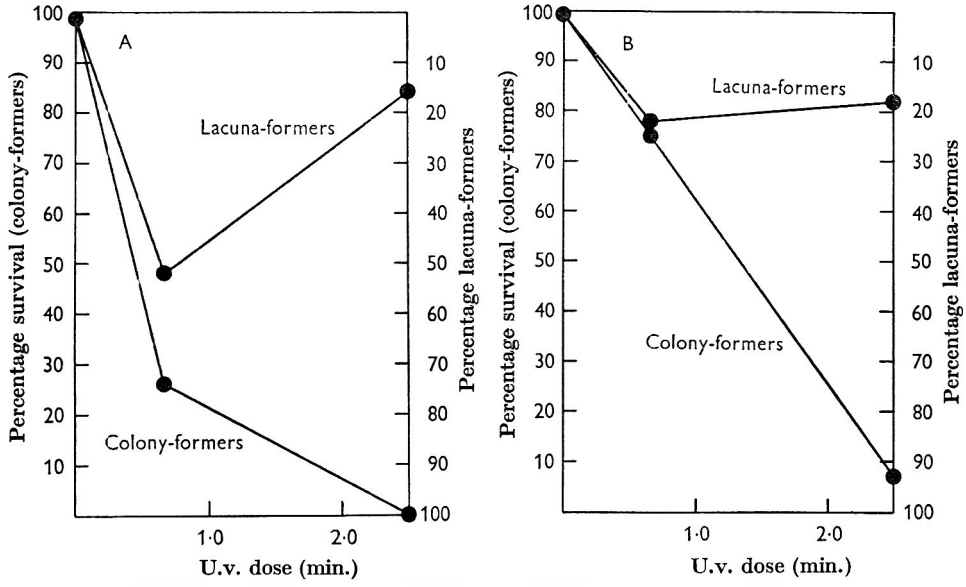

Fig. 4. Influence of *colI* on the u.v. induction of *colE2* in *Salmonella typhimurium* LT2. Results given in graph A were obtained using strain *cysD-36* (*colE2*) and those in graph B using *cysD-36* (*colE2*) (*colI*). Both show the percentage of bacteria surviving (colony-formers) and the percentage of bacteria induced to form colicine E2 (lacuna-formers) after u.v. irradiation for 40 sec. and 2 min., 30 sec. (Note: arithmetic scales). Saline suspensions for irradiation contained about 4×10^7 bacteria /ml. A high-pressure mercury arc was used as a source of u.v. (see Methods). After irradiation samples were diluted in saline and plated on peptone agar for viable counts; a further 0·1 ml. sample was diluted in 5 ml. pre-warmed broth at 37° and incubated for 90 min. to permit synthesis of colicine by induced bacteria. This suspension was further diluted in broth to yield about 8×10^3 bacteria/ml. and 0·1 ml. samples plated in streptomycin soft-agar, seeded with about 10^8 indicator bacteria, on peptone agar plates. The indicator strain used was a colicine-sensitive, streptomycin-resistant derivative of *Escherichia coli*, strain CL104.

Influence of colI on the ultraviolet induction of colE2

Ozeki *et al* (1959) in an experiment on an LT2 line carrying *colE2*, found that after u.v. irradiation giving about 30 % survival, more than half the bacteria, that is the majority of those which did not survive, were induced to form colicine E2. Colicine

produced by a single bacterium formed a clear spot or 'lacuna' in the lawn of a colicine-sensitive indicator strain. At the same dose of u.v. irradiation, the survival of a strain carrying *colI* is about 80 %, much higher than a strain not carrying this factor. As colicine production is a lethal process (Ozeki *et al.* 1959) it would therefore be expected that increased survival caused by the presence of *colI* in bacteria also carrying *colE2* would be accompanied by a decrease in the proportion of bacteria induced to form colicine E2—that is of bacteria forming lacunae. This prediction

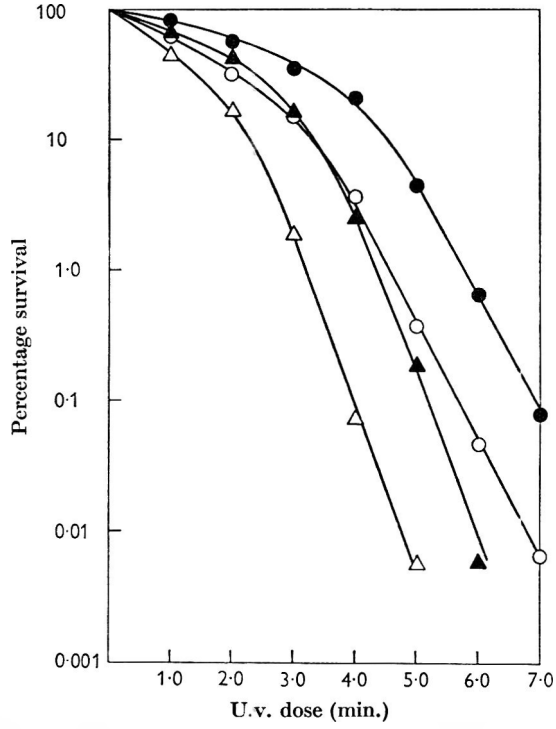

Fig. 5. Ultraviolet-survival curves of non-lysogenic, non-colicinogenic strain *Escherichia coli* K12 HfrC and its lysogenic and/or colicinogenic derivatives: ○, strain HfrC λ^- *col*$^-$; ●, λ^- (*colI*); △, (λ) *col*$^-$; ▲, (λ) (*colI*). Lysogeny for λ phage is denoted by (λ) and colicinogeny for colicine I by (*colI*); absence of the two characters is indicated by λ^- and *col*$^-$ respectively. Saline suspensions for irradiation contained about 6×10^7 bacteria/ml. For further details see legend to Fig. 1.

was tested using the method of Ozeki *et al* (1959). Strains *cysD-36* (*colE2*) and *cysD-36* (*colE2*) (*colI*) were u.v. irradiated for 30 sec. and for 2 min. 30 sec., doses expected to give about 30 % survival for the strain carrying *colE2* alone and that carrying *colE2* and *colI*. After u.v. irradiation samples were plated for viable counts; after 90 min. incubation in broth, to permit synthesis of colicine by induced bacteria, lacuna counts were made by plating bacteria in a streptomycin soft-agar layer seeded with a streptomycin-resistant colicine-sensitive indicator strain. The lacunae formed after incubation for 8 hr each represented a single bacterium which had synthesized colicine E2. The proportion of survivors (colony-formers) and of induced bacteria (lacuna-formers) are plotted, on arithmetic scales, in Fig. 4. The presence of *colI* increased the survival of the bacteria irradiated for

40 sec. from 26% to 75%; and, as predicted, the proportion of lacuna-formers at this dose was correspondingly depressed, from 52% to 22%. Survival of the strain carrying *colE2* and *colI* at 2 min. 40 sec., 7%, was lower than the anticipated value, 30%. Repeated tests of the u.v. sensitivity of this strain showed it to be slightly more sensitive than a strain carrying *colI* alone; and this was most apparent when dilute suspensions were irradiated.

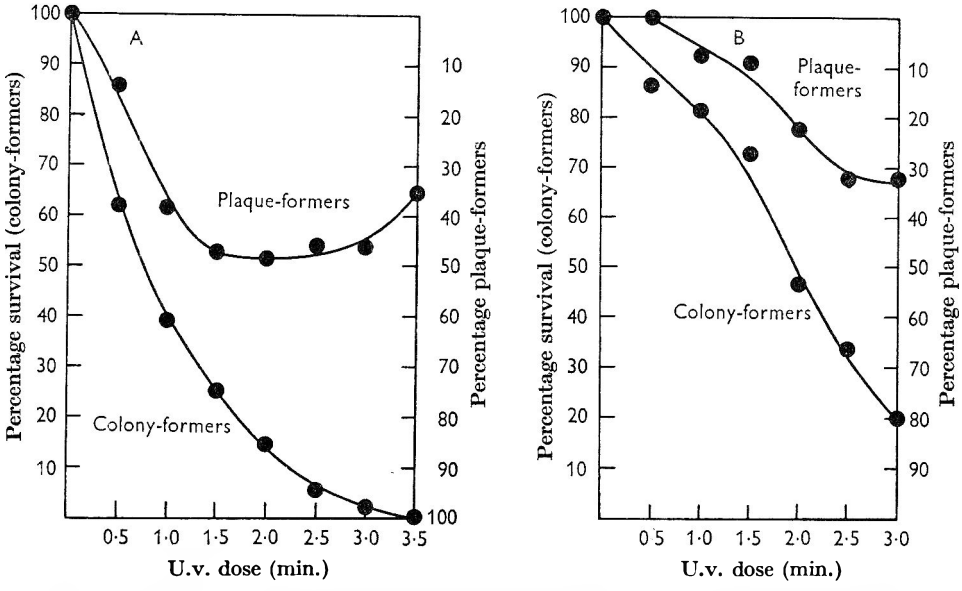

Fig. 6. Influence of *colI* on the u.v. induction of λ phage in *Escherichia coli* K12 HfrC. Results given in graph A were obtained using a lysogenic, non-colicinogenic derivative (λ) *col*⁻ and those in graph B using a lysogenic colicinogenic derivative (λ) (*colI*). Both graphs show the percentage of bacteria surviving (colony-formers) and the percentage of bacteria induced to form phage (plaque-formers) with increasing doses of u.v. (Note: arithmetic scales.) Saline suspensions for irradiation contained about 10^7 bacteria/ml. A high-pressure mercury arc was used as a u.v. source (see Methods). After irradiation samples were diluted and plated for viable counts on peptone agar; a further 0·1 ml. sample was diluted in 9·9 ml. of pre-warmed broth at 37° and incubated for 30 min. to allow synthesis of phage in induced bacteria. This suspension was then further diluted to yield about 1000 bacteria/ml. and 0·1 ml. samples plated in a soft-agar layer, seeded with about 5×10^7 indicator bacteria, on peptone agar. The indicator strain was a derivative of strain HfrC, resistant to colicine I.

Influence of colI on ultraviolet induction of prophage in Escherichia coli K12

As *colI* reduced the u.v. induction of *colE2* its effect on the inducible prophage λ of *Escherichia coli* K12 was investigated. The presence of λ prophage in this strain greatly increases its sensitivity to the killing effect of u.v. irradiation, due to phage induction (Lwoff, 1953). Sub-lines HfrC (λ) *col*⁻, λ⁻ (*colI*) and (λ) (*colI*) were prepared from a stock of strain HfrC, which is normally non-lysogenic for prophage λ. For each of these four strains the survival curve showed a pronounced shoulder (Fig. 5). As expected, the survival of the lysogenic non-colicinogenic strain (λ) *col*⁻ was lower at all doses than that of its non-lysogenic non-colicinogenic parent, λ⁻ *col*⁻. Furthermore, the presence of *colI* in the lysogenic strain (λ) (*colI*)

resulted in increased u.v. resistance to about the same extent as it did in the non-lysogenic strain λ^- (*colI*). Thus *colI* confers some protection against u.v. killing in *E. coli* K12, both when it is non-lysogenic and when it carries the inducible prophage λ. Since the completion of this work Monk & Devoret (1964) have reported similar increased resistance to u.v. irradiation of non-lysogenic strains of *E. coli* K12 carrying *colI*. To see whether the presence of *colI* in the lysogenic strain would decrease the proportion of bacteria induced to form phage an experiment was made similar to that described in the previous section using the two strains HfrC (λ) *col*$^-$ and (λ) (*colI*). Ultraviolet irradiated samples were incubated in broth for 30 min. to permit phage synthesis in induced bacteria and were then plated in soft-agar layers seeded with a phage-sensitive colicine-resistant indicator strain. The results (Fig. 6; note that the scales are arithmetic) showed that the lysogenic strain carrying *colI* gave a higher proportion of survivors at all doses tested, and that the proportion of bacteria induced to phage production by a given dose of u.v. was reduced by the presence of *colI*; e.g. from 47% to 9% in the 90 sec. samples. On comparison of the curves it is also apparent that for some u.v. doses bacteria which would have formed plaques in the absence of *colI* were recovered as colony-formers in its presence; e.g. in the 90 sec. sample 47% of the non-colicinogenic lysogenic bacteria formed plaques, but 73% of the colicinogenic lysogenic bacteria survived as colony-formers. Thus the presence of *colI* partly prevented the lethal inducing effect of u.v. irradiation on bacteria carrying prophage λ. However, comparison of the survival curves of the two colicinogenic strains shows that, even in the presence of *colI*, the lysogenic strain is much more u.v. sensitive than the non-lysogenic strain—that is, the protection conferred by *colI* against lethal induction of prophage λ is incomplete.

DISCUSSION

One way in which the *colI* factor might affect radiation sensitivity would be by increase in the chain length of bacteria, which would cause a shoulder on the survival curve. However, this possibility is not supported by direct microscopic examination which reveals no obvious differences in morphology between colicinogenic and non-colicinogenic bacteria. Secondly, it is possible that the *colI* factor increases the ploidy of the bacteria; this again would be expected to cause a shoulder on a survival curve. Thirdly, one might suppose that *colI* increases, in some way, the proportion of bacteria with a pool of RNA and protein precursors adequate to allow these bacteria to withstand lethal damage (Hill, 1963). Gillies & Alper (1960) found that cultures in the stationary phase of growth, where a large intracellular pool of precursors would be expected, gave a survival curve with a pronounced shoulder. It is also possible that *colI* factor of *Shigella sonnei* strain P9 carries, in addition to the structural gene for colicine production and the gene or genes concerned in its ability to confer maleness, a further gene for decreased u.v. sensitivity, perhaps comparable to the genetic loci controlling u.v. sensitivity in *Escherichi coli* (Adler & Copeland, 1962; Howard-Flanders, Boyce, Simson & Theriot, 1962; Rörsch, Edelman & Cohen, 1963; Greenberg, 1964). Such a gene may be a structural gene for an enzyme responsible for the repair of u.v. damage or alternatively it may cause de-repression of a host gene controlling the production of a reactivating enzyme. Sauerbier (1962) has proposed an enzymic mechanism to explain the host cell

reactivation of u.v. irradiated phage. Furthermore, Rupert (1961) demonstrated that photo-reactivation is an enzymic process. There is no evidence to support any one of these hypotheses rather than another. The following considerations raise a further possibility.

(1) The mechanism of the lethal effect of u.v. irradiation is not well understood, but in at least one instance, that of *Escherichia coli* K12 lysogenic for phage λ, much of the lethal effect is due to induction, since the lysogenic strain is much more sensitive to killing by u.v. irradiation than its non-lysogenic parent. In bacteria carrying defective forms of inducible prophage, irradiation will be expected to cause lethal induction, even though, because of the defect in the genome of the prophage, no plaque-forming units are released. Defective prophages are apparently not uncommon as laboratory mutants and also occur naturally (Lwoff & Siminovitch, 1951; Appleyard, 1954; Jacob & Wollman, 1956). It is, therefore, possible that much of the lethal effect of u.v. irradiation on the apparently non-lysogenic strains we have investigated (*Escherichia coli* K12 HfrC non-lysogenic for phage λ; and *Salmonella typhimurium* LT2 cured of a B phage) results from induction of undetected defective prophages in these strains—or perhaps inducible defective plasmids of some other sort, perhaps colicine factors, although under conditions used for u.v.-survival tests u.v. inducible colicine factors *colE1* and *colE2* do not appear to increase sensitivity.

(2) The presence of one sort of plasmid in an irradiated bacterium may prevent induction of a plasmid of some other sort which is also present (Hamon, 1959); and experiments described in this paper (Figs. 4–6) show that the presence of *colI* considerably reduces the induction of prophage λ in *Escherichia coli* K12 and of *colE2* in *Salmonella typhimurium*.

(3) It is therefore proposed, as a speculative hypothesis, that the effect of *colI* in protecting apparently non-lysogenic and non-colicinogenic strains against the lethal effects of ultraviolet irradiation results from its ability to suppress the lethal induction of a defective plasmid in these strains without itself being induced. Nevertheless, *colI* is inducible in sublines of *Escherichia coli* K12 as shown by Amati (1963) and Monk & Clowes (1964). Experiments have shown that ultraviolet induction of the production of colicine E2 in *Salmonella typhimurium* is partly prevented by *colI*, but this effect can hardly account for the increased survival of bacteria carrying *colI* as well as *colE2*; because, though *colE2* is inducible, its presence does not increase the susceptibility of *S. typhimurium* LT2 to the lethal effects of irradiation. The reasons for this are not known; but if, as is hypothesized, strain LT2 carries an inducible defective plasmid then perhaps the presence of *colE2* in u.v. irradiated bacteria results in the lethal induction of *colE2* instead of the postulated defective plasmid.

The hypothesis that *colI* protects by suppressing induction of defective plasmids in the strains concerned predicts that, unlike photo-reactivation, it will decrease the lethal but not the mutagenic effect of irradiation. Preliminary experiments have shown that the presence of *colI* in some auxotrophic mutants of *Salmonella typhimurium* strain LT2 did not result in a decrease of the number of revertants for a given dose of u.v. radiation but an actual increase (Howarth, to be published).

The author wishes to express gratitude to Professor B. A. D. Stocker for help and encouragement during the course of the work; for suggestion of the 'inducible plasmid' hypothesis put forward in the Discussion and for criticism of the manuscript.

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J. gen. Microbiol. (1965), **40**, 57–70
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Phage-Associated Lysins Affecting Group N and Group D Streptococci

By J. D. ORAM and B. REITER
National Institute for Research in Dairying, University of Reading

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SUMMARY

Phage lysins were prepared from high titre phage lysates of 3 strains of *Streptococcus lactis*, one of which was purified 500-fold by treatment with cold acetone, chromatography on Amberlite CG 50 ion-exchange resin, and fractional precipitation from potassium phosphate buffers. The lysins were activated by monovalent cations and were similar in physical properties. Viable streptococci of groups N and D were lysed, but not those in groups A, B and C. Different strains of group N streptococci were lysed at different rates, the rate of lysis being characteristic for individual phage lysins. A lytic enzyme, prepared from purified phage particles by freezing and thawing or by ultrasonic treatment, showed the same pH optimum and specificity as the corresponding phage lysin. Streptococci were lysed-from-without in the presence of high multiplicities of phage. The type of lysin produced in phage-infected culture was genetically determined by the phage. Lysis of streptococci by phage lysin was inhibited by an unidentified substance present in crude phage lysates. The lysis of different strains of streptococci by particulate phage, phage-tail enzyme and phage lysin occurred at the same relative rates. This is interpreted as evidence for the localization of the phage-lysin substrate within the phage receptor site. Possible reasons for the different specificities of phage lysins from streptococci of groups N and C are discussed.

INTRODUCTION

It has long been known that phage lysates contain a lytic agent which can act on the host bacterium, but is distinct from particulate phage (Bronfenbrenner & Muckenfuss, 1927). Evans (1934) demonstrated that a strain of group A streptococcus normally resistant to a group C phage, was lysed when grown in mixed culture with the phage-infected group C strain. This so-called 'nascent' phage reaction was shown by Maxted (1957) to be due to a lytic agent present in crude phage lysates of this particular strain of group C streptococcus; the lytic agent was active against group A, C and E streptococci. Naylor & Czulak (1956) suggested that the 'nascent' phage phenomenon shown by group N streptococci (the lactic acid streptococci) was caused by a lysin. These factors are similar to the 'facteur bactériostatique des lysates bactériophagique' of *Streptococcus lactis* reported by Wahl & Josse-Goichot (1950). The occurrence of phage-associated lysins (in the sequel phage-lysins) active against group D streptococci (Bleiweis & Zimmerman, 1961) and other bacterial genera such as *Bacillus megaterium* (Murphy, 1957), *Escherichia coli* (Brown, 1956; Adams & Park, 1956; Koch & Jordan, 1957) and *Staphylococcus aureus* (Ralston, Baer, Lieberman & Krueger, 1955) is well established.

The presence of phage-bound enzyme which attacked the host cell-wall was first observed with coliphage T2 by Weidel (1951) and has since been described for phages of *B. megaterium* (Murphy, 1960) and *Micrococcus lysodeikticus* (Brumfitt, 1960).

The present communication describes methods for the isolation of lysins from phages and phage lysates of some lactic acid streptococci, and some of the properties of these phage-lysins.

METHODS

Organisms

The following strains of streptococci were used (National Collection of Dairying Organisms, NCDO catalogue numbers are given in brackets):

Group N: *Streptococcus cremoris*: E8 (1196), HP (607), US3 (1197), Z8 (1199), R6 (764), KH (1198), TR (1200), 924 (924), 803 (803), 972 (972); *S. lactis*: ML3 (763), C10 (509), 712 (712); *S. diacetilactis*: DRC1 (1007).

Group A: *S. pyogenes*: 3/3A, Richards (923), Pope (1716).

Group B: *S. agalactiae*: A178 (1351); NCTC 8187 (1336); 8080.

Group C: *S. dysgalactiae*: C4 (1357); K64 (939); S51.

Group D: *S. bovis*: Pearl 11 (597); *S. zymogenes*: Mahoney (586); *S. durans*: 98/D (596); *S. liquifaciens*; Elv/2025 (588); *S. faecalis*: 775 (581).

Bacteriophage strains: homologous to their appropriate strains of group N Streptococci: *ml3*, *c10*, 712.

Media and propagation. Glucose Lemco (GL) broth consisting of (g./l.): Evans peptone, 10; Oxoid Lab-Lemco, 10; glucose, 10; NaCl, 5; water to 1000 ml.; adjusted pH to 7·2; glucose Lemco (GL) agar was prepared from GL broth by adding 1·5% agar. Strains of groups A, B, C and D streptococci were incubated at 37°; strains of group N at 30°. Growth was estimated by readings of extinction with a Hilger Biochem Absorptiometer at 580 mμ (E_{580}): and E_{580} of 1·0 corresponded to about 9×10^8 cocci cm^{-2}. Viable streptococci were enumerated by the method of Miles & Misra (1938). Phage counts were made by adding 20–200 phage particles (in 1·0 ml.) to 3·0 ml. of $\frac{1}{2}$ strength GL agar containing 0·025% (w/v) $CaCl_2$ and 0·1 ml. of a 24 hr culture of the homologous streptococcus at 45°, and poured on GL agar. The efficiency of plating was about 0·1.

Preparation of cell-wall suspensions. Cocci were harvested from GL broth, washed twice in distilled water and broken by shaking with ballotini beads (Chance no. 12) in a Mickle disintegrator in the cold room. After shaking for 2 hr the cell walls were freed from cytoplasmic impurities by treatment with trypsin, pepsin and ribonuclease (Cummins & Harris, 1956).

Assay of phage-lysin. Cocci grown for 18 hr in GL broth were washed in $\frac{1}{4}$ strength Ringer's solution, resuspended in 0·1 M-K-phosphate buffer (pH 6·7) and adjusted to give an E_{580} of 0·8 when diluted fivefold; 1·0 ml. of the standardized suspension pre-warmed to 37° was added to 4·0 ml. of enzyme preparation in the same buffer also at 37°; lysis was followed by measuring the decrease in extinction. It was found that lysis followed first-order reaction kinetics, the velocity constant K being given by the relationship:

$$K = \frac{2{\cdot}3}{t} \log 10 \frac{a}{a-x}$$

where $a = E_{580}$ at 0 min., and $x = E_{580}$ at t min. One unit of activity was defined as the amount of enzyme required to give $K = 1$ under the conditions defined above. Figure 1 shows that there was a linear relationship between concentration and activity of the *ml 3* enzyme.

Purification of phage-lysins

Preparation of phage-lysates of streptococci. 20 l. of GL broth, pre-warmed to 30°, were given a 10% (v/v) inoculum of a 12 hr culture of appropriate streptococcus and incubated at 30°. When the extinction had risen to 0·6 (in about 3 hr) the cocci were harvested and resuspended in 100 ml. of $\frac{1}{4}$ strength Ringer's solution. This suspension was added to 3·5 l. double-strength GL broth at 30° which contained sufficient phage to give a phage:coccus ratio of about 6. The infected culture was stirred at 30° and maintained at pH 6·5. After 2 hr about 90% of the cocci were lysed. A few milligrams of DNAase were added to the viscous lysate, and after another 5 min. at 30° the lysate was centrifuged for 20 min. at 2000*g* to remove unlysed cocci and some cell debris. The supernatant fluid was chilled to 0° and all subsequent steps were done in a cold room at 4°.

Removal of phage by precipitation with acetone. Acetone at −20° was added to 1 l. batches of coccal lysate to give 40% (v/v) acetone; the temperature was kept at 0° by immersing the flask in an ice+salt mixture, which was left overnight at 4° and then filtered through Whatman no. 40 paper. The original phage titre of 10^{12}/ml. was decreased to 10^{9}/ml. in the filtrate by this procedure.

Chromatography on Amberlite CG 50 resin. The clear filtrate from the previous step was passed through a 50 × 2 cm. column of Amberlite CG 50 ion-exchange resin equilibrated with a 60+40 by vol. mixture of 0·002 M-K-phosphate (pH 5·5)+acetone. The resin was washed with about 10 bed-volumes of 0·002 M-K-phosphate (pH 5·5) and then eluted with 0·2 M-K-phosphate (pH 7·3), 10 ml. fractions being collected and assayed. The active fractions were pooled, shell-frozen and finally freeze-dried.

Fractional precipitation from potassium phosphate buffers. The freeze-dried powder was shaken with 30 ml. distilled water at 37°. After about 1 min. the clear red-brown supernatant fluid was decanted from a small quantity of insoluble material and left overnight at 4°. A light-yellow precipitate (P1) was removed by centrifugation, washed with ice-cold 1·5 M-K-phosphate (pH 6·0) and dissolved in 0·1 M-K-phosphate (pH 6·7). This solution (S1) on standing at 4° overnight gave a second precipitate (P2) of inactive material which was removed by centrifugation. The colourless supernatant solution (S2) contained the enzyme.

Table 1 shows that a 500-fold purification of *ml 3* phage-lysin was achieved by the above procedure. Attempts at further purification on Sephadex G 100, DEAE cellulose and carboxymethylcellulose led to considerable losses of activity. The purified enzyme was relatively unstable: storage for 6 days at 4° resulted in 50% loss of activity. The freeze-dried preparation retained much of its activity when stored for 18 months in a desiccator at 4° and has been used routinely to prepare cell-free extracts of group N streptococci.

A shorter method of purification was used in some experiments. Coccal lysates were freed from phage by centrifugation for 2 hr at 100,000*g* and the phage-lysin in the supernatant fluid precipitated by adding solid ammonium sulphate.

The *ml 3* phage-lysin precipitated at between 50 and 75% saturation; the *c 10* enzyme was precipitated at 25–50% saturation. These precipitates were dissolved in 0·1 M-K-phosphate (pH 6·7) and de-salted by passage through columns of Sephadex G25. The phage-lysins prepared by the two methods appeared to have the same properties.

Table 1. *Purification of ml 3 phage-lysin*

Step	Volume (ml.)	Units/ml.	Total units	mg./protein /ml.
Crude phage lysate	3,400	14·5	49,200	10·0
Eluate from Amberlite CG50 column	455	47·1	21,400	2·1
Precipitate P1 from 2 M-potassium phosphate	7·5	1,400	10,500	2·34
Supernatant fluid S2	7·5	875	6,550	1·20

Step	Specific activity	Purification	Yield (%)	Total phage
Crude phage lysate	1·45	—	—	$9·5 \times 10^{14}$
Eluate from Amberlite CG50 column	22·4	15·4	43·5	$8·1 \times 10^{11}$
Precipitate P1 from 2 M-potassium phosphate	600	414	21·4	$3·3 \times 10^{9}$
Supernatant fluid S2	730	503	13·3	$1·0 \times 10^{9}$

Table 2. *Isolation of a lytic enzyme from ml 3 phage particles*

Step	Units/ml.	Volume (ml.)	Total units	Phage/ml.	Total phage
Washed phage particles	2·56	2·5	6·4	$3·6 \times 10^{11}$	9×10^{11}
Frozen-thawed phage					
Before centrifugation	0·64	10	6·4	—	—
Supernatant fluid	0·224	10	2·24	$2·5 \times 10^{9}$	$0·25 \times 10^{11}$
Deposit	2·21	2·5	5·5	$1·33 \times 10^{11}$	$3·34 \times 10^{11}$
Phage-treated with ultra-sonic vibrations					
Before centrifugation	0·63	10	6·3	—	—
Supernatant fluid	0·36	10	3·56	4×10^{9}	$0·4 \times 10^{11}$
Deposit	1·21	2·5	3·02	$8·8 \times 10^{10}$	$2·2 \times 10^{11}$

Amount of enzyme in original phage suspension: 6·4 units
Amount of enzyme released into solution: 5·8 units
Amount of enzyme remaining in phage particles: 3·25 units
Total amount of enzyme recovered 10·5 units
That is a recovery of 157%

Preparation of a lytic enzyme from purified ml 3 phage particles. Phage *ml 3* particles were freed from cell debris and phage-lysin by centrifugation (three cycles of 8000*g* for 30 min. followed by 100,000*g* for 2 hr). A lytic enzyme was then released from a concentrated suspension of the particles in 0·1 M-K-phosphate (pH 6·7) by the freezing and thawing method of Weidel & Primosigh (1957). After freezing and thawing 20 times, about 70% of the phage was non-infective although no increase in viscosity was observed. After centrifugation at 100,000*g* for 2 hr the deposit, which presumably contained phage ghosts as well as infective particles, was ultrasonically treated at 0° in a Dawe Soniprobe Type 1130A (Dawe Instruments Ltd., London). Six exposures for 30 sec. at position 6 were separated by

cooling periods of 2 min.; insoluble material was deposited by centrifugation (100,000*g*). The amount of enzyme released into the supernatant by these methods is given in Table 2.

RESULTS

Physical properties of ml 3 and c 10 phage-lysins

Effect of pH value. The effect of pH value on the activity of the two phage-lysins was determined in 0·1 M-K-phosphate. Both enzymes showed a broad peak of activity in the range pH 6–7·5 with an optimum at pH 6·5–6·8.

Cations. Various authors (e.g. Doughty & Hayashi, 1962; Ghuysens & Strominger, 1963) have emphasized the importance of cations for the activity of enzymes which act on bacterial cell walls. Both *ml 3* and *c 10* phage-lysins were activated by Na^+, Li^+, and NH_4^+. Figure 2 shows the influence of Na^+ concentration on the activity of *c 10* phage-lysin. There was a sharp optimum at ionic strength 0·15; higher concentrations were inhibitory.

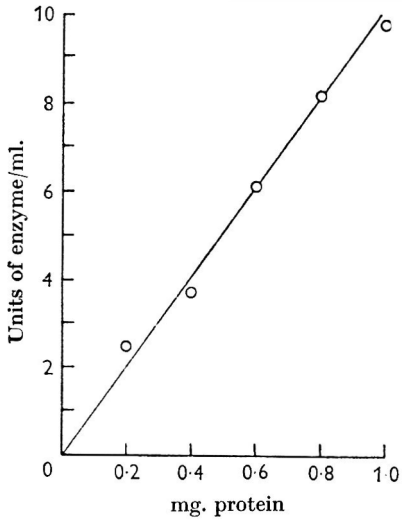


Fig. 1

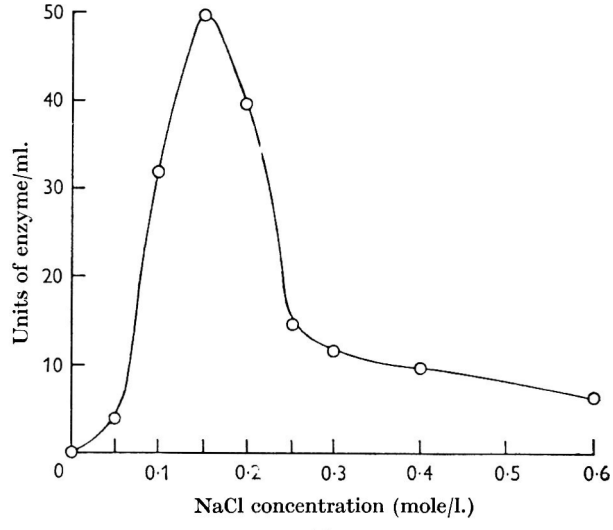


Fig. 2

Fig. 1. *ml 3* phage-lysin activity measured as a function of protein concentration. Activity was measured with vacuum dried cells of *Streptococcus cremoris* E8.

Fig. 2. The influence of ionic strength on the activity of *c 10* phage-lysin against *Streptococcus cremoris* KH in NaCl solutions buffered with 0·005 M-phosphate, (pH 6·7).

Divalent cations Ca^{2+} and Mg^{2+} also activated the phage-lysins, but were less effective than monovalent cations although not inhibitory at higher ionic strengths.

Influence of reducing agents. The *ml 3* phage-lysin was relatively unstable; losses of the order of 50% occurred on dialysis at 4° or on standing in the refrigerator for a few days. Other phage-lysins have been shown to be susceptible to oxidation or to require reducing agents for maximum activity (Maxted, 1957; Bleiweis & Zimmerman, 1961; Doughty & Hayashi, 1962). However, none of the group N phage-lysins was affected significantly by cysteine, ascorbic acid or thioglycollic (mercapto-acetic) acid in the concentration range 10^{-1}–10^{-4}M, nor was it possible to reactivate aged preparations with reducing agents.

Heat activation and inactivation. Both of the phage-lysins had high temperature coefficients (Q_{10} = 4·9) and possessed almost identical energies of activation: 28·0 kcal. mole^{-1} for the *ml 3* and *c 10* phage-lysins, respectively. At temperatures above 37°, rapid heat inactivation occurred. Figure 3 shows the effect of pH value on the heat inactivation of the two phage-lysins. Although *c 10* phage-lysin was more resistant to heat than the *ml 3* phage-lysin, the inactivation of both had the same dependency on pH value.

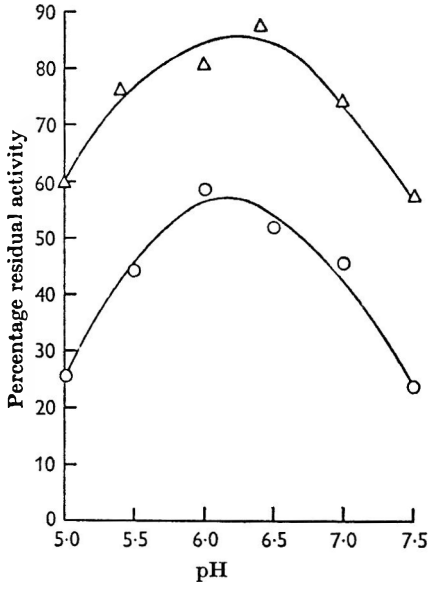


Fig. 3

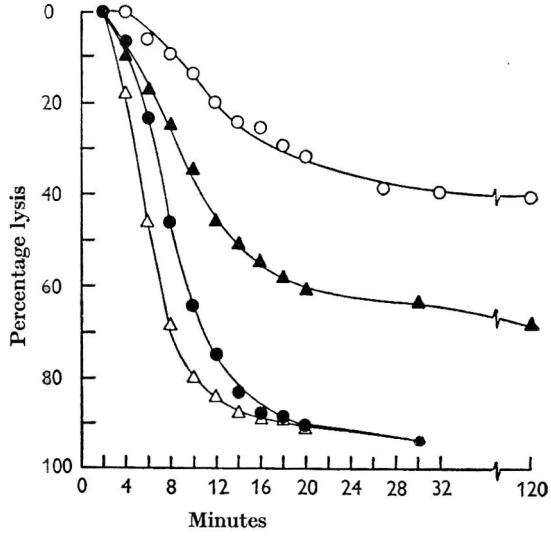


Fig. 4

Fig. 3. The effect of pH on the heat inactivation of *ml 3* and *c 10* phage-lysins. The lysins (in 0·1 M-phosphate) were held at 45·5° for 3 min. and then rapidly cooled to 0°. Residual activity was measured with *Streptococcus cremoris* KH for *c* 10 lysin, and *S. cremoris* E8 (vacuum dried) for *ml 3* lysin. ○ = *ml 3* phage-lysin; △ = phage-lysin.

Fig. 4. Lysis of *Streptococcus cremoris* KH by *c* 10 phage-lysin—the effect of ageing of the cocci on the rate and extent of lysis. ○ ——— ○, 96 hr culture; ● ——— ●, 12 hr culture; △ ——— △, 12 hr culture aged 7 days at 0° in 0·1 M-phosphate pH 6·7; ▲ ——— ▲, 12 hr culture aged 5 days at 20° in 0·1 M-phosphate pH 6·7.

Effect of age of streptococci on their susceptibility to phage-lysin

Doughty & Hayashi (1962) reported that ageing of group A streptococci led to a progressive resistance towards lysis by the group C phage-lysins. A similar effect has been found in the lysis of group N streptococci; the effect of age on the lysis of *Streptococcus cremoris* KH by the *c 10* phage-lysin is illustrated in Fig. 4. Cocci from 4-day cultures were much less readily lysed than those from a log-phase culture (12 hr). The storage of cocci at 4° resulted in no loss of susceptibility, whereas those kept at room temperature became significantly more resistant.

Lysis of viable streptococci and the specificity of phage-lysins

Phage-lysin from *ml 3* was tested against a representative selection of streptococci of groups A, B, C, D and N. The conditions of assay were as given under

'Methods' except that a much wider range of phage-lysin concentration was used (up to 105 units). The first column in Table 3 represents the activity of the phage-lysin towards each streptococcal strain; the second column gives the activity expressed as a ratio of that towards a reference strain, *Streptococcus cremoris* E8 (see below).

Table 3. *The specificity of ml 3 phage-lysin towards strains of streptococci in Groups A, B, C, D and N*

Group	Strain	Activity/ml.*	Relative activity/ml.†
	S cremoris E8	76·5	19·1
N	*S cremoris* E8 (vacuum dried)	401	100
N	*S cremoris* HP	8·95	3·98
N	*S cremoris* US3	25·8	6·27
N	*S cremoris* Z8	5·6	1·35
N	*S cremoris* R6	35·6	8·85
N	*S lactis* ML3	6·05	1·50
N	*S lactis* C10	1·52	0·38
N	*S lactis* 712	18·3	4·55
N	*S diacetylactis* DRC1	9·45	2·36
A	*S pyogenes* 3/3A	0	0
A	*S pyogenes* Richards	0	0
A	*S pyogenes* Pope	0	0
B	*S. agalactiae* A178	0	0
B	*S. agalactiae* 8187	0	0
B	*S. agalactiae* 8080	0	0
C	*S. dysgalactiae* C4	0	0
C	*S. dysgalactiae* K64	0	0
C	*S. dysgalactiae* S52	0	0
D	*S. bovis* Pearl 11	0 39	0·10
D	*S. zymogenes* Mahoney	1 09	0·27
D	*S. durans* 98/D	0 24	0·06
D	*S. liquefaciens* Elv/2025	1·13	0·28
D	*S. faecalis* 775	0 94	0·23

* The activity was measured as described under 'Methods'.

† The relative activity is defined as the ratio:

$$\frac{\text{Activity towards a strain}}{\text{Activity towards the reference strain (vacuum dried E8)}}.$$

Table 4. *A comparison of the specificities of ml 3 and c10 phage-lysins*

	ml 3 phage-lysin		*c 10* phage-lysin	
Strain	Activity/ml.*	Relative activity/ml.†	Activity/ml.*	Relative activity/ml.†
S. cremoris KH	4·33	1·00	50·3	1·00
S. cremoris 924	6·41	1·48	5·75	0·114
S. cremoris E8	13·1	3·22	4·35	0·087
S. cremoris TR	5·79	1·32	10·35	0·206
S. lactis M2S1	4·95	1·14	6·32	0·126

* The activity was measured as described under 'Methods'.

† Relative activity is defined as the ratio:

$$\frac{\text{Activity towards a strain}}{\text{Activity towards the reference strain (KH)}}.$$

Strains of groups A, B and C streptococci were unaffected by *ml 3* phage-lysin, but the groups D and N strains were all lysed. Strains within a group varied in their susceptibility to the phage lysin. *Streptococcus cremoris* E8 was the most readily lysed and, dried over $CaCl_2$ in a dessicator at 4°, was a useful reference organism for use in assaying *ml 3* phage-lysin. *Micrococcus lysodeikticus* was not lysed by *ml 3* phage-lysin.

When several strains of group N streptococci were examined for their sensitivity to the *c 10* phage-lysin, the pattern of susceptibility to the *c 10* phage-lysin was found to be different from that to the *ml 3* phage-lysin (Table 4). The differences are most apparent when activities are expressed relative to the activity towards one particular strain (in this case *Streptococcus cremoris* KH). Strain KH was the most susceptible strain for the *c 10* phage-lysin and the least affected by the *ml 3* phage-lysin, proving that the two phage host systems produced two different phage-lysins. Although these have many physical properties in common, they can be differentiated by their kind of specificity patterns and also in their differing solubilities in ammonium sulphate solutions (see Methods).

The effect of phage-lysin and lysozyme on viable streptococci and cell walls

Viable cocci of lactic streptocci were lysed at very different rates by the *ml 3* phage-lysin (Table 3). This variation was also found with isolated cell walls of different streptococcal strains. Figure 5 shows that cell walls of the very sensitive strain of *Streptococcus cremoris* E8 were lysed much more rapidly than those of the relatively resistant *S. lactis* C10. Cell walls and viable cocci have the same relative susceptibilities to phage-lysin: this indicated that cell-wall structure was more important than other factors (e.g. osmotic fragility) in determining the inter-strain variations in sensitivity to the phage-lysin.

Egg-white lysozyme lyses group N streptococci only in the presence of EDTA (Brown, Sandine & Elliker, 1962) and has no effect on group A streptococcus cell-walls (Krause & McCarty, 1961). In Fig. 6 a comparison is made between the action of *ml 3* phage-lysin and lysozyme on viable cocci and cell walls of *Streptococcus cremoris* 972. Although lysozyme in the absence of EDTA was without apparent action on whole organisms, it was more effective in lysing cell walls than was *ml 3* phage-lysin at concentrations which gave rapid lysis of viable cocci. The similarity between the effects of EDTA on viable cocci and modifications of the cell surface during the preparation of cell walls on their sensitivity to lysozyme is being investigated.

The relationship between ml 3 phage-lysin and a lytic enzyme obtained from ml 3 phage particles

It is not known whether the lytic enzyme prepared from *ml 3* phage particles was obtained as discrete protein molecules or whether it was still attached to phage-tail subunits not sedimented by the centrifugation procedure. It is noticeable from Table 2, that in addition to lysis by the soluble phage-tail enzyme, *S. cremoris* E8 is also lysed by the infective particles of *ml 3* phage. This was due to a lysis-from-without phenomenon. During the isolation of the phage-tail enzyme, an increase in the total amount of lytic activity occurred. This might have been due to a variety

of factors. The irreversible adsorption of the phage to the bacterial surface might have resulted in a 'blocking' of the activity of the phage-bound tail enzyme but not of the soluble phage-tail enzyme. The release of more than one molecule of enzyme from each phage particle during the preparation of the phage-tail enzyme would be expected to give an increase in the effective enzyme concentration.

The pH optimum of the phage-tail enzyme was measured with dried *Streptococcus cremoris* E8 and found to be identical with that of *ml 3* phage-lysin. A comparison of the specificities of the two enzymes is given in Table 5. From these results, the two enzymes appear to be identical.

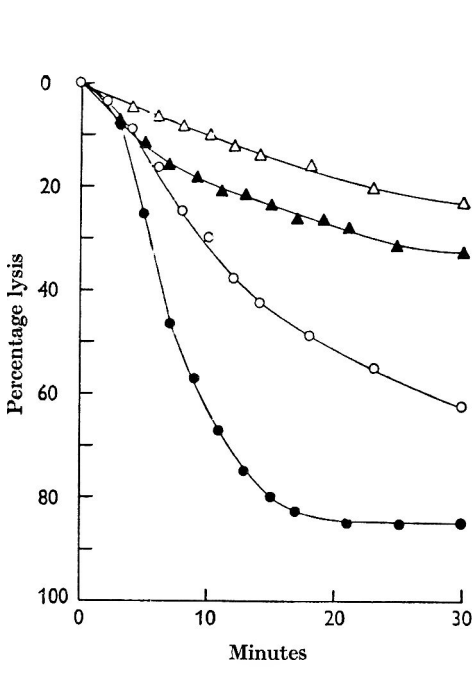


Fig. 5

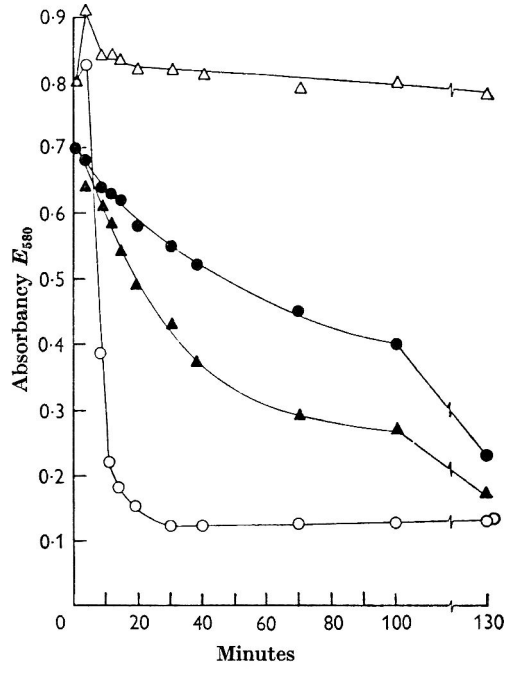


Fig. 6

Fig. 5. Lysis of viable cocci and cell walls by *ml 3* phage-lysin. 10 mg. (dry weight) of cells or cell walls were suspended in 5 ml. of 0·1 M-phosphate buffer, pH 6·7 containing 1·0 unit of *ml 3* phage lysin. ○ ——— ○, E8 cell walls; ● ——— ●, E8 viable cells; △———△, C10 cell walls; ▲———▲, C10 viable cells.

Fig. 6. Lysis of viable cocci and cell walls of *Streptococcus cremoris* 972 by *ml 3* lysin and lysozyme. 10 mg. (dry weight) of viable cocci or cell walls was suspended in 5·0 ml. 0·1 M-phosphate buffer, pH 6·7, containing either *m l3* phage-lysin (12 units) or lysozyme (2·5 mg.). ○ ——— ○, Viable cocci + phage-lysin; △———△, viable cocci + lysozyme; ● ——— ●, cell walls + phage-lysin; ▲———▲, cell walls + lysozyme.

Phage control of lysin production

Since the two phage-lysins (*ml 3, c 10*) differed in certain respects, and since no lytic activity was detected in cell-free extracts of non-infected cocci an experiment was devised to illustrate the role of phage in lysin production. *Streptococcus lactis* ML3 is susceptible to two phages: *ml 3*, which forms large plaques and *712* which gives minute plaques. The same host was used to prepare phage-lysins by infection with either phage race. Whereas phage *ml 3* produced large quantities of phage-

lysin, phage *712* gave lysates with little or no activity towards viable cocci. Treatment of cocci with acetone had been found to increase their sensitivity to *ml 3* phage-lysin. When the *712* lysate was tested against acetone-treated cocci, a weak

Table 5. *A comparison of the specificities of ml 3 phage-lysin and the lytic enzyme from ml 3 phage particles*

	Phage lysin		Enzyme from thawed and thawed phage	
Strain	Activity units/ml. × 10	Relative activity*	Activity units/ml. × 10^2	Relative activity*
S. cremoris KH	1·86	1·0	2·62	1·0
S. cremoris E8	3·16	1·70	4·38	1·68
S. cremoris 972	1·0	0·53	1·46	0·56
S. lactis ML3	0·62	0·37	0·62	0·24
S. lactis C10	0·53	0·28	0·88	0·33
	Phage lysin		Enzyme from sonicated phage	
	Activity units/ml. × 10	Relative activity*	Activity units/ml. × 10^2	Relative activity*
S. cremoris KH	2·48	1·0	1·86	1·0
S. cremoris 803	2·18	0·88	1·63	0·88
S. lactis 712	1·23	0·50	0·73	0·39
S. lactis C10	0·63	0·25	0·41	0·22

* The relative activity is defined as the ratio:

$$\frac{\text{Activity towards a strain}}{\text{Activity towards the reference strain (KH)}}.$$

Table 6. *The influence of phage type on the properties of phage-lysins produced in Streptococcus lactis* ML *3: comparison of the specificities of ml 3 lysin, 712 lysin, and* ML *3/712 lysin (*ML *3 lysed by 712—see text)*

A. Lysis of viable cocci (activity/ml.)

Strain	*ml 3* lysin	*712* lysin	ML *3/712* lysin
S. lactis ML3	$2·7 \times 10^{-2}$	0	0
S. lactis C10	$1·2 \times 10^{-2}$	0	0
S. lactis 712	$6·5 \times 10^{-2}$	0	$1·2 \times 10^{-3}$
S. cremoris KH	$1·8 \times 10^{-1}$	0	0
S. cremoris E8	$2·7 \times 10^{-1}$	0	0
S. cremoris HP	$1·2 \times 10^{-2}$	0	$1·6 \times 10^{-3}$
S. cremoris 803	$3·3 \times 10^{-1}$	0	0

B. Lysis of acetone treated cocci (activity/ml.)

Strain	*ml3* lysin	*712* lysin	ML *3/712* lysin
S. lactis ML3	$1·3 \times 10^{-1}$	$7·8 \times 10^{-3}$	$2·6 \times 10^{-2}$
S. lactis C10	$2·2 \times 10^{-2}$	0	0
S. lactis 712	$2·3 \times 10^{-1}$	$1·4 \times 10^{-2}$	$4·6 \times 10^{-2}$
S. cremoris KH	$3·0 \times 10^{-1}$	0	0
S. cremoris E8	$8·1 \times 10^{-1}$	$3·8 \times 10^{-2}$	$1·7 \times 10^{-1}$
S. cremoris HP	$3·7 \times 10^{-2}$	$1·1 \times 10^{-2}$	$5·7 \times 10^{-2}$
S. cremoris 803	1·03	$3·6 \times 10^{-2}$	$1·1 \times 10^{-1}$

The *ml 3* was a partially purified preparation (1 mg./ml.); the *712* and ML *3/712* lysins were crude phage lysates freed of phage by centrifugation.

lytic activity against some but not all strains was detected. In Table 6 a comparison is made between the lytic activity of lysates of the host *S. lactis* ML3 lysed by phage *ml 3* or phage *712*, and of *S. lactis* 712 lysed by phage *712*. It is evident that the lysin found in lysates of *S. lactis* ML3 lysed by phage *712* was very similar to that of lysates of *S. lactis* 712 lysed by phage *712*, and not at all like the lysate obtained when *S. lactis* ML3 was lysed by its homologous phage, *ml 3*. This shows that the lysin produced in lysates of *S. lactis* ML3 was under the genetic control of the infecting phage.

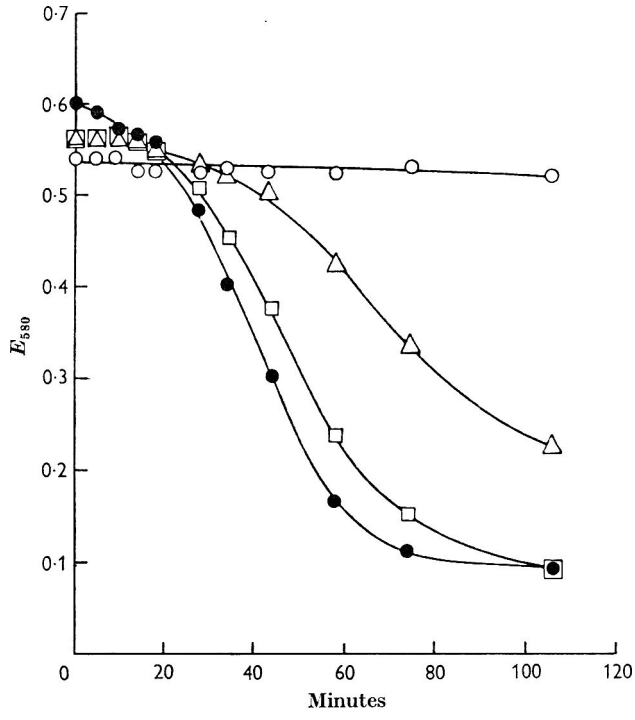


Fig. 7. The lysis-from-without of *Streptococcus lactis* ML3 by *ml 3* phage. Cocci were suspended in 0·1 M-K-phosphate (pH 6·7) at 37° and phage added to give the multiplicities; ○———○, 0 (control); △———△, 13·5; □———□, 27; ● ——— ●, 54.

Effect of particulate phage on the lysis of viable streptococci lysis-from-without

In a preliminary account of this work (Reiter & Oram, 1963) evidence was given that the lysis of streptococci by *ml 3* phage-lysin could be inhibited by the homologous phage. These results appeared convincing because increasing multiplicities of phage infection caused a progressive inhibition of lysis. Furthermore, *ml 3* phage-lysin was not inhibited by heterologous phages.

At that time we had not observed lysis-from-without by phage: this was not surprising as this phenomenon had been previously reported only for Gram-negative organisms. However, recent attempts to cause lysis-from-without were successful (Fig. 7). High multiplicities of *ml 3* phage lysed not only *S. lactis* ML3, but also a number of heterologous strains of group N streptococci. Lysis-from-without of these strains occurred at different rates, but the relative rates of lysis followed the

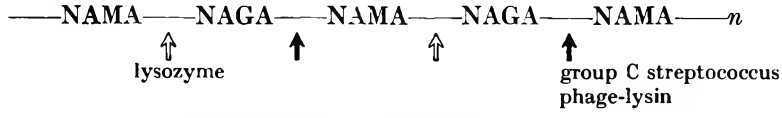
same specificity pattern as those obtained for *ml 3* phage-lysin and *ml 3* phage-tail enzyme.

Since these results were in direct contradiction to the inhibition of lysis by homologous phage, the earlier experiments were repeated. An *ml 3* phage lysate freed of phage by centrifugation (100,000*g* for 2 hr) lysed all the heterologous strains which were tested, but was inactive against *S. lactis* ML3. However, this strain was sensitive to a purified preparation of *ml 3* phage-lysin, indicating the presence of an inhibitor in the crude preparations. Since phage *ml 3* particles could be freed of the inhibitor by several washes with $\frac{1}{4}$ strength Ringer's solution, it now seems probable that our earlier phage preparations were contaminated with the inhibitor.

DISCUSSION

In common with a number of other phage-lysins the group N streptococcus phage-lysins were activated by certain cations and inhibited by reagents which inactivate SH groups. However, unlike the phage-lysins from a group C streptococcus (Doughty & Hayashi, 1962) and a group D streptococcus (Bleiweis & Zimmerman, 1961) the lysis of streptococci by the *ml 3* and *c 10* phage-lysins was not enhanced by reducing agents.

The specificity of the *ml 3* phage-lysin is quite different from that of the phage-lysin of a group C streptococcus (Maxted, 1957). Barkulis, Smith, Botralik & Heymann (1964) reported that a phage-lysin, prepared from a group C streptococcus differed from egg-white lysozyme in the nature of the susceptible chemical bond; whereas lysozyme acts as a muramidase, breaking the bond between the *N*-acetylmuramic acid (NAMA) and the *N*-acetylglucosamine (NAGA) residues in the mucopeptide polymers of the cell-wall, the group C streptococcal phage-lysin acted as a glucosaminidase, breaking the NAGA–NAMA linkage:



Our work (unpublished) shows that the *ml 3* phage-lysin is, like egg-white lysozyme, a muramidase. It seems possible that the specificity difference between the phage-lysins of the group C and group N streptococci may be due to differences in both the cell-wall structure and the site of enzyme action. The lysis of group D streptococci by the *ml 3* phage-lysin is not surprising in view of the serological similarities between the streptococci of groups D and N (Sharpe, 1952).

Whereas the streptococcal phage-lysins act on viable streptococci, the phage-lysins of *Bacillus megaterium* (Murphy, 1957) and of *Staphylococcus aureus* (Ralston, Baer, Liebermann & Krueger, 1957) were not active against viable bacteria unless particulate phage was also present. Ralston (1963) proposed that teichoic acids in the phage receptor sites mask regions in the staphylococcal wall which are sensitive to phage-lysin. Since the removal of teichoic acids with hot 5% trichloroacetic acid left the cocci sensitive to phage-lysin, she suggested that the adsorption of phage involved changes in the arrangement of teichoic acid, leading to the exposure of the lysin-sensitive sites. Teichoic acids are absent from the cell walls of the group N streptococci (Oram & Reiter, unpublished) and to our knowledge have not been

found in the cell walls of group A streptococci, lysis of which by group C streptococcus phage-lysin is also independent of phage sensitization (Krause, 1958).

The lysis-from-without of the group N streptococci by phage is similar to that by the phage-tail enzyme and by phage-lysin. The relative rates of lysis for different strains of streptococci are very similar in each case. It seems, therefore, that the three processes occur at the same site on the bacterial surface—the phage receptor. Both *ml 3* and *c 10* phages adsorb to a variety of streptococci within the group, without phage multiplication (Oram & Reiter, unpublished). The differences in the rate of lysis of a strain by the *ml 3* and *c 10* phage-lysins might well be due to attacks at different phage receptors. The proposal that the substrate of these phage-lysins lies within the phage receptor is supported by the inability of the phages to adsorb to mucopolysaccharide material released from the cell wall by phage-lysin. Lysozyme caused a similar loss in the integrity of the phage receptors of *Bacillus megaterium* KM (Salton, 1956) and of *Micrococcus lysodeikticus* (Brumfitt, 1960).

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Centrifugation Studies on the Infectivities of Cellular Fractions Derived from Mouse Brain Infected with Scrapie ('Suffolk strain')

By D. L. MOULD, W. SMITH and A. McL. DAWSON
Animal Diseases Research Association, Moredun Institute, Gilmerton, Edinburgh

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SUMMARY

Cellular components in homogenates of brain tissue from scrapie-affected mice have been separated by centrifugation in sucrose and caesium chloride density gradients with the objective of location of the scrapie agent and concentration of scrapie activity. Improvements in the relative activity of the fractions removed from sucrose gradients were small but recovery was high in the material sedimented through 0·88 M-sucrose. No peak of activity was observed in zones removed from a caesium chloride gradient and activity throughout remained strongly associated with particulate matter. Ultrasonic disruption had little effect on scrapie activity. Concentrated preparations of sufficient potency for characterization of virus particles by electron microscopy were not obtained. From various experimental evidence the scrapie agent, if a virus, appears to be of small size. The strong association of the agent with tissue debris suggests that the presence of a tissue component may be necessary in the experimental transmission of the condition.

INTRODUCTION

Recent experimental work on the condition of scrapie in animals has emphasized the strong association of the active agent with particulate cell fractions in brain homogenates (Mould & Smith, 1962; Hunter, Millson & Chandler, 1963; Hunter, Millson & Meek, 1964; Mould, Smith & Dawson, 1964). This has been further substantiated by chromatographic studies of tissue homogenates on DEAE-cellulose columns by Hunter & Millson (1965). It has also been pointed out that there may be species variations in the location and binding of the agent (Mould, Smith & Dawson, 1964), and that there are both definite histopathological (Zlotnik & Rennie, 1962, 1963) and possibly biochemical differences (Slater, 1965) in the brain lesions present in diseased mice according as to whether the source of inoculum was derived from a Suffolk sheep or from material passaged in goats.

The experimental procedures reported in this paper were all carried out with the 'Suffolk strain' of mouse scrapie (Zlotnik & Rennie, 1962, 1963) which produces histopathological lesions not identical with those produced by the 'drowsy goat strain' (Chandler, 1963) investigated by Hunter *et al.* Centrifugation studies of brain tissue homogenates in sucrose or caesium chloride density gradients have been made with the primary objective of location of the binding sites of the scrapie agent and concentration of scrapie activity. Some of the results have been reported in a brief communication (Mould, Dawson & Smith, 1964).

METHODS

Animal experiments. Moredun Institute stock Swiss white mice were used for all the experiments. The mice were 3–6 weeks old when inoculated. Groups of seven mice were inoculated (0·03 ml.) by the intracerebral route with serial tenfold dilutions of the various tissue preparations and each experiment terminated 5 or 6 months after the inoculation of the mice. Cases of scrapie were examined histopathologically for the presence of scrapie lesions and LD50 values were calculated according to the method described by Mould, Dawson & Smith, 1965.

Centrifugation of brain tissue homogenates in sucrose. Tissue was provided from the brains of mice in advanced clinical stages of mouse scrapie following the third passage in mice of the Suffolk sheep strain of scrapie. Crude particulate fractions from brain homogenates were prepared by differential centrifugation in 0·32 M-sucrose. Brains from three clinically affected mice (1 g.) were homogenized in 0·32 M-sucrose (10 ml.) and centrifuged for 5 min. at 500*g* to remove readily sedimentable tissue debris. The supernatant fluid was successively centrifuged for 10 min. at 1000*g*, 15 min. at 20,000*g*, and 75 min. at 100,000*g* to provide a nuclear, mitochondrial and ribosomal fraction respectively. The sediments were taken up in equivalent volumes of saline (0·85%) and serial dilutions in saline prepared from the suspensions and final supernatant fluid for inoculation in mice. The nuclear fraction was not tested.

A further experiment was designed to investigate the possibility of removing scrapie inactive material by a simple centrifugation procedure in sucrose density gradients. Sucrose solutions of increasing concentration were made up in a dilute phosphate-succinate buffer at pH 7·4 (Johnson, 1962; 0·004 M-K_2HPO_4, 0·001 M-KH_2PO_4, 0·004 M-sodium succinate). Brains from three clinically affected mice (1 g.) were homogenized in 0·1 M-sucrose (9 ml.) and centrifuged for 30 min. at 500*g*. The supernatant fluid was removed and maintained in a disperse state by gentle agitation throughout the remainder of the procedure. A sample (0·5 ml.) of the supernatant fluid was layered on 1·2 M-sucrose (4·5 ml.) in a small centrifuge tube (5 ml.). Three tubes were prepared and centrifuged for 30 min. at 50,000*g* (Spinco Model L, SW39 rotor; 25,000 rev./min.) The whole of the supernatant was rejected and the small sediment from each tube pooled and resuspended in saline (2·5 ml.). Another volume (0·5 ml.) of the original supernatant fluid was layered on a density gradient consisting of 1·2 M-sucrose (1·5 ml.), 1·0 M-sucrose (1·5 ml.), and 0·88 M-sucrose (1·5 ml.). Three tubes were again prepared and centrifuged for 30 min. at 50,000*g*. The cloudy zones that formed at the 1·2 M/1·0 M and 1·0 M/0·88 M interfaces were withdrawn through the side of the tube by a syringe and corresponding zones from each tube were pooled. The procedure was repeated in a preformed gradient of 0·88 M, 0·75 M and 0·60 M- sucrose and finally in a gradient of 0·60 M, 0·44 M and 0·30 M-sucrose. All the interfacial layers collected were dialysed against saline and saline dilutions prepared for inoculation into mice. Protein-N in each inoculum was estimated by a micro-Kjeldahl method.

Centrifugation of tissue homogenates in caesium chloride. Brains from four clinically affected mice (1·56 g.) were homogenized in 0·85% saline (15 ml.) and centrifuged for 30 min. at 500*g*. Equal volumes (5 ml.) of the supernatant fluid were centrifuged for 90 min. at 120,000*g* (SW39 rotor; 39,000 rev./min.). One sedimented

pellet was resuspended in saline for inoculation into mice and the other resuspended in saline (1 ml.) and agitated in an ultrasonic beam (30,000 cyc./sec.; 80 W.) for 20 min. The suspension was diluted (5 ml.) and a portion (1 ml.) layered on 40% (w/v) caesium chloride (4 ml.) in a 5 ml. centrifuge tube and centrifuged for 20 hr at 100,000*g* (SW39 rotor; 35,000 rev./min.). No attempt was made to remove visible zones from the tube after centrifugation. Equal volumes (1 ml.) were taken successively from the top of the gradient. The final sample included the small sediment packed at the base of the tube. The five fractions from the density gradient were dialysed against saline and serial dilutions prepared for inoculation into mice.

Separation in a caesium chloride gradient was repeated with a mitochondrial-sized fraction prepared from the brains of two scrapie-affected mice according to the method described by Hunter *et al.* (1964). The brains (0·72 g.) were homogenized in 0·25 M-sucrose containing 5 mM-$CaCl_2$. The homogenate (7 ml.) was layered on 0·88 M-sucrose (15 ml.), and centrifuged for 60 min. at 50,000*g* (SW25 rotor; 25,000 rev./min.). The sediment was resuspended in 0·25 M-sucrose (7 ml.), a sample taken for inoculation into mice, and the remainder (5·5 ml.) layered again on 0·88 M-sucrose and centrifugation repeated. The sediment was resuspended in saline (5·5 ml.), a further sample taken for inoculation into mice, and the remainder (4 ml.) agitated in an ultrasonic beam for 30 min. Equal volumes (1 ml.) were then layered on 40% (w/v) caesium chloride (4 ml.) and centrifuged for 24 hr at 100,000*g*. Successive layers of fluid of increasing density were withdrawn from the tube contents, dialysed against saline, and serial dilutions prepared for inoculation into mice.

Table 1. *Differential centrifugation of scrapie mouse brain homogenate in 0·32 M-sucrose*

Titres are calculated as $-\log_{10}$ (LD50) or in terms of protein content as $-\log_{10}$ (specific LD50). The experiment was terminated 5 months after inoculation.

Inoculum	LD50 ($-\log_{10}$)	Protein (g./ml. $\times 10^{-3}$)	Specific LD50 ($-\log_{10}$)
Initial 10% homogenate	5·03 ± 0·21	11·14	5·84 ± 0·21
Mitochondrial fraction	4·73 ± 0·40	6·09	5·86 ± 0·40
Microsomal fraction	4·46 ± 0·32	1·86	6·17 ± 0·32
Final supernatant fluid	2·07 ± 0·22	0·87	3·86 ± 0·22

RESULTS

Centrifugation of brain homogenates in sucrose

Table 1 shows the results of the titration of activity in the crude particulate fractions obtained by differential centrifugation in 0·32 M-sucrose. The LD50 of each inoculum is defined as that dilution of material which induced within the stated experimental period lesions in the brain consistent with those observed in mouse scrapie in half the animals inoculated. The specific LD50 of each inoculum is defined as the concentration of protein (g./ml.) which induced scrapie in half the animals inoculated. This remained essentially constant in the original homogenate and sediments. There was a significant fall in the scrapie activity of the final supernatant fluid. Complete removal of particulate material is improbable under the density conditions of the sucrose medium.

The distribution of scrapie activity after centrifugation in the pre-formed sucrose

density gradients is shown in Table 2 and Fig. 1. The data for each sucrose concentration refer to the scrapie activity present in the layer of tissue particles that penetrate a sucrose layer of the stated concentration. Although activity was present throughout all the various layers the highest recovery was in the material sedimenting through sucrose concentrations greater than 0·75 M. There was a small increase in the

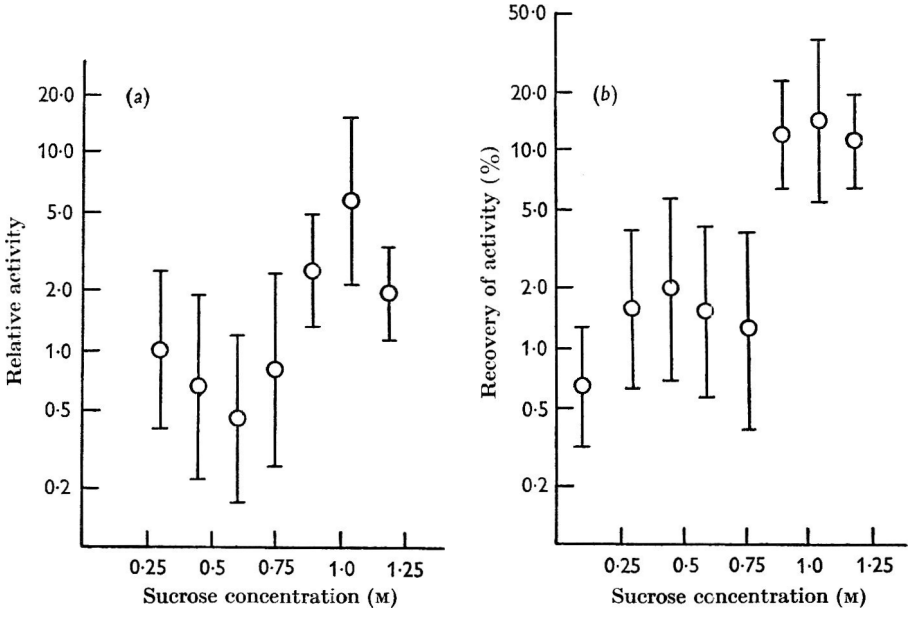

Fig. 1. The recovery of scrapie activity after centrifugation of mouse brain tissue homogenates in pre-formed sucrose density gradients. (*a*) Relative activity of zones as function of sucrose concentration penetrated. (*b*) Recovery of activity in zones as function of sucrose concentration penetrated.

Table 2. *Centrifugation of scrapie mouse brain homogenate in layered sucrose density gradients*

The data refer to the scrapie activity in the layer of tissue particles that penetrate a sucrose layer of stated concentration. The LD50 values are corrected to the same effective dilution as the 10% homogenate. Specific LD50 is expressed as protein concentration (g./ml.). The experiment was terminated 6 months after inoculation.

Sucrose cone. (M)	Density of sucrose soln.	LD50 ($-\log_{10}$)	Recovery of activity (%)*	Protein (g./ml. $\times 10^{-4}$)	Specific LD50 ($-\log_{10}$)	Relative activity†
0·10	1·012	2·05 ± 0·35	0·6	15·76	3·57 ± 0·35	0·01
0·30	1·037	2·44 ± 0·52	1·6	0·56	5·48 ± 0·52	1·0
0·44	1·056	2·55 ± 0·60	2·0	0·99	5·32 ± 0·60	0·7
0·60	1·075	2·43 ± 0·55	1·6	0·96	5·14 ± 0·55	0·5
0·75	1·094	2·34 ± 0·65	1·3	0·48	5·39 ± 0·65	0·8
0·88	1·113	3·32 ± 0·27	12·0	1·21	5·92 ± 0·27	2·7
1·0	1·132	3·40 ± 0·54	14·5	0·67	6·26 ± 0·54	5·9
1·2	1·151	3·29 ± 0·19	11·2	2·25	5·77 ± 0·19	1·9
10% homogenate		4·24 ± 0·26	100	56·20	5·49 ± 0·26	1·0

* Compared with homogenate as 100%.
† Specific LD50 of the homogenate divided by that of sample.

relative activity, calculated from the numerical values for specific LD50, in the more dense layers associated with a corresponding decrease in the less dense material. In Fig. 1 the relative activity and recovery of activity are plotted to a logarithmic scale with estimated deviations as an indication of the inherent error involved in the titration procedures.

Centrifugation of brain homogenates in caesium chloride

The appearance of the separated zones in the tubes after centrifugation are shown diagrammatically in Fig. 2. The experimental results from the complete sediment (Table 3) indicate that only about 0·01 % of the activity was not sedi-

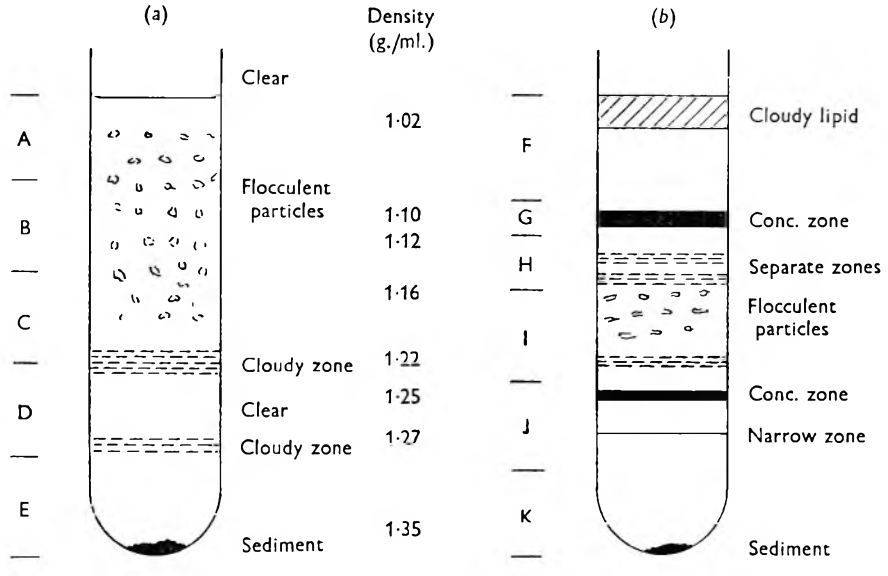

Fig. 2. Ultracentrifugation of scrapie mouse brain tissue homogenates in caesium chloride gradients. Appearance of zones of debris and light scattering zones with volumes of serial fractions (A–E; F–K) collected after centrifugation indicated. (*a*) Sonicated sediment from brain homogenate layered on caesium chloride. (*b*) Sonicated mitochondrial-sized fraction sedimenting through 0·88M-sucrose layered on caesium chloride.

mented under the centrifugation conditions used and that the activity was not affected by ultrasonic treatment of the resuspended sediment. Activity was scattered throughout the gradient but was highest in the low density region of the gradient where particulate matter predominated. The distribution was in general agreement with that already observed in the sucrose gradient. It has been reported (Mould, Dawson & Smith, 1965) that the potency of scrapie-active material was reduced by 1·8 log units by contact with caesium chloride for 24 hr comparable with the conditions applicable during the centrifugation procedure. A possible diminution to $10^{-3.7}$ of the effective LD50 titre of the applied zone of active material would therefore be expected after centrifugation in caesium chloride, further decreased to $10^{-3.4}$ by the dilution factor (1/5). The spread of activity in the zones collected from the gradient (Table 3) agreed with this estimated value and substantiated the previously reported effect of the strong salt solution.

The experimental titres for the various fractions derived from the portion of mouse brain homogenate sedimenting through 0·88 M-sucrose are listed in Table 4. There was good recovery of activity in the sediment with a high specific LD50. Ultrasonic treatment in this experiment reduced the total scrapie activity. After centrifugation in caesium chloride a number of well-defined zones were observed with a band of flocculated particles (fraction I) in the gradient over a density

Table 3. *Centrifugation of sediment from scrapie mouse brain homogenates in caesium chloride*

The experiment was terminated 6 months after inoculation.

Inoculum		LD50 ($-\log_{10}$)
10% homogenate		5·67 ± 0·17
Supernatant fluid above 100,000*g* sediment		1·50 ± 0·20
Material sedimenting at 100,000*g*		4·48 ± 0·56
Sediment after ultrasonic treatment		5·47 ± 0·31
Fractions from caesium chloride gradient as shown in Fig. 2*a*	A	3·76 ± 0·30
	B	3·79 ± 0·18
	C	3·35 ± 0·23
	D	3·08 ± 0·26
	E	2·79 ± 0·25

Table 4. *Centrifugation of material from scrapie mouse brain homogenate sedimenting through 0·88 M-sucrose*

The experiment was terminated 6 months after inoculation.

Inoculum		LD50 ($-\log_{10}$)	Protein (g./ml. $\times 10^{-3}$)	Specific LD50 ($-\log_{10}$)
Material sedimenting through 0·88 M-suerose		5·49 ± 0·37	5·40	6·61 ± 0·37
Material after repeat sedimentation through 0·88 M-sucrose		6·07 ± 0·35	7·53	7·23 ± 0·35
Sediment after ultrasonic treatment		4·97 ± 0·29	—	—
Fractions from caesium chloride gradient as shown in Fig. 2*b*	F	2·79 ± 0·55	—	—
	G	3·35 ± 0·23	—	—
	H	3·52 ± 0·35	—	—
	I	3·92 ± 0·26	—	—
	J	2·61 ± 0·50	—	—
	K	3·07 ± 0·24	—	—

range 1·16–1·22. A cloudy appearance and activity in the low density fraction (F) suggested that ultrasonic disruption of the particles must have taken place as low density material originally present in the brain homogenate had been removed by repeated sedimentation in 0·88 M-sucrose. Contact with caesium chloride again reduced the overall recovery of scrapie activity which was spread throughout all the fractions removed from the gradient. The highest activity was observed within the density range 1·10–1·22.

DISCUSSION

Although there are histopathological and biological transmission differences (Zlotnik & Rennie, 1962, 1963, 1965) between the 'Suffolk strain' and the 'drowsy goat strain' of mouse scrapie the sedimentation behaviour of the activities from mouse brain homogenates appear to be similar (Hunter & Millson, 1964). Any improvement in the purification or concentration of the scrapie agent as indicated by the relative activity (Table 2) of the various zones of activity arising in the sucrose gradients was small, but the recovery of scrapie activity was higher in the material sedimented through 0·88 M-sucrose in agreement with the results reported by Hunter *et al.* (1963, 1964). Activity throughout remained strongly associated with particulate matter. No pronounced peak of activity was observed in the zones successively removed from the caesium chloride gradients. At this preliminary stage of attempted fractionation the protein content of the zones was not determined, but visible particles were scattered throughout tested zones. The effect of ultrasonic treatment can be explained either by the disruption of active particles into smaller fragments retaining activity or the release of an active agent followed by strong readsorption to the smaller particles. Prolonged ultrasonics treatment of the mitochondrial fraction tended to reduce the scrapie activity.

The zones isolated from such gradients have all been examined on the electron microscope for morphological characteristics, but except in the case of identifiable mitochondria (Hunter *et al.* 1964) were of heterogeneous appearance. This difficulty in the application of zonal gradient centrifugation to the separation of cellular fragments from brain tissue with its relatively complicated cytological differentiation has been recently discussed (Eichberg, Whittaker & Dawson, 1964). If it be assumed that the scrapie agent is closely associated with a single type of subcellular structure in brain tissue, then following homogenization, ultrasonic disruption and differential centrifugation, it would be certain to appear over a wide range of fractions with varying degrees of activity determined only by the amount of bulk material distributed within those fractions.

Many fractions of known biological activity prepared from sheep, goat and mouse scrapie brain homogenates have been examined by electron microscopy for the possible presence of characteristic virus-like particles. At an earlier stage in the investigation this was done with more varied scrapie preparations than could experimentally be subjected to biological testing in sheep or goats. When quantitative measurements of biological activity and purification were achieved with the mouse it became obvious from the low titres in supposedly purified material that sufficient potency had hardly yet been attained such that unequivocal recognition by electron microscopy might be possible. Within this limitation no virus-like particle has been observed. An extensive application by Drs Mackay & Vantsis (personal communication) of tissue-culture techniques has failed to demonstrate any cytopathogenic effects associated with the addition of scrapie-infected inoculum.

The biological activity, however, of an unfractionated scrapie-affected mouse brain, determined by titration inoculation of serial dilutions of the brain tissue in groups of mice, is high. At a period of 9 months after inoculation the end-point of the titre of scrapie activity is consistently 10^{-8} g. wet tissue/ml. (0·03 ml. inoculum). If the causal agent of this level of activity is a virus particle with a particle

infectivity ratio of 100:1, a concentration of 10^{10} particles/ml. might be expected in a 10% (w/v) brain homogenate. In thin sections of infected tissue an even higher local concentration of virus associated with lesions might be feasible and the possibility of characterization of a particle 50–100 mμ diam. greatly enhanced.

A proposition that the scrapie agent is a very small virus (< 25 mμ) as yet uncharacterized is substantiated by its adsorption on or occlusion by small tissue particles and the progressive loss of activity in the supernatant fluid from mouse brain homogenates after repeated centrifugation (Mould, Dawson & Smith, unpublished). The apparent absence of biological activity in free suspension in the absence of tissue particles, combined with the relative stability of the active agent to chemical and physical action in the presence of tissue particles could indicate almost a state of chemical combination with a tissue component. It is significant that in experimental work consistently high activities or titres have only been reached with brain homogenates under physiological conditions of pH and salt concentration. A change in pH, addition of salts, solvent action or heat have all decreased the potency of the original scrapie activity (Hunter & Millson, 1965; Mould *et al.* 1965). This has been, in fact, one of the difficulties encountered during investigations into the preparation of mocula of increased potency.

Although the transmission of scrapie to animals is most successful when tissue is also transferred, experiments have been reported where this was not the situation. Thus dialysates derived from goat brain homogenates and cerebro-spinal fluid were active in goats (Pattison & Sansom, 1964); extracts in buffer of sheep brain homogenates were active in sheep (Mould & Smith, 1962; Mould, Smith & Dawson, 1964); and scrapie was transmitted to the host animal by cerebrospinal fluid from sheep and goat (Stamp *et al.* 1959; Pattison & Millson, 1962).

As an addition to the proposition that scrapie activity may be passaged by small free virus particles occluded in fragmented tissue sediments two further conceptions are suggested for experimental verification. First, that the actual transfer of a tissue component itself in the inoculum is necessary and, secondly, that the nature of this component is of importance in the mechanism of adsorption of the inoculum by the recipient tissue.

We are indebted to Mr J. C. Rennie for the histological examination of mouse brain tissue.

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The Enzymic Activity of the Outer Shell of *Lactobacillus arabinosus*

By H. A. COLE and D. E. HUGHES

Medical Research Council Unit for Research in Cell Metabolism, Biochemistry Department, Oxford, and the Microbiology Department, University College, Cardiff

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SUMMARY

Preparations of the outer shell of *Lactobacillus arabinosus* were made by crushing the bacteria in a Hughes press and removing the cytoplasm by washing and centrifugation. Such preparations had none of the enzymes associated with flavin-mediated oxidation; they had consistently adenosine triphosphatase (ATPase) activity. The kinetics and other properties of this enzyme were studied. The evidence suggested that the enzyme was bound to the cytoplasmic membrane, the bulk of which appeared to be within the cell wall. The preparation was thus similar to that from obligate facultative aerobes and which, in contrast to the preparations from lactobaeilli, contained enzymes associated with oxygen utilization mediated by the cytochrome hydrogen transport system. The bound ATPase had an optimum at pH 6·0 and was not markedly inhibited by ouabain or oligomycin: it was stimulated by 2,4-dinitrophenol. It was thus different from ATPase associated with ion transport but similar to that from mitochondrial membranes. There was no nucleotide pyrophosphorylase activity in the preparation but other nucleotide triphosphates and diphosphates and some inorganic phosphates were hydrolysed but at slower rates than was ATP.

INTRODUCTION

In previous work (Hughes, 1962; Francis, Hughes, Kornberg & Phizackerly, 1963) it was shown that the outer shells of many aerobic bacteria may be prepared by crushing the organisms in a Hughes (1951) press and removing the cytoplasmic contents by washing and centrifugation. Such preparations have been called cell-wall membranes because they have the form and chemical constituents of the cell wall and also the phospholipid components and enzymic activities associated with the cytoplasmic membrane. In the present work similar preparations were made by the same method from *Lactobacillus arabinosus*. This organism normally does not use oxygen as a final hydrogen acceptor for energy (ATP) production but can utilize oxygen to oxidize several substrates by flavin enzymes which were thought to be soluble; the exception was the pyruvate oxidase which was particulate (Strittmatter, 1959). However, the enzyme preparations from *L. arabinosus* were made by prolonged ultrasonic treatment, a process which comminutes the outer shells of bacteria into fine fragments and solubilizes some enzymes associated with them. The cell-wall membranes were found not to contain these flavin enzymes which were all in the soluble fractions, but did contain an adenosine triphosphatase

(ATPase). The properties of this enzyme have now been investigated. The evidence presented here supports the view that the ATPase is bound to the cytoplasmic membrane. While this work was in progress an ATPase has been found in similar preparations from the obligate anaerobes *Clostridium welchii* and *C. sporogenes*. It was also present in membrane preparations 'ghosts' from spheroplasts of this latter organism (Galli & Hughes, 1965). The enzyme is also present in membrane preparations and cell-wall membranes of aerobes. So far it has not been possible to assign a role to this membrane-bound bacterial ATPase.

METHODS

Growth of Lactobacillus arabinosus. *L. arabinosus* strain 17–5 was grown in a medium (Barton-Wright, 1952) containing amino acids, B-group vitamins, glucose and salts. In 1 l.; 20 g. Oxoid tryptone; 6 g. Oxoid yeast extract; 20 g. glucose; 10 g. sodium acetate; 4·5 g. KH_2PO_4; 26 ml. N-NaOH; 5 ml. essential salts solution; 4 g. $MgSO_4.7H_2O$; 0·1 g. $MnSO_4.4H_2O$; 0·04 g. anhydrous $FeCl_3$ in 100 ml. dilute hydrochloric acid; adjusted to pH 7·6. Stock cultures of the organism were subcultured every 2 weeks, grown at 30° and stored at 2°.

Large quantities of the organism were grown in 10 l. bottles nearly full of medium which were inoculated with a growing culture. After incubation for 18–20 hr, when the medium had become about pH 4·5, the bacteria were collected with a Sharples continuous centrifuge. The bacteria were then washed well with 0·1 M-tris + maleate buffer (pH 7), centrifuged again and resuspended in a small volume of this buffer. The average yield was 7 g. wet wt. bacteria/l. medium.

Preparation of cell-wall membranes. In a typical preparation, about 60 g. wet-weight bacteria, suspended in a small volume of 0·1 M-tris + maleate buffer (pH 7), were crushed at −25° in a Hughes (1951) press. The material obtained was thawed, suspended in 2 vol. of a mixture of equal volumes of 0·1 M-tris + maleate buffer (pH 7) and 0·9 % NaCl in a loose-fitting glass homogenizer and incubated for 30 min. at about 2° with 100 μg. deoxyribonuclease to decrease the high viscosity of the preparation and facilitate emptying of the broken bacteria. The degree of breakage was judged at this stage by electron microscopy (Pl. 1, fig. 1). The preparation was then centrifuged at 30,000 *g* at 2° for 30 min. and separated into a supernatant fraction and a solid residue. The residue consisted of two layers, the uppermost being composed of cell-wall membranes (CWM). This layer was removed by using a curved spatula and resuspended in buffer saline mixture and centrifuged at 30,000 *g* for 30 min. twice more. The material thus obtained was usually equivalent to about 1–3 g. dry wt. This material was resuspended in about 75 ml. of 0·1 M-tris + maleate buffer (pH 7) and stored at 2°. The preparation then consisted of electron-transparent hulls of bacteria, containing very little electron-dense material (Pl. 1, fig. 2).

An attempt was made to produce cell-wall membranes by using a French press (Milner, Lawrence & French, 1950) in which a 20 % (v/v) suspension of bacteria in buffer at 4° was broken by liquid shear at pressures of 15×10^3 lb./sq.in. Electron microscopy showed that a suspension had to be passed through the French press at least six times to produce the same degree of breakage as was produced by one treatment in the Hughes press. This resulted in further comminution of the cell-wall membranes and so the method was not used further.

Further fragmentation of the cell-wall membranes by ultrasonic treatment. The suspension (25 ml.) of washed cell-wall membranes (about 0·2 g. wet wt./ml.) in 0·1 M-tris + maleate buffer in a 100 ml. beaker cooled in ice was disrupted by ultrasonic treatment with a 600 W. Mullard magnetostricter oscillator operating at a power level 6·9 V. at a setting of 65/100 for 45 min. Electron microscopy showed that the cell-wall membranes after this treatment were considerably fragmented (Pl. 1, fig. 3). The solid fragments were removed by centrifugation at 100,000 *g* for 90 min. at 0° in a Spinco model L preparative ultracentrifuge. The clear supernatant fluid was separated from the residue and the latter resuspended in 25 ml. of the original buffer.

Electron microscopy. Cell fractions for routine work were dried on to Formvar-covered grids and examined by transmission. To gain a more accurate knowledge of the shape of the particles in the fractions, the samples were fixed with 1 % osmic acid and then shadowed with 40 % Au + 60 % Pd. All material was examined in an Akashi TR 50 Tronoscope at initial magnifications between 600 and 15,000.

Assays of adenosine triphosphatase activity. It was assumed that the enzyme hydrolysed ATP according to the equation: $ATP + H_2O \rightarrow ADP + H_3PO_4$. Thus assays for either the phosphate ion or the first highly acidic hydrogen ion of H_3PO_4 both estimated the activity of the enzyme.

Assay of phosphate release. In most experiments about 15 mg. wet weight solid or 0·1 ml. of supernatant fluid were incubated at 37° with ATP (0·3–20 mM); $MgCl_2$ at the same concentration as that of ATP; 0·5 ml. 0·1 M-tris + maleate buffer (pH 7·0); in total volume of 1–2 ml. Incubation times varied between 15 min. and 2 hr. After incubation the tubes were cooled to 2° and 1 ml. of cold 5 % (w/v) perchloric acid added. Tubes were then centrifuged at about 5000 *g* in cooled centrifuge cups to remove precipitated protein. Duplicate samples of the supernatant fluid were added to 8 ml. water in 10 ml. graduated tubes and the inorganic phosphate estimated by the method of Fiske & SubbaRow (1925). The reducing agent was 1-amino-2-naphthol-4-sulphonic acid, sodium sulphite and bisulphite. A blank without reagents and a standard containing 1 μmole orthophosphate were also treated with the reagents. E_{700} was read in glass cuvettes in a Unicam S.P.600 spectrophotometer. The amount of phosphate was calculated from standard curves and expressed as a mean of the two samples from which the inorganic phosphate of ATP and the enzyme was deducted ($< 0{\cdot}2$ μmole).

Assay of hydrogen-ion release. Continuous titrations were carried out at 37° with NaOH in a Radiometer automatic titrator, with a combined calomel and hydrogen electrode. The initial volume of cell-wall membrane and $MgCl_2$ was 1 ml.; no buffer was added to the incubation mixture, which was kept constant at pH 7·4. This is slightly higher than the optimum pH value, but consistent results were not obtained at pH 6·0.

Typical mixtures contained 0·4 mM-$MgCl_2$; 50 mg. wet weight cell-wall membrane, in a total volume of 1 ml. This mixture was at about pH 6; 0·1 N-NaOH was added to bring to pH 7·4. The mixture was kept at 37° and stirred with CO_2-free air. Various volumes of 0·2 M-ATP were then added to start the reaction and the microburette added sufficient 0·01 N-NaOH to maintain the mixture at pH 7·4. The volume of NaOH added was recorded as a function of time; the slope of this curve was therefore proportional to the rate of reaction.

Chemical analysis of cell fractions. Protein was estimated by the method of Lowry, Rosebrough, Farr & Randall (1951) with the Folin–Ciocalteau reagent and measurement of the extinction (E_{625}) of the blue colour in a Unicam S.P. 600 spectrophotometer. For carbohydrate analysis, fractions were hydrolysed by 4 N-sulphuric acid at 100° for 2 hr in a sealed tube, after which insoluble material was removed by centrifugation at 5000 *g*. In this supernatant fluid, total carbohydrate was estimated by the method of Dische (1929); methylpentoses by the method of Dische & Shettles (1948) and glucosamine by a modified method of Aminoff, Morgan & Watkins (1951), Levvy & McAllen (1959). Deoxyribonucleic acid in the original samples was estimated by the method of Burton (1956) and ribonucleic acid in the same samples by the method of Mejbaum (1939).

Lipid was extracted with a 2 + 1 mixture of redistilled chloroform + methanol and the extract washed twice with 0·5 vol. of water. After drying the chloroform + methanol extract with anhydrous sodium sulphate, the extract was evaporated under nitrogen and then under vacuum to dryness in weighed beakers by an infra-red lamp. Samples of this anydrous material were then saponified with methanolic (50 %) 2 N-potassium hydroxide solution and esterified in light petroleum (40–60°) with diazomethane in ether, then applied to a gas chromatography column, as described below.

Chromatographic methods. Nucleotides and inorganic phosphate were separated by paper chromatography by the method of Krebs & Hems (1953).

For the chromatography of sugars, samples of carbohydrate, hydrolysed with H_2SO_4, were neutralized with saturated baryta, the barium sulphate was removed by centrifugation and the solutions evaporated to 0·5 ml. under a Magda infra-red lamp. The solvents used were phenol + water and *n*-butanol + acetic acid + water (Woiwod, 1949). A standard sugar sample was placed on each chromatogram (Putman, 1957) *m*-phenylenediamine hydrochloride was used as the developing agent (Chernick, Chaikoff & Abraham, 1951). The relative R_F values used were those of Partridge & Westall (1948).

Methyl esters of fatty acids were separated by gas chromatography in an Argon Chromatograph (Pye Co. Ltd., Cambridge). The support was acid and alkali-washed Celite 535 (Johns-Manville Co. Ltd., London, S.W. 1). Apiezon L high-vacuum grease (10 %) was the non-polar stationary phase. Samples (0·1 μl.) of dry ester were placed on the column by the method of Renshaw & Biran (1962). Peak areas were determined by triangulation according to Kouleman (1957). The chromatographic peaks were identified by comparison of the retention volumes with known acids and by hydrogenation and chromatography of the products.

The measurement of radioactive phosphorus. ^{32}P in solution was estimated in a conventional Geiger–Miller 10 ml. counting tube type M 6 (supplied by 20th Century Electronics Ltd., Croydon, Surrey), operating at 1090 V. in a lead castle from E.R.D. Engineering Co. Ltd., Slough, Middlesex. The scaling unit was type N 530 F obtained from Ekco Electronics Ltd., Southend-on-Sea, Essex.

For the measurement of radioactivity on paper chromatographs, an end-window counter operating at 1625 V. attached to a Panex Scaling Unit type D 657 was used. Radioactive phosphorus on chromatograms was measured by shielding the spot with a lead plate 2 mm. thick with a hole 2 cm. + 3 cm. in the centre.

Chemicals. ^{32}P-labelled adenosine triphosphate was made by the method of

Glynn & Chappell (1964). Starting with 1 mc of orthophosphate, the final activity of the terminal phosphate group of the ATP was 300 μc; very little activity appeared in the β-phosphate group. Chromatograms indicated that some labelled AD^{32}P was also present.

All other chemicals used were commercial preparations and were not purified unless so stated.

RESULTS

The rate of hydrolysis of ATP by the various preparations from *Lactobacillus arabinosus*, when measured by the formation of orthophosphate or hydrogen ions, proceeded linearly until about half the ATP was hydrolysed; the rate then decreased gradually (Fig. 1). The release of phosphate from increasing amounts of ATP

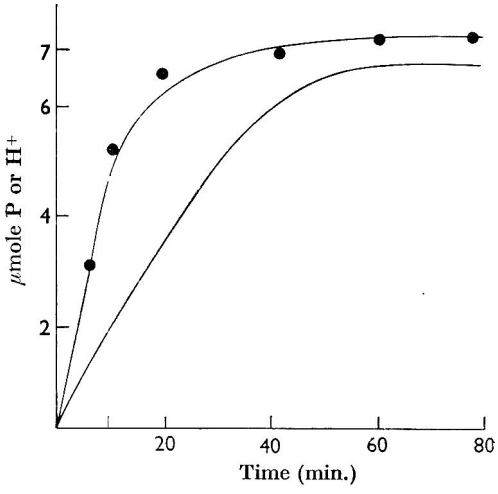

Fig. 1

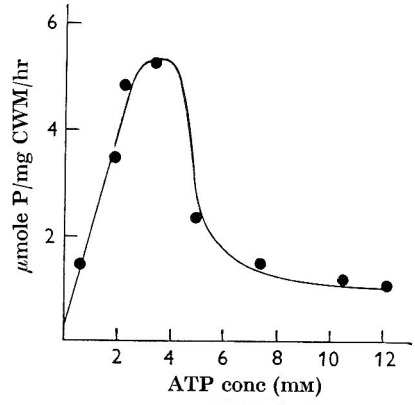

Fig. 2

Fig. 1. Reaction curves of ATPase measured by phosphate release and by titrating the H^+ formed. The cell-wall membrane preparation was incubated with ATP (10 μmole) and phosphate released and H^+ formation measured as described in the text. ●——●, Phosphate release; ———, H^+ formation.

Fig. 2. The effect of ATP concentration on phosphate release by the ATPase of cell-wall membrane preparations from *Lactobacillus arabinosus* 17–5. A cell-wall membrane preparation was incubated with various concentrations of ATP and the phosphate released estimated after incubation for 30 min.

with constant amounts of cell-wall membrane was measured under standard conditions. Consistent results were obtained only when the molar concentration of Mg^{2+} was the same as that of ATP. When the concentrations of ATP and Mg^{2+} were equal, plots of phosphate release against ATP concentration increased to a maximum and then decreased (Fig. 2). This suggested that ATP at high concentration was inhibitory. The concentration of ATP needed to produce the maximum rate of hydrolysis varied with different batches of cell-wall membranes and was not proportional to their specific activity. Lineweaver–Burk plots gave an approximate value of K_m of 3 mM and of V of $1{\cdot}7 \times 10^5$ μmole P/g. dry wt./hr. at low values of substrate concentration. Similar results were obtained when the hydrogen ion formation was measured. The rate of the initial reaction then was plotted as a function at the

concentration of ATP and gave a K_m of 6–12 mM. The ATPase showed a marked optimum at pH 6·0 when measured in tris + maleate buffer (Table 1).

Hydrolysis of compounds other than ATP. Suspensions of cell-wall membrane hydrolysed ADP at 20–30 % of the rate of that of low concentrations of ATP. No maximum or minimum was observed in the curve of phosphate release against concentrations of ADP up to 16 mM (Table 2). The value of K_m was 12 mM and V was 1×10^4 μmole P/g. dry wt. cell-wall membrane/hr. To test that the same enzyme was acting on the two adenine nucleotides, equal concentrations of ATP and ADP alone and mixed were incubated with cell-wall membrane. The release of phosphate was not additive; the release from ATP with ADP was less than with ADP alone. This suggests that the same enzyme hydrolyses both ATP and ADP.

Table 1. *Variation with pH value of ATPase activity of cell-wall membrane preparations from Lactobacillus arabinosus* 17–5

Tubes contained 0·4 ml. tris + maleate buffer (pH 7); 0·1 ml. 50 μmole ATP/ml.; 0·1 ml. 50 μmole $MgCl_2$/ml.; cell-wall membrane (equiv. 15 mg. dry wt.). Measured amounts of 0·2 M-acetic acid or sodium hydroxide were added to bring the mixtures to between pH 4·6 and 10 as measured on a pH meter. The pH value of control tubes without enzyme or ATP was adjusted similarly. The tubes were then incubated at 37° for 20 min. and phosphate was determined as described previously.

pH value of mixture before incubation	Phosphate release (μmole P/g. dry wt. cell-wall membranes/hr.)
4·6	880
5·1	2080
5·6	2460
6·2	2500
6·8	2300
7·5	1690
7·8	1870
8·4	1750
9·0	1380
10	1300

Table 2. *The effect of ADP concentration on the rate of hydrolysis of adenosine diphosphate by cell-wall membranes*

ADP at various concentrations was incubated under the usual conditions and phosphate release estimated as described in the text.

ADP concentration (mM)	Phosphate release (μmole P/g. dry wt. cell-wall membranes)
4·0	180
6·0	300
8·0	350
9·0	470
12·0	590
13·5	980

To estimate the effect of released ADP on the value of K_m found for the hydrolysis of ATP, various amounts of ATP were incubated with cell-wall membrane for various times up to 40 min. The value of K_m for each time was found by a Lineweaver–Burk plot. The straight lines produced in each case all cut the x-axis at the same point, giving a value of K_m of about 10 mM. Thus increasing concentrations of

ADP produced by the hydrolysis of ATP had no appreciable effect on the value of K_m found by this method.

Suspensions of cell-wall membrane had no effect when incubated with adenosine monophosphate, other nucleoside diphosphates, sugar phosphates and NAD, at the times and concentrations normally used with ATP. All the nucleoside triphosphates tested were hydrolysed at a slow rate by the cell-wall membrane. Inosine triphosphate was the most rapidly attacked: the rate of hydrolysis was 50 % of that of ATP under the same conditions (Table 3).

Table 3. *The hydrolysis of nucleotide phosphates by cell-wall membrane preparations from Lactobacillus arabinosus* 17–5

Tubes contained 0·47 and 1·41 mM nucleotides. Comparison tubes held similar concentrations of ATP and ADP, and a similar concentration of Mg^{2+}; 20 mg. wet wt. cell-wall membrane preparation; 0·5 ml. 0·1 M-tris + maleate buffer (pH 7); total volume 1·2 ml. The tubes were incubated at 37° for 1 hr and phosphate was determined in the usual way, in duplicate. The amount of phosphate released is expressed after the deduction of blanks without cell-wall membrane or nucleotide.

	Phosphate release*	
Nucleotide	Nucleotide conc. (0·47 mM)	Nucleotide conc. (1·41 mM)
Adenosine monophosphate	0	0
Adenosine diphosphate	27	32
Cytidine diphosphate	0	0
Inosine diphosphate	0	0
Uridine diphosphate	0	0
Guanosine diphosphate	0	0
Cytidine triphosphate	18	15
Inosine triphosphate	42	57
Guanosine triphosphate	32	46
Thymidine triphosphate	40	26
Uridine triphosphate	40	45

* Expressed as $\frac{\text{Phosphate release from nucleotide}}{\text{Phosphate release from ATP under identical conditions}} \times 100\,\%$.

The cell-wall membrane preparations also hydrolysed condensed inorganic phosphates. Pyrophosphate was hydrolysed at the same rate as ATP under the same conditions; tripolyphosphate and tetrametaphosphate were hydrolysed slowly; hexametaphosphate was attacked at significant rates (Table 4).

The reversibility of the reaction was tested by incubating the cell-wall membranes with ATP and ADP in the presence of P_i or $AT^{32}P$. The nucleotides and inorganic phosphate were separated by paper chromatography and the activity of each was found. When the cell-wall membrane was incubated with inorganic radioactive orthophosphate, only the phosphate spot was labelled on the chromatogram; no exchange had taken place. There was no increase of activity in the ADP spot when cell-wall membrane preparations were incubated with $AT^{32}P$; the bulk of the activity appeared in the orthophosphate. There was thus no indication of reversibility of the hydrolysis of ATP by these cell-wall membrane preparations.

$AT^{32}P$ was also incubated with other nucleotides and their activity measured after separation by paper chromatography. Consistent labelling was found in uridine triphosphate and cytidine triphosphate only. The amount of ^{32}P incorporated

represented only 1 % of that added as $AT^{32}P$ but the specific activity, although not measured, appeared to be high.

Inhibition of the ATPase. 2,4-Dinitrophenol decreased the inhibition of the rate of hydrolysis by excess ATP and at high concentrations of DNP (4 mM) appeared to activate the ATPase. Chloramphenicol had a similar effect. There was no apparent stimulation at concentrations of ATP which did not inhibit.

Table 4. *The hydrolysis of inorganic phosphate anhydrides by cell-wall membrane preparations from Lactobacillus arabinosus 17–5*

Tubes contained various amounts of the phosphate anhydrides or of ATP and $MgCl_2$ at the same concentrations; about 20 mg. wet wt. of cell-wall membrane. At the two highest concentrations of ATP, 0·5 ml. of tris maleate + buffer (pH 7) was in a total volume of 1·5 ml.: at the other concentrations of ATP, total volume was 1 ml. Where the time and concentration of ATP is the same as that of a phosphate compound, the two incubations were carried out at the same time. Controls for the phosphate content of the compounds and the cell-wall membrane have been deducted as usual.

Substance	Final concentration (μmole/ml.)	Time of incubation (hr)	Release (μmole P/g. dry wt./hr)	Hydrolysis of phosphate compound (%)
ATP	2	0·33	1480	62
	2	1	250	55
	2	2	240	107
	3·75	1	735	65
	15	1	2570	57
$Na_4P_2O_7$	2	0·33	623	13
	2	1	477	53
	2	2	261	58
	3·75	1	855	42
	15	1	113	1
$Na_5P_3O_{10}$	2	0·33	0	0
	2	1	7·2	16
	2	2	5·0	22
$(NaPO_3)_4$	2	0·33	0	0
	2	1	10·8	18
	2	2	3·0	13
$(NaPO_3)_6$	2	0·33	0	0
	2	1	527	117
	2	2	362	181

Oligomycin and ouabain had no effect on the hydrolysis of ATP by the cell-wall membrane at 10 μg. dry wt./ml. These concentrations were sufficient to inhibit the Na^+ stimulated and K^+ stimulated brain ATPases almost completely.

The effects of the sulphydryl-blocking reagents phenylmercuriacetate and *p*-chloromercuribenzoate were complicated. Further inhibition was found at some concentrations of ATP which were alone sufficient to cause inhibition of the ATPase. At a higher concentration of ATP there was apparent stimulation (Table 5). Sodium deoxycholate had similar effects to those of sulphydryl-blocking agents. Hexadecyltrimethylammonium bromide (Cetavlon) had no effect on the ATPase at or below 0·1 mM. At 1 mM, that is, above the critical point of micelle formation, the phosphate release decreased to 10–20 % of its normal value. This inhibitory concentration was a hundred times that required to prevent growth of the *Lactobacillus arabinosus* strain used. Ammonium sulphate had an inhibitory effect at high concentrations. Fluoride (5 mM) inhibited the enzyme strongly over all concentrations of ATP tested.

Ultrasonic treatment of the cell-wall membrane. Treatment of suspensions of cell-wall membrane preparations in the MSE Mullard cell disintegrator released various amounts of ATPase and comminuted the membranes into increasingly finer fragments (Pl. 1, fig. 3), depending on the time of treatment. After centrifugation at 100,000 *g* for 2 hr of a preparation treated ultrasonically for 45 min. the supernatant fluid, although clear, was enzymically active (Table 6). The solid deposit and the supernatant fluid both showed about a threefold increase in their maximum

Table 5. *The effect of phenylmercuriacetate (PMA) and p-chloromercuribenzoate (CMB) on phosphate release from ATP by cell-wall membrane preparations*

Tubes contained various amounts of ATP; cell-wall membrane (15 mg. wet wt.); Mg^{2+} at the same concentration as ATP; 0·5 ml. 0·1 M tris+maleate buffer (pH 7); 0·75 mmole mercury compound in a final volume of 1·5 ml. The tubes were incubated at 37° for 45 min., after which the phosphate release was estimated in the usual way.

	ATP conc. (mM)		
	(3·3)	(6·7)	(13)
	P release (μmole P/g.dry wt. cell-wall membrane/hr.)		
ATP alone	2760	4120	760
ATP+PMA	2140	3600	2350
ATP+CMB	2020	3540	3260

Table 6. *The effect of various concentrations of ATP on ultrasonically treated cell-wall membrane of Lactobacillus arabinosus* 17–5 *and its supernatant fluid*

Tubes contained different concentrations of ATP; ultrasonically treated cell-wall membrane (20 mg. wet wt.) or the corresponding 100,000 *g* supernatant fluid (equiv. 4 mg. dry wt.); Mg^{2+} at the same concentration as ATP; 0·5 ml. of 0·1 M-tris+maleate buffer (pH 7) in a total volume of 1·5 ml. After incubation for 30 min. at 37° phosphate release was estimated in the usual way.

	Phosphate release by μmole P/g. dry wt./hr.	
Conc. of ATP (mM)	ultrasonically treated cell-wall membranes	100,000 *g* supernatant fluid
3·3	14,700	15,100
6·7	20,600	15,900
13·3	2380	1110

specific activity. The value of K_m for the supernatant fluid was 0·6 mM. In other properties the ATPase in the supernatant fluid appeared very similar to that in the untreated cell-wall membrane preparation. This included the inhibitory effects of high concentrations of ATP, and the effect of 2,4-dinitrophenol. Further centrifugation of this supernatant fluid for 16 hr at 100,000 *g* did not yield a solid pellet, nor distinct layering. The supernatant fluid was separated into upper, middle and bottom fractions. The bottom fraction was considerably enriched in both protein and enzymic activity (Table 7). The addition of ammonium sulphate up to 80% saturation of the clear 100,000 *g* supernatant fluid precipitated the bulk of the enzyme activity. The first precipitate, up to 50% saturation with ammonium sulphate would not redissolve in water or various buffers; the other fractions did resuspend to give clear liquids. There was no increase in specific activity in any fraction obtained by this treatment.

The effect of enzymes on ATPase activity. Cell-wall membrane preparations were incubated with various hydrolytic enzymes at values between pH 5 and 8. After incubation the particulate material was removed by centrifugation and the ATPase of this deposit and the supernatant fluid compared with controls incubated without the addition of enzymes (Table 8). In most cases there was a marked decrease of ATPase activity, except in the case of material digested with pancreatic lipase where there was a marked increase in activity in the supernatant fluid but no decrease of

Table 7. *The protein and ATPase distribution after the long centrifugation of the* 100,000 *g supernatant fluid of the ultrasonically treated cell-wall membranes of Lactobacillus arabinosus* 7–5

Protein determination was by the usual method. For the ATPase determination, tubes contained ATP and Mg^{2+} (8·4 mM); a fraction of the supernatant fluid (0·2 ml.); 0·5 ml. of 0·1 M-tris + maleate buffer (pH 7) in a total volume of 1·3 ml. After incubation for 3 min. at 37°, phosphate release was estimated in the usual way. The figures are not corrected for the negligible phosphate content of the supernatant fractions.

Layer of supernatant fluid	Phosphate release (μmole P/ml./hr)	Protein content (mg./ml.)
Lower	19·6	16·5
Middle	1·8	4·5
Upper	1·3	4·3

Table 8. *The effect on the cell-wall membrane preparations from Lactobacillus arabinosus* 17–5 *of incubation with various enzymes*

Cell-wall membranes (50 mg. wet wt.) were incubated with about 1 mg. of the enzymes shown for 1·5 hr at 37° in buffer of the shown pH value. After centrifugation at 10,000 *g* for 5 min., the supernatant fluid was separated from the solid cell-wall membrane and both fractions were tested for ATPase activity. A control tube was incubated containing no external enzyme at pH 7. These fractions were then incubated with 3 mM ATP and Mg^{2+} and 0·5 ml. 0·1 M-tris + maleate buffer (pH 7) in a total volume of 1 ml., for 45 min. at 37°. Phosphate release was then estimated in the usual way. Blanks were run as usual for the phosphate content of the ATP and the incubated fractions. Phosphate release is expressed as a percentage of the untreated control.

Enzyme	pH value of incubation mixture	Relative phosphate release (%)	
		Supernatant fraction	Cell-wall membrane fraction
Papain	6	14	49
Hyaluronidase	5	19	30
Mycozyme	7	38	44
Lysozyme	7	94	107
Trypsin	8	11	14
Rattlesnake venom	7	51	33
Phospholipase D + Ca^{2+}	8	—	62
Lipase (pancreatic)	8	510	92

activity in the solid deposit. It is possible that this result indicates a release of ATPase activity accompanied by an activation of the released enzyme. As with the preparations partially solubilized by ultrasonic treatment, it was not found possible to fractionate further the enzyme released by lipase by the usual precipitation methods.

Chemical composition of cell-wall membrane. The cell-wall membranes contained

20–23 % protein compared to 27–30 % present in the whole bacteria (calculated on dry wt.). The total lipid varied from 7 to 11 % and represented the bulk of that contained in the bacteria; that remaining in the supernatant fluid was separable by further centrifugation. The lipid after extraction contained about 4 % phosphorus and can thus be assumed to be mainly phospholipid. Methyl esters of the fatty acids were prepared after saponification and examined by gas chromatography. There was no significant difference in the carbon number of the fatty acids from whole bacteria, cell-wall membrane or the supernatant fluid fractions. The bulk of the acids had carbon numbers between C_{15} and C_{18}, but were not examined in detail. The total carbohydrate content of the cell-wall membranes was 37 % compared to 14 % of the whole bacteria. The glucosamine determinations in whole bacteria were not very consistent, but the carbohydrate of the supernatant fluid contained about 0·8 % glucosamine while that of the cell-wall membranes contained 2·4 %. Both supernatant fluid and cell-wall membranes contained considerable amounts of rhamnose which is often used as an indication of the presence of bacterial cell-walls; cell-wall membranes contained 9–12 % rhamnose. The DNA content of the cell-wall membranes was usually negligible, but some batches contained up to 0·8 %; this amount when present was usually associated with electron-dense granules trapped in relatively unfragmented cell-wall membranes. The RNA content of the cell-wall membranes was 7 % when DNA was mostly absent, but increased when the DNA content was higher and was presumably due to the trapping of cytoplasmic contents. The chemical analysis of fragments of cell-wall membrane isolated after ultrasonic treatment was substantially the same as that of the intact cell-wall membranes. A preliminary examination by paper chromatography of the amino acids obtained by acid hydrolysis of cell-wall membrane showed the complete range of typical proteins. The chemical analysis therefore confirms the idea that a large part of the cytoplasmic membrane is present together with the cell wall in these preparations.

Cellular distribution of enzymes other than ATPase. Supernatant and cell-wall membrane fractions were examined in some detail for the presence of the riboflavin-mediated oxidation of pyruvate, lactate and glycerophosphate, glucose and glycerol (Strittmatter, 1959). Oxygen uptake with these substrates by the fractions was measured manometrically in the Warburg apparatus, with and without methylene blue and riboflavin. In all cases the enzymic activity was in the supernatant fractions and the cell-wall membrane was without significant activity. The activity in the supernatant fractions was not removed by centrifugation at 25,000 *g* for periods up to 2 hr. Similar results were obtained with supernatant fractions from bacteria broken in a 'sonomec' shaker (Rodgers & Hughes, 1960) or by ultrasonic treatment for 15 min. There was some pyruvate oxidase in the solid fractions from bacteria treated ultrasonically for longer periods (up to 60 min). This is similar to the findings of Strittmatter who disintegrated bacteria in the Raytheon disintegrator for 60 min., and was possibly due to denaturation of soluble protein (Hughes, 1961). Other enzymes found in the supernatant fraction and not in the cell-wall membranes were hexokinase, myokinase and malic enzyme. Enzymes located in the supernatant fractions in previous work include nicotinamide deamidase (Hughes & Williamson, 1953), pantothenate and pantothine phosphorylase (Pierpoint, Hughes, Baddiley & Mathias, 1955), cytidine pyrophosphorylase (Shaw 1961), lactic dehydrogenase (Don & Kaplan, 1960). It is also likely that most, if

not all, of the glycolytic enzyme system is soluble (Hughes, 1951). None of the cell-wall membrane preparations had polynucleotide phosphorylase activity when tested by the method of Abrams & McNamara (1962).

DISCUSSION

The outer shell or hull of *Lactobacillus arabinosus* strain 17–5 has been prepared by crushing the bacteria in the Hughes press and removing the cytoplasmic contents by washing. Such preparations formed inorganic phosphate from ATP at rates of 1·0–6 mmole P/g. dry wt./hr. Phosphate was also released from ADP at slower rates and kinetic evidence suggests that the same enzyme was involved in both reactions. There was also a phosphate release from various inorganic phosphates but it was not certain whether this too was due to the nucleotide phosphatase which hydrolysed ATP and ADP as follows: $ATP + H_2O \rightarrow ADP + H_3PO_4$. It is noteworthy that so far this is the only enzymic activity definitely associated with outer shells of *L. arabinosus*, whereas similar preparations from obligate and facultative aerobes contain a wide range of enzymes associated with energy production and electron transport (Hughes, 1962). The cell-wall membranes from a wide range of aerobically grown bacteria have also been found to have an ATPase similar to that reported here, and the enzyme has also been found in membrane preparations from *Streptococcus faecalis* (Abrams, McNamara & Johnson, 1960) and in preparations from *L. casei* supplied to us by Dr J. Shockman. More recently the enzyme has been found in cell-wall membranes and empty spheroplasts ('ghosts') of *Clostridium sporogenes* (Galli & Hughes, 1965) and in particulate fractions of *Chlorobium thiosulphatophilum* (Cole & Hughes, 1965). The hull preparations from *L. arabinosus* contained from 7 to 11 % total lipid of which the bulk appeared to be phospholipid since it contained about 4 % phosphorus. Most of the fatty acids previously reported to be present in this organism (Hofman, Lucas & Sax, 1952) appeared to be present in this hull lipid. A variable amount of ATPase activity was found in the supernatant fractions after centrifugal deposition of 'hulls', but the bulk of this could be recovered as a solid pellet by more prolonged centrifugation. This pellet also contained lipid and carbohydrate in about the same proportion as in the 'hulls' and it is assumed to be the result of their further comminution during disintegration; similar fragments were obtained by ultrasonic treatment. Up to now no satisfactory method has been found for solubilizing the ATPase of these hulls or of markedly increasing its specific activity. Of a number of enzymes, only pancreatic lipase brought about a release of some part of the enzymic activity from the particulate preparations, but this released enzyme could be regarded as insoluble since it was concentrated by centrifugation. The evidence therefore strongly supports the idea that the ATPase is bound to the protoplasmic membrane and that the membrane or large parts of it are associated with the wall in these preparations. They have therefore been assumed to be similar to cell-wall membranes previously described (Hughes, 1962). In the whole bacteria the ATPase cannot utilize exogenous ATP, presumably because the latter cannot enter.

Membrane bound ATPases are widely distributed in nature (Novikoff, Essner, Goldfischer & Heus, 1962) and are associated with ion transport (Whittam, 1964) changes in conformation (Kielly, Kimura & Cooke, 1964) and energy production

(Jagendorf, Hind & Neuman, 1964). The ATPase from *Lactobacillus arabinosus* differs from those associated with ion transport in that it is not markedly inhibited by ouabain and oligomycin; in experiments not reported here it was not markedly affected by Na^+ or K^+. Its pH optimum was also markedly lower (pH 6·0) than ATPases associated with ion transport. On the other hand it is stimulated by DNP, as is the ATPase of fragmented mitochondrial christae associated with oxidative phosphorylation.

Experiments with labelled ATP and lactobacillus ATPase preparations did not transfer terminal phosphate to any acceptor added, but the possibility cannot be ruled out that the ATPase is concerned in a phosphorylation associated either with cell-membrane or cell-wall synthesis. The lactobacillus preparations had no nucleotide pyrophosphorylase activity such as reported for membranes of *Streptococcus faecalis* (Abrams & McNamara, 1962). The consistent presence of RNA in the preparations of lactobacillus cell-wall membranes however lends support to the many suggestions that some membranes in bacteria play a role in protein synthesis in association with ribosomes or ribosomal RNA (Hunter, Brookes, Crathorn & Butler, 1959; Nisman & Fukuhara, 1961). It is possible that this ubiquitous ATPase is also involved. Up to the present however no definite role can be ascribed to this enzyme.

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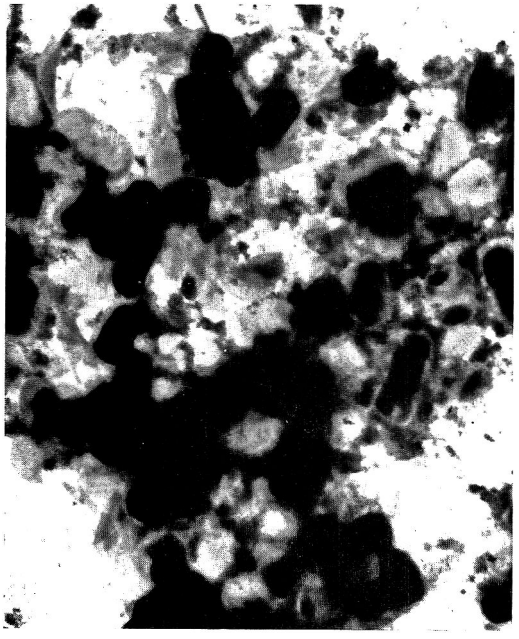

Fig. 1

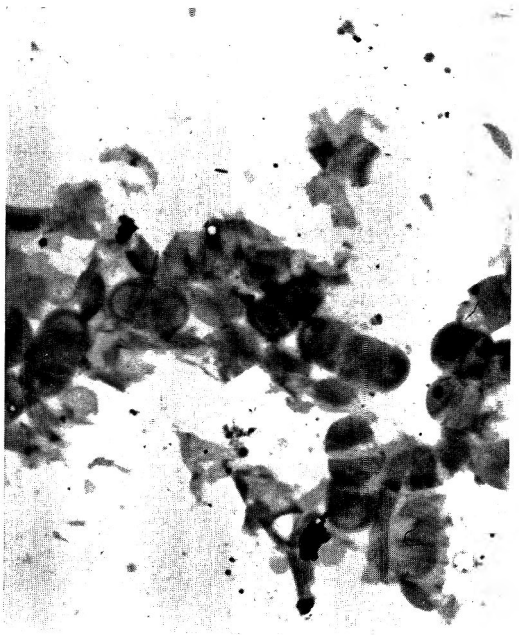

Fig. 2

Fig. 3

(*Facing p.* 95)

EXPLANATION OF PLATE

Fig. 1. Disintegrated *Lactobacillus arabinosus* 17–5 before fractionation by centrifugation. Bacteria were disintegrated by crushing in the Hughes press and treated with DNAase, fixed and mounted for examination without further treatment. × 6500.

Fig. 2. Cell-wall membranes from *Lactobacillus arabinosus* 17–5 after purification. The disintegrated bacteria shown in fig. 1 were fractionated by centrifugation as described in the text. × 6500.

Fig. 3. Cell-wall membranes of *Lactobacillus arabinosus* 17–5 after ultrasonic treatment. A purified preparation of cell-wall membranes was treated for 10 min. in the M.S.E. cell disintegrator, fixed and mounted as before. × 8500.

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A Study of some Pediococci and their Relationship to *Aerococcus viridans* and the Enterococci

By R. WHITTENBURY
Department of Bacteriology, School of Agriculture, Edinburgh

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SUMMARY

A collection of tetrad-forming bacteria was divided into five groups by physiological and biochemical tests. Of these groups four were identified as species of the genus *Pediococcus* according to the classification of Nakagawa & Kitahara (1959). Useful differential tests were: pseudocatalase activity, catalase activity in the presence of heated blood, fermentation of glycerol and maltose, salt tolerance, pH sensitivity, final pH value in glucose broth culture, growth temperatures, arginine hydrolysis. The remaining group, composed of the type culture of *Aerococcus viridans* and a strain of *Pediococcus urinae-equi*, was identified as *A. viridans*. The pediococci were compared with enterococci and two species, *P. pentosaceus* and *P. acidilactici*, appeared to be physiologically and biochemically more like *Streptococcus faecium* than is *S. faecalis*.

INTRODUCTION

Pediococci are the least studied group of the homofermentative coccal lactic acid bacteria. They are similar to the streptococci in many respects and can only be completely differentiated from them by their mode of division, pediococci forming tetrads and streptococci chains. Pediococci are found most often on plant materials, in various foods and in alcoholic beverages such as beer. In *Bergey's Manual* (1957) only two species are recognized, *Pediococcus cerevisiae* Balcke and *P. acidilactici* Lindner, and they are distinguished from one another on the basis of hop tolerance and optimum growth temperature.

At least two problems about the taxonomy of the pediococci have to be considered. The first concerns the placing of pediococci and streptococci into separate genera. Besides a difference in mode of division, a difference in the optical rotation of the lactic acid produced by the two groups has been used to justify their separation; pediococci were thought to form only inactive lactic acid and streptococci to form only L(+) lactic acid. Orla-Jensen (1943), however, recognized an L(+) lactic acid-forming organism as a pediococcus. Originally Orla-Jensen (1919) called this organism Tetracoccus no. 1 but subsequently (Orla-Jensen 1943) accepted it as *Pediococcus halophilus* Mees (1934). More recently Nakagawa & Kitahara (1959) and Deibel & Niven (1960) have identified L(+) lactic acid-forming tetracocci as pediococci. Even though there is some doubt about the identification of these organisms as pediococci, the finding of Langston & Bouma (1960) that two strains of *Streptococcus liquefaciens* (*S. faecalis*) formed inactive lactic acid instead of the expected L(+) lactic acid, rules against the usefulness of the rotation of lactic

acid as a generic characteristic in this instance. Mode of division, therefore, remains as the only characteristic which completely separates the pediococci from the streptococci. Evidence will be presented here to suggest that it is hard to justify the separation of the two genera, *Streptococcus* and *Pediococcus*, on the basis of this one characteristic.

The second problem concerns the classification of species within the genus *Pediococcus*. No generally acceptable system exists. The one proposed by Pederson (1957), separation on the basis of hop tolerance and optimum growth temperature, does not distinguish some obviously different types of organisms. A study by Nakagawa & Kitahara (1959), however, does provide a useful basis for classification; separation of species is based on ability to grow at different pH values, growth temperatures, weak catalase activity, CO_2 requirement, salt tolerance, hop tolerance and range of substrates fermented. Two of the species, *P. cerevisiae* and *P. acidilactici*, accommodate the species of those names recognized by Pederson. A third species, *P. pentosaceus* Mees, seems to be similar to *P. acidilactici* Lindner as described by Pederson, but has a lower maximum growth temperature than *P. acidilactici* (Lindner) (Nakagawa & Kitahara 1959). The two remaining species, *P. urinae-equi* Mees and *P. halophilus* Mees, differ considerably from the previous three. Both are tolerant of highly alkaline conditions, are acid-sensitive and form L(+) lactic acid instead of inactive lactic acid. *P. halophilus* is unusual among lactic acid bacteria in that it can tolerate 18–20% NaCl. Another system of classification was used by Günther & White (1961) who grouped pediococci according to optimum growth temperature, variation in colony diameter, degrees of growth turbidity, and times at which growth began after inoculation. This group of characters is of doubtful taxonomic significance and it seems clear that the work of Nakagawa & Kitahara (1959) provides a more useful basis for a classification of the pediococci.

The present investigation of the physiological and biochemical properties of some pediococci provides further information on the classification of these organisms. The organisms studied included the type culture of *Aerococcus viridans*, a species which Deibel & Niven (1960) transferred to the genus *Pediococcus* and included in the species *P. homari* (composed of L(+) lactic acid-forming cocci) which they proposed.

METHODS

Organisms. The organisms examined and their sources are given in Table 1. Those obtained during this investigation were isolated from fresh grass and silage macerates plated in the acetic acid + acetate agar described below.

Media. Basal medium agar, 'inoculum' medium, liquid medium, soft agar, heated blood + *o*-dianisidine agar and heated blood agar were those described previously (Whittenbury, 1963, 1964). Acetic acid + acetate agar was a modification of the medium proposed by Keddie (1951) as being selective for lactobacilli and contained meat extract (Lab Lemco), 0·5 g.; peptone (Evans), 0·5 g.; yeast extract (Difco), 0·5; Tween 80, 0·05 ml.; glucose, 1 g.; agar (Davis), 1·5 g.; tap water to 90 ml.; adjusted to pH 5·4 and autoclaved at 121° for 15 min. Before plating the medium 10 ml. of 2 M-acetic acid + sodium acetate buffer (pH 5·4) were added. Blood agar was 100 ml. melted basal medium agar at 45° to which was added 1 g.

NaCl and then 5 ml. defibrinated ox blood. Lysed blood agar was 100 ml. melted basal medium at 45° to which was added 5 ml. of a 1+1 by vol. mixture of ox blood and sterile water.

Tests. Tolerance of potassium tellurite, arginine hydrolysis, relationship to oxygen and fermentation tests in soft agar, reduction of 2, 3, 5-triphenyltetrazolium chloride, methylene blue and litmus, ability to grow at various temperatures,

Table 1. *Sources and identification of tetrad-forming organisms*

Identified as*	Designation when received	Source
Pediococcus pentosaceus	W1, W2, W3, W4, W5, W50	Herbage and silage
	M1, M11, M12	Silage (Cunningham & Smith, 1940)
	P2, P3	Silage. From Dr A. C. Stirling
	Leuconostoc citrovorum 7837	NCIB
	L. citrovorum 8106	NCIB
P. acidilactici	P4, P5, P7	Silage. From Dr A. C. Stirling
	L. mesenteroides 8018	NCIB
P. cerevisiae	S13, S14	Silage (Cunningham & Smith, 1940)
	Pediococcus 1250	NCDO
P. halophilus	*Pediococcus soyae* 1673	IAM
Aerococcus viridans	*Aerococcus viridans* 8251	NCTC
	Pedioccoccus urinae-equi 1684 (original culture of Mees)	IAM

NCIB = National Collection of Industrial Bacteria, NCDO = National Collection of Dairy Organisms, NCTC = National Collection of Type Cultures, IAM = Institute of Applied Microbiology (Tokyo).

* Pediococcus species were identified according to Kitahara & Nakagawa (1959).

catalase activity on heated blood agar and pseudocatalase activity on glucose (0·05%, w/v) nutrient agar, formation of H_2O_2 on heated blood + *o*-dianisidine agar and the dissimilation of citrate and malate were tested as described previously (Whittenbury, 1963, 1964, 1965). The ability to grow at pH 8·0 and above was tested by the method of Chesbro & Evans (1959), the final pH value of the medium being adjusted as required. The ability to grow lower than pH 8·0 was tested in glucose (0·5%, w/v) soft agar. The final pH value reached in glucose (1%, w/v) liquid medium initially at pH 6·5 was measured electrometrically after 1 week at 30°. Aesculin hydrolysis, hippurate hydrolysis and motility were tested by the methods of Gemmell & Hodgkiss (1964).

Incubation. Unless otherwise stated, incubation was aerobic at 30°.

RESULTS AND DISCUSSION

The Gram-positive tetrad-forming organisms were divided into groups and subgroups (Table 2). No organism reduced nitrate to nitrite, liquefied gelatin, produced gas from glucose, formed dextran from sucrose, reduced tetrazolium significantly, tolerated tellurite, hydrolysed starch, dissimilated citrate, reduced methylene blue or litmus in milk, or was catalase-positive on media containing

Table 2. *Properties of the organisms*

Property	Group* 1 (13)	2a (3)	2b (1)	3 (3)	4 (1)	5a (1)	5b (1)
	Reactions						
Fermentation of							
Arabinose	±	+	+	−	−	−	−
Xylose	±	+	+	−	−	−	−
Rhamnose	±	+	−	−	−	−	−
Melibiose	±	±	−	−	−	−	+
Sucrose	−	±	−	±	+	+	+
Lactose	±†	±	+	±	−	+	+
Trehalose	+	−	+	+	+	+	+
Maltose	+	−	−	+	+	+	+
Melezitose	−	±	−	−	−	−	−
Cellobiose	+	±	+	+	+	−	+
Raffinose	−	−	−	−	−	−	+
Glycerol	a‡	a	a	−	+	a	a
Sorbitol	−	−	−	−	−	a	a
Mannitol	−	−	−	−	−	a	a
Salicin	+	+	+	+	+	−	+
Hydrolysis of							
Arginine	+	+	+	−	−	−	−
Hippurate	−	−	−	−	−	+	+
Aesculin	+	+	+	+	+	−	−
Growth in glucose soft agar	f	f	f	pan	f	pa	pa
Growth and acid at							
40°	+	+	+	−	−	+	+
45°	+	+	+	−	−	−	−
50°	−	+	+	−	−	−	−
Survival at 63°/30 min.	±	±	−	−	nt	nt	nt
Final pH (glucose broth)	3·6	3·6	3·6	3·4–3·8	4·5	5·2	5·2
Growth on acetic acid + acetate agar	+	+	+	±	−	−	−
H_2O_2 formed on							
Basal HBD§ agar	+	+	+	±	−	+	+
Glucose HBD agar	−	−	−	±	−	+	+
Glycerol HBD agar	+	+	+	±	−	+	+
Dissimilation of malate in the presence of glucose	+	+	+	+	+	−	+
Growth initiated at							
pH 5·0	+	+	+	+	−	−	−
pH 8·0	+	+	+	−	+	+	+
pH 9·6	−	−	−	−	−	+	+
Pseudocatalase	±	−	−	−	−	−	−
Catalase (haem requiring)	−	+	−	−	−	−	−
NaCl % (w/v) tolerated	8–10	8–10	8	4	18	10	10

+ = All strains positive; − = all strains negative; ± = some strains positive and some negative; a = fermentation aerobic only; pa = preferentially aerobic; pan = preferentially anaerobic; f = facultative; nt = not tested.

* Group 5a is *Aerococcus viridans* NCTC 8251 and group 5b is *Pediococcus urinae-equi* IAM 1684; number of strains in each group is in parentheses.

† Some strains give rise to lactose-fermenting mutants.

‡ Some strains give very weak reaction.

§ Heated blood + *o*-dianisidine agar.

glucose (1 %, w/v) but lacking haem compounds. All the organisms except *Aerococcus viridans* dissimilated malate in the presence of glucose but none used malate as a source of energy. All grew and formed acid from glucose at 10° and 37°, and fermented fructose, galactose and mannose. None fermented *meso*-inositol or inulin.

All the organisms except those in group 3 grew uniformly throughout the aerobic and anaerobic portions of glucose soft agar adjusted to pH 6·8. One of the group 3 strains, Pediococcus NCDO **1250**, first grew in the anaerobic portion. As acid diffused towards the surface, however, growth appeared in the aerobic portion, indicating that a lowering of pH value overcame the organism's requirement for anaerobic conditions. This interpretation appeared to be confirmed by the following observations. Acid-forming growth developed simultaneously and uniformly throughout glucose soft agar adjusted to pH 5·0 and the organisms grew aerobically on glucose agar adjusted to pH 5·0 but not on glucose agar adjusted to pH 7·0. The remaining two strains in this group also showed an initial preference for anaerobic conditions in media at the higher pH value but, in contrast to the other strain, they both grew aerobically at pH 7·0.

Identification of groups 1, 2a, 2b, 3 and 4. Several problems arise in the application of names to these groups, apparently similar organisms being given different names by different investigators. The genus *Pediococcus* was introduced by Balcke (1884) when he gave the name *Pediococcus cerevisiae* to tetrad-forming cocci which multiplied in beer. Additional names have been applied to 'beer-sarcinae', although the organisms were not clearly distinguished from the original species of Balcke (1884), and several species have been proposed for pediococci isolated from sources other than beer. A definite advance in the taxonomy of this genus was made by Nakagawa & Kitahara (1959) who examined strains, some of historical importance, which they received from various sources and compared them with their own isolates. The whole collection was divided into five species of which one, *P. cerevisiae*, was distinguished from the others principally by ability to grow in hopped wort and beer, requirements for low temperatures, acid media and CO_2, a preference for anaerobic conditions, and the range of substrates fermented. The fact that these organisms could multiply in beer provides strong evidence of their identity with Balcke's original *P. cerevisiae*, the type species of the genus. The four remaining species of *Pediococcus* accepted by Nakagawa & Kitahara were derived from materials other than beer and they were found to be susceptible to the action of hops. More recently Günther & White (1961) and Coster & White (1964) applied species names quite differently and introduced a new binomial, *P. parvulus*, for one group. On the important question of their use of the name *P. cerevisiae* Coster & White (1964, p. 27) erroneously attribute this binomial combination to Pederson, and they put forward the inadmissible contention that their cultures were more typical of the genus than the less common strains from breweries.

Inevitably the strains examined in the present work were named according to Nakagawa & Kitahara (1959). Table 3 shows the features that appeared to be the most suitable for differentiating the species. *Pediococcus acidilactici* and *P. pentosaceus* seem to be quite similar to each other and they might eventually be grouped as one species. If this were done the name *P. acidilactici* would have priority.

It is difficult to relate this classification exactly to others proposed in the

recent literature because of the different methods used in the various investigations Nevertheless, an attempt is made in Table 4 to show approximately the equivalence of names and designation which have been published since the year 1959. The fact that one species of some investigators appears to be similar to two or three species or designated groups of others illustrates the present disagreement in opinion on the classification of pediococci.

Table 3. *Properties useful in the differentiation of the pediococci*

Results expressed as in Table 2

Property	Species: *P. pentosaceus* group 1	*P. acidilactici* group 2a, 2b	*P. cerevisiae* group 3	*P. halophilus* group 4
Fermentation of				
Maltose	+	−	+	+
Glycerol	a*	a	−	+
Growth and acid at				
45°	+	+	−	−
50°	−	+	−	−
Arginine hydrolysis	+	+	−	−
H_2O_2 formed on basal HBD† agar but not on glucose HBD agar	+	+	−	−
Pseudocatalase	± (11/13+)	−	−	−
Catalase	−	± (3/4+)	−	−
NaCl % (w/v) tolerated				
8·0	+	+	−	+
18·0	−	−	−	+
Growth initiated at pH 5·0	+	+	+	−
pH 8·0	+	+	−	+

* Some strains give a very weak reaction. † heated blood + *o*-dianisidine.

Table 4. *Approximate correspondence of recently published names and designations of pediococci*

Nakagawa & Kitahara (1959)	Deibel & Niven (1960)	Langston & Bouma (1960)	Günther & White (1961) and Coster & White (1964)	Whittenbury (this paper)
P. cerevisiae	.	Group 4	*P. damnosus*, *P. parvulus*, group III	*P. cerevisiae*
P. pentosaceus	.	Groups 1–3	*P. cerevisiae*	*P. pentosaceus*
P. acidilactici	.	Group 5	*P. cerevisiae*	*P. acidilactici*
P. halophilus	.	.	*P. halophilus*	*P. halophilus*
P. urinae-equi	*P. homari*	.	*P. urinae-equi*	*Acrococcus viridans*

Identification of the group 5 organisms. The results in Table 2 show that the two organisms in this group are very similar. One, 5*b*, is *Pediococcus urinae-equi* IAM 1684. It is the original culture of Mees (Mees, 1934) and was studied by Nakagawa

& Kitahara (1959) who expanded its description and retained it in the genus *Pediococcus*. The other organism, 5a, is *Aerococcus viridans* NCTC 8251, and is the type culture of the genus *Aerococcus* described by Williams, Hirsch & Cowan, 1953. It is considered by Deibel & Niven (1960) to be a pediococcus and was included by them with similar organisms in the species *P. homari*. Deibel & Niven (1960) thought this species was very similar to *P. soyae* as described by Sakaguchi (1958) which is synonomous with *P. halophilus*, Mees (Nakagawa & Kitahara, 1959). The results of the present investigation suggest that *P. homari* (assuming the culture of *A. viridans* studied here to be typical of this species) and *P. urinae-equi* are strains of the same species and that *P. homari* is a synonym of *P. urinae-equi* and not of *P. halophilus*.

Although the two organisms in this group 5 are similar to pediococci in that they form tetrads and ferment sugars, they have properties not normally associated with lactic acid bacteria—i.e. the ability to grow well on nutrient agar lacking sugars, acid-sensitivity and slight acid-forming powers in weakly buffered media. They also differ from previously described pediococci in that they have a preference for aerobic conditions. Anaerobic growth in sugar soft agars is slight and develops after a more dense acid-forming growth has appeared in the aerobic portion of the medium. With glycerol, mannitol and sorbitol as energy sources growth is strictly aerobic. Hydrogen peroxide is a product of the aerobic metabolism of these organisms and tends to inhibit growth on solid agar media unless it is destroyed as it is formed by the inclusion of manganese dioxide or catalase in the medium. The large areas of greening (α-haemolysis) produced by these organisms on blood agar are presumably caused by H_2O_2 as these areas matched exactly the H_2O_2 zones formed on blood agar containing *o*-dianisidine poured to cover half the blood agar plate.

To include these organisms in the genus *Pediococcus* would not be helpful at the present time. Although they have a superficial resemblance to the pediococci they also have physiological properties in common with respiratory-deficient mutants of staphylococci unable to synthesise iron porphyrins. Until more is known about these possible relationships the species *Aerococcus viridans* should be maintained. *Pediococcus urinae-equi* is here allotted to that species.

The similarity of Pediococcus pentosaceus and P. acidilactici to Streptococcus faecium. Towards the end of this investigation it became clear that *Pediococcus pentosaceus* and *P. acidilactici* were physiologically and biochemically similar to some enterococci which were studied earlier (Whittenbury, 1965). A comparison of these two species with *Streptococcus faecalis* and *S. faecium* showed that a closer resemblance existed between these pediococci and *S. faecium* than between *S. faecalis* and *S. faecium*. An example is given in Table 5 where the properties of a strain of *P. pentosaceus* are compared with those of a strain of *S. faecalis* and a strain of *S. faecium*.

A serological study (Norris & Whittenbury, unpublished) with precipitin tube and gel-diffusion tests seemed to confirm this relationship. Lancefield group D sera, obtained from Burroughs Wellcome and Co. or prepared with disintegrated *Streptococcus faecalis* H 69 D 5 and with *S. faecium* IMEC, reacted with HCl extracts of strains of *Pediococcus pentosaceus* and *P. acidilactici* but not with HCl extracts of other species of pediococci or of group N streptococci. A serum prepared with

Table 5. *Comparison of the properties of a strain of Pediococcus pentosaceus with those of Streptococcus faecium* SD *20 and Streptococcus faecalis* H69 D5

Properties common to *P. pentosaceus*, *S. faecium* and *S. faecalis*	
Fermentation of	
Glucose	+
Fructose	+
Sucrose	−
Arabinose	−
Maltose	+
Cellobiose	+
Galactose	+
Raffinose	−
Melibiose	−
Mannose	+
Lactose	+
Trehalose	+
Inositol	−
Salicin	+
Hydrolysis of	
Aesculin	+
Arginine	+
Growth at	
10°	+
45°	+
Tolerance of	
6·5% NaCl	+
63°/30 min.	+

Properties separating *P. pentosaceus* and *S. faecium* from *S. faecalis*		
	P. pentosaceus *S. faecium*	*S. faecalis*
Fermentation of		
Melezitose	−	+
Rhamnose	−	+
Sorbitol	−	+
Mannitol	−	+
Glycerol	aerobic	+
Hydrolysis of		
Hippurate	−	+
Vigorous reduction of		
Tetrazolium	−	+
Litmus milk	−	+
Tolerance of		
0·1% MB in milk	−	+
Tellurite	−	+
Growth with		
Malate	−	+
Citrate (pH 8·0)	−	+
Dissimilation of malate in presence of 1·0% glucose	+	−
Catalase (in presence of heated blood)	−	+
H_2O_2 formed on		
Basal HBD* agar	+	−
Glycerol HBD agar	+	−
α-Haemolysis (greening)	+	−

Properties separating *S. faecium* and *S. faecalis* from *P. pentosaceus*		
	S. faecium *S. faecalis*	*P. pentosaceus*
Growth at		
pH 9·6	+	−
Final pH (glucose broth)	4·1	3·6
Growth on acetic acid + acetate agar	−	+
Pseudocatalase	−	+
Tetrads formed	−	+

* Heated blood + *o*-dianisidine agar

a strain of *P. pentosaceus*, however, was unreactive with bacterial extracts other than of itself.

Apart from tetrad formation, which is not always easy to detect, a few tests (Table 6) still distinguish *Pediococcus acidilactici* and *P. pentosaceus* from the enterococci and should prove useful in the routine identification of enterococci isolated from materials likely to contain these pediococci.

Table 6. *Physiological properties separating Streptococcus faecalis and S. faecium from Pediococcus pentosaceus and P. acidilactici*

	Organisms	
Property	*S. faecalis* and *S. faecium*	*P. pentosaceus* and *P. acidilactici*
Growth at pH 9·6	+	−
Final pH in glucose broth	4·0 or higher	3·6
Growth on acetic acid + acetate agar	−	+

This example of a similarity between certain types of pediococci and streptococci seems reasonable evidence for suggesting that it would be more logical to group these organisms in one genus rather than in two genera. A comparative study of pediococci and streptococci with this possibility in mind should precede any formal proposals on the definition of the genus *Pediococcus* and of species within the genus.

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Some Effects of Nystatin on the Growth of Four Aspergillus Species

By VALERIE C. STANLEY* and MARY P. ENGLISH
Mycology Laboratory, Bristol Royal Hospital, General Hospital Branch, Bristol 1

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SUMMARY

At subinhibitory concentrations nystatin exerted its main effect on four species of Aspergillus in the lag phase of growth. Subsequent growth and sporulation appeared to be normal, indicating the lack of permanent damage to survivors. Starting with an inoculum of spores the increased lag period was caused by the inhibition of swelling and germination; starting with an inoculum of mycelium, by the temporary cessation of hyphal elongation. At low concentrations of nystatin the lag period increased in proportion to the increase in initial nystatin concentration. Under these conditions the length of the lag was limited by the natural breakdown of the antibiotic in the medium rather than by any metabolic activity or adaptive response of the fungi. However, the lag period increased sharply, as the inhibitory nystatin concentration for each species was approached, to values greater than can be explained solely by the increased concentration of antibiotic. This was not due to the time taken for growth by any especially resistant spores but to the greatly decreased viability and slow outgrowth of normal survivors. This loss in viability was due to factors other than K^+ leakage. Plasmolysis of germinated spores and mycelium occurred in some Aspergillus species at high concentrations of nystatin. The action of nystatin was relatively slow; no significant loss of viability or lengthening of the lag phase was noted until after exposure to the antibiotic for at least one hour, even at very high concentrations. Since the fungicidal effect on *Aspergillus terreus* and *A. fumigatus* was appreciably slower than on *A. niger* and *A. flavus*, it is possible that differences in the rate of absorption of nystatin in part determine sensitivity to nystatin. Spore germination took place at higher nystatin concentrations than did hyphal growth. The lethal dose for spores, which was three to four times higher than the fungistatic value, was also higher than for the mycelium.

INTRODUCTION

Nystatin, a polyene antifungal antibiotic obtained from *Streptomyces noursei*, is absorbed in significant amounts only by micro-organisms which are sensitive to it, that is, by certain yeasts, algae and filamentous fungi (Lampen, Morgan, Slocum & Arnow, 1959; Lampen & Arnow, 1961), their growth and utilization of various substrates being inhibited. These effects have been studied, in the main, in yeasts, relatively little work having been done with filamentous fungi. According to Lampen, Arnow, Borrowska & Laskin (1962), 90 % of the absorbed nystatin is bound by the yeast cell membrane. The membrane is damaged in the binding process,

* Present address: Bacteriology Department, Queen Charlotte's Maternity Hospital, Goldhawk Road, London, W. 6.

which leads to leakage of cytoplasmic constituents, inhibition of glucose utilization and eventual death. In the filamentous fungus, *Neurospora crassa*, the loss of cytoplasmic constituents and lysis of the protoplasts by the action of nystatin have been demonstrated by Kinsky (1961, 1962), indicating again that the primary action of the antibiotic is due to a direct action on the membrane leading to an alteration of permeability.

During an examination of the effectiveness of nystatin as an antifungal antibiotic for the treatment of otomycosis (a fungal disease of the external auditory canal and of postoperative aural cavities) studies have been made of the action of nystatin on the growth of various Aspergillus species. Experiments were made to determine the cause of the delay in growth which occurs at subinhibitory nystatin concentrations and attempts made to discover whether this delay was due to the breakdown of nystatin to a concentration at which growth can occur, to the reversal of damage caused by nystatin, to adaptation by the fungi, or to a combination of these factors.

METHODS

The following fungi were used: one isolate each of *Aspergillus flavus*, *A. terreus*, *A. niger* and *A. fumigatus* all obtained from cases of otomycosis; a laboratory isolate of *A. fumigatus* (*A. fumigatus* NyR) with increased resistance to nystatin; a baking strain of *Saccharomyces cerevisiae*.

Stock cultures were grown for 48 hr at 37° on glucose + peptone agar (GP agar) slopes (4% glucose, 1% peptone, 2% agar, pH 5·2), and then maintained at room temperature.

Spore suspensions were prepared by rubbing a 48 hr slope culture on GP agar with a solution of 0·05% (v/v) Tween 80 in distilled water. Spore counts were made in a haemocytometer and the suspension adjusted to the required strength by adding more Tween 80 solution. A spore suspension of 6×10^7 spores/ml. (standard spore suspension) was used in all experiments unless stated otherwise.

In all experiments cultures were incubated at 37°.

Suspensions of germinated spores were obtained by mixing 2 ml. of a standard spore suspension with 2 ml. of glucose + peptone broth (GP broth) and incubating until microscopic examination showed that the majority of spores had germinated. For the production of mycelial balls, 0·1 ml. of a standard spore suspension was inoculated into 50 ml. flasks containing 20 ml. of GP broth. The flasks were then incubated on a reciprocating shaker for 24 hr and the mycelial balls produced were harvested, washed and resuspended in fresh medium.

Thin seeded agar discs for radial growth experiments were prepared by inoculating 0·1 ml. of a standard spore suspension into 10 ml. of molten GP agar, cooled to 45°. The tubes were well mixed and plates poured. Discs of 0·9 cm. diameter were cut out with a cork borer. When similar discs containing mycelium with the minimum degree of sporulation were required, the seeded agar was covered with cellulose film and the plates incubated for 24 hr.

The size of colonies in plate cultures was measured along two diameters, crossing at right angles, marked on the back of the plate and the average diameter of the colony calculated. To determine the growth rate this measurement was repeated at intervals of time.

To determine the percentage germination of spores in liquid culture a sample was mounted in lactophenol cotton blue in a haemocytometer and the number of germinated spores in a total of 500 counted. A spore was considered to be germinated when the germ tube was as long as it was wide.

Nystatin solutions were prepared by dissolving commercial Squibb nystatin (3000 units/mg.) in propyleneglycol to give stock solutions of 12,000 units/ml. These were freshly prepared as required and all further dilutions were made in propyleneglycol. In all experiments, unless stated otherwise, 1 ml. nystatin solution was added to 19 ml. of molten GP agar cooled to 45°, or to 19 ml. of GP broth, to give final concentrations of 600, 400, 200, 100, 50, 25, 12·50, 6·25, 1·60, 0·80 units/ml. An equal volume of solvent was added to the controls.

The sensitivity to nystatin of the Aspergillus species used in the present work was measured by a method (standard sensitivity test) described briefly here but in more detail elsewhere (English & Stanley, in preparation). Molten GP agar incorporating nystatin at different concentrations, prepared as described above, was inoculated with 0·1 ml. of a standard spore suspension, mixed, and plates poured. The highest nystatin concentration at which microscopic examination showed a few spores to have germinated after incubation for 48 hr was taken as the minimal inhibitory concentration (MIC; see Table 1).

Table 1. *The minimal inhibitory concentration (MIC) of nystatin for four Aspergillus species determined by the standard sensitivity test*

Nystatin (1 ml.) solution at a range of concentrations together with 0·1 ml. of a standard spore suspension were added to 19 ml. molten cooled GP agar, well mixed and plates poured. The lowest concentration inhibitory to germination was noted after incubation for 48 hr. at 37°.

	MIC at 48 hr./(units nystatin/ml.)
Aspergillus flavus	12·5
A. niger	25
A. fumigatus	25
A. fumigatus NyR	50
A. terreus	200

RESULTS

The stability of nystatin

It is well known that nystatin is unstable under conditions likely to be used in experimental work (Kubitsa & Derse, 1959; Eisenberg *et al.* 1956; Stewart, 1956). Since most of the experiments done in the present work were long-term growth studies it was necessary to know the rate of breakdown of nystatin during handling and under experimental conditions. Kinsky (1961) stated that the re-growth of the mycelium of *Neurospora crassa*, after 4 hr at 25° in the presence of the minimal inhibitory concentration of nystatin in liquid medium was due to photo-oxidation of the antibiotic. To determine the importance of the effect of light, flasks containing GP broth and plates of GP agar, both incorporating from 0 to 25 units nystatin/ml. were prepared. One series of each was stored for 24 hr at room temperature in daylight and one in the dark. All series were then inoculated with a suspension of *Saccharomyces cerevisiae* and incubated for a further 24 hr in the dark. In liquid culture the apparent amount of nystatin necessary to inhibit growth after

storage in the light was double that necessary after storage in the dark. On the solid medium no difference was noted between plates stored in the light and in the dark. However, in all subsequent work nystatin solutions and plates were protected from light during manipulation, and incubation was always in the dark.

To determine the rate of breakdown of nystatin in the dark, series of GP agar plates containing from 0 to 600 units/ml. were prepared in triplicate. For each Aspergillus species one plate at each concentration was inoculated with 0·05 ml. of a standard spore suspension spread over a circular area 2·5 cm. in diameter previously marked on the agar. The second and third series of plates were similarly inoculated after incubation for 24 and 48 hr respectively. Further inoculations were made on each plate 24 and 48 hr after the first. The minimal inhibitory concentrations for each series of inocula were noted at daily intervals. The results, which are the average of three experiments, are shown in Fig. 1. Other similar experiments were done with one series of plates only, the results of which confirmed those presented above. Therefore, using the minimal inhibitory concentration as a measure of the amount of nystatin remaining in the medium after incubation, it was apparent that the potency of the antibiotic was halved every 24 hr.

At nystatin concentrations which allowed growth during the first 48 hr, and after equal periods of incubation, the same amount of growth was obtained from inocula on plates on which the fungus was already growing as on those on which it was not. This indicates that loss in the potency of the nystatin was independent of the growth of the fungi and was due to spontaneous decomposition. In contrast to our results, Kubitsa & Derse (1959) showed that there was a greater loss of potency of nystatin in plates on which a previous inoculum of *Candida albicans* had grown than on uninoculated plates. It is possible that in the present investigation the inocula were insufficiently close to one another for their zones of influence to interact, but since the distance between the edges of the original and subsequent inocula was only *c.* 1 cm. any metabolic action of the Aspergillus species on nystatin must be slight.

At nystatin concentrations which prevented germination of Aspergillus spores during the first 48 hr (i.e. 200 units/ml. for *Aspergillus terreus* and 50 units/ml. for *A. niger*, *A. fumigatus*, *A. flavus*) a different effect was observed. The first inoculum on a fresh plate showed much less growth after a longer incubation period than later inocula on the same plate. The first inoculum would receive the full dose of nystatin, but even when this had deteriorated to a subinhibitory concentration further time was presumably required for the recovery of the majority of the spores or for the outgrowth of a few survivors. To confirm these results experiments were made using the length of the lag phase of growth (see next section) as a measure of the nystatin remaining in the medium. Plates of GP agar incorporating 0–200 units nystatin/ml. were prepared in triplicate. After incubation for 0, 24, 48 hr one plate at each concentration was inoculated with *A. fumigatus* in the form of a seeded agar disc. The size of each colony was measured at intervals of time and the growth rate and length of the lag period before normal growth began were determined from the appropriate graphs. The results are shown in Fig. 2.

The length of the lag phase at each nystatin concentration decreased with increasing age of the plates and after the lag was overcome the final growth rates

all reached those of the controls. At the lower concentrations the amount of antibiotic remaining after 24 hr had fallen to a value which had no effect on the lag phase. Examination shows that the curves through each set of points are identical in shape and differ only in displacement along the log concentration axis; that is, the sole factor which affected the growth of the fungus was the loss in potency of the antibiotic with storage time. Read from the graph, the displacements of the curves for 24 and 48 hr storage, respectively, are approximately log. 0·4 and log. 0·5, indicating that the potency of the nystatin was about halved every 24 hr.

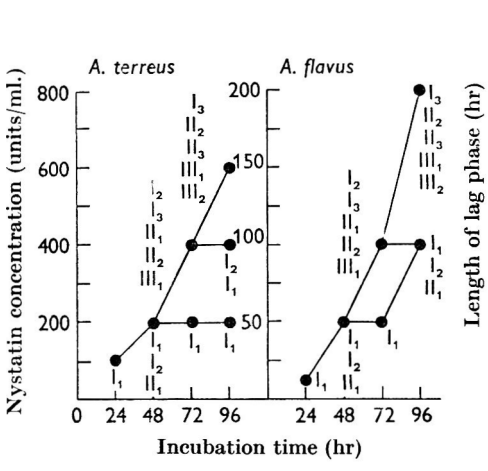

Fig. 1

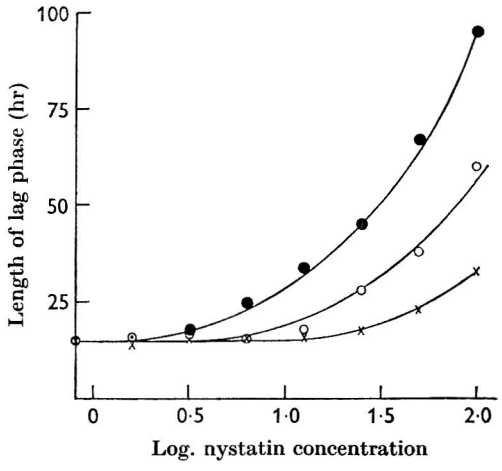

Fig. 2

Fig. 1. The change in the minimum concentration of nystatin necessary to inhibit *Aspergillus terreus* and *A. flavus* caused by ageing the antibiotic for varying periods at 37°. Plates of GP agar incorporating from 0 to 600 units nystatin/ml. were prepared in triplicate and a plate at each concentration inoculated after the interval indicated with 0·05 ml. of a standard spore suspension spread over a circular area 2·5 cm. diameter. The MIC for each series of inocula was noted at 24 hr intervals. Time of inoculation after ageing plates at 37°: 1st series of plates: (I_1), 0 hr; (I_2), 24 hr; (I_3), 48 hr. 2nd series of plates: (II), 24 hr; (II_2), 48 hr; (II_3), 72 hr. 3rd series of plates: (III_1), 48 hr; (III_2), 72 hr.

Fig. 2. The breakdown of nystatin, incorporated into GP agar at 37°, as demonstrated by the lag in growth rate of *Aspergillus fumigatus*. Plates of GP agar incorporating from 0 to 200 units nystatin/ml. were prepared, and plates at each concentration inoculated with a seeded agar disc (0·9 cm. diameter) at the times indicated. The diameters of the colonies were measured at intervals of time and the length of the lag phase determined from the growth curves. Time of inoculation after ageing plates at 37°: (●), 0 hr; (○), 24 hr; (×), 48 hr.

The effect of nystatin on the length of the lag phase

The nature of the initial delay in growth caused by subinhibitory nystatin concentrations was investigated. For each Aspergillus species two series of GP agar plates were prepared, incorporating from 0 to 600 units nystatin/ml. Agar discs containing ungerminated spores were placed on one series of plates and similar discs containing mycelium on the other. The growth rate from each disc was measured. From the growth curves at each nystatin concentration the length of the lag phase was determined; the results are plotted in Figs. 3 and 4. In each case, the final growth rates were the same as those of the controls (Fig. 3). Above a certain

threshold value peculiar to each species the length of the lag increased to values which could not be explained solely by the increase in nystatin concentration. Figure 4 shows, for *Aspergillus terreus* at low nystatin concentrations, that the lag phase is longer for spores than for mycelium. This was also true of the other species tested, and is probably explained by the fact that spores neither swell nor germinate at inhibitory concentrations so that, after the nystatin has broken down to subinhibitory levels, additional time is necessary for this to occur before growth can begin. It also appears from Fig. 4 that the relationship between the length of the lag phase and the log. concentration of nystatin, while usually a smooth curve, may approach linear. Duplicate experiments showed that this occurred most often with *A. flavus* and *A. niger:* Lampen *et al.* (1959) noticed a similar phenomenon.

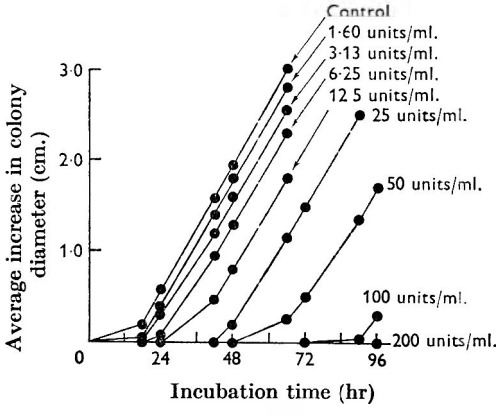

Fig. 3

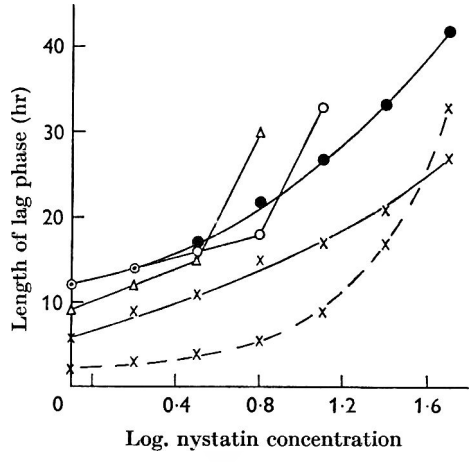

Fig. 4

Fig. 3. Effect of nystatin (units/ml.) incorporated into GP agar at concentrations indicated, on the radial growth of *Aspergillus fumigatus*. Plates of GP agar incorporating from 0 to 600 units nystatin/ml. were inoculated with discs seeded with spores or mycelium of *Aspergillus fumigatus* and the diameter of the colonies measured at intervals of time.

Fig. 4. Effect of nystatin incorporated into GP agar on the lag phase in growth of *Aspergillus* species. The length in lag phase in growth was determined from growth curves obtained as described in Fig. 3. Inoculum spores: (×), *A. terreus;* (○), *A. niger* (△), *A. flavus;* (●), *A. fumigatus*. Inoculum mycelium: (×), broken line, *A. terreus*.

Since from this experiment it appears that the main effect of nystatin at sub-inhibitory concentrations was to lengthen the lag phase, further tests were made in an attempt to determine whether the ending of the lag was entirely due to the breakdown of nystatin or partly to adaptation by the fungi.

Test tubes of GP broth incorporating from 0 to 6000 units nystatin/ml. were inoculated with 1 ml. of a standard spore suspension of *Aspergillus fumigatus* to a total vloume of 6 ml. and incubated on a reciprocating shaker. After exposure for 0, 5, 15, 30 min. and 1, 4, 24 hr to nystatin, 1 ml. samples were taken, washed three times with large volumes of sterile distilled water to remove unabsorbed nystatin, and resuspended in 0·5 ml. water. One loop of this suspension was spread over a circular area (0·9 cm. diameter) on GP agar plates, the growth rate of the colonies measured, and the length of the lag phase calculated from the growth curves. After

the lag phase, growth rates all reached that of the control. Exposure to nystatin, at all concentrations tested for periods up to 1 hr resulted in only small increases in the lag period. After exposure for 4 hr the lag period increased by a significant amount at all concentrations and the increase was greater at the higher concentrations. The spores of *A. fumigatus* at the concentration used were killed by 6000 units nystatin/ml. but not until they had been exposed for longer than 4 hr. Since in yeasts the inhibition of glycolysis caused by low concentrations of nystatin was annulled by K^+ (Cirillo, Harsch & Lampen, 1964) the above experiment was repeated using 1 % (w/v) KCl for the washing and resuspension of the spores. Comparison with controls washed in water shows that K^+ had no significant effect on the length of the lag phase.

Table 2. *Length of lag period of growth of Aspergillus fumigatus exposed to nystatin at 37° after removal of unabsorbed antibiotic by washing in water*

Nystatin in GP broth (units/ml.)	Time of exposure to nystatin: 1 min.	5 min.	15 min.	30 min.	1 hr	4 hr	24 hr
	Lag period (hr)*						
0	13	13	14	13	13	12	9
6·25	13	13	16	15	18	21	28
12·5	14	15	15	16	17	23	35
25	13	14	15	14	18	22	24
50	13	13	15	15	18	21	27
100	14	15	15	15	19	22	34
200	14	15	15	16	19	23	30
600	14	13	15	16	17	25	36
1000	14	16	15	16	16	28	47
6000	17	17	18	17	19	38	†

* The length of the lag phase was calculated from growth curves obtained from spores treated as described in Fig. 5.

† Lethal.

Spore germination in the presence of nystatin

Three series of 50 ml. flasks containing GP broth incorporating 0—100 units nystatin/ml. were prepared in duplicate, inoculated with 0·1 ml. of standard spore suspensions of *Aspergillus niger*, *A. fumigatus* and *A. fumigatus* NyR, respectively, and incubated on a reciprocating shaker. Samples were taken at intervals and the percentage germination determined. The average diameter of the spores was also measured. Since mycelium formed both in control flasks and at low nystatin concentrations after 18 hr, the final count was made at 12 hr. The results are plotted in Fig. 6. Nystatin delayed swelling and germination of the spores of each species. The numbers germinated at each time interval decreased with increasing nystatin concentration in a linear fashion, until a concentration was reached at which no swelling of the spores occurred (50 units/ml. for *A. fumigatus* and *A. niger*, 100 units/ml. for *A. fumigatus* NyR). After incubation for 36 hr the numbers of spores germinating at subinhibitory concentrations of nystatin had increased, and a few had germinated at a concentration which had been inhibitory at 12 hr. This was probably due to the normal decomposition of the nystatin, but the small numbers of spores which germinated at high concentrations suggests a possible fungicidal effect.

Fungicidal activity

At low concentrations nystatin is fungistatic to a wide variety of yeasts and moulds, but at higher concentrations it becomes fungicidal (Bradley & Jones, 1960). To determine the point at which this occurred with the Aspergillus species used

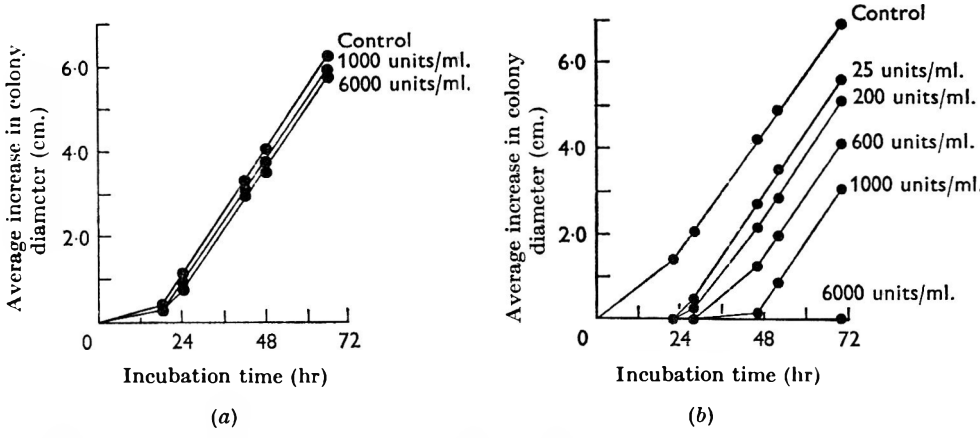

Fig. 5. Effect of time of exposure to nystatin (units/ml.) at the concentrations indicated on the growth rate of *Aspergillus fumigatus*. (*a*) Growth after exposure to nystatin for 5 min.; growth at intermediate concentration not shown as all values were very similar. (*b*) Growth after exposure to nystatin for 24 hr. 5 ml. of GP broth incorporating from 0 to 6000 units nystatin/ml. were inoculated with 1 ml. of a standard spore suspension of *A. fumigatus* (total vol. 6 ml.). 1 ml. samples were taken after 5, 15, 30 min. and 1, 4, 24 hr, the spores washed in water, resuspended in 0·5 ml. water, spread over a circular area of 0·9 cm. diameter on GP agar and the diameters of the colonies measured at intervals of time.

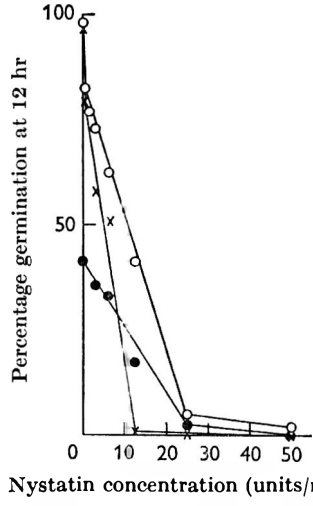

Fig. 6. Percentage germination of spores of *Aspergillus* species in the presence of nystatin incorporated into GP broth. GP broth incorporating from 0 to 100 units nystatin/ml. was inoculated with 0·1 ml. of a standard spore suspension (total vol. 20 ml.) and incubated on a reciprocating shaker. Samples were taken at intervals of time and the % germination determined by counting in a haemocytometer. (●), *A. niger*; (×), *A. fumigatus*; (○), *A. fumigatus* NyR.

here, 1 ml. of a standard spore suspension of each Aspergillus species was inoculated into a series of test-tubes of GP broth +0 to 6000 units nystatin/ml. and incubated on a reciprocating shaker. Samples (1 ml.) were taken after exposure for 1, 4, and 24 hr, washed as before and resuspended in 0·5 ml. sterile distilled water; 0·2 ml. samples of this suspension were spread over each of duplicate GP agar plates and the total number of colonies which developed within a week counted. The results are given in Table 3. No significant fungicidal action was noted after exposure for 1 hr to any nystatin concentration used. After 4 hr about 99·9 % of the spores of *Aspergillus flavus* and *A. niger* were killed at all concentrations tested. The fungicidal action on *A. fumigatus* and *A. terreus* was less rapid. After 24 hr at concentrations of over 100 units/ml. no spores of *A. flavus* or *A. niger* survived, but a few colonies of *A. fumigatus* developed after exposure to 1000 units/ml. and of *A. terreus* to 6000 units/ml. Lampen, Arnow & Safferman (1960) have shown that even when 99·9 % of yeast cells are killed, the remainder can grow when the unabsorbed nystatin is removed. Heavy inocula are known to be far less sensitive to fungicides than light inocula (Horsefall, 1954); in the present experiment clumping of the spores in shaken liquid culture might produce small foci of very heavy inocula which could explain the survival of a small number of spores for longer periods than the majority. It is clear, however, that the main fungicidal effect occurred at concentrations of nystatin only a few times higher than the fungistatic concentration.

Table 3. *Death of Aspergillus spores caused by exposure to nystatin in glucose + peptone broth at 37° for different times, followed by removal of the unabsorbed antibiotic by washing with water.*

		Concentration of nystatin (units/ml.)					
	Time of exposure	0	100	200	500	1000	6000
Organism	(hr)	No. of colonies					
Aspergillus flavus	1	∞	∞	∞	∞	∞	∞
	4	∞	60*	42*	40*	24*	18*
	24	∞	8*	0	0	0	0
A. niger	1	∞	∞	∞	∞	∞	∞
	4	∞	167*	112*	89*	92*	75*
	24	∞	5*	0	0	0	0
A. fumigatus	1	∞	∞	∞	∞	∞	∞
	4	∞	∞	∞	∞	∞	∞
	24	∞	31*	9*	16*	19*	0
A. terreus	1	∞	∞	∞	∞	∞	∞
	4	∞	∞	∞	∞	∞	∞
	24	∞	155*	112*	122*	78*	98*

∞ = Colonies too numerous to count; * = about 99·9% killing.

The effect of more prolonged exposure to moderate concentrations of nystatin was investigated for three growth phases. Standard suspensions of ungerminated and germinated spores of each Aspergillus species were streaked on GP agar + 600 units nystatin/ml. After incubation for 48 hr the inocula were scraped off and plated on fresh GP agar. The ungerminated spores of all species and the germinated spores of *A. terreus* all gave numerous colonies although growth was delayed from 1 to 2 days; but in comparison with the controls the colonies were less numerous, suggesting

some killing. All the germinated spores of *A. niger* were killed, but a few colonies developed from those of *A. flavus* and *A. fumigatus* after 3–5 days. This might have been due to the presence of a few ungerminated spores in the inoculum or to clumping. When forty mycelial balls of each species were exposed to 600 units nystatin/ml. in GP broth (total volume 6 ml.) for 48 hr, then washed and replated on GP agar, growth occurred only from the mycelium of *A. terreus*.

Microscopical changes in Aspergillus germ tubes after exposure to nystatin at inhibitory concentrations

To obtain Aspergillus spores at different stages of development, pieces of cellulose film laid over GP agar were inoculated with spores of each Aspergillus species and incubated. Pieces of film were removed at intervals and placed on the surface of GP agar + 100 units nystatin/ml. for *Aspergillus niger*, *A. flavus* and *A. fumigatus* and 400 units/ml. for *A. terreus*. After incubation for 24 hr the cellulose strips were mounted in lactophenol cotton blue and examined microscopically. In each case growth was inhibited at the stage reached before contact with nystatin. The germ tubes of *A. niger* and particularly of *A. flavus* were greatly plasmolysed in comparison with refrigerated controls. The germ tubes of *A. fumigatus* contained numerous refractile granules not seen in the controls. No differences were observed in *A. terreus*.

Induction of resistance to nystatin

The possibility that the growth shown by the Aspergillus species at high nystatin concentrations after the long lag phase was due to induced resistance to nystatin was examined. Gradient plates (Szybalski, 1952) at a maximum strength of 600 units nystatin/ml. were prepared; 0·1 ml. of a spore suspension of each Aspergillus species was spread over the plates which were then incubated for 72 hr. Colonies which developed at the highest nystatin concentration were picked off and subcultured on further gradient plates at the same concentration of nystatin, in the manner just described. After ten and twenty transfers the sensitivity of the isolates was determined by the standard sensitivity test. There was no increase of resistance in *Aspergillus niger* or *A. flavus*, but a strain of *A. fumigatus* with double the resistance of the parent strain was obtained, i.e. with the same resistance as *A. fumigatus* NyR. No further increase of resistance was observed in *A. fumigatus* NyR. As a further check on the possible occurrence of adaptation or the selection of resistant strains, spores which survived exposure to high concentrations of nystatin in some of the experiments previously described were tested by the standard sensitivity test, but no increase in resistance was found.

DISCUSSION

Direct experiments on the sensitivity of the fungi suggested that, within each Aspergillus species, the level of nystatin necessary to inhibit spore germination was the same as that needed to inhibit hyphal growth; but experiments on the lag phase showed that mycelium was more sensitive than spores, and the lethal concentration of the drug was also shown to be lower for mycelium than for spores. The probable explanation of this inconsistency is that the doubling increments of nystatin used throughout this work were too large to detect small differences in the

minimal inhibitory concentration. This could also account for our finding no difference in the minimal inhibitory concentration in liquid and in solid media, contrary to the results of Stewart (1956) who, working with yeasts, found that more nystatin was required to inhibit growth in solid than in liquid media. An indication in our experiments that this is, in fact, true for Aspergillus species was that, as nystatin deteriorated with time, more spores germinated in initially inhibitory solid media than in liquid media containing the same amount of nystatin. Better contact between spore and antibiotic in shaken, liquid cultures than in solid ones is the probable explanation.

Marini, Arnow & Lampen (1961) have demonstrated that in *Saccharomyces cerevisiae*, even if the inhibition of glycolysis which is caused by nystatin is reversed by the addition of K^+ etc., there may still be a complete loss of viability of some cells: these workers showed that this irreversible change was due to the additional loss of cytoplasmic constituents other than those necessary to maintain glycolysis. This probably also explains our finding of the marked lengthening in the lag phase of the Aspergillus species at concentrations approaching the inhibitory level, and the high proportion of spores killed by nystatin at concentrations only a few times greater than those causing fungistasis.

The necessity for a minimum length of exposure of Aspergillus spores to nystatin before any effect at all can be observed, even at the highest antibiotic concentrations, is consistent with the findings of Lampen, Arnow, Borowska & Laskin (1962) that in yeasts the intact cell-wall delays binding and that the binding sites do not appear to be readily accessible. Nystatin absorbed on to the organism is not removed by washing with buffer or acetone, or by adjusting the pH (Lampen *et al.* 1959), and in yeasts the rate of absorption and total amount absorbed increases as the nystatin concentration is raised (Lampen & Arnow, 1959). This suggests that the lengthening of the lag phase of Aspergillus species is also due to increased absorption of nystatin, this phase eventually being terminated when the absorbed nystatin breaks down to a subinhibitory level.

Differences in the rate at which nystatin is absorbed at the cell surface, combined with the decay of the absorbed antibiotic with time, might explain differences in the behaviour of the individual Aspergillus species in the presence of the antibiotic. In the standard sensitivity test, where incubation was for a period of 48 hr in continued contact with nystatin, maximum absorption must occur and germination be inhibited at a relatively low external concentration. In the fungicidal tests, on the other hand, unabsorbed nystatin was removed after limited exposure periods, and if absorption were slow in some species, recovery of survivors, even after exposure to very high concentrations, might be expected. This would appear to be the case with *Aspergillus terreus*, but cannot alone explain the comparative resistance of *A. fumigatus* to the fungicidal action of nystatin, when coupled with its extreme sensitivity to fungistasis.

Strains of filamentous fungi or yeasts which have developed resistance to nystatin have rarely been reported in the literature and the general opinion is that resistance of the type occurring with other antibiotics is not met with (Stout & Pagano, 1956). Where resistance does occur, as in *Aspergillus fumigatus* (Manning & Robertson, 1959), *Candida albicans*, *Cerastomella ulmi* (Stout & Pagano, 1956) and *Candida* spp. (Littman, Pisano & Lancaster, 1957–58), it is only two or three times that of

the original strain and it does not appear possible to increase it further. These observations have been confirmed with the four Aspergillus species used in this investigation.

Since it has been shown that nystatin undergoes natural degradation at 37°, that there is survival of a small number of spores at high nystatin concentrations, and that these survivors have the normal growth rate and no increased resistance, it would appear that the growth which follows exposure to nystatin at high concentrations for long periods of time is not due to adaptation, or to the selection of resistant spores, but to the slow outgrowth of a few normal survivors as the nystatin loses potency.

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Cephalosporinase and Penicillinase Activity of Gram-negative Bacteria

By G. A. J. AYLIFFE*

Department of Bacteriology, Postgraduate Medical School of London, Ducane Road, London, W. 12

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SUMMARY

Sensitivity to ampicillin and cephalothin, the formation and induction of penicillinase and cephalosporinase, and the role of cephalosporinase in resistance to cephalothin were investigated in nine strains of Gram-negative bacteria. Naturally occurring strains resistant to cephalothin formed cephalosporinase, and ampicillin-resistant strains formed penicillinase. Cephalothin-resistant strains were all resistant to ampicillin, but the ampicillin-resistant strain of *Proteus mirabilis* was sensitive to cephalothin and did not form cephalosporinase. Penicillinase and cephalosporinase activity were induced by benzylpenicillin in five strains of Gram-negative bacteria and cephalosporinase was also induced by cephalothin and 6-aminopenicillanic acid in four of the inducible strains examined. Methicillin and quinacillin were variable in effect as inducers and cloxacillin partially inhibited cephalosporinase activity of two strains. Intrinsic resistance to cephalothin was high in four of five naturally occurring resistant strains.

INTRODUCTION

Recently isolated derivatives of cephalosporin C, namely cephalothin (sodium salt of 7-(thiophene-2-acetamido)-cephalosporanic acid) and cephaloridine (7-[(2-thienyl) acetamido]-3-(1-pyridylmethyl)-3-cephem-4-carboxylic acid betaine), show antibacterial activity against several Gram-negative bacteria. Nevertheless, many strains of Gram-negative bacteria are resistant to these antibiotics and form an enzyme which inactivates cephalosporin (Chauvette *et al.* 1962; Fleming, Goldner & Glass, 1963; Barber & Waterworth, 1964; Muggleton, O'Callaghan & Stevens, 1964). It has been shown that ampicillin resistance can usually be correlated with penicillinase formation, although most ampicillin-resistant strains of Gram-negative bacteria also show some degree of intrinsic resistance (Ayliffe 1963; Percival, Brumfitt & de Louvois, 1963; Sutherland, 1964). Ampicillin is readily inactivated, as is benzylpenicillin, by the penicillinases of *Staphylococcus aureus* and *Bacillus cereus* (Smith & Hamilton-Miller, 1963), whereas cephalosporin C is inactivated slowly by these enzymes (Crompton *et al.* 1962). It was thought of interest to determine the sensitivity to ampicillin and cephalothin of some Gram-negative bacteria of clinical importance and to compare penicillinase and cephalosporinase activity and induction in these strains. The role of cephalosporinase in determining resistance to cephalothin was also investigated. Penicillinase and cephalosporinase activity in this paper refers to the hydrolysis of benzylpenicillin and cephalothin respectively, as assayed by an iodometric method.

* Present address: M.R.C. Hospital Infection Research Laboratory, Summerfield Hospital, Birmingham 18.

METHODS

Organisms. Strains of *Proteus morganii*, *P. vulgaris*, *P. rettgeri*, *P. mirabilis*, *Klebsiella aerogenes*, *K. edwardsii* var. *edwardsii*, *Enterobacter* (*Aerobacter*) *aerogenes*, *Escherichia coli* and *Pseudomonas aeruginosa* (*pyocyanea*) were used in the experiments. These strains were isolated from clinical specimens and were classified according to the tables of Cowan & Steel (1961).

Culture media. A beef digest broth (pH 7·4) containing 0·5% (w/v) glucose was used in all enzyme experiments. Digest broth was solidified with 1·4% (w/v) agar (Oxoid no. 3) for the sensitivity tests.

Minimum inhibitory concentrations. The minimum inhibitory concentrations (MIC) of cephalothin and ampicillin were determined on nutrient agar plates containing serial dilutions of the antibiotics. These plates were inoculated with a standard 4 mm loopful of an 18 hr broth culture diluted 1/500 in nutrient broth (about 5000 organisms) and the lowest concentration of antibiotic which showed no growth after incubating for 18 hr at 37° was recorded as the MIC.

The effect of inoculum size on growth in cephalothin. Dilutions of 18 hr broth cultures of 7 strains of Gram-negative bacteria were made in sets of 8 tubes containing 4·5 ml. nutrient broth and cephalothin, 0·5 ml. (about 5×10^8 organisms) of the broth culture was added to 4·5 ml. of nutrient broth containing cephalothin and serial tenfold dilutions were made from 1/10 to $1/10^8$. The final concentration of cephalothin in each tube was 100 μg./ml. The cultures were incubated at 37° and examined for growth at 24 and 48 hr.

Induction by benzylpenicillin. Two 6 in. × 1 in. test tubes containing 9 ml. glucose broth were each inoculated with 1 ml. of an 18 hr broth culture of the same organism and shaken for 6 hr at 37°. After 2 hr incubation, benzylpenicillin was added to one of the two cultures, in an amount such that with 7 strains the final concentration was 200 μg./ml., with one ampicillin-sensitive strain it was 20 μg./ml. and with one strain of *Pseudomonas aeruginosa* it was 2·0 mg./ml.

Induction by various penicillins and cephalothin. Benzylpenicillin, methicillin, cloxacillin, quinacillin, 6-aminopenicillanic acid and cephalothin were added to final concentration 200 μg./ml. to shaking broth cultures as described in the previous section. Whole broth cultures were used for the cephalosporinase assay.

Preparation of enzyme and assay. After incubation for 6 hr the two broth cultures were centrifuged and the organisms resuspended in phosphate buffer (pH 6·5) to the same turbidity (equiv. about 5×10^9 bacteria/ml.); 5 ml. of each suspension was then treated in an M.S.E. ultrasonic disintegrator for 25 min., the tube containing the suspension was surrounded by ice to prevent any excessive rise in temperature during the sonic treatment. Penicillinase and cephalosporinase activities were estimated in the supernatant fluid, in whole organism suspensions, disintegrated organisms or whole broth cultures by an iodometric method, which assays β-lactamase and not amidase activity. Iodine is reduced by penicilloic acids and by the degradation products of cephalosporinase as soon as the β-lactam bond is ruptured; approximately 8 equivalents of iodine are equivalent to 1 mole penicilloic acid, and 4 equivalents of iodine are equivalent to 1 mole cephalosporin after hydrolysis (Fleming *et al.* 1963). In the assay, samples (0·5–2·0 ml.) of enzyme preparation were added to 5 ml. substrate solution (2·5 mg benzylpenicillin or

cephalothin/ml. in 0·2 M-$KH_2PO_4 + Na_2PO_4$ buffer (pH 6·5) containing 50 μg. chloramphenicol/ml. to inhibit the growth of organisms in the assay where this might occur). The destruction of benzylpenicillin or cephalothin was then determined in these samples by titration against standard iodine with appropriate controls (see Perret, 1954). The tests were repeated with different broth cultures of each strain.

RESULTS

The amount of penicillinase and cephalosporinase activity in the whole organism suspensions, disintegrated organisms and supernatant fluids of cultures of several Gram-negative species grown in the presence or absence of benzylpenicillin, together with the minimum inhibitory concentrations of ampicillin and cephalothin for these organisms, are shown in Table 1. The strains examined may be divided into 5 main groups according to their response to inducer.

The strains of *Proteus morganii*, *P. rettgeri*, *P. vulgaris*, *Enterobacter aerogenes* and *Pseudomonas aeruginosa* were resistant to ampicillin and to cephalothin and formed inducible cephalosporinase and penicillinase. The cephalosporinase values were higher than the penicillinase values which were especially low in the strains of *P. rettgeri*, *E. aerogenes* and *Ps. aeruginosa*. Strains of *Ps. aeruginosa* required a much higher concentration of benzylpenicillin to demonstrate induction of cephalosporinase and penicillinase activity than was the case with the other organisms tested.

The strain of *Escherichia coli* was resistant to both antibiotics and formed both enzymes, but enzyme activity was not increased by growth in the presence of benzylpenicillin.

The strain of *Klebsiella aerogenes* was resistant to ampicillin but moderately sensitive to cephalothin. Both substrates were hydrolysed, but the cephalosporinase activity was less than the penicillinase activity; neither enzyme was inducible.

The strain of *Proteus mirabilis* was resistant to ampicillin and sensitive to cephalothin. Only penicillinase activity was detected; this was not increased by growth in presence of benzylpenicillin.

The strain of *Klebsiella edwardsii* var. *edwardsii* was sensitive to ampicillin and cephalothin. Enzyme activity was low in both cases and was not induced by benzylpenicillin 20 μg./ml. Higher concentrations of benzylpenicillin inhibited growth in this organism.

Table 1 shows that *Proteus rettgeri* was the only organism which gave an appreciably higher cephalosporinase activity after cell disintegration, whereas the strains of *P. mirabilis*, *P. morganii*, *Klebsiella aerogenes* and *Escherichia coli* all showed higher penicillinase activity after disintegrating the bacteria. Both enzymes were mainly cell-bound, although moderate cephalosporinase activity was obtained in the supernatant fluid of some strains after induction. The penicillinase activity in the supernatant fluid of the strain of *K. aerogenes* was similar to that of the intact organisms, but cephalosporinase activity in the supernatant fluid was very low.

Induction of cephalosporinase activity

Table 2 shows the cephalosporinase activity of two strains of *Proteus morganii* and one each of *P. vulgaris*, *Enterobacter aerogenes* and *Klebsiella aerogenes* after growth in the presence (200 μg./ml.) or absence of various inducers. Growth in the

Table 1. *Penicillinase and cephalosporinase activity of certain Gram-negative bacteria*

Organism	Ampicillin Minimal inhibitory concentrations (μg./ml.)	Cephalothin Minimal inhibitory concentrations (μg./ml.)	Inducing agent benzylpenicillin (μg./ml.)	Penicillinase and cephalosporinase activities (μmole/ml./hr) supernatant fluid Pen.*	supernatant fluid Ceph.*	whole organisms Pen.	whole organisms Ceph.	disintegrated organisms Pen.	disintegrated organisms Ceph.
Proteus morganii	256	> 256	—	0·2	0·4	0·3	1·0	0·3	0·6
			200	0·3	0·6	6·5	12·2	13·3	14·8
P. vulgaris	64	128	—	0·3	0·4	0·4	0·6	0·2	0·4
			200	0·8	4·0	7·6	30·8	7·6	30·0
P. rettgeri	> 256	> 256	—	0·3	0·4	0·3	6·8	0·2	4·2
			200	0·3	8·0	2·1	9·8	1·9	15·8
P. mirabilis	> 256	4	—	0·3	< 0·2	3·0	< 0·2	10·8	< 0·2
			200	0·3	< 0·2	3·4	< 0·2	8·4	< 0·2
Klebsiella aerogenes	> 256	16	—	3·6	0·2	3·9	3·0	13·5	3·0
			200	4·0	< 0·2	4·6	4·0	13·6	4·0
K. edwardsii var. *edwardsii*	2	2	—	< 0·2	< 0·2	0·2	< 0·2	0·2	< 0·2
			20	< 0·2	< 0·2	0·2	< 0·2	0·3	< 0·2
Enterobacter aerogenes	128	128	—	0·3	0·6	0·5	5·8	0·5	5·4
			200	0·2	3·0	1·1	16·0	1·2	18·0
Escherichia coli	64	256	—	0·5	0·8	4·5	7·7	7·4	7·1
			200	0·5	1·3	4·4	7·1	8·8	6·7
Pseudomonas aeruginosa (*pyocyanea*)	> 256	> 256	—	< 0·2	0·4	< 0·2	0·6	< 0·2	0·2
			2000	0·25	2·2	1·9	7·2	1·2	9·6

— = No inducing agent. * Pen. = Penicillinase activity. Ceph. = Cephalosporinase activity.

presence of benzylpenicillin, cephalothin or 6-aminopenicillanic acid led to increased cephalosporinase activity of all strains tested except *K. aerogenes* which was unaffected by any of the compounds at the concentration used. Methicillin caused a similar increase in activity with strain 1 of *P. morganii* and the strain of *P. vulgaris*, but not with the other organisms tested. Cloxacillin increased the activity of the same two organisms but to a lesser degree, and with strain 2 of *P. morganii* and the strain of *E. aerogenes* the activity was apparently decreased after growth in its presence. Quinacillin increased the activity of strain 1 of *P. morganii* but did not affect that of the other organisms.

Table 2. *Induction of cephalosporinase*

Cephalosporinase activity (μmole/ml. of broth culture/hr.) of five strains of Gram-negative bacteria after growth in the presence of six potential inducers.

Inducing agent (200 μg./ml.)	*Proteus morganii* 1	*Proteus morganii* 2	*P. vulgaris*	*Enterobacter aerogenes*	*Klebsiella aerogenes*
Nil	1·6	1·6	1·2	6·8	3·0
Benzylpenicillin	30·2	21·6	21·2	25·2	3·6
Cephalothin	30·4	14·0	20·4	15·6	3·8
Methicillin	27·6	4·8	21·6	4·0	3·4
Quinacillin	28·0	1·6	3·6	7·2	3·8
6-A.P.A.	26·8	19·6	14·8	30·8	4·0
Cloxacillin	12·8	0·8	8·8	0·8	3·6

Table 3. *The effect of inoculum size on growth of certain Gram-negative bacteria in presence of cephalothin*

The smallest dilution of an 18 hr broth culture showing growth in broth containing 100 μg. cephalothin/ml.

Organism	Minimum inhibitory concentration cephalothin* μg./ml.	Inoculum size 24 hr incubation	Inoculum size 48 hr incubation
Proteus morganii	> 256	10^{-8}	10^{-8}
P. vulgaris	128	10^{-8}	10^{-8}
P. rettgeri	> 256	10^{-8}	10^{-8}
P. mirabilis	4	$< 10^{-1}$	$< 10^{-1}$
Enterobacter aerogenes	128	10^{-5}	10^{-7}
Klebsiella aerogenes	16	10^{-2}	10^{-2}
Escherichia coli	256	10^{-8}	10^{-8}

* Inoculum = 1/500 dilution of an 18 hr culture.

The effect of inoculum size on growth in cephalothin

The smallest inoculum of each strain which showed growth in broth containing 100 μg. cephalothin/ml. is shown in Table 3. With three strains of Proteus and the strain of *Escherichia coli* showing a minimum inhibitory concentration of 128 μg./ml. or more when tested with the standard inoculum (1/500 dilution of an 18 hr culture), growth occurred in the presence of 100 μg./ml. within 24 hr with the smallest inoculum used (about 50 organisms). The strain of *Enterobacter aerogenes* showed a similar degree of resistance with the standard inoculum, but the highest dilution of an 18 hr broth culture from which an inoculum gave growth in the presence of 100 μg./ml. was $1/10^5$ after 24 hr or $1/10^7$ after 48 hr.

DISCUSSION

In this investigation, cephalosporinase and penicillinase were considered as separate enzymes but this may not be so and the results only show that Gram-negative bacteria varied in their relative hydrolytic activity against benzylpenicillin and cephalothin. Ampicillin-resistant strains showed a predominant penicillinase activity, whereas cephalothin-resistant strains predominantly hydrolysed cephalothin. Among the strains tested, ampicillin-resistant strains of *Proteus mirabilis* hydrolysed penicillin but not cephalothin, whereas *P. rettgeri* and *Enterobacter aerogenes* hydrolysed cephalothin more readily than penicillin; *P. morganii* readily hydrolysed both penicillin and cephalothin. The Gram-negative bacteria studied are examples of organisms commonly isolated from routine clinical specimens. The sensitivity to ampicillin and cephalothin is typical for most strains of Klebsiella and Enterobacter, for *Pseudomonas aeruginosa* and for Proteus species other than *Proteus mirabilis*. Strains of *P. mirabilis* isolated in this laboratory as elsewhere were usually sensitive to ampicillin and cephalothin. A strain of *Klebsiella edwardsii* var. *edwardsii* was the only strain examined which was sensitive to both antibiotics; examples of other sensitive strains were not included as it was found that their penicillinase and cephalosporinase activities were low or non-existent.

All but one of the cephalothin-resistant organisms grew in broth containing 100 μg. cephalothin/ml. when tested with a small inoculum suggesting a high intrinsic resistance; the exception was the strain of *Enterobacter aerogenes*. Small inocula of this organism did not grow in 100 μg. cephalothin/ml., whereas large inocula did. This type of response to cephalothin is similar to that of some penicillinase-producing strains of Proteus and Aerobacter which had little intrinsic resistance to ampicillin but which were resistant by virtue of their penicillinase formation when tested with a large inoculum (Sutherland, 1964). It should be emphasized that naturally occurring cephalothin-resistant strains differ in their cephalosporinase activity from strains made resistant *in vitro* by subculture on plates containing cephaloridine. The habituated strains although equally resistant to cephalothin showed no greater cephalosporinase activity than did sensitive strains (Barber & Waterworth, 1964).

Until recently the penicillinases of Gram-negative bacteria have not been regarded as inducible enzymes, but it is now clear that there are exceptions to this. Induction of penicillinase activity has been demonstrated in a strain of *Proteus morganii* (Hamilton-Miller, 1964), that of cephalosporinase activity in a strain of *Pseudomonas aeruginosa* (Jago, Migliacci & Abraham, 1963) and that of both in Proteus species (Ayliffe, 1964). The present investigation shows that penicillinase and cephalosporinase activity may also be induced in other Gram-negative species, but with some strains induction of these enzymes was not obtained even with high concentrations of potential inducers. Different antibiotics differ in their effectiveness as inducers of cephalosporinase activity. Benzylpenicillin, cephalothin and 6-aminopenicillanic acid were effective inducers, but the other antibiotics showed variable effects. Methicillin and cloxacillin are good inducers of staphylococcal penicillinase (Smith, Hamilton-Miller & Knox, 1962). In the present investigation methicillin and to a lesser degree cloxacillin were found to be effective inducers of the cephalosporinase activity of 1 of 2 strains of *P. morganii* (strain 1) and a strain of *P. vulgaris*

but not that of three other organisms tested, including a second strain of *P. morganii*. Quinacillin is a poor inducer of staphylococcal penicillinase (Richards, Housley & Spooner, 1963); in the present study it was found to induce the cephalosporinase activity of strain 1 of *P. morganii*, but not that of the four other organisms tested. The difference obtained with methicillin, quinacillin and cloxacillin in the 2 strains of *P. morganii* indicates that the effectiveness of these penicillins cannot be predicted even with the same species. Methicillin and cloxacillin have been found to inhibit penicillinase in several Gram-negative species (Hamilton-Miller & Smith, 1964; Sutherland & Batchelor, 1964) and cephalosporinase in *Pseudomonas aeruginosa* (Jago *et al.* 1963). In the experiments reported in this paper, partial inhibition of cephalosporinase activity occurred when one of the strains of *P. morganii* and the strain of *Enterobacter aerogenes* were grown in the presence of cloxacillin.

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The Biosynthesis of Carbamoyl Phosphate in *Saccharomyces cerevisiae*

By F. LACROUTE

Laboratoire de Génétique Physiologique de C.N.R.S., Gif-sur-Yvette, Seine et Oise, France

and A. PIÉRARD, MARCELLE GRENSON and J. M. WIAME

Laboratoire de Microbiologie, Université de Bruxelles and Institut de Recherches du C.E.R.I.A., Bruxelles 7, Belgium

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SUMMARY

Mutants of *Saccharomyces cerevisiae* impaired in the biosynthesis of carbamoyl phosphate were obtained. Genetical, physiological and enzymic studies of these mutants showed the existence in this organism of two independent enzymic systems which catalysed the synthesis of carbamoyl phosphate from HCO_3^-, glutamine, ATP and Mg^{2+}. One system provides carbamoyl phosphate for the arginine pathway, the other plays a similar role for the pyrimidine pathway. Carbamoyl phosphate from one pathway is freely available for the other. The mutations have been mapped in three unlinked loci. Two loci determine the arginine-specific carbamoyl phosphate synthesizing system. The third locus corresponds to the pyrimidine-specific system. Mutations in either of the two genes concerned with the arginine pathway lead to a deficiency in the activity of that pathway to synthesize carbamoyl phosphate. Crossing a mutant deficient in one of the two arginine loci with a mutant deficient at the other produces a diploid in which complementation occurs. Also, *in vitro* activity may be partially regained by combining the cell-free extracts of the two single mutants. The physiological significance of the two enzymic systems is established by the study of their regulation. The carbamoyl phosphate synthesizing activity of the arginine pathway was repressed to 10% of its value in minimal medium by addition of excess of arginine to the growth medium. The activity corresponding to the pyrimidine pathway was only slightly repressed by uracil but was subject to feed-back inhibition by uridine-5′-triphosphate. These results are compared with the data available for other micro-organisms.

INTRODUCTION

Interest in the mechanism of the biosynthesis of carbamoyl phosphate is justified by its key role as a common precursor of two important biosynthetic pathways: that of arginine and that of the pyrimidines. For this reason, a complex regulation of the biosynthesis of carbamoyl phosphate may be expected. The nature of the reactions responsible for this biosynthesis is not well known in micro-organisms.

The reaction catalysed by carbamoyl phosphokinase (ATP: carbamate phosphotransferase; E.C. 2. 7. 2. 2),

$$NH_2COO^- + ATP \overset{Mg^{2+}}{\rightleftharpoons} NH_2\text{–}CO_2PO_3^{2-} + ADP,$$

was the first to be proposed as the route of formation of carbamoyl phosphate in micro-organisms (Jones, Spector & Lipmann, 1955; Gorini & Kalman, 1963; Davis, 1963). Others see in Levenberg's glutamine-dependent reaction (Levenberg, 1962):

$$\text{glutamine} + \text{ATP} + HCO_3^- \xrightarrow{Mg^{2+}} NH_2\text{–}CO_2PO_3^{2-} + \text{ADP} + \text{glutamate},$$

the functional mechanism of that biosynthesis (Piérard & Wiame, 1964).

In *Escherichia coli* one-step mutants simultaneously auxotrophic for arginine and uracil have been obtained (Roepke, 1946; Davis, B. D., 1962). Other mutants, which require arginine when uracil is present in the growth medium, are known (Novick & Maas, 1961; Gorini & Kalman, 1963). All of these mutants are located within the same small genetic region (Piérard, Glansdorff & Mergeay, in preparation; Beckwith, Pardee, Austrian & Jacob, 1962). Thus, it would appear that a single enzymic system synthesizes carbamoyl phosphate for both pathways in this organism. A different situation exists in Neurospora. Indirect but extensive evidence from genetic studies led to the opinion that there were two independent enzymic syntheses of carbamoyl phosphate in Neurospora, one specific for the arginine, the other for the pyrimidine pathway (Davis, R. H., 1962; Reissig, 1960, 1963; Charles, 1962). Carbamoyl phosphate formed by one reaction is not freely available for the other pathway (Davis & Woodward, 1962; Davis, 1963). This channelling leads the mutants to be auxotrophic either for arginine or for pyrimidine. Supporting this view Davis (1963) has identified the reaction specific for the arginine pathway as mediated by a carbamoyl phosphokinase which is repressible by arginine and which is not present in the *arg-3* mutant. This enzyme affected by the mutation concerning the pyrimidine-specific pathway, has not yet been identified.

The data obtained in the present work show that in *Saccharomyces cerevisiae* the enzymic reaction leading to carbamoyl phosphate uses glutamine as the nitrogen donor (Levenberg, 1962). Direct enzymic evidence will be presented for the existence of two different enzymes. Mutations which affect each one separately have been found, thus allowing the study of them independently. One enzyme is related to the pyrimidine pathway, since it is strongly retro-inhibited by uridine-5′-triphosphate (UTP); the other, while unaffected by UTP, is strongly repressed by arginine. Either enzymic system alone permits growth, showing that there is no channelling of carbamoyl phosphate in *S. cerevisiae*. As expected, strains unable to conduct both reactions required to be given arginine + uracil for growth. A preliminary report of this work has appeared (Lacroute, Piérard, Grenson & Wiame, 1964).

METHODS

Organisms. The yeast strains used are haploid clones of *Saccharomyces cerevisiae* which are heterothallic. Mutants *cpu* and cpa_2 were obtained by using nitrous acid as mutagen, mutants cpa_{1-1} and cpa_{1-2} by using X-rays. (Mutant *cpa* was named E_1^- and mutant *cpu*, E_2^- in our preliminary note; Lacroute *et al.* 1964; Lacroute, 1964). Mutant DE_{14} (ar_3) was kindly provided by Dr R. K. Mortimer and identified by us as blocked in ornithine carbamoyltransferase (E.C. 2. 1. 3. 3).

Genetical methods. Diploids were obtained and isolated, and tetrad analysis was done as described by Hawthorne & Mortimer (1960).

Media used and conditions of growth. The basal medium for growth was a defined basal medium (no. 140) containing (per l. medium): $MgSO_4.7H_2O$, 0·7 g.; KH_2PO_4, 1 g.; $CaCl_2$, 0·4 g.; NaCl, 0·5 g.; $(NH_4)_2SO_4$, 1·2 g.; citric acid, 10·5 g.; 10 M-KOH, 16 ml.; trace metals solution, 1 ml. One l. of trace metals solution contained: H_3BO_3, 500 mg.; $CaSO_4.5H_2O$, 40 mg.; KI, 100 mg.; $FeCl_3.6H_2O$, 5 g.; $MnSO_44H_2O$, 400 mg.; $Na_2\ MoO_4.2H_2O$, 200 mg.; $ZnSO_4.7H_2O$, 720 mg.; citric acid, 10 g.

The medium was adjusted to pH 6·15 before sterilization. After autoclaving (121°, 20 min.), glucose (to final concentration 3 g./100 ml.) and a solution of vitamins (final dilution 1/100) were added to the medium. The vitamin solution was composed as follows (per l.): DL-calcium panthotenate, 200 mg.; biotin, 250 μg.; nicotinic acid, 100 mg.; thiamine, 100 mg.; riboflavin, 100 mg.; inositol, 500 μg.; *p*-aminobenzoic acid, 50 mg.; pyridoxine, 100 mg.; folic acid (sterilized separately by filtration), 400 μg.

The cultures were grown aerobically on a rotary shaker at 30°.

Growth curves. Each organism was grown on the four following media: minimal medium (M); minimal medium + uracil (100 μg./ml.); minimal + L-arginine 100 μg./ml.; minimal + uracil + arginine. The growth was followed by measuring the extinction in a Beckman model C colorimeter with an interference filter (765 mμ). Two different extinction values were used at inoculation: 0·100 and 0·001 (calculated) to obtain a sufficient number of generations in the exponential phase of growth.

Preparation of organisms for enzyme assays. Minimal medium was supplemented when started with 200 μg. L-arginine, uracil or both together/ml. Organisms were harvested during the exponential phase (extinction 0·8–1·6, with a Hilger-Spekker colorimeter, equivalent to 0·4–0·8 mg. dry wt. organism/ml.).

Double mutants FL 80-2C (*cpa*$_2$, *cpu*) and Σ 1320c (*cpa*$_1$, *cpu*) were grown on limiting concentrations of arginine (15 μg./ml.) and uracil (7 μg./ml.). The organisms were collected one doubling-time after the end of the exponential phase of growth, to allow for de-repression. The organisms were collected by centrifugation, washed with distilled water and kept frozen until the preparation of cell-free extracts.

Cell-free extracts. Organisms harvested from 1·5 l. culture, were resuspended in 8 ml. 0·05 M phosphate buffer (pH 7·5) and broken in a Nossal cell disintegrator (MacDonald Engineering Co., 1725 Fall Avenue, Cleveland 13, Ohio, U.S.A.) with 8 g. glass beads; the breakage period was for 30 sec. twice with cooling with a stream of CO_2 (Somlo, 1962). After centrifugation at 20,000 *g* for 30 min. in a Servall SS-1 centrifuge, the supernatant fluid obtained (referred to as the crude extract), was passed once and in some experiments twice through a column of Sephadex G 25 (pre-treated with 0·05 M-phosphate, pH 7·5). Enzymic activities were determined shortly after the preparation of the cell-free extracts to avoid loss of the rather unstable carbamoyl phosphate synthesizing activity.

Assay of enzymes. The activities of carbamoyl phosphokinase and of glutamine-dependent carbamoyl phosphate synthetase were determined by converting carbamoyl phosphate formed from radioactively labelled bicarbonate to acid-stable citrulline according to a modification of the method developed by Levenberg (1962) for the mushroom system.

The assay system for carbamoyl phosphokinase activity contained (in total

volume 1 ml.): ATP, 20 μmole; $MgCl_2$, 25 μmole; ornithine, 10 μmole; tris buffer (pH 8·5), 200 μmole; NH_4Cl, 50 μmole; ^{14}C-Na_2CO_4 (specific activity from 2 to 4×10^4 c.p.m./μmole), 50 μmole; excess of ornithine carbamoyltransferase partially purified from *Escherichia coli*; crude extract. The reaction was started shortly after the addition of ^{14}C-Na_2CO_4 by introducing the extract. Incubation was for 30 min. at 30°. The reaction was stopped by adding 1 ml. of 2N-HCl, followed by boiling for 10 min. After removal by centrifugation of the precipitated proteins, samples of the reaction mixture were plated on copper planchets. The samples were evaporated to dryness and the radioactivity in the acid-stable samples counted in a (Nuclear Chicago) low background gas-flow counter.

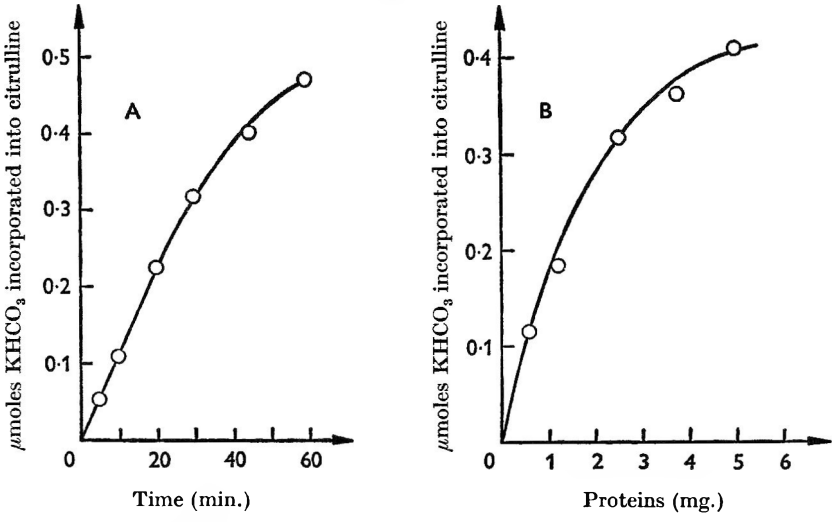

Fig. 1. Glutamine-dependent carbamoyl phosphate synthetase activity. Dependence of incorporation of $KHCO_3$ in citrulline on time (A) and amount of Sephadex-treated extract (B) of *Saccharomyces cerevisiae* mutant FL 82-25C (*cpu*). In A, the amount of protein is 2·52 mg. In B, incubation time 30 min.

The constituents of the reaction mixture for the assay of the glutamine-dependent carbamoyl phosphate synthetase were as follows: in 1 ml. total volume: phosphate buffer (pH 7·5), 85 μmole; ATP, 12 μmole; $MgCl_2$, 12 μmole; ornithine, 6 μmole; glutamine, 6 μmole; ^{14}C-$KHCO_3$ (specific activity from 4 to 8×10^4 c.p.m./μmole), 30 μmole; excess of ornithine carbamoyltransferase partially purified from *Escherichia coli*; cell-free extract. Incubation and counting procedures were as described for the carbamoyl phosphokinase activity. Under these conditions, a reasonable linearity of citrulline production with time and amount of extract was obtained (Fig. 1). All the values given have been corrected for controls where the reaction was stopped at zero time. Under the conditions used, no corrections for self-absorption were necessary. Specific activities are expressed as mμmoles radioactive bicarbonate incorporated into citrulline/hr/mg. protein.

Identification of ^{14}C-labelled products. The identity of the ^{14}C-labelled products obtained by coupling carbamoyl phosphate synthesis with ornithine carbamoyltransferase or with aspartate carbamoyltransferase, was confirmed by the use of high voltage (5 kV.) electrophoresis in acetate buffer (pH 3) and by paper chromato-

graphy with 2-butanol + 88 % formic acid in water + water (75 + 15 + 10, by vol.) as solvent (Bishop & Campbell, 1963). The paper strips were passed through a Nuclear Chicago chromatogram scanner. Comparison with samples of authentic ^{14}C-ureido compounds confirmed that the radioactivity from ^{14}C-bicarbonate was incorporated into citrulline or ureidosuccinic acid. A third radioactive peak corresponded to a non-identical degradation product of these compounds; this through a non-enzymic reaction favoured by ATP. Nevertheless, the results were not quantitatively affected because this degradation product was acid-stable.

RESULTS

Genetic analysis and physiological properties of the mutants

To study the biosynthesis of carbamoyl phosphate *Saccharomyces cerevisiae*, we tried to isolate mutants which required simultaneously uracil and arginine for growth. We obtained such a strain (FL 80-2C) but genetic analysis showed that it had undergone two mutations. The physiological consequences of these mutations and the phenotypes of all the mutants obtained from it are described in Table 1. The independence of the two mutations is shown in Table 2 (cross no. 1). One of these

Table 1. *Growth behaviour of various mutants of Saccharomyces cerevisiae deficient in the biosynthesis of carbamoyl phosphate*

S. cerevisiae		Culture medium			
		Minimal	Minimal + arginine	Minimal + uracil	Minimal + arginine + uracil
		Growth			
strain	Genotype				
FL 100	Wild	+	+	+	+
Σ 1330a	cpa_1	+	+	−	+
FL 80-2A	cpa_2	+	+	−	+
FL 82-25C	*cpu*	+	±	+	+
Σ 1320c	cpa_1, *cpu*	−	−	−	+
FL 80-2C	cpa_2, *cpu*	−	−	−	+
Σ 1461b	cpa_1, *cpa*	+	+	−	+

mutations (mutant cpa_2) led in the presence of uracil to a requirement for arginine, while the phenotype was of wild-type in the absence of added uracil. The growth rate of *S. cerevisiae* strains which carried the second mutation (*cpu*) was slightly lower in the presence of arginine and this partial inhibition was annulled by uracil. Once isolated each of the mutations showed a regular 2:2 segregation when crossed with a wild-type strain. When both mutations were present in the same strain, an ur^- ar^- phenotype was obtained. These properties are seen in Fig. 2, which shows the growth curve of the four types of strains. Figure 2D shows the rapid inhibition of growth of a mutant cpa_2 after adding uracil to a minimal medium (upper curve). In the same experiment, starting from a lower extinction value (0·001, calculated) no detectable growth occurred in minimal medium + uracil. Figure 2C shows that, in contrast with the preceding case, the effect of arginine on a *cpu* mutant was much more delayed and less important.

Two other mutants with the same phenotype as mutant cpa_2 (requirement for

Table 2. *Genetical analysis of mutants of Saccharomyces cerevisiae*

PD = parental ditype; NPD = non-parental ditype; T = tetratype.

Cross		Gene pair	Types of tetrads PD	NPD	T	Composition of a tetratype tetrad as an example*: Phenotype-growth on minimal medium supplemented with: *ar*† / *ur*‡	– / –	– / *ur*	*ar* / –	Genotype
(1) FL 18-1a (+ + +)	× FL 80-2c (+ *cpa*$_2$ *cpa*)	*cpa*$_2$–*cpu*	5	5	12	+	+	+	+	+ +
						+	+	+	+	+ *cpu*
						+	+	–	+	*cpa*$_2$ +
						+	–	–	–	*cpa*$_2$ *cpu*
(2) FL 80-2A (+ *cpa*$_2$ +)	× Σ 1330a (*cpa*$_{1-2}$ + +)	*cpa*$_{1-2}$–*cpa*$_2$	0	3	9	+	+	+	+	+ +
						+	+	–	+	*cpa*$_1$ +
						+	+	–	+	+ *cpa*$_2$
						+	+	–	+	*cpa*$_1$ *cpa*$_2$
(3) D 46-4 (*cpa*$_{1-1}$ + +)	+ FL 80-2A (+ *cpa*$_2$ +)	*cpa*$_{1-1}$–*cpa*$_2$	0	1	5	id. (2)				
(4) FL 82-18B (+ + *cpu*)	× D 46-4 (*cpa*$_{1-1}$ + +)	*cpa*$_{1-1}$–*cpu*	3	1	8	+	–	–	–	*cpu* *cpa*$_1$
						+	+	+	+	+ +
						+	+	–	+	+ *cpa*$_1$
						+	+	+	+	*cpu* +
(5) FL 38-11 B (+ *cpa*$_2$ *ur*$_2$)	× FL 80-2C (+ *cpa*$_2$ *cpu*)	*cpu*–*ur*$_2$	28	0	0	+	–	–	–	*cpa*$_2$ *cpu*
						+	–	–	–	*cpa*$_2$ *cpu*
						+	–	–	–	*cpa*$_2$ *ur*$_2$
						+	–	–	–	*cpa*$_2$ *ur*$_2$
(6) DE 14 (*ar*$_3$)	× FL 80-2A (+ *cpa*$_2$ +)	*ar*$_3$–*cpa*$_2$	2	1	3	+	–	–	+	*ar*$_3$ *cpa*$_2$
						+	–	–	+	*ar*$_3$ +
						+	+	–	+	+ *cpa*$_2$
						+	+	+	+	+ +
(7) DE 14 (*ar*$_3$)	× Σ 1328d (*cpa*$_{1-2}$ + +)	*ar*$_3$–*cpa*$_1$	0	2	4	id. (6)				

* Except for (5) where only PD are found. † *ar* = arginine; ‡ *ur* = uracil.

arginine in presence of uracil) were isolated independently. Each of them contained a single mutation showing the normal 2:2 segregation. The two mutations were allelic, as shown by the absence of complementation and by the mutant phenotype of the haploid descendants from a cross between them; we call these mutants cpa_{1-1} and cpa_{1-2}. A strain of *Saccharomyces cerevisiae* with a similar inhibition of growth by uracil, which was annulled specifically by arginine was described by Miller & Harrison (1950).

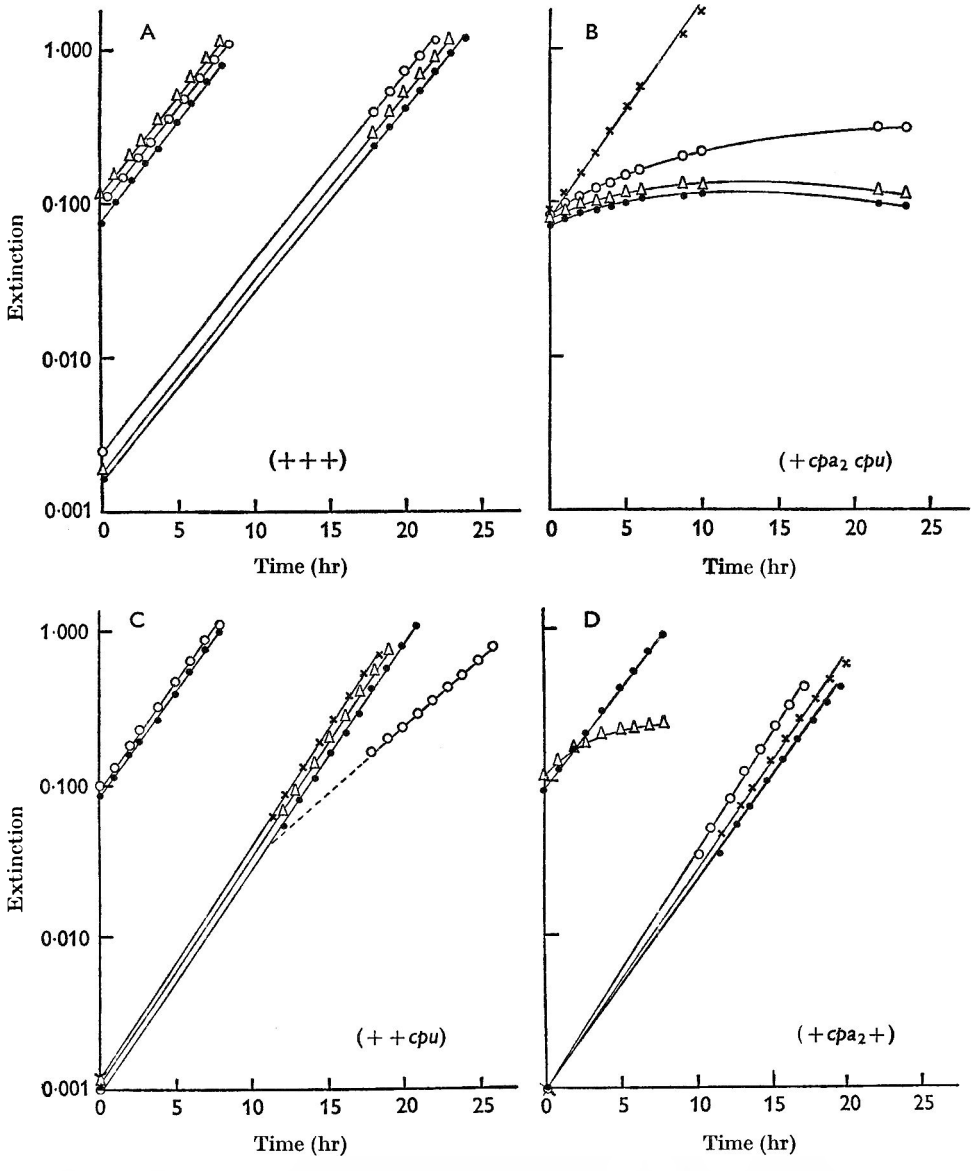

Fig. 2. Growth behaviour of the mutants. Two sets of curves are given for each strain, one starting from extinction 0·100 and one from extinction 0·001 (calculated). ●, Minimal medium (M); △, M+uracil (100 μg./ml.); ○, M+L-arginine (100 μg./ml.); ×, M+L-arginine+uracil.

Strains of *Saccharomyces cerevisiae* carrying the mutation cpa_{1-1} and cpa_{1-2} were crossed with cpa_2 mutants to determine whether or not these mutations affected the same locus. Complementation tests and tetrad analysis showed that mutants cpa_1 and cpa_2 affected two different genes which segregated independently (Table 2, nos. 2 and 3). The simultaneous presence of cpa_{1-1} or cpa_{1-2} and *cpu* in a strain gave the phenotype $ur^- ar^-$, as in the cases of mutants cpa_2 and *cpu* (Table 2, no. 4). Finally, *cpu* was shown to be allelic or very closely linked to ur_2 (Table 2, no. 5), the structural gene of aspartate carbamoyltransferase (E.C. 2. 13. 2; Lacroute, 1964), whereas neither cpa_1 nor cpa_2 were genetically linked to ar_3, the structural gene of ornithine carbamoyltransferase (Table 2, nos. 6, 7).

Based on the genetic analysis and the physiological properties of the mutants obtained, we formulate the hypothesis that two independent carbamoyl phosphate synthesizing systems exist in *Saccharomyces cerevisiae*, one specific for the arginine pathway, the other for the pyrimidine pathway, genes cpa_1 and cpa_2 corresponding to the arginine system and gene *cpu* to the pyrimidine system. In the following sections, we give further evidence in support of this hypothesis.

Nature of the reaction responsible for the biosynthesis of carbamoyl phosphate

In the study of the mechanism of the biosynthesis of carbamoyl phosphate in *Saccharomyces cerevisiae* we considered two possible reactions, one catalyzed by carbamoyl phosphokinase, the other by the glutamine-dependent carbamoyl phosphate synthetase. These carbamoyl phosphate synthesizing activities were

Table 3. *Specific activities of carbamoyl phosphokinase and of glutamine-dependent carbamoyl phosphate synthetase of various mutants of Saccharomyces cerevisiae*

Conditions are as described in 'Methods'. For carbamoyl phosphokinase activity NH_4^+ was at 5×10^{-2} M, glutamine at 6×10^{-3} M. For the glutamine-dependent carbamoyl phosphate synthetase, both NH_4^+ and glutamine were at 6×10^{-3} M.

		Carbamoyl phosphokinase activity		Glutamine-dependent carbamoyl phosphate synthetase activity	
Mutant	Amino group donor	Crude extract	Sephadex-treated extract	Crude extract	Sephadex-treated extract
FL 100	None	19	1	99	2
(wild)	NH_4^+	40	12	78	5
	Glutamine	32	33	100	78
FL 80-2A	None	3	1	29	1
(cpa_2)	NH_4^+	4	0	26	1
	Glutamine	3	1	36	15
FL 82-25C	None	17	1	27	2
(*cpu*)	NH_4^+	49	13	58	5
	Glutamine	46	55	85	100
FL 80-2C	None	3	2	9	3
(cpa_2, *cpu*)	NH_4^+	4	2	8	4
	Glutamine	3	2	9	3
Σ 1320C	None	—	0	—	3
(cpa_1, *cpu*)	NH_4^+	—	0	—	3
	Glutamine	—	0	—	3

determined for the various mutants described in the preceding section. For each strain, the activities for the crude and for the Sephadex-treated extracts were estimated; the results are shown in Table 3.

Crude extracts of the wild-type strain (FL 100) of *Saccharomyces cerevisiae* exhibited considerable activity for both reactions. However, these activities were little dependent on the presence of added ammonium ion or glutamine. After Sephadex treatment, the carbamoyl phosphokinase activity was only partially

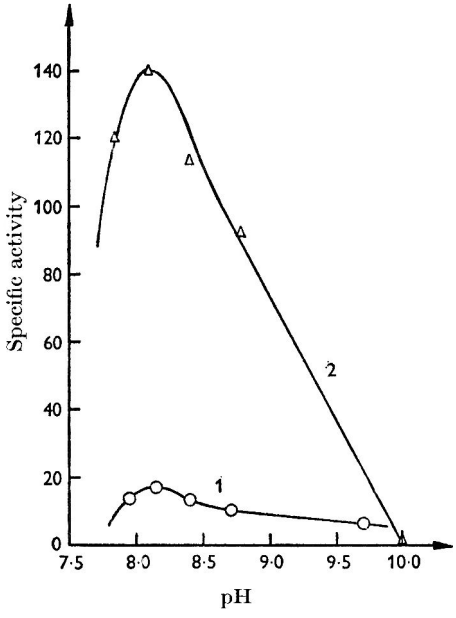

Fig. 3

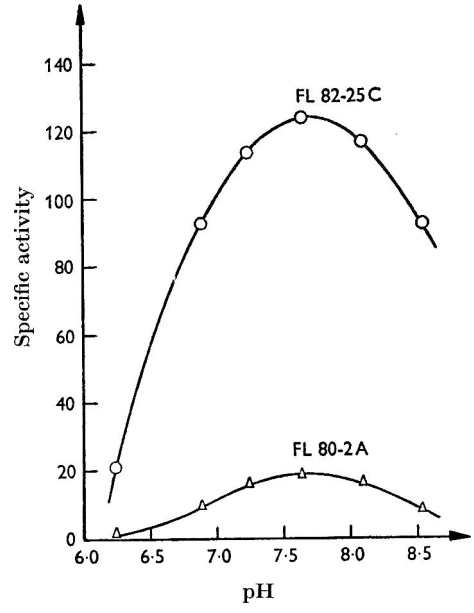

Fig. 4

Fig. 3. Variation with pH value of activities of carbamoyl phosphate synthesis with NH_4^+ or glutamine. Conditions described in Methods for estimation of carbamoyl phosphokinase activity (curve 1). In curve 2, NH_4^+ replaced by 6×10^{-3}M-glutamine. The extract was treated twice with Sephadex G 25, pH value measured at the beginning of incubation.

Fig. 4. Variations with pH value of the activity of glutamine-dependent carbamoyl phosphate synthesis of *S. cerevisiae* mutants FL 82-25C (*cpu*) and FL 80-2A (cpa_2). Conditions as described in Methods. Extract treated with Sephadex G 25; pH value measured at the beginning of incubation.

recovered in presence of NH_4^+ (5×10^{-2}M), but was fully restored when NH_4^+ was replaced by 6×10^{-3}M-glutamine. Under the assay conditions of the glutamine-dependent carbamoyl phosphate synthetase activity, glutamine (6×10^{-3}M) was not efficiently replaced by an equivalent concentration of ammonium ion. A similar situation was observed with mutants FL 80-2A (cpa_2) and FL 82-25C (*cpu*). An important difference in the amount of carbamoyl phosphate synthetase of these two mutants should be noted (Table 3). Finally, the double mutants, FL 80-2C (cpa_2, *cpu*) and Σ 1320c (cpa_1, *cpu*) were almost totally deficient in both activities (Table 3).

Most of the carbamoyl phosphokinase activity seems due to the presence of glutamine in the crude extract which is eliminated by the Sephadex treatment.

Nevertheless, the Sephadex-treated extract showed a weak activity with NH_4^+, activity better explained by assuming a small affinity of the synthetase for this ion. This is well illustrated in Fig. 3, where the variations of the activities with pH value were recorded with glutamine or NH^+_4 as the nitrogen donor. The activity with NH_4^+ varied in parallel with the activity with glutamine. On the basis of the data presented here, it seems reasonable to conclude that in *S. cerevisiae*, the activity ascribed to carbamoyl phosphokinase corresponds to a weak activity with NH_4^+ of glutamine-dependent carbamoyl phosphate synthetase.

Table 4. *Requirements for substrates and co-factors of the reactions catalysed by glutamine-dependent carbamoyl phosphate synthesizing activity of Saccharomyces cerevisiae mutants FL 80-2A and FL 82-25C.*

The experimental procedure is described under Methods. Extracts treated twice on Sephadex G 25 column were used.

Reaction mixture	Mutant FL 82-25C (*cpu*)	Mutant FL 80-2A (cpa_2)
	Specific activities	
Complete	134	11
without glutamine	2	2
without ATP	0	0
without Mg^{2+}	1	0
without ornithine	10	5

Properties of the glutamine-dependent carbamoyl phosphate synthetase activities

To study independently the carbamoyl phosphate synthesizing activities related to the arginine and the pyrimidine pathways, we used mutants FL 82-25C (*cpu*) and FL 80-2A (cpa_2). The substrates and cofactors requirements of both activities were determined (Table 4). Incorporation of radioactivity from ^{14}C-bicarbonate into citrulline by a cell-free extract of mutant FL 82-25C (*cpu*) was dependent on glutamine, ATP, Mg^{2+} and ornithine; ornithine was required for the conversion of carbamoyl phosphate to citrulline through ornithine carbamoyltransferase. The activity of mutant FL 80-2A (cpa_2), although weaker than that of FL 82-25C (*cpu*), exhibited the same requirements. Acetylglutamate, an activator of the mammalian carbamoyl phosphate synthetase (Cohen, 1962) did not enhance the activity of the yeast carbamoyl phosphate synthetases. The weak activity of mutant FL 80-2A led us to consider that this activity might differ in some way from that of mutant FL 82-25C. We therefore tested asparagine as an amino group donor in the reaction. However, asparagine behaved as a poor amino donor for carbamoyl phosphate synthesis with both mutants. As shown in Fig. 4, the activities of mutants FL 82-25C (*cpu*) and FL 80-2A were not differentiated by their pH optima. The acid-stable product of both activities was citrulline in presence of ornithine, and ornithine carbamoyltransferase or ureidosuccinate in the presence of aspartate and aspartate carbamoyltransferase. The identification of these products is described above.

Regulation of the two carbamoyl phosphate synthetases

To obtain evidence of the physiological meaning of the two glutamine-dependent carbamoyl phosphate synthetases, we studied the effect of arginine and uracil in the growth medium on these activities; the results are given in Table 5. The activity of the single mutant FL 82-25C (*cpu*), which retained only the carbamoyl phosphate synthetase related to the arginine pathway, was strongly repressed by arginine in

Table 5. *Influence of the addition of arginine and uracil to the growth medium on the degree of glutamine-dependent carbamoyl phosphate biosynthesis by mutants of Saccharomyces cerevisiae*

Arginine and uracil in the growth medium were at 200 μg./l.

	S. cerevisiae		
	FL 100 (wild type)	FL 82-25C (*cpu*)	FL 80-2A (cpa_2)
Growth medium	Specific activity		
Minimal	141	146	20
Minimal + arginine	20	11	19
Minimal + uracil	140	—*	—
Minimal + arginine + uracil	9	—	8

* —, Not measured.

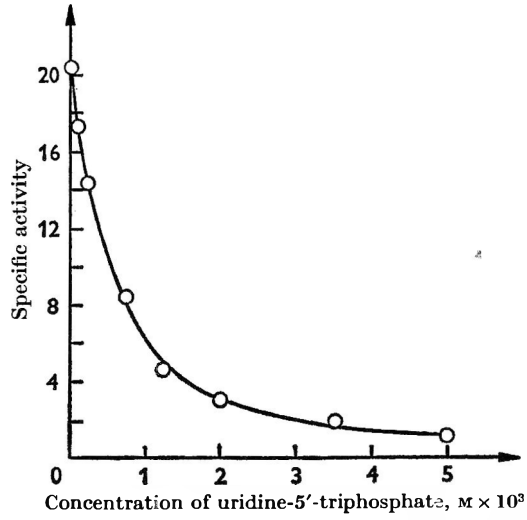

Fig. 5. Inhibition of UTP of the glutamine-dependent carbamoyl phosphate synthetase of mutant FL 80-2A (cpa_2). Experimental procedure as described in Methods; the extract used was treated twice with Sephadex G 25.

the growth medium. On genetic grounds, we supposed that the mutant FL 80-2A (*cpa*) possessed only the activity specific for the pyrimidine pathway. Indeed, no repression of this activity by arginine was noted, but a limited repression by uracil was observed (Table 5). The activities of the wild-type strain can be explained by

combination of the values given for the single mutants (within experimental error).

The repression by arginine of the carbamoyl phosphate synthetase specific for its own pathway allows an explanation of the behaviour of the single mutant FL 82-25 C (*cpu*): due to repression by arginine, the activity of carbamoyl phosphate synthesis becomes too low to support a normal growth rate. The action of arginine appears only several generations after the addition of arginine to the medium, in other words, when the repressed value has been reached. This delayed action of arginine is consistent with the large difference in activity between the carbamoyl phosphate synthesizing systems specific to the two pathways.

An additional feature of the regulation of these activities was obtained from the observation of a feed-back inhibition by uridine-5'-triphosphate (UTP), and to a lesser degree by uridine-5'-monophosphate (UMP), of the carbamoyl phosphate biosynthesis linked to the pyrimidine pathway. UTP completely inhibited the activity at 5×10^{-3}M (Fig. 5); arginine or citrulline did not exert a similar action on the activity of the arginine pathway. This latter activity was not affected by UTP. The feed-back inhibition by UTP explains the inhibition by uracil of the growth of mutants blocked in the arginine-specific carbamoyl phosphate synthesizing system. When uracil was present in the medium, such mutants were unable to satisfy their requirements for carbamoyl phosphate needed for biosynthesis of arginine.

Table 6. *In vivo and in vitro complementation of Saccharomyces cerevisiae mutants cpa_1 and cpa_2 observed in cell-free extracts*

Experimental procedure as described under 'Methods'. The cell-free extracts were treated twice on Sephadex G 25.

S. cerevisiae mutant strain	Specific activity of glutamine-dependent carbamoyl phosphate synthetase
FL 80-2C (*cpa_2*, *cpu*)	2
Σ 1320c (*cpa_1*, *cpu*)	1
FL 80-2C + Σ 1320c*	17
Diploid mutant FL 80-2C × Σ 1320c (*cpa_2 cpu × cpa_1 cpu*)	28

* The two extracts were mixed just before starting the incubation.

Complementation between mutants cpa_1 and cpa_2

We observed that the two non-allelic mutations cpa_1 and cpa_2 both lead to a block in the arginine-specific carbamoyl phosphate synthesizing system. However, these mutants were able to complement in the diploid strain which resulted from their cross. Cell-free extracts of the diploid strain obtained by crossing double mutants (*cpa_1*, *cpu*) and (*cpa_2*, *cpu*) showed an appreciable carbamoyl phosphate synthetase activity (Table 6). A significant activity was recovered by mixing the cell-free extracts of the same double mutants, which by themselves were devoided of such an activity (Table 6).

DISCUSSION

Interest has been devoted recently to the study of the synthesis of carbamoyl phosphate in micro-organisms. These studies have been focused on the nature of the enzymic reaction and on the problems raised by the double function of carbamoyl phosphate biosynthesis.

The nature of the enzymic reaction responsible for the biosynthesis of carbamoyl phosphate is interesting to consider as against the reaction responsible for its breakdown. The latter function occurs in some species of Lactobacteriaceae and Pseudomonas. Carbamoyl phosphokinase, present in Lactobacteriaceae is inducible by arginine; the equilibrium is thermodynamically in favour of the breakdown of carbamoyl phosphate (Jones *et al.* 1955). The enzyme is responsible for the degradation of this compound (Jones, 1963; Thorne & Jones, 1963). This enzyme catalyses the synthesis of carbamoyl phosphate *in vitro*, but its physiological meaning for biosynthesis is questionable. The recent finding by Grisolia *et al.* that acetylphosphokinase (ATP: acetate phosphotransferase; E.C. 2. 7. 2. 1) can catalyse a reaction of the carbamoyl phosphokinase type, stresses the difficulty which may arise in identifying *in vitro* results with *in vivo* mechanisms (Grisolia & Harmon, 1962; Grisolia, Amelunxen & Raijman, 1963). The identification of an enzymic reaction with a physiological function is best established by the cumulative evidence from enzymic, genetical and regulation studies. In this connexion, the enzymic system which utilizes glutamine as nitrogen donor for the synthesis of carbamoyl phosphate in *Escherichia coli*, similar to the Agaricus system (Levenberg, 1962), satisfies all the above criteria (Piérard & Wiame, 1964). Acetylphosphokinase has not been detected in yeast (Ochoa & Stern, 1952). This permits the study of carbamoyl phosphate synthesis in Saccharomyces, without the interferences arising from the presence of that enzyme. The present work has shown that, after removal of the metabolites of the intracellular pool, the synthesis of carbamoyl phosphate is much greater with glutamine than with NH_4^+ as nitrogen donor. This is true even under the optimal conditions for the assay of carbamoyl phosphokinase. If NH_4^+ can serve as a nitrogen donor in the reaction, the speed and probably the affinity are probably much weaker than with glutamine.

In Neurospora the only donor of nitrogen for carbamoyl phosphate synthesis seems to be NH_4^+ (Dr. R. H. Davis, personal communication). In *Escherichia coli*, the work of Yashphe & Gorini, 1965 shows that after removal of acetylphosphokinase there remains a carbamoyl phosphokinase which, in regard to mutation and regulation, behaves like the glutamine-dependent reaction described by Piérard & Wiame (1964). This would mean that in *E. coli* the biosynthetic enzyme is not absolutely specific for glutamine but is able to use NH_4^+ with a lower affinity. This favours the idea that glutamine is the true physiological substrate. The *E. coli* system thus appears as intermediate between *Saccharomyces cerevisiae* and the Neurospora systems, so far as the nitrogen donor is concerned. That one enzyme, according to its source, can utilize NH_4^+ or glutamine or both, as nitrogen donor for an amidation or amination reaction is not unusual. Meister (1962) and Levenberg (1962) pointed out several cases of such dual sources of nitrogen, particularly in the synthesis of purine and pyrimidine nucleotides.

Carbamoyl phosphate is a common donor of the carbamoyl group for the synthesis

of arginine and pyrimidines, thus constituting a branching point in metabolic pathways. This raises difficulties for understanding the regulation of these biosyntheses, a problem solved in similar cases (reviewed by Stadtman, 1963; Wiame, 1965) by the existence of independent enzymes responsible for the synthesis of the common product. The existence of two enzymes permits the independent regulation of two syntheses and as a further refinement may lead to different structural associations of these enzymes in relation to their respective functions. Different kinds of evidence may suggest the existence of two different enzymes when two functions have to be

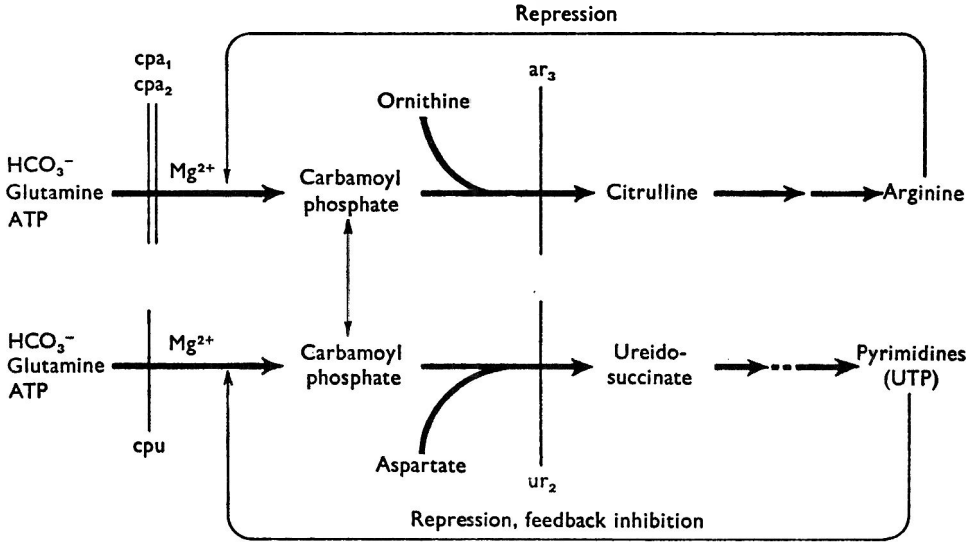

Fig. 6

fulfilled. The most conclusive proof is the demonstration of two physically separable enzymes, each affected by a specific mutation and regulated in accordance with their function. In the case of *Saccharomyces cerevisiae*, the data presented in this paper and summarized in Fig. 6 permit the conclusion that there exist two enzymes responsible for the synthesis of carbamoyl phosphate. They have not been physically separated but both can be affected by specific mutations (*cpa*, *cpu*) and in this way they can be studied separately with respect to their regulation. Both are much more active with glutamine than with NH_4^+. The enzyme present in the strain (*cpu*, *cpa*$^+$) is strongly repressed by arginine. The enzyme of strain (*cpa*, *cpu*$^+$) is markedly retro-inhibited by uridine-5′-triphosphate. The strains which carry both mutations *cpu* and *cpa* have no activity, and are auxotrophic for arginine and pyrimidines. The properties of the enzymes leave no doubt about their function: one is related to the biosynthesis of arginine, the other to the biosynthesis of pyrimidines. However, carbamoyl phosphate formed by either enzyme is available *in vivo* for both syntheses and the two types of mutants are able to grow on a minimal medium. There is no indication of a channelling, despite genes *cpu* and ur_2 (for aspartate carbamoyltransferase) being allelic, as in Neurospora (Woodward & Davis, 1963). The enzymes corresponding to both these genes are sensitive to feed-back inhibition by UTP (Lacroute, 1964).

In *Escherichia coli*, since one mutation leads to double auxotrophy for arginine

and uracil, it has to be admitted that there is a common catalytic element for the carbamoyl phosphate synthesis by both pathways. This raises a problem since it seems that both arginine and uracil have cumulative effect in the repression of this activity (Piérard & Wiame, 1964).

In Neurospora (Dr R. H. Davis, personal communication), it was anticipated since 1960 that there would be two different enzymes for carbamoyl phosphate synthesis, one for arginine, the other for pyrimidines. The basis for such an idea came from the study of Neurospora mutants *arg*-3 and *pyr*-3a. These mutants are, respectively, auxotrophic for arginine and a pyrimidine. Nutritional studies locate their deficiencies in the synthesis of carbamoyl phosphate, leading to the hypothesis that not only are there two independent systems for carbamoyl phosphate synthesis but also that the carbamoyl phosphate which arises from each system is channelled to its related function. Experiments to be published have shown that carbamoyl phosphate can be shunted to the other function by a mutation which affects its normal route of utilization. A question left open in Neurospora is the nature of the reaction which supplies carbamoyl phosphate for the pyrimidine pathway. The significance of mutant *arg*-2, not allelic but phenotypically identical with *arg*-3, remains to be elucidated. While mutant *arg*-3 exhibits a complete loss of carbamoyl phosphokinase activity, *arg*-2 retains this activity. Two non-allelic genes (cpa_1, cpa_2) determine the arginine specific carbamoyl phosphate biosynthetic system of *Saccharomyces cerevisiae*. However, in contrast to Neurospora mutants *arg*-2 and *arg*-3, both *S. cerevisiae* mutations cpa_1 and cpa_2 lead to a deficiency in the arginine-specific carbamoyl phosphate synthesis. Several hypotheses may be put forward to explain these facts. A first explanation would be that one gene is the structural gene, the other being a regulator gene. However, this hypothesis must be discarded since the combination of the cell-free extracts of single mutants carrying these two mutations regain activity. Two alternatives are left: (i) existence of two enzymic steps, each gene determining one enzyme; (ii) a single enzyme made up of two kinds of subunits, each kind corresponding to one of the genes. Preliminary experiments do not allow a distinction between the two possibilities.

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Microbial Identification*

BY THE LATE K. J. STEEL

National Collection of Type Cultures, Central Public Health Laboratory, Colindale Avenue, London, N.W. 9

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SUMMARY

Identification is the practical application of taxonomic knowledge. Dichotomous keys and diagnostic tables form the backbone of everyday identification, but computers will be used in the future. Standardization of methods for characterizing tests, the development of multiple inoculation apparatus, and the use of mass cultures will enable more reliable tests to be carried out and more strains to be tested.

Introduction

In an earlier paper (Steel, 1962) I considered classification to be an art and identification a science; recent developments in numerical taxonomy have lessened this distinction between them. Taxonomy, here equated with Simpson's (1961) definition of systematics, consists of (i) classification, (ii) nomenclature, (iii) identification; and the components should be taken in this order so that communication of results is made possible. Cowan (1965) regards the practice of identification as the utilitarian aspect of systematics or taxonomy and I propose to concentrate on this, and I shall use the terms 'identification' and 'diagnosis' as synonyms.

Aims of diagnosis

The identifier or diagnostician aims to identify a micro-organism accurately in the shortest practical time but when a pathogenic organism is to be identified he is often under pressure for a quick report; however, speed must always be secondary to accuracy. Nungester (1963) stated five objectives in identifying micro-organisms: (1) to determine quickly the susceptibility to antimicrobial drugs, (2) to gain information which may have prognostic value for physicians, (3) to identify pathogens in terms of their potential danger to people in contact with patients, (4) to aid epidemiologists in tracing sources of infections, (5) to accumulate data of interest to those studying infectious diseases. These objectives are primarily for the clinical microbiologist but can be adapted and amended for all concerned with microbial identification, irrespective of their field of study.

Ideally, every specimen for identification should be treated as a research problem, but time and facilities generally preclude this. Consequently the diagnostician

* Based on a paper 'Microbial identification: theory and practice', read at Quebec in August 1964 a few weeks before Dr Steel died, and edited by his colleagues in the National Collection of Type Cultures. As presented to the meeting organized by the Canadian Committee on Culture Collections the paper contained several extracts from Cowan & Steel (1965) and Cowan (1965); these were removed as that material can be read in the original; cross headings have been added.

has developed presumptive tests for those micro-organisms believed to be important, and relegates those he considers unimportant to ill-defined dump-heaps with labels such as 'achromobacter', 'paracolon', or 'non-pathogen'. The magnitude of the diagnostician's task can be gauged by the routine work of the Public Health Laboratory Service of England and Wales which, in 1963, examined two-and-a-half million specimens. Complete identification may not always be warranted and the extent to which identification will be carried will depend on the nature of the specimen and the purpose for which it was submitted.

Practice of identification

The identification of a micro-organism involves comparison of an unknown and a known unit, and eventually giving a name to the former. Both processes depend on adequate information for characterizing the known unit and many ways have been proposed for making such information readily available. Some are based on the use of dichotomous keys and others on diagnostic keys and tables; one recent scheme which uses a computer as an automatic library facility may soon become a practical proposition (Payne, 1963).

Dichotomous keys. Skerman (1959) devised a comprehensive key which enables the searcher to place an unknown in its genus, but for further differentiation and diagnosis of species the supplementary keys in *Bergey's Manual* (1957) are required. Difficulties in interpretation of keys arise where strains behave inconsistently in some respect, and to make allowances for the variable reactions given by such strains Manclark & Pickett (1961) developed flow charts in which a strain may then appear in more than one place at the extremities of the chart.

Some characters are almost invariably positive or negative, but characters of such constancy are usually shared by similar organisms and, although they are important in characterizing an organism, have little value in distinguishing it from its neighbours. On the results of a limited number (up to ten) of selected cytological and physiological tests, Cowan & Steel (1961, 1965) found that most organisms encountered in clinical bacteriology could be placed in a genus or group of genera; this constituted the first stage of identification with their diagnostic tables.

Diagnostic keys and tables. These have one important limitation, the specimen under examination must belong *a priori* to the group of organisms for which the scheme was devised; otherwise, mis-identification or failure to identify will result. If the diagnostician has ready access to an electronic computer he could use an almost infinite number of characters, but with tables he is restricted by memory or is limited by his ability to recognize similarities and differences when making comparisons simultaneously. These limitations led to the construction of the Determinator and the compilation of tables suitable for use with it (Cowan & Steel, 1960, 1961, 1965).

Another way of comparing the characters of known and unknown micro-organisms is to use punched cards; the characters of the unknown are punched on a card which is then compared, mechanically or by hand, with a series of cards containing the characters of known organisms.

Diagnosis by computer. The use of an electronic computer to assist in the identification of micro-organisms is a new venture, but its potential usefulness in bacteriology has already been proved. Fundamentally the computer is provided with a

'microbial memory'. This consists of a table of microbial characters but with the usual plus and minus signs replaced by numerical data which express the constancy of the reactions quantitatively; the names of the organisms and of the reactions are stored in numerical form.

The results of tests on an unknown organism are fed into the computer, which then compares the pattern of these results with the pattern of results for the same tests already held in its 'microbial memory'. The computer selects from its memory those organisms whose behaviour most closely resembles that of the unknown. Finally the computer is programmed to consider the remaining tests for their potential value in differentiating between the suspected organisms.

Diagnostic tests

Of the three approaches to identification (Cowan, 1965), the third or progressive method aims to determine a few fundamental characters so that an isolate can be placed in one genus or small group of genera; other appropriate tests are then carried out so that specific identification can be made. Additional tests for the better identification of a species, variety or biotype may be required.

The diagnostician and the taxonomist are dealing with similar material and both aim to decrease subjective bias and increase objectivity; phylogenetic speculation is out of order for both workers, but only the diagnostician may properly use character weighting. The variable weighting attached to these characters is based largely on experience, but it is likely that the assimilation of data from a wide range of organisms and subsequent computer analysis will, in the future, enable the value of a character to be expressed objectively in a quantitative manner.

The tests used should be those which give the most reproducible results; many reactions are influenced by factors that are difficult to control and, in the absence of agreed standard methods, test methods should be those recommended in published manuals (Cowan & Steel, 1965; Skerman, 1959; Society of American Bacteriologists Committee on Bacteriological Technic, 1957). The need for this is well illustrated among the enteric bacteria where, under the conditions that provide adequate –SH compounds few fail to produce H_2S; when tested under other conditions only strong H_2S producers are recorded as positive and the test then has good diagnostic value. Acetoin production is another example of a test whose sensitivity is easily altered; many 'soft rot coliforms' appear to be V-P positive when grown in O'Meara's fumarate broth and Barritt's method is used to detect acetoin.

The characters used should be independent of subjective criteria such as rate of growth, odour and recognition of the finer shades of pigments; when too great a reliance is placed on pigment production the occurrence of non-pigmented organisms (whether natural or artificially induced) poses a dilemma. Colonial morphology will vary with the conditions under which the organism is grown and is seldom of diagnostic value; for example, non-rhizoid variants of the typically rhizoidal *Bacillus mycoides* are common.

Special tests. Mention must be made of special tests, usually described for the distinction of similar micro-organisms or for use only within a particular group or genus. The diagnostic and taxonomic value of characters revealed by such tests is diminished when they are known only for a restricted number of micro-organisms. In the National Collection of Type Cultures we try to avoid using special tests for

particular organisms and, in evaluating newly described tests, apply them to a wide range of bacteria; this frequently produces unexpected and interesting results and permits the inclusion of the test in routine identification work.

Ideally perhaps, diagnostic characters should be expressions of the occurrence of a particular gene in a micro-organism; current genetical knowledge does not permit this and we are fortunate indeed to have a few tests which are detecting the presence of a single enzyme (for example the β-galactosidase test). Similarly, it is not known how many or which genes influence a particular enzyme and it may well be that some of the tests used are each reflecting the same gene. Certainly it does not seem defensible to record the production of both acid and gas from a range of carbohydrates when gas production in all of them revolves around the presence of the same enzyme. Again, it is not unreasonable to assume that when an organism produces acid from maltose it will usually produce it from glucose (*Pseudomonas maltophilia* is a notable exception); the converse is however untrue, since a glucose-attacking organism may or may not attack maltose. Similarly, flagellated organisms may be expected to be motile, but even when non-flagellated some organisms may show gliding motion.

The diagnostician must be fully aware of the importance of adaptation and ecology, and consider a microbial culture as a population rather than a collection of cells; the host range, which is of epidemiological importance, is also an ecological problem, but such factors cannot be satisfactorily tabulated or weighted.

Construction of diagnostic schemes

For many years diagnosticians have distinguished three species within the genus *Brucella* by their dye sensitivity, H_2S production, and agglutination with mono-specific sera; the specialist worker recognizes the same named species but on their oxidative metabolic pattern and bacteriophage sensitivity. Thus what is *B. melitensis* to the diagnostician may be a biotype of *B. abortus* to the specialist in this genus. This example raises the question, need a diagnostic scheme be taxonomically correct? Some schemes are entirely artificial and do not bear any resemblance to accepted classifications; examples are those used by water bacteriologists, based upon IMViC reactions, and some of those intended for identification of the rhizosphere flora. They are useful but often static and may fail to take account of recent developments. Much present-day identification is based upon schemes and keys that are monothetic; such keys are analytical tools rather than classifications but are constructed in the manner of an artificial classification, being based upon a few discriminating criteria that happen to provide a ready means of subdivision. We cannot identify micro-organisms at a glance as can often be done with higher organisms. If we are to identify speedily, which is one of the aims of the diagnostician, we must rely on the determination of selected characters (differentiae or key characters) fewer in number than would be needed for classification. Although identification and the construction of diagnostic schemes logically follows classification, the importance of prominent single characters to the diagnostician has reflected back and such characters have been assumed to be important in constructing taxa.

Does the possibility of producing natural classifications affect identification? A monothetic classification makes the preparation of diagnostic keys easy, but there

is always a risk of mis-identification when an organism which is aberrant in one of the key characters selected is encountered; this difficulty is less likely to arise with tables which are essentially polythetic. Tables have the further advantage that it is possible to obtain some idea of the 'nearest fit' for an aberrant strain.

Polythetic taxa resulting from taxonomic methods based upon overall similarity must be augmented by identification schemes that also reflect the current taxonomic trend. In the creation of natural taxa on phenetic evidence it is possible that a single common character may not be found; this is disturbing for the diagnostician but the dilemma may be resolved by the adoption of a polythetic diagnostic scheme in which the possession of any one character is not essential for identification.

The basic data upon which such schemes are made could be fed into a computer; by suitable programming this information could be retrieved in a form suitable for the preparation of keys. This might best be done by using the discriminant analysis of Fisher (1936) in which each character is given a weighting such that there is the least probability of mis-identifying a specimen taken at random. Another approach is based on the method of Brisbane & Rovira (1961) who calculated the association coefficients for all character-pair combinations and then arranged characters with the highest association coefficients in a dichotomy; this arrangement was unfortunate as dichotomies may lead to error, and the application of cluster analysis to reveal clusters of characters with a high degree of mutual association would be better.

The theory and practice of the preparation of diagnostic keys has been discussed in some detail by Ainsworth (1941), Metcalf (1954), and mathematically by Maccacaro (1958). Hill & Silvestri (1962) and Möller (1962) introduced the concept of probability into the construction of keys; their method permits the ascription of a specimen to a taxon on a probabilistic basis. The choice of tests used for the key was decided on their information content, and for each taxon the mean probability of identification was calculated.

The future?

What does the future hold for microbial identification? About 1880, Koch was working with crude fluid media and gelatin-solidified media but his bacteria grew about as quickly as ours do today. Without forced aeration of liquid cultures, which involves technical problems, we are unlikely greatly to accelerate microbial growth. How then are we to answer the challenge for speedier identification? The use of computers to aid identification has been mentioned. Spot tests and micro-reactions may well play an important role; latex-fixation reagents provide the pathologist with rapid screening tests for agammaglobulinaemia, hypofibrinogenaemia, systemic lupus erythematosus, and other conditions; similar rapid tests would be of value to microbiologists.

The number of organisms submitted for identification increases annually and the examination of larger numbers of strains envisaged in numerical taxonomy puts a considerable burden on the laboratory. If this challenge is to be met, attention must be paid to techniques for the mass handling of cultures as well as to the handling of data resulting from such action. Replica plating, plate inoculators, and apparatus such as that used in colicine and phage typing will play a more important part in the microbiologist's work. However, these methods may be insufficient and thought must be given to ways of relieving the tedium of inocu-

lating scores of cultures into perhaps hundreds of tubes. A method that comes to mind is the application of the fraction collector used in chromatography. Automation has become an accepted tool in clinical biochemical determinations; when the problems of possible cross-contamination and of sterilization of the apparatus have been overcome we may expect automated microbial identification to become a practical proposition. With the possible exception of fluorescent antibody techniques, all diagnostic methods require that the unknown micro-organisms be isolated in pure culture.

These considerations of future developments do not assume that the diagnostician will be unnecessary; rather they suggest that he should be in a better position to identify more efficiently and accurately, to devise new test methods, and to aid the study of ecological and epidemiological patterns.

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