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Cultural Characters of *Streptococcus pluton* and its Differentiation from Associated Enterococci

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SUMMARY

Strains of *Streptococcus pluton* (White) from widely separate parts of the world were very similar culturally and serologically and differed greatly in these respects from associated enterococci. *S. pluton* was satisfactorily isolated only on a yeast + glucose + potassium phosphate + starch medium incubated anaerobically $+CO_2$. Oxidation potentials in agar of this medium were very stable for which the high concentration of phosphate, together with yeast and glucose, was necessary. In deep agar incubated aerobically, *S. pluton* started to grow only within a narrow range of Eh values. It did not grow on ordinary bacteriological media but was adapted to grow on the special agar in air $+CO_2$. Adapted strains grew on the special agar in a limited volume of air and produced CO_2.

INTRODUCTION

Since the first account of the isolation and cultural characteristics of *Streptococcus pluton* (White) from honey bee larvae with European foul brood disease (Bailey, 1957), further work on several isolates from different parts of the world has been done. Information was sought that would facilitate the differentiation of *S. pluton* from organisms resembling *Streptococcus faecalis*, which often occur in diseased larvae and are still confused by bacteriologists with *S. pluton*.

METHODS

Mid-guts of living diseased larvae were removed aseptically, smeared on sterile microscope slides, covered, allowed to dry and stored at room temperature. *Streptococcus pluton* stays viable in such smears for at least 15 months and associated bacteria do not (Bailey, 1959). Material of this kind was collected from Southern England (E); Wisconsin (W), New York (N) and Louisiana (L) U.S.A.; Argentina (A) and Switzerland (S); strains from these sources are referred to below by the single letter. One strain from each source and an additional unusual strain, P, from England were tested.

The usual medium, referred to as 'basal' below, was as follows: 1 g. yeast extract (Difco); 10 ml. M-KH_2PO_4 adjusted to pH 6·6 with KOH; 2 g. agar; made up to 100 ml. with distilled water. Ten ml. lots were distributed into screw-capped bottles and autoclaved at 116° for 20 min. immediately before use.

Unless otherwise stated, cultures were incubated anaerobically at 34° in McIntosh and Fildes jars containing hydrogen + about 10 % (v/v) CO_2.

Organisms identified as *Streptococcus faecalis* were isolated from diseased larvae

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from Argentina, Wisconsin (U.S.A.) and Switzerland: strains A′, W′, and S_1, S_2, S_3, S_4, respectively.

Failure to grow on modified or different media is reported only when similar inoculation gave growth on a suitable medium.

Antisera were prepared by injecting rabbits with washed formolized suspensions of strains N and W′. Four intravenous injections were given during 14 days, and the rabbits bled 12 days after the last injection. Serological assays were made by agglutination tests in tubes containing suspensions of bacteria equiv. 0·25 mg. dry wt./ml. Qualitative tests were made by a micro gel-diffusion method (Mansi, 1958) with bacterial suspensions and with extracts made from them by the method described by Lancefield (1933).

RESULTS

Cultural characters

For growth of *Streptococcus pluton*, basal medium stored for a few weeks was inferior to basal medium freshly prepared, and the bacteria that grew on it were very pleomorphic, sometimes being mostly rod-like. Basal medium (100 ml.)+ 0·5 g. Neopeptone (Difco) deteriorated quicker than did basal medium alone. Growth in pour plates without starch sometimes failed even when streaks on similar plates with the same inoculum produced colonies. Many of the organisms which did grow in pour plates were rod-like. Good growth occurred in pour plates of basal medium + starch. The easiest way to isolate *S. pluton* from natural material containing enterococci was to inoculate such pour plates with decimal dilutions of bacterial suspensions. Enterococci were fewer than *S. pluton* even in diseased larvae which had decomposed in a moist state for some days, and were easily diluted out. Colonies of enterococci were larger than those of *S. pluton* and were surrounded by a clear halo. Small inocula of *S. pluton* grew well in pour plates of basal medium + starch; most of the organisms grew from inocula containing about fifty bacteria. Slopes of basal medium were superior to plates or broths for subculturing *S. pluton*, growth often appearing within 2 days.

Many attempts were made to isolate the aerobic rod-forms observed with the first isolates (Bailey, 1957) from all sources of *Streptococcus pluton*, but all failed. Growth often occurred in aerobically incubated broths, particularly in narrow or sealed tubes, but it was in streptococcal form or in chains of mixed rods and cocci that could not be subcultured aerobically on the surface of nutrient agar. Nevertheless strains tested adapted to oxygen when they were subcultured on slopes of basal medium in an atmosphere of (%, v/v): 5 air + 85 H_2 + 10 CO_2 in sealed 3 l. jars. They could then be subcultured on 10 ml. slopes in tubes sealed with about 20 ml. of air (in which they produced CO_2) or on slopes in the large jars with up to 90 % (v/v) air + 10 % (v/v) CO_2. No growth occurred in the large jars without CO_2.

The growth of *Streptococcus pluton* in deep agar media (Table 2) was the same with inocula from dried smears or from pure cultures. The concentration of the inoculum made little difference to the depths at which growth appeared. The upper limit of the lower bands appeared at the level where methylene blue (2 mg./l.) incorporated in similar sterile incubated media was reduced. This boundary of reduction was very stable: in deep agar tubes of basal medium it was about 10 mm. from the surface and moved down to 15 mm. after aerobic incubation for 3 weeks. In basal medium,

Table 1. *Growth of* Streptococcus pluton *and associated enterococci in various media at* 34°

Media constituents and environmental conditions*																		Growth**									
																		S. pluton								Enterococci	
																		Strains								Strains	
1 % yeast extract (Difco)	1 % glucose	1 % starch	0·1 M-K phosphate, pH 6·6	0·01 M-K phosphate, pH 6·6	0·1 M-K phosphate, pH 9·0	0·1 M-Na phosphate, pH 6·6	0·1 M-KHCO$_3$ + 75 % v/v CO$_2$ (pH 6·6)	0·1 M-KCl	0·1 M-NaCl	0·2 M-NaCl	Aerobic	Anaerobic	10 % CO$_2$	Litmus milk	Ordinary media†	0·5 % Na taurocholate	60° for 30 min. before incubating	S	E	W	N	L	A	P	Remarks	A′ W′ S_1 S_2 S_3 S_4	Remarks
/	/	/	/									/	/					+	+	+	+	+	+	+	Growth after 2 or more days. No acid	++	Growth within 24 hr. Final pH 4·0
/	/		/									/	/					+	+	+	+	+	+	+	Primary isolates sometimes failed	++	
/	/	/	/									/						−	−			−	−	−		++	
/	/	/		/								/	/					±	−					+	Type P strains were rare in natural material		
/	/											/	/					−						−		+	
/	/	/										/	/					−	−	−	−	−	−	+			
/	/	/			/							/	/					+	−	−	−	+	−	−	Only 1 of 2 replicates of L grew	++	
/	/	/				/						/	/					−	−	−	−	−	−	−		++	
/	/	/					/					/	/						−	−	−			+			
/	/	/						/				/	/					+	+	+			+		Primary isolates feeble. Final pH 5·2		
/	/	/							/			/	/					−	−	−			−	±		+	
/	/	/								/		/	/											−		+	
/	/	/	/								/		/						+	+	+		+	±	Training necessary. Catalase negative	++	Prompt growth
/	/	/	/								/							−	−	−	−		−			++	
												/	/	/				−	−	−	−	−	−	−		+	Coagulation; some reduction and peptonization
	/											/	/		/			−	−	−	−	−	−	−		+	
	/										/				/			−	−	−	−	−	−	−		+	
											/				/			−	−	−	−	−	−	−		±	
/	/	/										/	/			/		−	−	−	−	−	−	−	All strains insoluble in 1 % Na taurocholate	++	
/	/	/	/									/	/				/	+		+			−	+	Growth delayed about 24 hr.	++	

* /, medium constituent or environmental condition present.
** ++, strong; +, moderate; ±, slight; −, nil.
† Nutrient broth no. 2 (Oxoid) or brain heart infusion (Difco).

with glucose added after autoclaving, methylene blue appeared less abruptly reduced at a depth of about 20 mm., and the boundary moved down about 10 mm. after incubation for 3 weeks. The yeast extract, glucose and phosphate each contributed substantially to the stability of the oxidation/reduction potential; starch in the medium made no difference. In basal medium + 5 g. Neopeptone (Difco)/l. the boundary of methylene blue reduction was deeper and less well defined than in ordinary basic medium. (The phosphate and glucose autoclaved together without yeast yielded a well-poised agar: methylene blue was reduced at 15–20 mm. below the surface with no change after a week.) Media containing Nile blue remained blue to the bottom of the

Table 2. *Growth of* Streptococcus pluton *and associated enterococci in deep agar**

	Medium	*Streptococcus pluton* (strains, E, L, N)†	Enterococci (all strains)
(1)	g./l.: yeast extract (Difco), 10; glucose, 10; 0·1 M potassium phosphate (pH 6·6); autoclaved in screw-capped bottles at 116° for 20 min.	Colonies after several days from 3 to 8 mm. below surface, trailing to variable depths. Thin band (< 1 mm) of optimal growth at upper level, sometimes with a second similar band about a mm. above. Growth sometimes feeble or absent	Strong, even growth from surface to bottom within 24 hr.
(2)	(1) with glucose autoclaved separately	Colonies after several days from 10 to 25 mm. below surface, trailing to variable depths, and usually optimal at the upper level; followed by a second band a few mm. above, ending a few mm. below surface and usually optimal at its lower level. A third band ocasionally appeared over the second. Growth sometimes feeble or absent	As above
(3)	As (1) or (2) but autoclaved in open tubes	As in (1) or (2) but growth restricted to the optimal regions	—
(4)	(1), (2) or (3) + 10 % v/v CO_2 in air	Little change in (1) or (2); growth restrictions observed in (3) removed	—
(5)	(1) or (2) + starch	As in (1) or (2) but optima and banding less distinct although growth more reliable	—
(6)	(1) + Neopeptone (Difco)	As in (1) but with wider single band of optimal growth	—

* Molten agar media inoculated, poured into tubes and incubated aerobically at 34°.
† See text.

tube. *S. pluton* grew well in deep basal agar with methylene blue and double-banded growth occurred as shown in Table 2. When deep basal agar tubes which had been inoculated and incubated aerobically for 2 days were extruded from their tubes before growth was visible and were then re-incubated aerobically, colonies appeared in the position that the lower band of growth normally occupied in deep agar tubes. Such deep basal agars with methylene blue and inoculated with *S. pluton* became blue throughout when extruded.

Wille (1960) reported difficulty in culturing *Streptococcus pluton*, much of which was undoubtedly caused by absence of CO_2 (he used alkaline pyrogallol to achieve anaerobiosis) and starch. He reported that added trace elements (1 % v/v Hoag-

land solution Hoagland & Snyder, 1931) improved matters. Hoagland solution was tried with all the various strains of *S. pluton* except W but did not improve growth in the basal medium, with or without starch, on plates or slopes, or in liquid media and did not cause growth in such media incubated without CO_2.

Serology

The serological tests confirmed the results of the cultural tests described above. The type of immunizing antigen used and the timing of the injections resulted in antisera which contained predominantly 'type' antibodies. Agglutination tests showed that all *Streptococcus pluton* isolates were serologically related to one another but not to the associated enterococci (Table 3). Not all the enterococci were similar; the strain W′ antiserum reacted with strain W′ and strain A′, but not with strains S_{1-4}.

As the antisera contained few group antibodies, the lines of precipitate formed in gel diffusion tests were usually very faint, even when concentrated preparations of antigen and antiserum were used. The results, however, confirmed those of the

Table 3. *Agglutination tests of bacterial isolates*

Antigen: bacterial suspension 0·25 mg./ml.

Antigen	Antiserum titre*	
	Strain N antiserum	Strain W′ antiserum
S. pluton strains		
E	1024	< 2
W	512	< 2
N	1024	< 2
L	256	< 2
A	512	< 2
P	1024	4
S	1024	8
S. faecalis strains		
A′	< 2	256
W′	4	1024
S_1, S_2, S_3, S_4	< 2	< 2

* Reciprocal of dilution end point after incubation for 4 hr. at 37°.

agglutination tests. Both antisera sometimes gave a faint line of precipitate, in the gel close to the antiserum well, against all isolates; this line was clearest when suspensions of bacteria were used, and was indistinct or absent when extracts were used. In addition the strain N antiserum gave a second faint line of precipitate against concentrated extracts of *Streptococcus pluton* isolates, and the strain W′ antiserum gave a dense line of precipitate against strain W′ extracts, a weaker line against strain A′ extracts, and sometimes a faint line against strain S extracts. Strain W′ antiserum reacted with strains A′ and W′ in gel diffusion and in agglutination tests, showing that these isolates were closely related serologically. However strain W′ antiserum only reacted with strains S_{1-4} in gel diffusion tests with bacterial extracts, which shows that they were less closely related; perhaps at the group level.

DISCUSSION

The *Streptococcus pluton* strains used were not all tested together in every culture trial, but the only considerable cultural differences seem to be between the rare strain P and the others. In its CO_2 requirement and its growth in deep agar *S. pluton* may seem to resemble *Brucella abortus* (Wilson, 1931). Like *B. abortus* it probably grew in deep agar without an external supply of CO_2 because it produced its own, but, unlike *B. abortus*, the bands of growth of *S. pluton* were little influenced when CO_2 was supplied. *S. pluton*, therefore, seems truly micro-aerophilic. Further, when *B. abortus* gave double-banded growth the lower band appeared later and arose, Wilson concluded, because the medium near the growing organism became alkaline and absorbed CO_2. Double-banded growth of *S. pluton*, however, seemed likely to be caused by over-reduction of the medium above the lower band: the upper band probably appeared where the oxygen diffusing from above counter-balanced reduction from below to maintain a critical Eh value long enough for growth to start. This Eh value is probably in the less negative regions of the range -50 to -100 mV., according to the observations with indicators. That growth sometimes failed to reach the bottom of deep agars, was less in deep agar autoclaved in open tubes than in similar agar autoclaved in closed tubes containing air, and was feebler in deep agar or anaerobic broths autoclaved with glucose, than in the same media with glucose added after autoclaving, was further evidence that growth was inhibited in over-reduced media.

The growth that appeared in extruded deep agars, equivalent to the lower band in tubed agar, suggests that the Eh value is most critical for initiation of growth; once begun growth continued in a more oxidized medium. Growth on plates started successfully in an anaerobic atmosphere because the medium may have been rather stably oxidized while setting after pouring and then was gradually reduced through the range of optimal Eh under anaerobiosis. Growth was quicker on slopes than on plates, perhaps because slopes were less exposed to the atmosphere in preparation, and oxidation may have been nearer the optimum. Frequent failure to grow in pour plates of basal medium may have occurred because it was too irreversibly oxidized below the surface, as suggested by the development of rod-like forms when growth did occur. Basal medium + Neopeptone probably deteriorated quickly because it was less well poised than ordinary basic medium and became too oxidized. The band of growth in deep agars containing Neopeptone was wider than in ordinary basal medium, probably because the zone of optimal Eh for growth initiation was wider. The high potassium phosphate concentration is probably required by the ordinary strains of *S. pluton* to give a high K/Na ratio, but the poising effect of phosphate may also be important as media with equivalent concentrations of bicarbonate or chloride seemed inadequate.

Failure to produce fully aerobic forms of *Streptococcus pluton* may suggest that those apparently obtained in the original isolates (Bailey, 1957) were contaminants. This cannot be ruled out but is considered unlikely because the original (basal) medium was developed by using these rod-producing cultures, and rod-like forms often arose from all strains of *S. pluton* exposed to oxygen or when plated on aged (presumably oxidized) media. Successful adaptation to fully aerobic growth may require a combination of conditions of inoculum and medium, obtained by chance

in the many changes of medium in the early trials. Some strains more tolerant of oxygen may also have been selected in the early work. The formation of rod-like forms in aerobic conditions, or in oxidized media, may be analogous to the formation of diphtheroid forms in extreme aerobiosis by an α-haemolytic streptococcus (Dubos, 1945).

It is now clear that starch is necessary to ensure isolation of *Streptococcus pluton* from natural material. The need is less with subcultures, however, which undoubtedly led to the original conclusion that it was unnecessary (Bailey, 1957). Presumably starch neutralizes growth inhibitors in the medium, as it does for other fastidious organisms (Foster *et al.* 1950; Ley & Mueller, 1946).

The fastidiousness of *Streptococcus pluton* seems to distinguish it from associated enterococci. In particular the facultative anaerobic growth of enterococci on a variety of ordinary media without CO_2 and their tolerance of sodium separates them sharply from *S. pluton*. Serologically, all the strains of *S. pluton* examined clearly differ from the enterococci. The serological tests indicate that, unlike the enterococci, all strains of *S. pluton* are closely related in spite of their widely separate geographical origins, which probably reflects their specific association with honey bees.

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Bacterial Spore Antigens: a Review

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SUMMARY

Agglutinogens and precipitinogens have been detected in the spores of many species of aerobic and anaerobic spore-formers and these antigens are distinct from those of the vegetative growth phases of the corresponding organisms. Spore antigens are heat resistant and antibody response is elicited by the injection of autoclaved spores into rabbits. Injection of living spores leads to the development of antibodies reacting with vegetative cell antigens in addition to spore antibodies.

Agglutinogens tend to show subspecies distribution in some aerobic species but spore precipitinogens appear to be mainly species specific in aerobes. Less information is available about anaerobes where a similar pattern of specificity is suggested. Data about the antigenic structure of the spore and the antigenic changes accompanying sporulation and germination are scant; it is in these aspects of the subject that the most significant progress is to be anticipated.

INTRODUCTION

Do bacterial spores possess antigens distinct from those of the vegetative organism? If so, what is their taxonomic value? Do spores carry any of the antigens found in the vegetative forms? These questions have been the theme of much of the work on spore antigens and part of the present task will be to consider how far they have been answered—in short, to summarize our knowledge about the distribution of antigens between spore and vegetative form and of particular spore antigens among different spore-forming bacteria. Today interest is being focused on the detailed antigenic structure of the bacteria. Techniques for separating cells into their component parts and for studying antigens both *in situ* in the cells and in separated cell components are developing rapidly and are opening new approaches to the problems of cell analysis. By their very nature, spores would seem to be well suited to this type of study. We know very little indeed of the 'antigenic architecture' of spores or of the antigenic changes which accompany the formation and germination of spores. The appearance, in the last few years, of papers touching on these aspects of the subject suggests that the time is ripe for a thorough investigation and I hope that this review, by presenting the information available and suggesting possible lines of extension, may act as a stimulus for further work.

Technical problems; the pre-treatment of spore antigen

Observations on spore antigens date almost from the beginning of the century; some thirty papers have dealt directly with the subject. There are technical difficulties in work of this kind which stem from the nature of the spore itself and from

the mode of its production. The success of the different studies and the significance of the results obtained are largely reflexions of the extent to which these difficulties have been overcome. The earliest paper dealing with spore antigens, that of Defalle (1902), which forms an admirable starting point for investigation of the subject, clearly shows that the author was aware of most of the technical problems which have since caused trouble.

The use of living spores as antigen. The use of viable spores for injection and for agglutination tests is open to criticism on several different accounts. Defalle (1902) suggested that living spores may germinate within the body of the animal after injection. This was subsequently shown to happen by several workers, notably Teale & Bach (1919) who found that spores of *Bacillus anthracis*, *B. mycoides* and *B. subtilis* germinated in large numbers when injected into animals. The resulting vegetative forms did not normally multiply or produce toxaemia but the possibility that these may stimulate the production of vegetative cell antibodies must be taken into account when the results of immunological experiments are being evaluated.

The agglutination test, in its usual form, involves a fairly long period of contact between antigen and antiserum at an elevated temperature and under these conditions living spores may well germinate—a change which is clearly undesirable when spore antigens are to be studied. Noble (1919) described a rapid method of performing the agglutination test in which shaking the reactants together at high concentration was followed by dilution. The results with a number of species compared well with those obtained by using standard techniques; Lamanna (1940*a*, 1940*b*), Lamanna & Eisler (1960), Lamanna & Jones (1961) and Norris & Wolf (1961) used a modification of Noble's method to study the agglutination of viable spores. The method decreases the agglutination time to 30 min. and diminishes considerably the possibility of germination interfering with the result. Moussa (1956), working with anaerobic spore-formers, had little success with this technique.

Auto-agglutination of spores. The surface of the bacterial spore appears to be strongly hydrophobic (Lamanna & Eisler, 1960) and several workers have noted that spores of certain strains tend to agglutinate spontaneously when suspended in saline. Noble (1927) showed that when a suspension of vegetative *Bacillus anthracis* was shaken vigorously for 12 hr. and then allowed to stand overnight, coarse aggregates of bacteria settled out, leaving a homogeneous suspension which could be used for agglutination. Norris & Wolf (1961) used a similar method for preparing spore suspensions for agglutination. Nevertheless, some spore suspensions, even when treated by Noble's method, show almost immediate clumping in the controls and it has been impossible to study a certain small proportion of these organisms by agglutination techniques. The addition of 5 mg. bovine serum albumin/ml. to the diluent to decrease spontaneous agglutination and the tendency for spores to be trapped in a meniscus was advocated by Lamanna & Jones (1961).

Contamination of spores with vegetative cell material

The most important factor which has caused confusion in the study of the antigenic structure of the spore has been the use of impure material for injection and agglutination. Growth on normal laboratory media rarely results in anything like 100% sporulation with the aerobic spore-formers; with the anaerobes the situation is even less satisfactory. Suspensions of old cultures must inevitably contain con-

siderable amounts of material derived from vegetative cells. Several workers have injected such preparations and have obtained antisera containing a mixture of spore and vegetative cell antibodies. There appear to be two approaches to this problem: the production of cultures consisting solely of spores or the active removal of vegetative cell debris.

Complete sporulation. Howie & Cruickshank (1940) described an asparagine agar medium which gave complete sporulation of an organism resembling *Bacillus cereus* and of *B. mesentericus*. Injection of a suspension of such a culture resulted in the production of antibodies specific for the spore stage and the antisera did not agglutinate vegetative cells. Occasionally complete sporulation has been claimed with more conventional media (Davies, 1951).

Active removal of vegetative debris. Mellon & Anderson (1919) used 'antiformin' to effect the lysis of vegetative cells and KOH was used by Krauskopf & McCoy (1937), Schweinsberg (1951) and Schlossberger (1951) for the same purpose. Antiformin consists of equal volumes of *liq. sod. chlorinat.* (B.P.; an aqueous solution of sodium hypochlorite containing 2·5–3·0% available chlorine) and a solution of caustic soda (150 g./l.). The use of such vigorous agents is open to criticism, since they will almost certainly cause changes in the serological properties of the spores, paralleling the drastic chemical effects which are readily observed. This has led several workers to seek gentler methods of removing vegetative debris while leaving the spores intact and viable.

Delpy & Chamsy (1949) used autolysis in the presence of thiomersalate to remove vegetative material from old cultures of *Bacillus anthracis* and found that the spores remained viable and antigenically unchanged. Norris & Wolf (1961) used this method with considerable success for the purification of spores of several *Bacillus* species; it was not successful with all species. Norris (1957) described a bacteriolytic principle derived from *B. cereus* which effectively lysed the vegetative cells of all species of aerobic spore-forming bacteria against which it was tested. Spores of these organisms were not affected by the lytic principle and antigenically 'clean' spores were readily obtained. Walker (1959) successfully used lysozyme to remove vegetative debris from spore suspensions of thermophilic aerobic spore-formers. Pancreatic extract was used by Moussa (1956, 1959) with some success to free spores of *Clostridium septicum* and *C. botulinum* from vegetative cell remnants. Meisel & Rymkiewicz (1957, 1958*a*, *b*, 1959) used purified preparations of lysozyme, deoxyribonuclease and trypsin to remove vegetative material from spores of *C. tetani* and other *Clostridium* species but, although this treatment prevented spore suspensions from being agglutinated by antisera produced against vegetative cells, vegetative cell remnants could still be seen under the microscope and the injection of enzymically purified spores yielded antisera containing both spore and bacillary antibodies. *B. megaterium* is highly sensitive to lysis by lysozyme and Tomcsik & Baumann-Grace (1959) experienced no difficulty in using this reagent to free *B. megaterium* spores from vegetative cell remnants.

The nature of the spore antigen and its relationship to the antigens of the vegetative cell

The first observations on the antigenicity of bacterial endospores appear to be those of Defalle (1902) who injected old agar cultures of *Bacillus mycoides*, '*B. mesentericus vulgatus*', *B. subtilis*, *B. alvei* and two attenuated strains of the anthrax

bacillus into rabbits and tested the resulting antisera by slide agglutination. The cultures which he injected contained few, if any, vegetative forms. Antiserum produced against *B. mycoides* spores agglutinated viable and autoclaved spores as did a serum prepared by injecting autoclaved spores. Vegetative cells gave rise to no spore agglutinins on injection although antibodies which agglutinated the bacilli were readily produced. Spore antisera showed some ability to agglutinate vegetative cells, a fact which the author attributed to the presence of vegetative debris in the suspensions used for injection.

Chimera (1916) found no evidence for the production of specific agglutinins within ten days of the inoculation of spores of *Bacillus anthracis* and *B. subtilis* into rabbits. Agglutinins active against bacilli had no action on the spores.

Mellon & Anderson (1919) injected two types of antigen in an investigation of the serological relationships between spores and vegetative cells of *Bacillus subtilis*. Their spore antigen was a formolized suspension of an old culture on nutrient agar; a formolized suspension of vegetative cells constituted their bacillary antigen. The resulting antisera showed complete cross-reaction, each agglutinating both types of antigen. Antiformin was then used to dissolve vegetative material from the spore suspension. After exposure for 18 hr. to this agent the vegetative material had lysed and many of the spores had lost the ability to retain the spore stain. The resulting preparation, after washing, was agglutinated by the spore antiserum but not by the bacillary antiserum, indicating that the cross-reactions had been due to the presence of bacillary remnants in the spore suspensions rather than to the sharing of common antigens by the two growth phases; this was confirmed by cross-absorption experiments.

Krauskopf & McCoy (1937) attempted to demonstrate the presence of bacillary H antigen in spores of *Bacillus niger* (presumably *B. subtilis* var. *niger*). Two types of antigen were injected: whole vegetative cells; spore suspensions treated with KOH to dissolve any vegetative material which may have been present. Both of the resulting antisera agglutinated bacillary O antigen, whole vegetative cells, KOH-treated spores and untreated spores (the latter suspension almost certainly containing much vegetative cell material). Absorption experiments gave complex results which are difficult to analyse. The main facts to emerge were: (*a*) KOH-treated spores absorbed antibodies specific for themselves from both antisera: (*b*) whole vegetative cells apparently absorbed all of the antibodies from the treated spore antiserum. The main conclusion—that the spore contained no specific antigen —would seem to be unwarranted in view of the fact that no antiserum to normal untreated spores was examined. Ferry & Fisher (1924) showed that organisms dissolved in antiformin or NaOH still retained their antigenicity and Professor J. W. Howie (personal communication) found that KOH did not remove vegetative antigen from spore suspensions of Krauskopf & McCoy's organism which normally contained large amounts of vegetative cell material. It is not clear from Krauskopf & McCoy's paper how thoroughly their injection preparations were washed; inadequate removal of dissolved vegetative material might have been responsible for some of their results.

Howie & Cruickshank (1940) reported an investigation of the antigenic properties of spores of aerobic and anaerobic bacteria. Antisera prepared against old cultures of *Clostridium sporogenes* contained both spore and bacillary agglutinins. H and O

bacillary antibodies could be absorbed by the corresponding antigens leaving intact specific spore agglutinins. An organism resembling *Bacillus cereus* spored completely on asparagine agar and gave rise, on injection, to a specific spore antiserum. *B. mesentericus* (probably *B. pumilus* or *B. subtilis*) behaved similarly and with this organism it was shown that autoclaving did not destroy the spore agglutinogens.

Lamanna (1940*a*, *b*) injected old agar cultures of aerobic spore-formers into rabbits and demonstrated agglutinins specific for the spore stage in the resulting antisera by differentially absorbing out bacillary antibodies. Later Lamanna (1942) used an acid extraction method to obtain precipitating antigens from spores of *Bacillus subtilis* and *B. vulgatus* and used them to clarify the relationship between these two organisms.

Bekker (1944) prepared antisera to living and autoclaved spores of *Bacillus anthracis*, *B. mesentericus* and *B. ubiquitarius* and to H and O vegetative antigens of the same organisms. The spore antisera in general showed high agglutinin titres for the homologous antigens and low anti-bacillary titres. Living spores of *B. mesentericus*, however, gave rise, on injection, to considerable amounts of vegetative H antibody which the author attributed either to vegetative material in the spore suspension or to germination occurring after injection. Autoclaved *B. mesentericus* spores gave no such H antibody response. In no case was the antigenicity of the spores affected by autoclaving.

In an extensive investigation involving *Bacillus cereus*, *B. subtilis*, *B. vulgatus*, *B. agri*, *B. brevis* and *B. sphaericus* Doak & Lamanna (1948) showed that treatment of spores with KOH brought about changes in their serological properties. In addition to a high titre of spore agglutinins, antisera to viable spores of *B. cereus* also showed a small amount of bacillary antibody which was attributed to the presence of vegetative debris in the spore suspension. After treatment with KOH a similar spore preparation on injection gave bacillary antibodies in amounts comparable with those of the spore agglutinins. The authors concluded that the spore had antigenic components in common with the vegetative cell but that vigorous treatment was needed to expose them. Prolonged injection of living vegetative cells gave rise to the production of small amounts of spore agglutinins but the authors' conclusion, that this indicated the presence of spore antigens in the vegetative cells, should perhaps be regarded with caution; the possibility of spores being present in small numbers in the injection material needs to be taken into account. Absorption experiments indicated that the antigenic structure of the spore was complex; one strain of *B. cereus* had at least three surface antigens. Both spores and vegetative cells induced the formation of precipitins and these were absorbed simultaneously with the agglutinins. In two cases agglutinins were absorbed by precipitinogens.

Schlossberger (1951) injected KOH-treated spores of *Bacillus sphaericus*, following Doak & Lamanna's (1948) method of purification, but found vegetative cell antibodies as well as spore antibodies in the resulting antisera. A bacillary O antiserum agglutinated KOH-treated spores and treated spores absorbed O agglutinins from antisera prepared against vegetative cell O antigen. O and H agglutinins were absorbed from spore antisera and antibody specific for the spore stage could then be demonstrated.

Schweinsberg (1951) used KOH to remove vegetative cell material from spore suspensions of *Bacillus sphaericus*. Spore suspensions were incubated at 37°

for 6 hr. with M-KOH and then washed three times. Spore antiserum agglutinated KOH-treated spores and also bacillary H and O antigens and the author concluded that spores contained H and O antigens identical with those of the bacilli while vegetative cells contained only minimal amounts of spore antigen. Schweinsberg's own results, however, show that KOH treatment did not inactivate bacillary antigens (especially not the O antigen); the period of washing may well have left residual antigenic material adsorbed to the spore surface. Indeed in this case it is unnecessary to postulate adsorption; *B. sphaericus* spores carry a legacy from the vegetative growth phase in the form of a tenacious sporangium wall remnant which envelops the spore and may well carry antigens derived from the vegetative cell.

Davies (1951) claimed that old nutrient agar cultures of *Bacillus polymyxa* consisted entirely of spores and used such material for the production of antisera. Injection of living spores resulted in the formation of high titre H agglutinins in addition to spore antibodies. This was not the case when heat-killed spores were injected and heated material was used throughout for the production of spore antisera. Bacillary O and H antigens and spore antigens were quite distinct showing no cross agglutination.

Norris & Wolf (1961) showed heat-resistant agglutinogens and precipitinogens in the spores of each of twelve *Bacillus* species and found them to be completely independent of the vegetative cell antigens. With four organisms (*Bacillus cereus*, *B. subtilis*, *B. licheniformis*, *B. alvei*) an examination of the relationship between spore and vegetative cell antigens was made. In the first three of these species the spore contained no vegetative antigens as surface components, but *B. alvei* spore suspensions did contain H antigen, the presence of which might have been associated with the persistence of the wall of the sporangium as an exterior membrane around the spore in this species. Injection of autoclaved spores did not result in the formation of vegetative cell antibodies with any of the twelve species studied. The effect of injecting viable spores was investigated with *B. cereus* and *B. subtilis* and in both cases vegetative H antibodies appeared along with spore antibodies in the resulting antisera. Spore suspensions of these two organisms were not agglutinated by specific H antisera prepared against their vegetative cells and the spores were incapable of absorbing H agglutinins from vegetative cell antisera. The spore antigens in general were not destroyed by autoclaving at 120° for 20 min. and autoclaved spore suspensions were used in the preparation of antisera for taxonomic purposes. Formamide extracts of the spores showed the presence of precipitinogens which reacted strongly with the specific antisera prepared against autoclaved spores. In many instances with both agglutinating and precipitating antigens evidence was obtained of the multiplicity of spore antigens.

Norris (unpublished work) has used gel diffusion techniques to study spore antigens of *Bacillus cereus*, and of crystal-producing *B. cereus* variants such as *B. cereus* var. *alesti* and has found one precipitinogen common to spores of all of these organisms. Several strains show more than one precipitinogen. A detailed study of *B. cereus* var. *alesti*, although showing several different antigens both in the spores and the vegetative cells, showed no sharing of antigens at all between the two growth phases.

Studies of anaerobic organisms have so far been fragmentary as compared with the work on aerobic species. Starin & Dack (1923) injected viable spores of *Clostri-*

dium botulinum into rabbits and obtained a serum which showed no ability to agglutinate vegetative cells of this organism. The serum was not apparently tested against a spore suspension. Moussa (1956, 1959) studied the spore and vegetative cell agglutinogens of *C. septicum* and *C. chauvoei*. No attempt was made to remove bacillary material from the spores before injection but spores for agglutination were incubated for 6 hr. with pancreatic extract—a process which removed most of the vegetative debris but did not impair the antigenicity of the spores. The spores possessed agglutinogens distinct from those of the vegetative cells and the two species shared a common spore antigen. Injection of viable spores resulted in the formation of antibodies reacting with vegetative H and O antigens; this was largely prevented by autoclaving the spores before injection. No attempt was made to study precipitinogens with these organisms.

Sussman (1959) worked with *Clostridium histolyticum* and *C. sporogenes* and showed these organisms to share a spore agglutinogen. Antisera prepared against vegetative cells did not agglutinate spores and antisera prepared by injecting autoclaved spores contained, in addition to spore agglutinins, vegetative cell O agglutinins but no vegetative cell H antibody. Precipitinogens were not studied. Mandia (1955) studied the antigenic structure of *C. tetani*, *C. sporogenes*, *C. parabotulinum* and *C. histolyticum* and noted a heat resistant antigen shared by these species. Although Mandia did not suggest that this antigen is associated with the spore, the antigen preparations he used certainly contained spores and Sussman (1959) suggested that Mandia's heat-resistant agglutinogen may have been a spore antigen.

In a series of publications since 1957, Meisel & Rymkiewicz have extended our knowledge of the spore antigens of anaerobes and have applied their findings to taxonomic problems. These authors (Meisel & Rymkiewicz, 1957) demonstrated spore agglutinogens in *Clostridium tetani*, but found that antisera produced by injecting spore suspensions contained agglutinins active against H and O antigens of the vegetative phase, even when the spores were heated 'to the limit of thermoresistance' before injection. Trypsin, lysozyme and deoxyribonuclease were used to free spores from the antigens of vegetative cells; but the majority of the spores were seen microscopically still to be retained within vegetative cell remnants. Antisera prepared against such enzyme-treated spores still agglutinated vegetative cell antigens as well as heated and enzyme-treated spores. Anti-H and anti-O sera, however, did not agglutinate enzyme-treated spores although they would agglutinate heated spores. The authors concluded on a basis of these findings that vegetative cell H and O antigens are present within spores of *C. tetani*. Similar results were obtained with *C. sporogenes* (Meisel & Rymkiewicz, 1958*a*) and it was shown that enzyme-treated spores gave rise to H and O antibodies even when these spores were autoclaved before injection, so that the result could not be explained by the germination of spores within the animal body. The same pattern of behaviour was later demonstrated in *C. histolyticum* and *C. botulinum*. The authors summarize their findings as follows '...while anti-H and anti-O sera no longer agglutinated enzyme-treated spore-containing suspensions, immunization with such enzyme-treated spores was followed by the formation of two types of antibodies, those reacting with the spore antigens and those reacting with the vegetative cell H and O antigens' (Meisel & Rymkiewicz, 1958*b*). While studying the kappa toxin of *C.*

welchii, Meisel, Albrycht & Rymkiewicz (1959) obtained the collagenase—which was neutralized by anti-welchii serum but not by anti-histolyticum serum or normal horse serum—in extracts of enzymically 'purified' spores.

The value of spore antigens in taxonomy

Taxonomy of the aerobic spore-formers. The classical work of Smith, Gordon & Clark (1952), while bringing order into the classification of the genus *Bacillus*, has led to the suppression of many species names. The interpretation of some of the earlier work on spore antigens is difficult since incorrectly labelled or inadequately described species were frequently used. Nevertheless, one cannot help but be impressed by the degree of consistency between the findings of different workers when these are analysed in the light of changing nomenclature and present-day taxonomy.

Defalle (1902) reported cross agglutination between spores of *Bacillus mycoides* and the anthrax bacillus. A *B. mycoides* serum gave high titre agglutination with spores of *B. mycoides*, *B. subtilis* and the anthrax bacillus, and low titre agglutination with those of '*B. mesentericus vulgatus*' and *B. alvei*. By using the complement-fixation technique cross-reactions were demonstrated between spores of *B. mycoides*, '*B. mesentericus vulgatus*' and *B. subtilis*. *B. mycoides* and the anthrax bacillus are now known to be closely related to one another and the finding that they possess a spore antigen in common is not surprising. Neither is the cross-reaction between *B. mycoides* and *B. subtilis* when it is realized that the strain of *B. subtilis* concerned was almost certainly one of the 'large-celled' (Michigan) strains, later shown by Conn (1930) to be *B. cereus*. '*B. mesentericus vulgatus*' would appear to have been *B. licheniformis*, *B. pumilus* or a true *B. subtilis* and the *B. alvei* used was probably correctly named. Thus the picture emerging from Defalle's work is not at variance with more recent ideas on classification.

The first extensive taxonomic work involving the use of spore antigens was that of Lamanna (1940*a*, *b*) who used a shaking method based on that of Noble (1919) to study spore agglutinogens. With the 'small celled' species Lamanna was able to demonstrate three clear-cut serological groups: a *Bacillus subtilis* group, a *B. mesentericus* group and a *B. agri* group. Included in the *B. mesentericus* group was *B. vulgatus* Flugge. Lamanna's *B. subtilis* was the Ford strain now known as *B. licheniformis* (Gibson, 1937, 1944). His *B. vulgatus* was the true (Marburg) strain of *B. subtilis* and his *B. agri* is now recognized as *B. brevis*. *B. mesentericus* appears to have been a *B. pumilus* and the cross-reaction between *B. pumilus* and *B. subtilis* serves to emphasize the close relationship which exists between these two species as judged from their morphological and physiological characteristics and is in agreement with the later findings of Norris & Wolf (1961). Less success attended the investigation of 'large celled' species. *Bacillus cereus* could be separated from other large-celled organisms and some strains of *B. mycoides* (which is now considered to be a variety of *B. cereus*) were shown to cross-react with this species. Later Lamanna prepared acid extracts from spores of the Marburg and Ford strains of *Bacillus subtilis* and from *B. vulgatus* and showed them to give precipitin reactions with the homologous spore antisera. Extracts from the Marburg strain and from *B. vulgatus* cross-reacted with one another, thus supporting the conclusion, arrived at by a study of their biochemical behaviour, that these two organisms were identical

and that the Ford strain was a distinct species (Lamanna, 1942). The Marburg strain is now recognized as the true *B. subtilis* and the Ford strain is re-named *B. licheniformis* (Gibson, 1937, 1944). Bekker (1944) studied only a few strains of *B. anthracis*, *B. mesentericus* and *B. ubiquitarius* but found spore agglutinogens to be species specific in his limited series of organisms. *B. mesentericus* in this case was probably *B. pumilus*. *B. ubiquitarius* cannot be identified.

Shattock (1955) quoted a personal communication from Davies & Proom to the effect that spore antigens are species specific in several species of aerobic spore-formers; Chu (1951) used spore agglutination reactions with some success to distinguish between *B. cereus* and the anthrax bacillus. These species had antigens in common but cross-absorption enabled useful diagnostic antisera to be produced.

Doak & Lamanna (1948) found cross agglutination between *B. cereus*, *B. brevis* and *B. sphaericus* spores and between those of *B. brevis* and *B. agri*, the latter organisms probably being identical.

The work of Davies with *Bacillus polymyxa* (1951) did much to focus attention on the taxonomic possibilities of spore antigens. Spores of all of the thirty-nine strains of *B. polymyxa* investigated were agglutinated by the seven spore antisera prepared and were capable of absorbing all of the homologous agglutinins. No cross-reactions occurred with the spores of fifteen other *Bacillus* species comprising eighty-one different organisms. Precipitinogens were not studied in this investigation.

Norris & Wolf (1961) studied twelve different *Bacillus* species in an attempt to extend the work of Davies with *B. polymyxa* and to assess the taxonomic value of spore and vegetative cell antigens in aerobic spore-formers. With some organisms, for example, *B. subtilis* and *B. licheniformis*, the spore agglutinogens proved to be species specific, being represented in each of a considerable number of strains of the species. But this was not the case with other species. For instance, with *B. cereus* an antiserum to autoclaved spores of one strain agglutinated spores of only seven of twenty-seven different strains. Spore precipitinogens on the other hand proved generally to be species specific. *B. cereus*, *B. megaterium*, *B. subtilis*, *B. pumilus*, *B. licheniformis*, *B. coagulans*, *B. polymyxa*, *B. circulans*, *B. alvei*, *B. brevis*, *B. laterosporus* and *B. sphaericus* each possessed a spore precipitinogen characteristic of each and every strain of the species studied. Spores of *B. pumilus* contained at least two agglutinogens one of which was shared with *B. subtilis*, thus tending to confirm the very close relationship between these two organisms suggested by their physiological properties. Faint cross-reactions involving precipitinogens were observed between spores of *B. cereus* and *B. megaterium*, *B. subtilis* and *B. pumilus*, *B. pumilus* and *B. coagulans*, and between *B. alvei* and *B. sphaericus*, but in each case these reactions were easily distinguished from the strong specific reactions. Spore extracts of *B. circulans* and antisera prepared against autoclaved spores of *B. circulans* showed a wide tendency to cross-precipitation with spore extracts and antisera of representatives of the other species; but, again, these reactions were readily distinguished from the strong reaction seen with strains of the homologous species.

Walker (1959) studied thermophilic *Bacillus* species and recognized three groups based on physiological characteristics. His group 1 could be subdivided into two clearly related but not identical biochemical types. He was able to show spore agglutinogens in groups 1*a*, 1*b*, and 2 but injection of spore material of representa-

tives of his group 3 led to the death of experimental animals and spores of these organisms could not be studied antigenically. The spore antigens of group 1*a* proved to be group specific, that is to say they were represented in all the strains studied; but this was not so with the corresponding antigens of group 1*b*, where evidence of at least two spore agglutinogens was obtained. A small proportion of group 1*b* strains was agglutinated by group 1*a* antisera. Spore agglutinogens of group 2 proved to be group specific and were not represented in members of the other groups. In spite of several attempts by different methods Walker was unable to extract precipitinogens from the spores of any of these thermophilic aerobic bacteria. He was also unable to demonstrate any cross-reaction between thermophilic and mesophilic members of the genus *Bacillus*.

Lamanna & Eisler (1960) used spore agglutinogens to attempt to distinguish between *Bacillus anthracis* and *B. cereus* but were unable to do so. Antisera prepared against washed spores and absorbed with the homologous vegetative cells showed widespread cross-reactions when tested for ability to agglutinate spores of several strains of the two organisms. Spore precipitinogens received only a cursory examination, but here also spore extracts from the two organisms reacted with antisera to both. Agglutination and agglutinin absorption tests were used by Lamanna & Jones (1961) to study the relationship between *B. cereus*, *B. cereus*-like insect pathogens and *B. anthracis*. Extensive cross-agglutination occurred and these organisms could not be distinguished from each other serologically. My own unpublished observations agree with these findings.

Dowdle & Hansen (1961) studied a phage-fluorescent antiphage staining system for identifying the anthrax bacillus and included some observations with fluorescent spore antibodies. Fluorescent staining of spores was obtained when a *Bacillus anthracis* spore antiserum was tested against all of twenty-nine strains of *B. anthracis*, thirty-three of thirty-six *B. cereus* including ten *mycoides* types, three of three *B. thuringiensis*, one of six *B. licheniformis*, one of seven *B. megaterium*, and two of thirteen *B. subtilis*. These results accord well with those of other workers but suggest a somewhat wider distribution of anthrax spore surface antigen among the various species.

Taxonomy of the anaerobic spore-formers. The classification of the anaerobic spore-formers, especially of the non-pathogenic species, still presents problems and the study of clostridial spore antigens is complicated by the technical difficulty of obtaining satisfactory spore yields. Studies with such antigens are as yet few, but the findings strongly suggest that, as with the aerobes, they may be of great value to the taxonomist. Moussa (1956, 1959) demonstrated a common spore agglutinogen in *Clostridium septicum* and *C. chauvoei* and Sussman (1959) made a similar observation with *C. histolyticum* and *C. sporogenes*. Meisel & Rymkiewicz (1959) investigated the applicability of spore agglutination reactions to the classification of various *Clostridium* species. With thirty strains of *C. sporogenes*, *C. histolyticum*, *C. botulinum* types A and B, *C. sordelli*, *C. bifermentans* and *C. tetani*, they showed species specific agglutinogens in each case and also detected minor antigens. The only instance of inter-species cross-agglutination occurred between *C. sporogenes* and *C. histolyticum*, a finding which agrees with that of Sussman (1959). The only studies involving clostridial spore precipitinogens appear to be the preliminary observations of Dr P. D. Walker (personal communication) who has compared the agglutinating

and precipitin reactions of several spore antisera. He has found that precipitinogens can readily be extracted from spores of anaerobes and that they show a pattern of species specificity in their distribution.

The antigenic architecture of the spore

The use of classical serological techniques of the kind discussed above provides the investigator with little information about the site and chemical nature of the antigens studied. Agglutinogens may be assumed to be surface components of the cell but the chemically-extractable precipitating antigens (almost certainly polysaccharide haptens in the extracted form) may be surface or deep seated components. Recently methods have been developed for studying in greater detail the distribution of antigens in different parts of the cell. A few attempts have been made to apply these techniques to the problems of antigen composition of spores. Tomcsik (1956) observed bacteria suspended in specific antisera under the phase-contrast microscope and obtained a great deal of information about the distribution of antigens at the cell surface and in particular cell components. Fowler & Harrison (1953) first noted that when spores of *Bacillus subtilis* reacted with specific agglutinating antisera at dilutions such that pre-zoning occurred an 'exudate' could be seen around the periphery of the spores under the phase-contrast microscope. Tomcsik & Baumann-Grace (1959) have taken this observation further with *B. megaterium*. By the use of lysozyme, pure spore suspensions were produced and spore antisera were prepared which agglutinated the homologous antigen. By phase-contrast specific antigen-antibody reactions could be seen around spores suspended in spore antiserum. Wet Indian ink preparations demonstrated a layer of viscous material surrounding spores of several strains and it was apparently antigens associated with this layer which were responsible for the observed reaction. Antisera prepared by injecting vegetative cell antigens failed to elicit this reaction. The authors referred to the slimy layer as the 'exosporium' and to the reaction as the 'specific exosporium reaction'. However, the term exosporium has been used loosely in the literature and should be reserved for the discrete membrane surrounding free-lying spores of certain species—particularly *B. cereus* and its varieties (Norris & Watson, 1960). Empty spore walls and incompletely germinated spores gave a specific 'spore coat' reaction analogous to the cell-wall reaction seen with vegetative cells in the presence of specific antisera. Agglutination tests revealed a complex cross-reaction pattern with spores of different strains of *B. megaterium*. There were no species specific spore agglutinogens and thirty-six strains could be divided into five antigenic types. Spore antisera did not react at all with vegetative cells.

Cherry & Freeman (1959) prepared antisera against encapsulated vegetative cells of the anthrax bacillus and found that these antisera gave a precipitin reaction with supernatant fluids from cultures of capsulated and non-capsulated strains. Spores resisted disintegration by freezing and thawing and by the use of a homogenizer. But they could be disintegrated by thorough shaking with glass beads, when precipitating antigen was released into the supernatant fluid and could be detected by antiserum prepared against capsulated organisms. It should be noted that the materials used for injection almost certainly contained small numbers of spores. The authors prepared fluorescent antibodies and used them primarily to detect bacillary antigen in cultures of the anthrax bacillus and in tissue sections

from infected animals, and from human anthrax cases. The main reactions observed were associated with capsular antigens. Intracellular spores did not take up fluorescent antibody but in some cases slight staining of the periphery of free-lying spores was observed. In some instances it was clearly shown that the spores retained on their surface capsular material of bacillary origin. Capsular staining was not given by fluorescent spore antiserum.

Vennes & Gerhardt (1959) carried out detailed studies of the antigenic characteristics of isolated structures and surface components of *Bacillus megaterium* and proposed to extend this work to include the spores of this species. In a brief note (Vennes & Gerhardt, 1957) these authors reported that they were unable to demonstrate common antigens between spore coats and vegetative cells in *B. megaterium*. The use of separated spore coats excluded the possibility of spores germinating on injection in these experiments.

Antigen changes at spore-formation and germination

Only one published observation refers to the antigenic changes associated with the passage of the cell from vegetative to spore phase or vice versa. Howie & Cruickshank (1940) tested spores of an organism similar to *Bacillus cereus* at different stages in germination, against spore and vegetative cell antisera. Spore agglutinogens decreased sharply during germination and bacillary antigens appeared.

Gel diffusion analysis and immuno-electrophoresis have enabled J. R. Norris & R. Przyborowski (unpublished observations) to show antigenic changes during sporulation of *B. cereus* and *B. cereus* var. *alesti* which show a pattern of behaviour more complicated than might have been assumed from a study restricted to agglutinogens. Both stages are antigenically complex, with several antigens readily demonstrable in both the vegetative and spore phases. The disappearance of bacillary antigens and the simultaneous appearance of spore antigens are clearly seen when cells at different stages in spore-formation are tested against vegetative cell, sporulating cell and spore antisera. Spore antigen appears first in a readily extractable form but becomes more intractable as the spore assumes its mature state.

Conclusions

That the bacterial endospore possesses antigens which are not represented in the vegetative growth phase is now well established; heat-stable agglutinogens and precipitinogens have been demonstrated as specific spore antigens in a wide variety of aerobic and anaerobic species, and indeed, considering the striking changes in morphology and chemical constitution which accompany sporulation, it would be surprising if there were not corresponding changes in the antigenic composition of the cell. It is more difficult to determine whether or not spores also contain antigens characteristic of vegetative cells. Injection of spores, living or dead, usually results in the formation of antibodies which react with bacillary antigens as well as specific spore antibodies, but the former must often result from the presence of vegetative debris in the spore preparations. Observations based on the use of spore suspensions from which bacillary material has been removed by chemical or enzymic treatments must be interpreted with caution since processes which result in the visual removal of cell remnants often do not inactivate the associated antigens. When we consider

the anatomy of mature spores we find that they are of three types: (i) naked spores where the spore escapes completely from the disintegrating sporangium; (ii) spores which escape from the sporangium but are completely surrounded by a membranous exosporium which is synthesized within the sporangium at sporulation so that the outer surface of the mature spore is no longer part of the spore wall proper; (iii) spores which remain permanently encased within the wall of the sporangium. Organisms of the first type (such as *Bacillus subtilis* and *B. megaterium*) show a clear-cut morphological break between the spore and vegetative phases; this is also true of *B. cereus* where the exosporium is a true 'spore structure' absent from young vegetative cells. Organisms of the third type, however (such as *B. alvei*, *B. sphaericus* and *Clostridium tetani*), show no such sharp distinction between the two phases and such information as we possess suggests that there is a corresponding carry-over of surface bacillary antigens into the spore stage of these organisms.

It would be reasonable to expect that the spore protoplasm would contain antigens or antigen precursors associated with the vegetative cell but claims that this has been demonstrated appear, on careful scrutiny, to be open to question and no definite conclusions can safely be drawn. Unequivocal proof seems most likely to come from an exhaustive analysis of spore and bacillary components of the type planned by Vennes & Gerhardt (1959).

Knaysi (1948), reviewing the state of knowledge of the bacterial endospore, concluded that 'In view of the sad state of the taxonomy of the spore-formers it is hoped that further study of the spore antigens will prove helpful in developing a sound knowledge of this interesting and important group of bacteria'. Since then order has come to the genus *Bacillus*, largely as a result of the work of Smith *et al.* (1952) in the U.S.A. and of Knight & Proom (1950) and of Proom & Knight (1955) in Britain and there remain relatively few taxonomic problems connected with the aerobic spore-formers. A few organisms are of uncertain status and some strains appear to be intermediate in type between recognized species, for instance, between *B. cereus* and *B. megaterium* and between *B. alvei* and *B. circulans*. It has been suggested that non-pathogenic strains of *B. cereus* can, by serial passage under appropriate conditions, be converted into pathogenic strains of crystal-forming insect pathogens (LeCorroller, 1958; Toumanoff, 1956). Norris & Wolf (1961) studied the spore precipitinogens of a few intermediate *Bacillus* types and were able to allocate them to definite species and it seems likely that a study of spore antigens could help in the solution of problems of this kind.

From the point of view of taxonomy it is with the anaerobes that spore antigens seem most likely to make valuable contributions. Progress is likely to be governed by the development of suitable media and methods for spore production which is often poor with the clostridia. Nevertheless, interesting results have come from the few studies so far reported. The anaerobic spore-formers offer a promising field for research into the nature and taxonomic significance of spore antigens.

The resistance of bacterial spores to drying and to degrees of heat rapidly fatal to vegetative cells is of great practical importance. But the resistance of spores, their dormancy and their ability to germinate (sometimes in a matter of seconds) when exposed to suitable conditions are also characteristics of fundamental biological importance. Much attention is being directed today towards the elucidation of the details of spore structure and of the processes of spore formation and germination.

Naturally enough cytologists and the biochemists are the most active participants in this work and recent symposia (Halvorson, 1957; Jacobs & Clegg, 1957) consist almost entirely of their contributions and bear witness to the extensive advances which are being made. The peculiar properties of the spore, however, are likely to be reflected in the nature of the proteins and other antigenic materials of which it is composed and a more detailed knowledge of these antigens and of their synthesis is eminently desirable. Serological techniques are extremely sensitive and their application to the investigation of spore structure could well be rewarding from several points of view. I hope that this review, by collecting together the information already available and suggesting lines along which the subject might profitably be developed, may stimulate research and contribute towards a better understanding of spore-forming bacteria and of the spores which they produce.

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The Effect of Amino Acid on the Uptake and Utilization of Tryptophan and other Precursors of Nicotinic Acid by *Neurospora crassa*

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SUMMARY

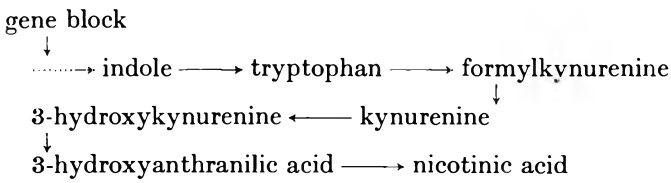
The influence of casein hydrolysate and certain amino acids on the uptake and utilization of tryptophan and other precursors of nicotinic acid biosynthesis by a mutant of *Neurospora crassa* which can grow on tryptophan or nicotinic acid was investigated. Whereas casein hydrolysate and certain amino acids inhibited the growth of the organism when tryptophan, kynurenine, or 3-hydroxykynurenine was present in the medium, they had no effect in the presence of other precursors, i.e. 3-hydroxyanthranilic acid or nicotinic acid. Studies have been made with wild strain and the mutant on the amount of tryptophan taken up from the medium at different periods of growth of the organism.

INTRODUCTION

One of the factors which seems to affect the conversion of tryptophan to nicotinic acid is the influence of certain proteins, when fed at concentrations with a tryptophan-low or nicotinic acid-free ration (Krehl, Sarma & Elvehjem, 1946). This relationship between the 'level of dietary protein' and 'nicotinic acid requirement' was attributed to the various amino acids present in the protein and in turn to their influence on the digestive tract (Hanks, Henderson, Brickson & Elvehjem, 1948). However, it was suggested by Rosen, Huff & Perlzweig (1946) and Goryachenkova (1951) that the effect of the protein or the amino acids contained therein might result from an inhibition produced in the conversion of tryptophan to nicotinic acid, involving certain enzyme systems participating in the conversion. Mathieson & Catcheside (1955) in studies on histidine-requiring mutants of *Neurospora crassa* observed that some related amino acids inhibited the uptake of histidine. It was reported by Shanmugasundaram & Sarma (1955) that certain protein hydrolysates when present in the medium inhibited the growth of a nicotinic acid-requiring mutant of *N. crassa*. In extending this observation, experiments have now been made to investigate the effect of casein hydrolysate, certain amino acids and B group vitamins on the uptake and utilization of tryptophan and its metabolites by a nicotinic acid-requiring mutant of *N. crassa*.

METHODS

Organism and media. The strain (no. 5297*a*) of *Neurospora crassa* and a mutant strain of it (no. 39401) kindly supplied by Dr H. K. Mitchell (California Institute of Technology, U.S.A.) were used in this work. The mutant strain is able to utilize tryptophan and other precursors for nicotinic acid synthesis shown below; the gene block is located before indole:



The organism was grown in Fries's medium as described by Horowitz & Beadle (1943); it had the following composition (g./l.) ammonium tartrate, 5; ammonium nitrate, 1·0; K_2HPO_4, 1·0; $MgSO_4.7H_2O$, 0·5; NaCl and $CaCl_2$, 0·1 (each); D-glucose, 20. The following trace elements (mg./l.) were also incorporated: B, 0·01; Fe, 0·2; Mo, 0·02; Cu, 0·1; Mn, 0·02; Zn, 2·0. In addition, biotin was added at 5 μg./l.

The pH value of the medium depended upon the tryptophan metabolite used. The medium was adjusted to pH 4·6 in the case of nicotinic and 3-hydroxyanthranilic acids and to pH 5·6 in the case of tryptophan, kynurenine and 3-hydroxykynurenine (Beadle & Tatum, 1945). With 3-hydroxyanthranilic and nicotinic acids, the effects of casein hydrolysate and amino acids were tried only at the optimum for growth, namely pH 4·6 (Bonner & Beadle, 1946), since they are less active at higher pH values, where dissociation becomes greater; at pH 5·6 the precursors become much less active and are not utilized quantitatively (Mitchell & Nyc, 1948). Ten or 50 ml. of medium with necessary substrates were dispensed in 50 ml. or 250 ml. conical flasks, respectively, and sterilized at 121° pressure for 15 min. The inoculum was prepared by suspending in water washed spores from a 5–6 day slope, and adjusting the turbidity to about 80 % transmission with a Lumetron photoelectric colorimeter model 400A. One or two drops of spore suspension containing *c.* $1{\cdot}5 \times 10^8$ spores/ml. were added per flask and incubation carried out at room temperature (28 to 30°) for 72 hr. The mycelia were then collected, washed, partially dried between folds of filter paper and then dried to constant weight. The mycelia were then weighed in a Roller–Smith torsion balance.

Studies of the effect of casein hydrolysate on the uptake of tryptophan were done either by (i) directly inoculating spores into experimental media or (ii) transferring mycelium grown for 48 hr. in medium supplemented with tryptophan into experimental media.

Preparation of casein hydrolysate. The casein hydrolysate was prepared as described by Snell (1950) and the nitrogen was determined by the conventional micro-Kjeldahl method.

Estimation of tryptophan. Tryptophan was estimated colorimetrically with p-dimethylaminobenzaldehyde (Spies & Chambers, 1948) and (N-naphthyl)-ethylenediamine dihydrochloride (Eckert, 1943).

RESULTS

The growth response of the mutant strain of *Neurospora crassa* (39401) to the various precursors of nicotinic acid was determined. The concentrations of the precursors for maximal growth are as follows (μg./10 ml.). L-tryptophan, 200; DL-kynurenine sulphate, 120; DL-3-hydroxykynurenine, 80; 3-hydroxyanthranilic acid, 25; nicotinic acid, 7·5.

The influence of casein hydrolysate, pure amino acids and certain B group vitamins on the utilization of tryptophan, etc., was studied at a concentration of precursor at or slightly above the optimum concentration, so that any decrease in growth caused by the addition of test substance (casein hydrolysate or amino acid) would be due only to the effect of the test substances and not to a partial deficiency with respect to tryptophan or other precursor.

Casein hydrolysate inhibited the growth of the *Neurospora crassa* mutant 39401 when tryptophan, kynurenine, or 3-hydroxykynurenine was the precursor added to the medium (Fig. 1); it had no effect even at high concentrations with 3-hydroxyanthranilic or nicotinic acids. Since casein hydrolysate is mostly a mixture of amino acids, the effects of individual amino acids were tested. Of twenty-one amino acids tested the following inhibited the growth of the mutant when tryptophan, kynurenine or 3-hydroxykynurenine were the precursors in the medium, namely: glycine, DL-alanine, β-alanine, L-cysteine, DL-threonine, DL-serine, DL-methionine, DL-valine, L-leucine, DL-isoleucine, DL-β-phenylalanine and L-tyrosine. These amino acids had no effect with 3-hydroxyanthranilic acid or nicotinic acid. Casein hydrolysate had no effect on the growth of the wild strain, even when added at the high concentration of equiv. 1·0 mg. N/ml. medium.

Here therefore is a case where an amino acid inhibited the growth of a mutant requiring tryptophan. A similar observation was made by Mathieson & Catcheside (1955) with a histidineless mutant of *Neurospora crassa*, where some amino acids (including L-arginine, DL-methionine, L-lysine, L-tryptophan, glycine, DL-β-phenylalanine, L-leucine, DL-valine, DL-isoleucine singly, and L-arginine+L-lysine) inhibited the growth of their mutant. The growth inhibition was due to interference with uptake of histidine. However, Bonner & Beadle (1946) showed that *N. crassa* mutant 39401 is a single gene mutant, with the genetic block placed before indole in the sequence of reactions leading to the formation of nicotinic acid from tryptophan. The enzymes and various factors affecting this biosynthetic pathway have been studied to some extent in other organisms. It appeared to be of interest to see whether the inhibition in growth by casein hydrolysate is due to inhibition of the tryptophan uptake or of the utilization of tryptophan in nicotinic acid biosynthesis.

To test whether the growth inhibition by casein hydrolysate was due to inhibition of tryptophan uptake, experiments were made on lines similar to those of Mathieson & Catcheside (1955). On the assumption that if inhibition of growth of the mutant by casein hydrolysate is because of interference with tryptophan uptake and not with its utilization, casein hydrolysate added to media inoculated with growing mycelia might be ineffective in preventing further growth, whereas it might prevent growth from a spore inoculum. The results of such experiments (Tables 1, 2, 3) with casein hydrolysate supplied alone and with tryptophan showed that casein hydrolysate inhibited the growth of the mycelia of the mutant (Tables 2, 3)

suggesting that the influence of casein hydrolysate might not be solely on the uptake of tryptophan. However, examination of the results in Tables 2 and 3 shows that casein hydrolysate had at least two effects: (i) it inhibited growth at all concentrations tested during the first 24 hr. after the addition; (ii) then after a lag depending on concentration, it caused a considerable growth stimulation, which except at the highest concentration after 2–3 days more than made up for the initial inhibition.

Table 1. *Influence of casein hydrolysate on the uptake of tryptophan; inoculation with spores of* Neurospora crassa *mutant* 39401

The experimental mixtures were made up from 25 ml. lots of double strength medium (Fries) + supplement + water to 50 ml. final volume. Results are the averages of replicates.

Supplement to medium	% initial tryptophan remaining in the medium at the end of			Weight (mg. dry wt.) of mycelium after 72 hr. growth
	6 hr.	24 hr.	48 hr.	
Nil (control)	—	—	—	—
1·25 mg. L-tryptophan	82·7	54·3	40·8	28·6
1·25 mg. L-tryptophan + equiv. 5 mg. N of casein hydrolysate	91·4	54·3	32·4	110·4
1·25 mg. L-tryptophan + equiv. 6 mg. N of casein hydrolysate	97·2	54·3	32·4	95·0
1·25 mg. L-tryptophan + equiv. 7·5 mg. N of casein hydrolysate	100·0	54·1	46·4	35·4
1·25 mg. L-tryptophan + equiv. 8 mg. N of casein hydrolysate	100·0	54·1	46·4	8·0

Table 2. *Effect of casein hydrolysate on uptake of tryptophan; inoculation with mycelium of wild strain of* Neurospora crassa

The experimental mixtures were made up from 25 ml. lots of double strength medium (Fries) + supplement + water to final volume 50 ml. Weight of mycelia as inoculum: 70·8 mg. Results are the averages of replicates.

Supplement to medium	% initial tryptophan remaining in culture filtrate at the end of			Weight (mg. dry wt.) of mycelium at the end of		
	24 hr.	48 hr.	72 hr.	24 hr.	48 hr.	72 hr.
Nil	0	8	8	110·4	174·6	194·6
1·25 mg. L-tryptophan	66·6	55·0	42·5	108·8	140·0	150·8
1·25 mg. L-tryptophan + equiv. 5 mg. N of casein hydrolysate	66·6	37·5	22·5	127·8	178·8	256·4
1·25 mg. L-tryptophan + equiv. 7 mg. N of casein hydrolysate	85·0	37·5	20·8	114·8	161·8	220·8
1·25 mg. L-tryptophan + equiv. 10 mg. N of casein hydrolysate	90·0	62·5	32·5	130·0	164·6	216·8

Table 3. *Effect of casein hydrolysate on uptake of tryptophan by growing mycelia of mutant strain of* Neurospora crassa (39401)

The experimental mixtures were made up from 25 ml. lots of double strength medium (Fries) + supplement + water to 50 ml. Results represent averages of replicates.

Supplement to medium	% initial tryptophan remaining in culture filtrate at the end of			Weight (mg. dry wt.) of mycelium at the end of		
	24 hr.	48 hr.	72 hr.	24 hr.	48 hr.	72 hr.
Nil (control)	—	—	—	5·6	10·0	14·6
1·25 mg. L-tryptophan	78·3	52·2	34·7	16·2	43·0	59·0
Equiv. 5·0 mg. N of casein hydrolysate	—	—	—	5·6	7·8	10·4
1·25 mg. L-tryptophan + 5 mg. N of casein hydrolysate	95·6	56·5	21·8	8·0	64·0	94·6
8 mg. N of casein hydrolysate	—	—	—	4·2	3·8	7·0
1·25 mg. L-tryptophan + 7 mg. N of casein hydrolysate	100·0	60·9	43·5	8·6	55·6	94·8
10 mg. N of casein hydrolysate	—	—	—	4·0	3·8	7·8
1·25 mg. L-tryptophan + 10 mg. N of casein hydrolysate	100·0	69·6	41·3	7·4	14·2	13·0

Table 4. *Influence of certain group B vitamins alone and in the presence of casein hydrolysate on the utilization of tryptophan, kynurenine and 3-hydroxykynurenine by* Neurospora crassa *mutant* (39401)

In all experiments to 5 ml. double strength concentrated medium (Fries), the supplements were added in solution and the final volume was adjusted to 10 ml. before autoclaving. The mycelia were removed after growth for 72 hr. Results represent averages of replicates.

B-group vitamin added	Metabolite: 250 μg. L-tryptophan/flask		120 μg. DL-kynurenine sulphate/flask		75 μg. DL-3-hydroxykynurenine/flask	
	Without casein hydrolysate	With casein hydrolysate equiv. 1·5 mg. N	Without casein hydrolysate	With casein hydrolysate equiv. 3·75 mg. N	Without casein hydrolysate	With casein hydrolysate equiv. 1·5 mg. N
	Mycelium harvested (mg. dry wt.)					
Control	24·1	14·1	28·2	12·1	27·0	13·9
Thiamine (1 mg.)	23·2	25·7	28·4	40·8	27·4	20·1
Riboflavin (1 mg.)	14·2	8·1	27·8	16·0	26·3	13·4
Pyroxidine (1 mg.)	23·0	26·2	28·3	18·6	26·5	14·1
p-amino-benzoic acid (1 mg.)	25·2	15·1	27·9	14·8	27·4	13·5
Folic acid (1 mg.)	25·0	27·1	27·6	—	26·3	—
Calcium pantothenate (1 mg.)	25·2	14·4	28·2	15·1	27·9	13·1
Choline (2 mg.)	25·4	26·0	28·0	43·7	27·1	21·5
Inositol (1 mg.)	25·0	13·8	28·5	15·5	27·1	13·6
Vitamin B12 (2 μg.)	24·6	13·6	27·9	16·1	27·3	13·3

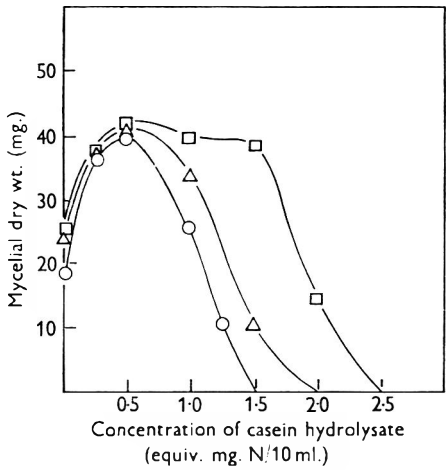


Fig. 1

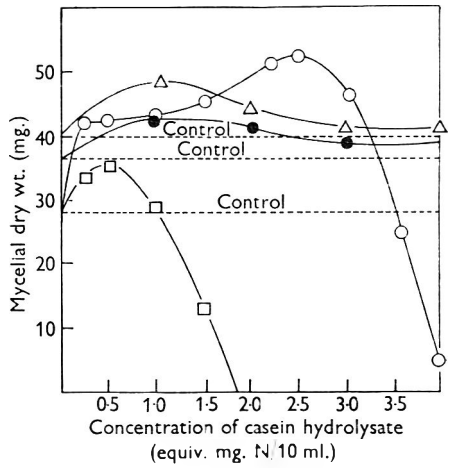


Fig. 2

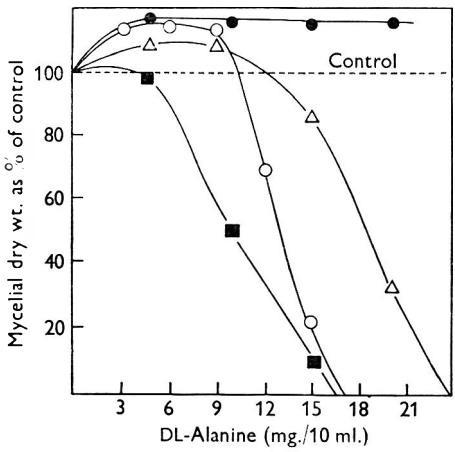


Fig. 3

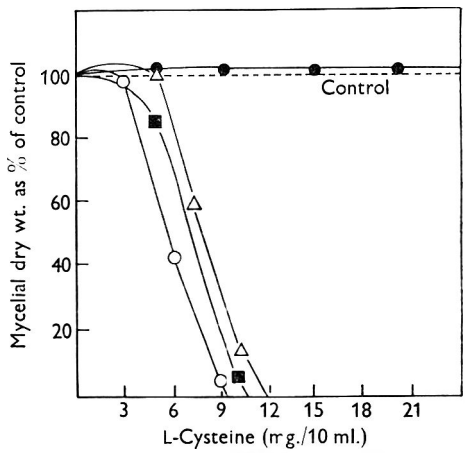


Fig. 4

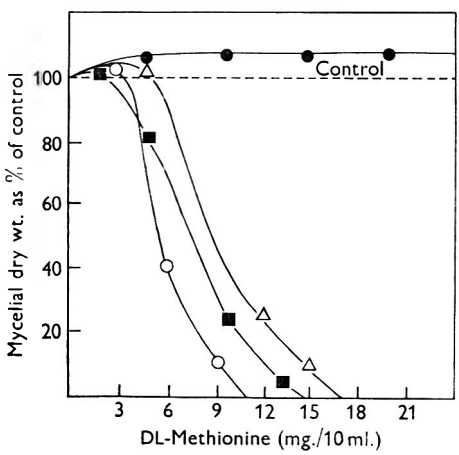


Fig. 5

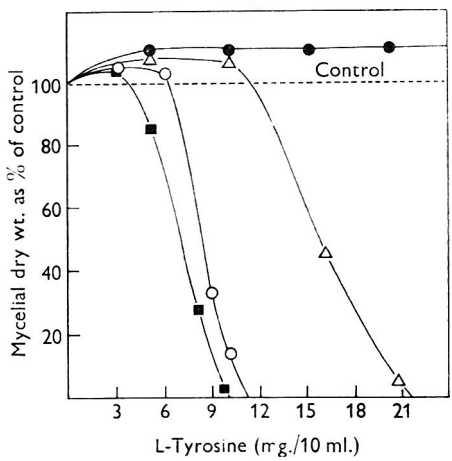


Fig. 6

For legends see foot of facing page.

The initial inhibition seems to be associated with an almost complete inhibition of tryptophan uptake.

Growth stimulation by casein hydrolysate or certain amino acids at lower concentrations can be seen in Figs. 1–6. However, at higher concentrations, growth was inhibited. As the concentration of tryptophan in the medium was increased, greater amounts of casein hydrolysate or amino acid were needed to produce inhibition of growth. This suggests the possibility of the existence of a competition between the amino acids in casein hydrolysate and tryptophan or its metabolites either for uptake or for enzymes normally involved in tryptophan metabolism after uptake. That casein hydrolysate and certain amino acids inhibited the activity of the enzyme, kynureninase in tryptophan metabolism was observed in rats by Goryachenkova (1951) and in *Neurospora crassa* by Jakoby & Bonner (1953). The inhibitory amino acids formed in the present work are more or less the same as those observed in the enzymic studies just mentioned, suggesting that the influence of casein hydrolysate and amino acids may be to some extent on the utilization of tryptophan.

The inhibitory amino acids are mono-amino mono-carboxylic acids containing an alanine side chain and the inhibited metabolites too (tryptophan, kynurenine, 3-hydroxykynurenine) have alanine side chains. This suggests an inhibitory function of the amino acids by competition involving the alanine moiety of the three metabolites. This seems to be supported by the absence of inhibition when the organism is supplied with other metabolites, namely 3-hydroxyanthranilic acid and nicotinic acid, where no alanine side chain is present.

Another possible effect of casein hydrolysate may be that it may produce in the *Neurospora crassa* some B group vitamin-requirement; it is known that some vitamins are involved in the conversion of tryptophan to nicotinic acid (Dalgleish, 1951). From Table 4 it can be seen that the inhibition produced by casein hydrolysate in the case of the metabolite tryptophan was annulled by thiamine, pyridoxine, folic acid or choline whereas kynurenine inhibition was annulled by thiamine or choline. Thiamine and choline decreased the inhibition with 3-hydroxykynurenine as metabolite. These vitamins do not have any effect when added alone and are normally synthesized by the organism.

Fig. 1. Influence of casein hydrolysate on the growth of *Neurospora crassa* mutant 39401 with tryptophan as nicotinic acid precursor.

Each flask contained 5 ml. double strength medium (Fries) with tryptophan and casein hydrolysate at various concentrations; final volume 10 ml. L-tryptophan 200 mg., ○—○; L-tryptophan 250 mg., △—△; L-tryptophan 300 mg., □—□.

Fig. 2. Influence of casein hydrolysate on the growth of *Neurospora crassa* mutant 39401 with tryptophan metabolites in the medium.

Each flask contained 5 ml. double strength medium, with the metabolites added for maximal growth and casein hydrolysate at various concentrations; final volume 10 ml. DL-kynurenine sulphate, ○—○; D-3-hydroxyanthranilic acid, △—△; DL-3-hydroxykynurenine, □—□; Nicotinic acid, ●—●.

Figs. 3–6. Influence of certain amino acids on the growth of *Neurospora crassa* mutant 39401 with tryptophan or other nicotinic acid precursors.

Each flask contained 5 ml. double strength basal medium with precursor added for maximal growth and the amino acid added at various concentrations as solution in water; final volume of 10 ml. L-tryptophan, ○—○; DL-kynurenine sulphate, △—△; 3-hydroxykynurenine, ■—■; nicotinic acid, ●—●. Observations for 3-hydroxyanthranilic acid are almost the same as for nicotinic acid and hence not presented in the Figures.

Our thanks are due to Dr C. P. Berg, Dr B. S. Schweigert and Professor A. Butenandt for the generous gift of tryptophan metabolites and to the University Grants Commission (India) for the award of a pre-doctoral merit scholarship to Miss R. Panicker. Some of the results presented herein were incorporated in an M.Sc. thesis submitted to the University of Madras by U. S. Acharya.

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Ploidal Inheritance in *Dictyostelium discoideum*: Stable Haploid, Stable Diploid and Metastable Strains

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SUMMARY

The existence of haploid and diploid *Dictyostelium discoideum* amoebae has been confirmed. Strains were isolated which displayed widely different ploidal compositions. These appear to fall into three general classes: stable haploid, stable diploid and metastable strains. Their ploidal compositions are clonally inherited. Procedures are described by which strains of differing ploidal composition can be isolated, one from the other. The ploidal composition of a clone is correlated with differences in spore and amoeboid size distributions as well as other morphological and morphogenetic properties.

INTRODUCTION

The chromosomal constitution of *Dictyostelium discoideum* was first described by Wilson (1952, 1953). He prepared aceto-orcein stained squashes of amoebae taken from migrating pseudoplasmodia and observed mitotic figures with haploid complements of 7 chromosomes. Bonner & Frascella (1952) reported a chromosome number of 4 with three extra arms but this difference was subsequently resolved and Wilson's conclusion has since been amply confirmed by Wilson & Ross (1957) and by R. R. Sussman (1961). The latter used a fixing and staining procedure which permitted immediate diagnosis of ploidy in exponentially growing organisms as well as those that had divided during the fruiting process. In 1957, Wilson & Ross observed the presence of diploid cells in *D. discoideum* cultures. Ross (1959) isolated strains which, although not subjected to clonal analysis, provided evidence that the diploid state is heritable. On several occasions he obtained haploid stocks from the diploid and vice versa, but was unable to define environmental conditions which could reproducibly induce or select the ploidal conversions. Sussman, Sussman & Ennis (1960) were able to isolate metastable strains containing appreciable numbers of both haploid and diploid cells. They arose as clones from I-cells, a minor component of the haploid myxamoebal population distinguishable by size, flatness, motility, granulation, etc. The capacity of these metastable strains to yield a ploidal mixture was found to be stably inherited through numerous subcultures and clonal re-isolations. The present communication describes the properties of the three ploidal varieties, in particular, the differences in the size distribution of spores and myxamoebae, I-cell frequencies, aggregative performances, and the stability of ploidal inheritance. It also specifies the environmental conditions which permit the isolation of one strain from another.

METHODS

Organisms and cultivation. *Dictyostelium discoideum* strain NC-4 is a stable haploid stock used in our laboratory for many years. Its genetic homogeneity was maintained by routine clonal re-isolations. Strain RA is a stable diploid stock which appeared in the laboratory of Dr K. B. Raper and which he kindly sent to us for study. The remaining strains were derived from these. Cultivation was accomplished on SM agar in association with *Aerobacter aerogenes* (Sussman, 1951).

Size distributions. Myxamoebae were harvested at the beginning of the stationary phase after incubation for 40–44 hr. at 22°, washed three times by centrifugation, suspended in water and dispensed in 0·01 ml. samples on washed agar at a density of 150–200 cells/mm^2. After incubation for 4–5 hr. at 22°, the amoebae were either measured directly with an ocular micrometer, or photomicrographs were taken using polaroid transparency film. These were projected on a screen and the major and minor cell diameters measured directly from the projected images and calibrated with a photomicrograph of an ocular micrometer. Both methods yielded the same size distributions. Care had to be taken to standardize the age of the organisms and conditions of cultivation and pretreatment in order to obtain reproducible data. Previous work (Bonner, 1960) had shown that amoebal size was significantly affected by these factors. Spores to be measured were taken from mass or clonal plates after incubation for 4–6 days and were measured with an ocular micrometer in wet mounts under an oil-immersion objective.

Chromosome stains. The procedure used is reported elsewhere (R. R. Sussman, 1961). The amoebae were fixed in Carnoy solution, brought to water through an ethanol series and were hydrolysed in N-HCl for 9–10 min. at 60°. A quick wash with 45 % (w/v) acetic acid was followed by staining in aceto-orcein. Squashed wet mounts could be examined immediately under phase contrast or sealed and retained for later examination.

Aggregative performance. This was determined as described by Sussman & Sussman (1961*b*). Myxamoebae were harvested from growth plates, washed three times by centrifugation, suspended in chilled water and counted in a Levy chamber. Appropriate dilutions were dispensed on washed agar in drop volumes ranging between 0·01 and 0·2 ml. in order to deliver a constant number of myxamoebae over a range of population densities. After incubation for 18–24 hr., counts were made of the number of aggregative centres.

RESULTS

Chromosome figures of stable haploid and diploid strains

Myxamoebae were harvested from growth in the exponential phase, suspended in salt solution and dispensed on a slide. The amoebae adhered to the glass and could be examined at intervals microscopically. During the following 3 hr., an appreciable proportion of the amoebae rounded up in a fashion characteristic of mitosis. They were immediately fixed in Carnoy solution and stained. When the fixation was timed correctly, considerable numbers of metaphase figures could be observed in the squashed stained preparations, thus permitting precise chromosome counts. In all, several hundred metaphase figures were scored. No haploids were encountered in strain RA, no diploids in strain NC-4.

Size distributions of strains NC-4 and RA

Preliminary data of Ross (1959) indicated a significant difference between the mean sizes of haploid and diploid myxamoebae. The size distributions of RA and NC-4 myxamoebae (see Fig. 1), confirmed this conclusion.

Figure 2 summarizes the size distributions of RA and NC-4 spores, and shows that spore size was also affected by ploidy. The major diameter in micrometer divisions (1 div. = 0·83μ), was used as the criterion of size since the minor diameters

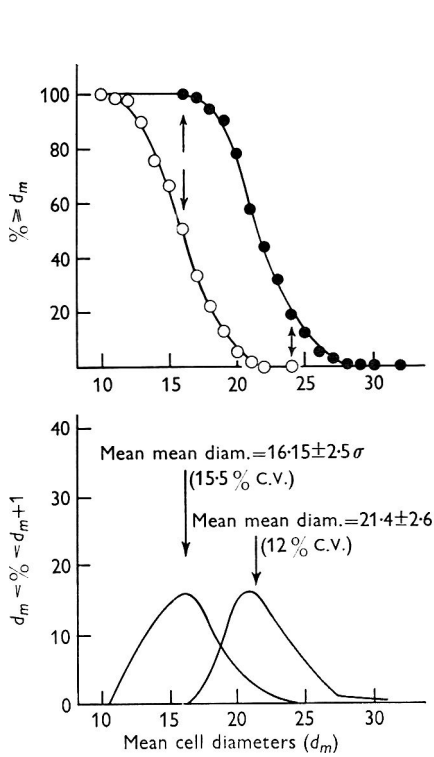

Fig. 1

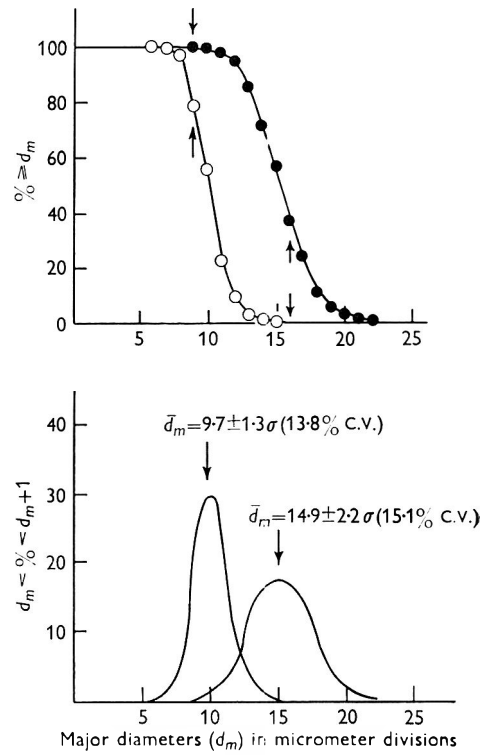

Fig. 2

Fig. 1. Size distributions of NC-4 and RA myxamoebae. Upper curves show the percentage amoebae whose mean diameters equalled or exceeded the values given in the abscissa. Lower curves are histograms derived from 1-A. They show the percentage amoebae falling within unit increments in the mean diameters given in the abscissa. The arrows delineate the zones of exclusivity, 49 % of the NC-4 amoebae were smaller than the smallest RA; 19 % of the RA amoebae were larger than the largest NC-4. ○, NC-4 (haploid) 262 amoebae; ●, RA (diploid) 326 amoebae.

Fig. 2. Size distributions of NC-4 and RA spores. The method of plotting is the same as used in Fig. 1 except that the units of the abscissae are major diameters in micrometer divisions (1 div. = 0·83μ). ○, NC-4 (haploid), 583 spores; ●, RA (diploid), 1049 spores.

differed only slightly (5·4 against 5·05 micrometer divisions). The zone of exclusivity at the low end of the scale included 23 % of the NC-4 spores and at the high end, 38 % of the RA spores. Considering the spores as perfect cylinders, the respective mean volumes were 201 and 115μ^3, yielding a volume ratio of about 1·75. The

photomicrographs of RA and NC-4 spores in Pl. 1, figs. 1 and 2, show differences in shape as well as size. The haploid spores are regular cylinders, whereas among the diploid spores many are banana- or boomerang-shaped and otherwise irregular in outline.

The data for strain RA shown in Fig. 2 were obtained in five separate experiments which yielded a grand mean major diameter of 14·9 div. The coefficient of variation was 3·7 %, indicating that examination of as few as 100–200 spores can provide a reliable estimate of mean cell size.

The frequency of I-cells

When myxamoebae, taken from log phase or early stationary phase cultures of strain NC-4, are inspected microscopically an occasional amoeba stands out in dramatic contrast to its neighbours by virtue of its large size, flatness, granulation and motility (Ennis & Sussman, 1958*b*). Frequently it contains one or more engulfed amoebae. For convenience, these exceptional myxamoebae were designated 'I-cells', and the remainder of the population 'R-cells'. It is not clear whether they represent a truly dichotomous type or are merely the extreme of a continuum of cell sizes and associated properties. Under the conditions used for measurement, a mean diameter of 28 μ was set arbitrarily as the lower limit for I-cells, and they have been seen to range up to 50 μ (in contrast to the mean of mean diameters = 16 μ for strain NC-4 as a whole). It is possible to show that the I-cells possess a capacity to initiate centres of aggregation greatly superior to that of the rest of the population, and that during their growth, they frequently undergo multiple fissions to yield three or four daughters. As shown below, they can yield clones which contain substantial numbers of both diploid and haploid myxamoeba.

In strain NC-4, the proportion of I-cells was found to be about 1 in 2000 under specified conditions of cultivation (Ennis & Sussman, 1958*b*). In strain RA under comparable growth and sizing conditions, about 1 % of the population had a mean amoebal diameter greater than or equal to 28 μ (see Fig. 1) and possessed a degree of flatness, granulation and explosive motility equivalent to the myxamoebae classified as I-cells within the haploid population. However, in strain RA the size distribution in this range was such that a diploid population, unlike its haploid counterpart, did not show a dichotomous appearance upon microscopical inspection.

Ploidal stability of strains RA and NC-4

As mentioned above, examination of mitotic figures led to the conclusion that the frequencies of diploid myxamoebae in strain NC-4 and of haploid myxamoebae in strain RA were probably of the order of one in several hundred myxamoebae or less. This point was investigated further. Both strains were plated clonally (Sussman, 1951) and wet mounts of spore samples, each from a single clone, examined microscopically. Major diameters of 20 randomly chosen spores were recorded for some of the clones, to obtain a rough measure of mean size. Thus, among 23 clones of RA, the mean of mean major diameters was 15·3 div., in good agreement with the value given in Fig. 2. In addition, a few hundred spores from each clone were scanned to detect the presence of abnormally small spores (major diameter less than 8 div.) in strain RA or abnormally large spores (major diameter greater than or equal to

18 div.) in strain NC-4. In practice the difference between stable haploid and diploid spore populations was clear enough to be readily apparent by simple inspection under the microscope and the spore measurements served merely to confirm the diagnoses numerically. Of 373 NC-4 clones examined in this fashion, none was diploid, i.e. they corresponded closely to the size distribution shown in Fig. 2 for strain NC-4 with respect to mean spore size and incidence of abnormally large spores, and with the spores shown in Pl. 1 with respect to shape. Of 410 strain RA clones, none was haploid, by the same criteria. In neither case were distributions intermediate between the two extremes observed.

During these and similar examinations, at least 10^4 spores of each strain were scanned without finding a single spore with major diameter less than 8 div. in strain RA or one greater than or equal to 18 div. in strain NC-4. Figure 2 indicates that about 10 % of the NC-4 spores had a major diameter less than 8 div. and about 10 % of the RA spores greater than or equal to 18 div. Therefore, it can be argued that since the incidence of small spores in strain RA and large spores in strain NC-4 was less than one in 10^4, the total incidence of haploid cells in RA and of diploid cells in NC-4 must have been of the order of one in a thousand or less.

The validity of either method of estimation described above rests on the assumption that a ploidal change would necessarily have been accompanied by a change in spore size; this assumption was checked without contradiction many times during the isolation of haploid and diploid strains against the actual chromosome complements. The inspection of RA and NC-4 clones with respect to spore size distributions thus extended the results obtained by direct chromosomal counts. The incidence in strain RA of myxamoebae capable of yielding haploid clones, and in strain NC-4 of myxamoebae capable of yielding diploid clones, must be less than one in several hundred. Also, the absence of abnormally large spores in strain NC-4 and of abnormally small spores in strain RA suggests that the incidence of ploidal variants may be of the order of one in a thousand or less.

Isolation of a stable diploid strain from NC-4

In light of the discovery (Ross, 1959) of stable diploid and metastable strains of *Dictyostelium discoideum*, stocks carried in a number of laboratories were re-examined for ploidal composition (Dr I. K. Ross; private communication). The prevalence of diploid stocks found by Dr Ross in some laboratories, regardless of the original source of the strains, suggested that the diplophase might be selected or induced by conditions of cultivation. We examined this point and this indeed turned out to be the case. Spores from a typical culture of strain NC-4 were inoculated at the centre of a cross streak of bacteria on SM agar after the method of Raper (1951). The myxamoebae grew out along the arms of the streak during the next 3–4 days. The resulting fruits revealed a spore-size distribution characteristic of the stable haplophase. Serial subculture with spores as inoculum did not alter this condition. When, however, the inoculum consisted of a mass of spores, stalks, partially complete fruits and unaggregated myxamoebae swept up from an agar surface and deposited at the centre of a cross-streak, the ensuing fruits often (not always) contained a small number (0·1–1·0 %) of abnormally large spores (major diameter greater than or equal to 17 div.), i.e. within the zone of exclusivity of stable diploids. The proportion was further enriched by a second serial cultivation

as described above. These spores were plated clonally and a majority of the clones displayed spore size distributions characteristic of the stable diplophase. The remaining clones were typically haploid. No intermediate distributions were detected. One of the presumptive diploid clones was retained and designated NC-4 dip-1; chromosome counts confirmed that this was indeed a stable diploid. Figure 3 demonstrates that the spore size distribution of this stock is identical to RA within the limits of experimental and sampling errors.

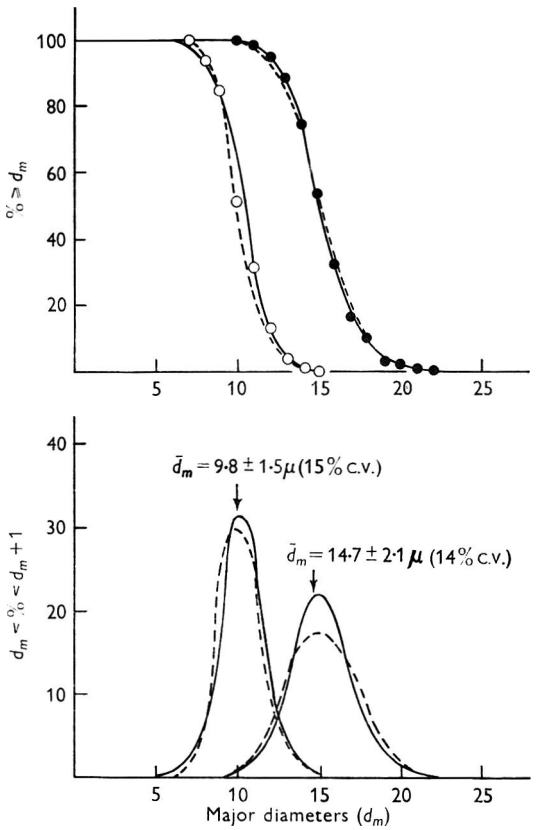

Fig. 3. Size distributions of spores from NC-4 dip-1 and NC-4 hap-1. For comparison, the distributions of NC-4 and RA spores taken from Fig. 2 are given as dotted lines. ○, NC-4 hap-1, 100 spores; ●, NC-4 dip-1, 109 spores.

Selection of stable haploid clones from strains RA and NC-4 dip-1 during sustained exponential growth

An inoculum of $1–2 \times 10^5$ RA myxamoebae spread with *Aerobacter aerogenes* on an SM agar plates grows exponentially with a generation time of 3·9 hr., attains the stationary phase after ten to twelve generations, and then aggregates and fruits. Strains RA and NC-4 dip-1 were both cultivated in this manner but were sub-cultured before the onset of aggregation once again, at an initial density of $1–2 \times 10^5$ myxamoebae/plate. The sampled plates were incubated further in order that the spores therefrom could be inspected microscopically. Four serial subcultures were

accomplished in this fashion. Table 1 shows the incidence of abnormally small spores in each subculture.

After the 4th subculture the spores were plated clonally. Of 19 clones examined, 14 were typical RA clones with respect to mean spore size and absence of abnormally small spores, while 5 were typically haploid in these respects. Three of the latter clones were retained for study. Chromosome counts yielded haploid figures only and examination of spores from 20 to 40 clones subsequently derived from each stock indicated that the haplophase was stably inherited. Stable haploid strains were also derived from the diploid stock NC-4 dip-1 (see the preceding section) recognized first on the basis of spore size and later confirmed by chromosome counts. The spore size distribution of one of these, designated NC-4 hap-1 (Fig. 3) was identical with the original parent (the haploid NC-4) from which the diploid NC-4 dip-1 had itself been derived.

Table 1. *Incidence of small spores in* Dictyostelium discoideum *strain RA after sustained logarithmic growth*

The symbol d_m = major diameter in micrometer divisions.

Subculture	Generations (cumulative)	Spores with $d_m \leqslant 9$		Spores with $d_m \leqslant 8$	
		Proportion	%	Proportion	%
0	0	3/1049	0·3	0/1049	0
2	22	5/421	1·2	3/421	0·7
3	33	10/430	2·3	6/430	1·4
4	44	16/234	6·8	7/234	3·0

Aggregative performances of diploid and haploid strains

Myxamoebae, washed free of bacteria and dispensed on washed agar, remain constant in number and can produce typical radiate aggregative patterns and, ultimately, normal fruits. Under these conditions, the number of centres of aggregation is a function of the number of myxamoebae present and their population density. The 'aggregative performance' of a particular strain was defined by Sussman & Sussman (1961*b*) as the specific relation between the number of centres and the parameters of cell number and population density. When carried out under comparable experimental conditions, this relation can serve as a measure against which can be assessed the effect of genetic and physiological variation, of added metabolites, enzyme poisons, etc., upon the morphogenetic sequence.

Table 2 compares numerically the aggregative performances of strains RA, NC-4, NC-4 dip-1 and NC-4 hap-1. The general shapes of the curves are published elsewhere (Sussman & Sussman, 1961*b*). Significant differences between strains NC-4 dip-1 and NC-4 hap-1 are apparent in respect to the optimal population density for centre formation (100 versus 200 myxamoebae/mm^2) and the ratio between the number of centres formed at the optimal population densities and the number of myxamoebae present (1:400 versus 1:2000). In contrast, the performance of NC-4 hap-1 was identical with that of the original parental stock, NC-4 (optimal population density = 200 myxamoebae/mm^2; R = 1:2200). Thus the shift from the stable haplophase (NC-4) to the stable diplophase (NC-4 dip-1) was accompanied by a considerable change in aggregative performance; and the subsequent reversion of NC-4 dip-1 to the haplophase (NC-4 hap-1) returned this performance to its

original condition. Because myxamoebal size has already been shown to be associated with differing morphogenetic capacities (Ennis & Sussman, 1958*b*) it may be that the myxamoebal size distribution is the determining factor here. It should be noted that the aggregative performance of strain RA was also found to be different from that of strain NC-4. However, the pedigree of RA is uncertain and since it is known that genetic alterations aside from ploidy can affect aggregative capacity (Sussman & Sussman, 1961*b*), the above difference cannot be ascribed uniquely to the change in ploidy.

Table 2. *Aggregative performances of haploid and diploid strains of* Dictyostelium discoideum

Optimal population density refers to the population density at which a maximum number of aggregative centres is formed. Centre:myxamoebae ratio refers to the ratio between the number of aggregative centres formed, and the number of cells present at optimal densities.

Strain	Optimal population density	Centre:myxamoebae ratio
NC-4	200	1:2200
NC-4 dip-1	110	1:400
NC-4 hap-1	200	1:2000
RA	150	1:800

Metastable strains

In previous work (Sussman *et al.* 1960), NC-4 myxamoebae classified as I-cells were isolated by micro-manipulation and incubated on agar with *Aerobacter aerogenes*. Examination of the resulting clones revealed that about 90 % were indistinguishable from those derived either from R-cell isolates or from the carried stock culture on the bases of size distributions of spores and myxamoebae, frequency of I-cells, aggregative performance and ploidy. (All countable metaphase figures were haploid.) In contrast, about 10 % of the I-cells yielded clones which differed from the norm in all the respects noted above and which inherited the anomalies in stable fashion. These anomalous stocks, hereafter termed 'metastable', have now been further studied; current information about them is summarized below.

Ploidal composition. Three metastable strains were examined cytologically; Table 3 shows the data. All displayed a mixture of haploid and diploid metaphase figures, but in very different proportions. The amplitude of transient variations in the proportion of haploid and diploid myxamoebae during the culture cycle is not known with precision but is probably considerable. Nevertheless, the strains tended to be sorted into two classes: those with a preponderance of haploid myxamoebae and those with a preponderance of diploid. These proportions tended to be maintained during serial subculture and clonal re-isolation.

Size distributions. Figure 4 shows the spore distribution encountered in strain I-2A, compared with the corresponding curves for NC-4 (the haploid stock from which I-2A was derived) and NC-4 dip-1 (a diploid stock of similar origin). In rough agreement with the cytological findings, the size distribution is seen to lie between the two extremes of ploidal stability. Figure 5 summarizes the spore-size distribution of three other metastable strains. I-47 had been isolated from a mutant

of *Dictyostelium discoideum*, designated Br-1, which produces the yellow pigment characteristic of the wild type in its fruits, but also forms a brown pigment which stains deeply both the fruits and the underlying agar. I-262 and W-7DH came from

Table 3. *Ploidal composition of metastable strains of* Dictyostelium discoideum

Strain	Metaphase figures scored	No. haploid	No. diploid
I-2A	40	3	37
I-47	112	100	12
W-7DH	44	1	43

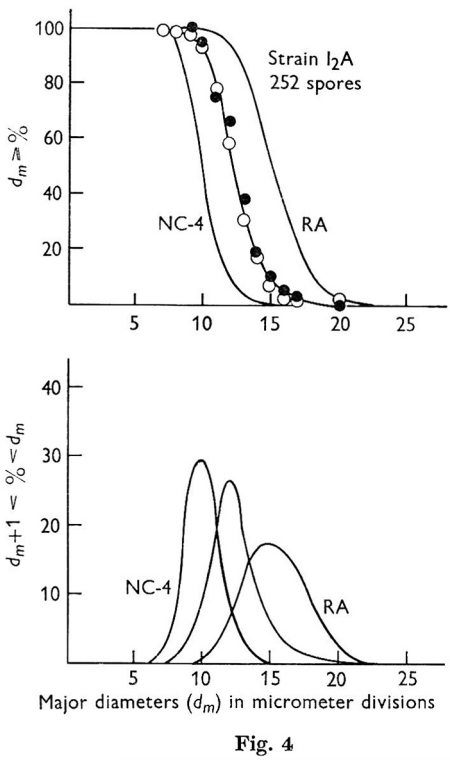

Fig. 4

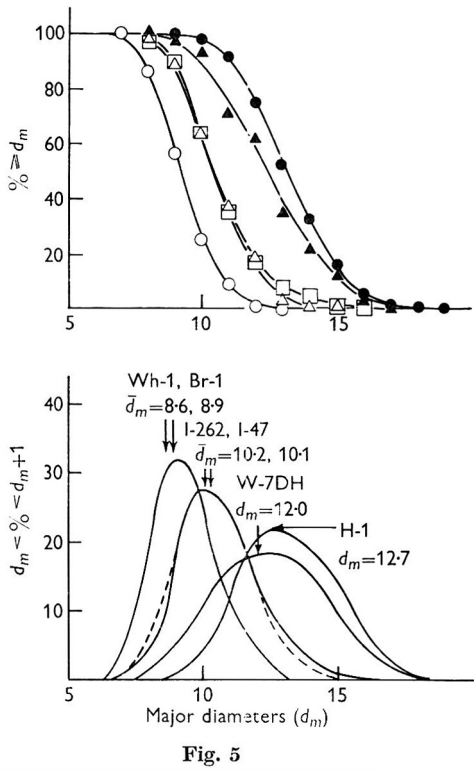

Fig. 5

Fig. 4. Size distribution of the spores of I-2A, a metastable strain. The open and closed circles represent data from separate determinations made 11 months apart. The distributions of NC-4 and RA spores, taken from Fig. 2, are given for comparison.

Fig. 5. Size distributions of spores from the following strains of *D. discoideum*: Br-1, a yellow-brown mutant, and Wh-1, a white mutant, both stable haploids; I-47, a metastable strain derived from Br-1; I-262 and W-7DH, metastable strains derived from Wh-1; H-1, a diploid strain isolated from a mixed culture of I-47 and I-262. ○, Wh-1, Br-1, 186 spores; ●, H-1, 721 spores; △, I-47, 253 spores; ▲, W-7DH, 150 spores; □, I-262, 135 spores.

a white mutant Wh-1 (Sussman & Sussman, 1953). Strain H-1 is a stable diploid; it was derived from a mixed culture of I-47 and I-262. Although most H-1 clones were diploid and had the wild-type pigmentation (yellow), haploid segregant clones appeared at low frequency (about 1/1000) which had either of the parental pheno-

types (white or yellow-brown) or either of the recombinant phenotypes (yellow, or white-brown). These phenotypes are described elsewhere (Sussman, 1961). Both parental stocks, Br-1 and Wh-1, formed spores slightly but significantly smaller than wild-type haploid NC-4. The derived H-1 spores were in turn correspondingly smaller than those of NC-4 dip-1, for example. The metastable spores were distributed intermediately between the two ploidal extremes of this series of stocks, in rough correspondence with the proportions of haploid and diploid cells observed cytologically.

Frequency of I-cells. Casual microscopic inspection of myxamoebae from metastable stocks revealed a degree of morphological heterogeneity more marked than in stable haploid populations (Pl. 1, fig. 3). This appeared to be due in part to a much greater frequency of myxamoebae classifiable as I-cells (as much as 1 % of the I-2A population) which appeared in dramatic contrast to their smaller neighbours and in part to the generally greater size of the metastable I-cells as compared with those in the haploid NC-4 stock.

Aggregative performance. Data published elsewhere (Sussman & Sussman, 1961*b*) showed that the performances of strains I-2A and I-47 were significantly different from that of the parental NC-4. Both metastable stocks showed an optimal population density less than 100 myxamoebae/mm^2 (as compared with 200 for NC-4). At optimal density the centre:myxamoebae ratios were 1:530 for I-2A and 1:830 for I-47 (as compared with 1:2200 for NC-4).

Ploidal stability. Clones produced by three metastable stocks (I-2A, I-47, and W-7DH) were examined for spore size. Of particular interest in this respect was I-47. Cytological examination of I-47 indicated a proportion of about 1 diploid myxamoeba to 10 haploids in growing populations. Yet when representative samples were plated out, every clone of over a hundred examined contained spores large enough (d_m = 17 divisions) to fall within the zone of exclusivity for diploids. No stable haploid clones were detected. It therefore appears that haploid myxamoebae in I-47 differed from haploid myxamoebae in NC-4 in terms of the rate at which they gave rise to diploid progeny. The term metastable seems to be an appropriate designation for such strains. Metastable stocks have been found to yield both stable haploid and stable diploid derivatives and by the same methods of cultivation as were described above. Stock I-2A was cultivated continuously in the log phase for twenty-four generations and a sample plated on SM agar with *Aerobacter aerogenes*. Six percent of the clones were diagnosed as stable haploids by spore size and confirmed by cytological examination. None of the zero-time clones was haploid. Stock I-47 was cultivated from an inoculum of spores, stalks, immature fruits and unaggregated cells. Stable diploid derivatives were isolated after a single subculture in this manner (about ten generations). During routine laboratory passage the stock W-7DH was discovered to have undergone a considerable heritable alteration in spore size distribution toward the haploid extreme. Cytological observation revealed only 3 diploid myxamoebae out of 12 countable metaphase figures. At this time myxamoebae classified as I-cells were isolated from the culture by micromanipulation. A low proportion of these yielded clones which had regained a preponderantly diploid state as indicated by spore size and cytological examination. Similarly, isolation of I-cells from I-47 yielded a low proportion of clones in which the diplophase predominated and the alteration was heritable.

DISCUSSION

Previous cytological examination of *Dictyostelium discoideum* had revealed the existence of haploid and diploid myxamoebae and had indicated that the ploidal condition might be stably inherited. The present work has confirmed these findings and supports the inference that ploidal constitution is a clonally inherited property in this species. It has also shown the existence of strains which display a wide range of ploidal compositions. For example, in strains NC-4, BR-1 and Wh-1, all scorable metaphase figures out of hundreds examined were haploid. Subsidiary evidence indicated that diploid cells might be present at a frequency of about 1 in 1000 or less in these strains. In strains RA, NC-4 dip-1 and H-1, all scorable metaphase figures were diploid and subsidiary evidence indicated that haploid myxamoebae might be present at a frequency of about 1 in 1000 or even less. In strains I-2A and W-7DH, both haploid and diploid figures were encountered but the diploid predominated (about 90–98 %) while in strain I-47, the haploid predominated (about 90 %). Subsidiary evidence suggests that in these strains the presence of both haploid and diploid cells in appreciable proportions is due to high rates of ploidal variation.

The three groups have been termed respectively, 'stable haploid', 'stable diploid', and 'metastable' strains and their ploidal properties have been found to be heritable and clonally distributed. It may be that a more detailed analysis of these and other stocks will demonstrate the existence of a continuous spectrum of ploidal compositions among isolates of *Dictyostelium discoideum*, in which case any categorization would be arbitrary. Nevertheless, the above nomenclature is offered as a convenient one and would seem to be justified at present since the relative proportions of haploid and diploid cells in the three groups differ by almost two orders of magnitude and they appear to retain these differences during serial passage under identical conditions of cultivation.

A correlation between cell size and ploidy has been reported for a wide variety of plant, animal, and microbial cells including at least two groups of Fungi (Sansome, 1949; Roman & Sands, 1953). It is therefore not unexpected to find this correlation operative in *Dictyostelium discoideum*. It should be emphasized, however, that size can be affected genetically without changes in chromosome number and the difference among the haploid stocks used in this study is a case in point. A previously encountered example is the mutant Agg-91, a dwarf aggregateless derivative of NC-4 (Ennis & Sussman, 1958*a*). In addition, the size of amoebae is to some extent governed by their physiological condition (Bonner, 1960). It is important, therefore, that comparisons between strains be made with these parameters in mind.

It is now possible to stipulate experimental procedures by which each of the three types of ploidal strains can be isolated, one from another. But it still remains unclear how the ploidal variants are thereby induced or selected. Furthermore, the genetic basis for inheritance of a particular range of ploidal composition is completely unknown and is an intriguing problem. Finally, neither the act of diploidization nor of haploidization is understood. One datum of significance is the existence of stable diploids like strain H-1, isolated from a mixed culture of two mutants and apparently heterozygous for both of the mutant markers which segregate during

haploidization (Sussman 1961). This indicates that at least some diploids can arise as the result of fusion and karyogamy between independent amoebae, as has been suggested by Wilson & Ross (1957). The ecological consequences of ploidal variation for *Dictyostelium discoideum* cannot be definitively estimated at present. The original species description of *D. discoideum* (Raper, 1935) suggests that the first isolate was a stable haploid. Stocks collected subsequently appear to have been either stable haploids or possibly metastable but certainly containing few diploids (Dr I. K. Ross, private communication). The only stable or largely diploid metastable strains now extant were derived in the laboratory from pre-existing haploids. However, only a relatively narrow range of natural habitats has thus far been investigated, by restricted methods of isolation, and only during certain seasons of the year (Raper, 1951). The realization that this species can exist in the diplophase may now prompt an examination of its ecological significance.

One final consideration is noteworthy. The data have demonstrated that ploidal variation is accompanied by a gamut of changes in morphology and morphogenetic behaviour. Because ploidal variants appear at appreciable frequencies in stock cultures, they constitute an additional danger to experimental reproducibility over and above the incidence of ordinary genetic variants.

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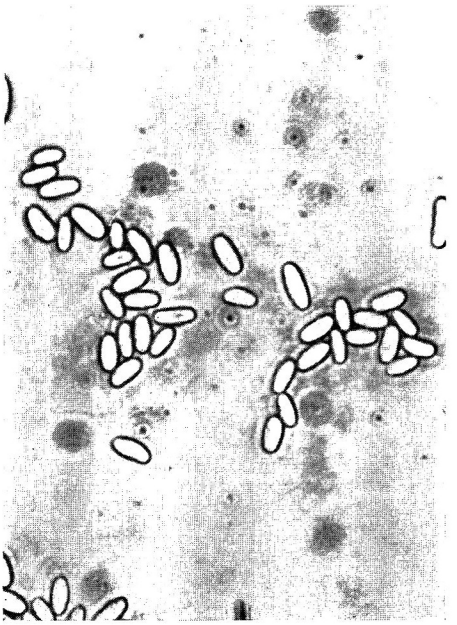

Fig. 1

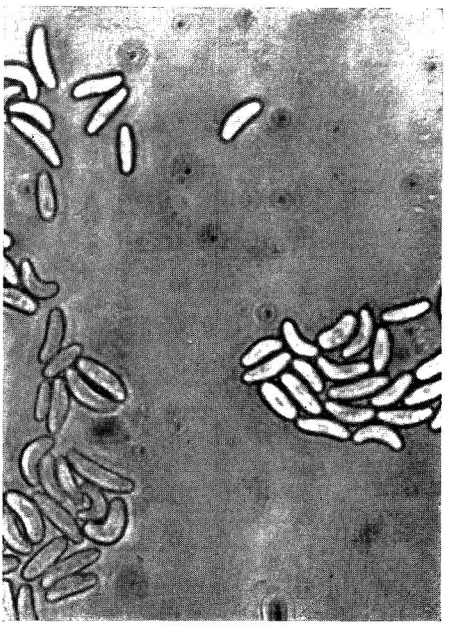

Fig. 2

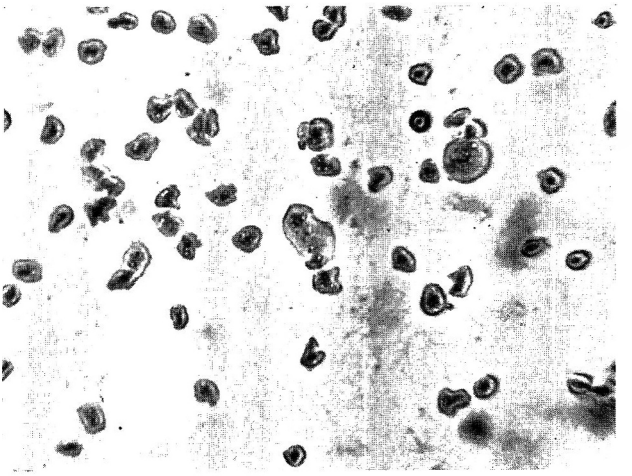

Fig. 3

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EXPLANATION OF PLATE

PLATE 1

Figs. 1 and 2. Photomicrographs of NC-4 spores (left) and RA (right) to show the differences in shape. Both are at × 1000.

Fig. 3. A photomicrograph of W-7DII myxamoebae. The mean diameter of these cells was *c.* 20 μ, but the two largest myxamoebae had mean cell diameters of 44 and 38 μ, respectively. × 240.

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Influences of β-Mercaptoethylamine and Oxygen Removal on the X-ray Sensitivity of Four Strains of *Escherichia coli*

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(*Received* 26 *August* 1961)

SUMMARY

In four strains of *Escherichia coli* B/r (ORNL), B (ORNL), B (Hill), Bs (Hill) protection by β-mercaptoethylamine (MEA) exceeded that observed for oxygen removal by nitrogen bubbling. Although only a slight decrease of protection was found with strain Bs (Hill) when oxygen was removed by nitrogen bubbling, the protection afforded by MEA in excess of that achieved by nitrogen bubbling was similar with all four strains. The data suggest that at least two mechanisms for protection by MEA may operate simultaneously in some bacteria and that one of these is a removal of oxygen equivalent to that achieved by nitrogen gas bubbling.

INTRODUCTION

Hollaender & Doudney (1954) and Elias (1961) showed that β-mercaptoethylamine (MEA) provided protection from X-rays in *Escherichia coli* B/r (ORNL) greater than that achieved by removal of oxygen by nitrogen gas bubbling. Other workers, with different techniques and possibly different strains, reported much lower protection with MEA (Marcovich, 1957). The present work was done with four strains of *E. coli* under standardized experimental conditions to determine whether MEA protected all strains to the same degree and to compare the effect of oxygen removal on these strains. We anticipated that if differences were found, these might be used in a study of MEA action. Such differences have been found and two mechanisms of action are proposed.

MATERIALS

The four strains used were *Escherichia coli* strains B/r (ORNL), B (ORNL), B (Hill), and Bs (Hill). Strains B/r (ORNL) and B (ORNL) were obtained originally from Dr Evelyn Witkin and were recently recharacterized by Adler & Haskins (1960). Strains B (Hill) and Bs (Hill) were provided by Dr Ruth Hill. Strain Bs (Hill) has been reported as very sensitive to both ultraviolet (Hill, 1958; Hill & Simson, 1961) and X irradiation (Hill & Simson, 1961; Engel & Adler, 1961).

The organisms were grown and X-irradiated by the technique described by Elias (1961). The bacteria were plated on Difco nutrient agar.

The refined nitrogen-gas technique used was similar to that of Birge & Tobias (1954). It consisted of passing tank nitrogen (High Purity dry, 99·98 %) over copper turnings in a quartz tube heated to 750°. The gas was then bubbled through a sterile water wash three alkaline pyrogallol solutions [600 g. KOH + 300 g. pyrogallic acid per l.], two additional water washes, and conducted to irradiation tubes.

Nitrogen gas was passed through the system for 15 min. before attaching the irradiation bubbling tubes; then suspensions were bubbled for 15 min. before irradiation and continuously during irradiation. No quantitative measurements were made of the purity of the nitrogen. Although the oxygen used was not purified, the bubbling treatment was similar to the nitrogen bubbling.

MEA was added to suspensions of organisms 30 min. before irradiation; incubation was at 0–1° in closed vials. Incubation was maintained at 0–1° until the irradiation was completed. At concentrations of MEA up to and including 0·12 M, no lethal effect was observed in the controls.

In experiments in which the protective effect of sodium dithionite ($Na_2S_2O_4$) was measured, the salt was present at a final concentration of 0·02 M, which provided maximum protection, according to Stapleton (1960). Samples were prepared by adding bacterial suspensions in saline solution directly to the dry sample of $Na_2S_2O_4$ immediately before irradiation.

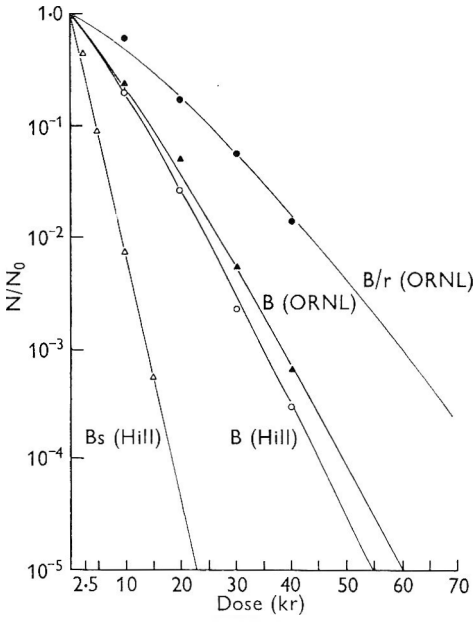

Fig. 1

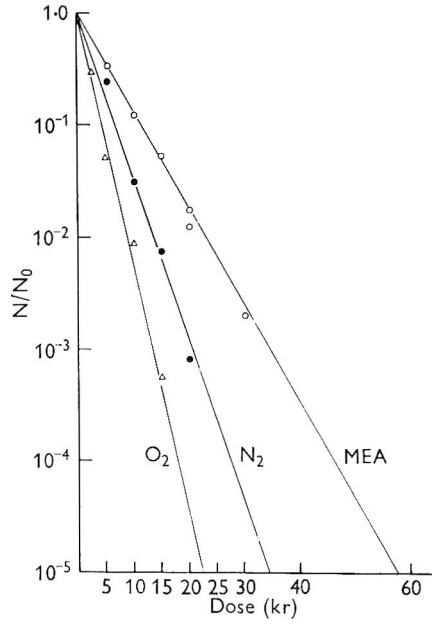

Fig. 2

Fig. 1. X-ray survival curves for four strains of *Escherichia coli* in a suspending medium of 8·5 g. NaCl/l. and bubbled with oxygen during irradiation. The points represent averages of three or more experiments.

Fig. 2. X-ray survival curves for *Escherichia coli* strain Bs (Hill) in oxygen, in nitrogen, and in 0·06 M-MEA (β-mercaptoethylamine). The suspending medium was 8·5 g. NaCl/l. The points represent the average of three or more separate experiments.

RESULTS AND DISCUSSION

The differences in sensitivity of the *Escherichia coli* strains used to ionizing radiation in the absence of protective agent are shown in Fig. 1. We confirmed the results of Elias (1961) with *E. coli* B/r (ORNL).

When we compared survival curves for MEA-treated organisms and those irradiated in oxygen (MEA/O_2), a DRF of approximately 6 was obtained whereas a dose reduction factor (DRF) of 3 was observed as between oxygenated suspensions and those made anoxic by nitrogen bubbling (N_2/O_2). The use of DRF (dose reduction factor defined by Alexander (1960) as the radiation dose which produces a given effect in the presence of protector divided by the radiation dose which produces a given effect without protector) to indicate relative sensitivity of cells in oxygen and MEA was appropriate since these curves, in agreement with the results of Elias (1961), seemed to be multiples of each other. In earlier work (Hollaender & Doudney, 1954), survival curves for cells in MEA were clearly not multiples of those for cells in oxygen. We are not able to account for this discrepancy.

The experiments done with strain Bs (Hill) using the same technique are shown in Fig. 2; the DRF for MEA/O_2 was about 2·6. The small magnitude of chemical protection observed with this strain indicated the possibility that the amount of MEA (0·06M) used was not optimum. Experiments on the concentration of MEA against survival to 20 kr of X irradiation (Fig. 3) showed that protection was not much increased beyond 0·06M MEA. These experiments also verified that MEA was not toxic to the cells up to 0·12M over the time period required for the experiments.

In another attempt to explain the small MEA effect in strain Bs (Hill), we examined the protection achieved by oxygen removal with nitrogen bubbling in this strain. It can be seen (Fig. 2) that the oxygen effect in this radiation-sensitive organism was considerably smaller than that obtained for *Escherichia coli* B/r (ORNL) by Elias (1961).

We examined the magnitude of the oxygen effect for two 'B' strains and found it to be intermediate between that for strains Bs (Hill) and B/r (ORNL). It can be seen (Table 1) that in this group of bacteria there was a correlation between radiation resistance, magnitude of the oxygen effect and magnitude of the MEA effect.

Table 1. *Oxygen and MEA effects on strains of* Escherichia coli

Strain of *E. coli*	$\frac{N_2}{O_2}$ DRF* value	$\frac{MEA\dagger}{O_2}$ DRF* value	LD50 (Kr)
B/r (ORNL)	3·2	6·0	14·0
B (ORNL)	2·4	3·9	7·0
B (Hill)	2·2	3·3	6·2
Bs (Hill)	1·5	2·6	2·2

* DRF is average figure for five determinations.
† MEA = β-mercaptoethylamine.

Attempts to enhance the oxygen effect in strain Bs (Hill) by further purification of the nitrogen resulted in only a small increase in the value of DRF N_2/O_2 from about 1·4 to 1·7. Although the reason for the small oxygen effect is not at present understood, the fact that it exists does aid in understanding the small MEA/O_2 DRF value observed for this organism. These observations also are consistent with the hypothesis that MEA protects in at least two ways. One offers protection equivalent to that obtained by nitrogen bubbling. Above this a second increment of protection was observed. This increment was about the same for both the Bs and B/r strains; the DRF value for MEA/N_2 was about 1·7. Thus, it is only the first

increment, obtainable with either nitrogen bubbling or low concentrations of MEA, that is small in the Bs (Hill) strain. Further support for the concept that MEA can protect by two mechanisms is presented in Fig. 4. Here relative radiosensitivity is plotted as a function of MEA concentration. If MEA operates solely as an agent to remove oxygen, this plot should be equivalent to that of Howard-Flanders & Alper (1957) in which relative radiosensitivity was plotted as a function of oxygen concentration. Indeed, over the range from about zero concentration to 0·012M MEA, this curve, being a linear function of MEA concentration, is equivalent. At higher concentrations of MEA, however, the slope changed and no longer resembled that of the earlier data. This inflexion occurred at a relative radiosensitivity of 1, obtainable either by exhaustive nitrogen bubbling or with 0·012M MEA under our conditions. The fact that the slope is different in the concentration range of 0·012M to 0·12M MEA supports the contention that a second mechanism is involved.

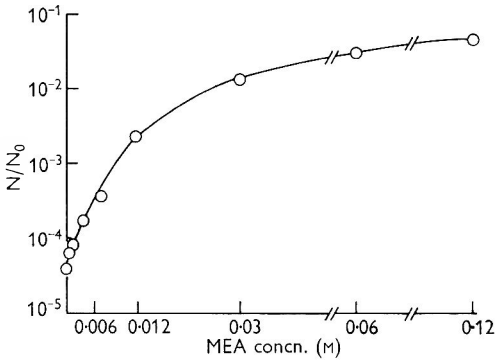

Fig. 3

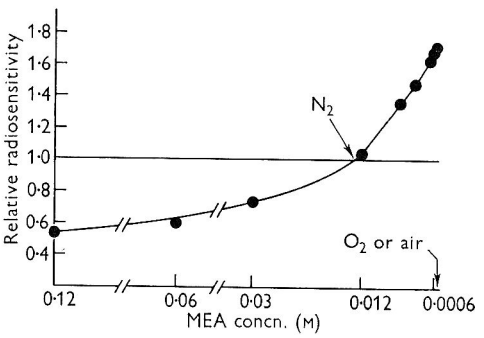

Fig. 4

Fig. 3. Surviving fraction of *Escherichia coli* strain Bs (Hill) to 20 kr as a function of MEA (β-mercaptoethylamine) concentration. The suspending medium was 8·5 g. NaCl/l. The points represent an average of five separate experiments.

Fig. 4. Relative radiosensitivity (Howard-Flanders & Alper, 1957) of *Escherichia coli* strain Bs (Hill) as a function of MEA (β-mercaptoethylamine) concentration.

This second mechanism might involve oxygen; if this be so, the oxygen must be bound to the cells in a manner not susceptible to removal by nitrogen bubbling. With this in mind, were made experiments in which $Na_2S_2O_4$ was used as the oxygen-removing agent. This compound is an excellent oxygen 'getter' and protects *Escherichia coli* strain B/r slightly better than oxygen removal by nitrogen bubbling. When $Na_2S_2O_4$ was used with *E. coli* strain Bs, the protection observed was as good as that achieved by nitrogen bubbling. However, the magnitude of the protective effect is limited by the toxicity of $Na_2S_2O_4$ for this strain. The indication is that an efficient chemical oxygen 'getter' does not duplicate the action of MEA.

The data presented here and elsewhere (Bacq & Alexander, 1961; Graevskii & Konstantinova, 1961) indicate that MEA can bring about protection by at least two mechanisms. One of these may be oxygen removal, and a complete understanding of this depends on an understanding of the oxygen effect. By using the Bs (Hill) strain of *Escherichia coli* in which the protective effect produced by oxygen removal is minimized, and the B/r strain, in which it is large, we hope to characterize the second mechanism.

One of the authors (H.L.C.) was a Research Participant at Oak Ridge National Laboratory operated by Union Carbide Corporation for the U.S Atomic Energy Commission.

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The Effect of Certain Steroids Upon the Growth of *Trichophyton rubrum*

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SUMMARY

The effect of thirty-seven different steroids on the growth of *Trichophyton rubrum* is reported. The effects varied from complete inhibition of growth to stimulation. Highly inhibitory compounds included androstan-3,17-dione, testosterone, 11-deoxycorticosterone, progesterone and 7-dehydrocholesterol. 17α-Hydroxypregnenolone was stimulatory but all other compounds with the 17α-hydroxyl group were inactive. Structural requirements for activity are discussed.

INTRODUCTION

Steroid derivatives have been reported to influence micro-organisms in a variety of ways—being essential growth factors for certain species (e.g. Edward & Fitzgerald, 1951; Butler & Knight, 1960; Van Wagtendonk, Conner, Miller & Rao, 1953); stimulators of growth for others (Buetow & Levedahl, 1957; Jefferson & Sisco, 1959); but most frequently growth inhibitors (e.g. Lester, Stone & Hechter, 1958). Included among the organisms for which inhibitions by steroids have been reported are several dermatophytes (Reiss, 1949; Tarbet, Oura & Sternberg, 1953; Casas-Campillo, Balandrano & Galarza, 1960). The dermatophytes are of particular interest since changes in the incidence of fungal infections have been noted at times of variation in the content of steroid hormones in the tissue. Microsporum infections usually cure spontaneously at puberty and dissemination of Trichophyton infections has been noted in association with Cushing's syndrome (Nelson & McNiece, 1959) and during cortisone therapy (Cremer, 1955). Thus steroids may influence either directly or indirectly the development of these infections and it seemed of interest to study in more detail the nature of the effect of these substances upon the fungi themselves. A preliminary report (Chattaway, Townsley & Barlow, 1959) showed the effect of a small number of steroids upon several dermatophytes. In the present paper this has been extended to a study of the effect of 37 steroids upon *Trichophyton rubrum*.

METHODS

Organism. The strain used was the OS strain of *Trichophyton rubrum* isolated from a foot infection in 1958.

Medium and inoculum. The growth medium contained (per 9·5 ml.): L-glutamic acid, 21 mg.; KH_2PO_4, 18 mg.; $MgSO_4.7H_2O$, 1 mg.; D-glucose, 100 mg.; D-biotin,

20 μg.; thiamine hydrochloride, 200 μg.; i-inositol, 200 μg. Aqueous solutions adjusted to pH 6·8 containing glutamic acid, vitamins and phosphate were autoclaved (121° for 10 min.) separately from those containing glucose and magnesium sulphate. Medium (9·5 ml.) was placed aseptically in 100 ml. conical flasks and its volume made 10 ml. by addition of the inoculum, which consisted of homogenized washed mycelium suspended in sterile water, having been grown initially on Sabouraud's maltose broth.

Steroids were added in chloroform ethanol (1 + 1, v/v) solution to the sterile 100 ml. conical flasks and the solvent removed by evaporation for 18 hr. at 33° before addition of medium.

Measurement of growth. This was done by determination of mycelial dry weight by filtering on to dried and weighed Whatman No. 1 filter paper (3·0 cm. diam.), washing three times with 50 ml. distilled water and drying at 105° for 18 hr. All such determinations were made in triplicate and after incubation at 33° for 7 days.

RESULTS

The earlier survey showed that 11-deoxycorticosterone was highly inhibitory and the effect of different concentrations showed that 50 % inhibition of growth occurred at 17 μg./ml. and maximal inhibition at 75 μg./ml.

For routine testing steroids were examined at 250 μg./ml. medium; the results are shown in Table 1. All compounds were tested on at least three separate occasions. Results are expressed as percentage inhibition or stimulation of growth in the absence of steroid. The normal growth was determined at the same time. Some of the compounds previously tested were included for completeness. While no close correlation of structure with activity can be discerned, some structural requirements can be seen. A considerable number of the compounds with high inhibitory activity are C_{17} or C_{19} steroids and all such compounds have a C_3 carbonyl group; reduction of this to a 3α- or 3β-hydroxyl group leads to considerable decrease or complete loss of inhibitory effect. Among inhibitory compounds the C_3 carbonyl group is often accompanied by unsaturation between C_4 and C_5, but this is not essential for activity since androstan-3,17-dione and 5β-androsten-3-one-17β-ol displayed high activity. The activity of the latter compound also suggests that the planarity of the steroid nucleus is not an essential feature. The nature of substituents at C_{17} afford some interesting comparisons. Thus high activity is seen with a C_{17} carbonyl, β-hydroxyl, β-acetyl and β-acetoxy groups and with an 8-carbon side-chain, but the presence of a 17α-hydroxyl group leads to complete loss of activity. Substitution at other sites (e.g. C_9 or C_{11}) also leads to reduction or loss of activity.

The series of sterols is of some interest since activity is seen with 7-dehydrocholesterol, lumisterol and ergosterol all of which have a $\Delta^{5,7}$-diene structure while the six compounds with the 3β-hydroxyl and Δ^5 double bond all show no inhibition or slight stimulation. Combination of this structure with a 17-hydroxyl group as in 17α-hydroxypregnenolone causes appreciable stimulation of growth.

There appears to be no parallel between inhibitory power and known function in mammals; thus 11-deoxycorticosterone displays high activity while 2α-methyl-9α-fluoro-hydrocortisone, the most active mineralocorticoid known, was without inhibitory effect. Similarly corticosterone displayed some activity while the much

more powerful glucocorticoid 16α-methyl-9α-fluoro-prednisolone was without activity. Also compounds such as androstan-3,17-dione showed high activity while possessing no great hormonal activity in the mammal.

Table 1. *Effect of steroids on growth of* Trichophyton rubrum, *strain OS*

Results are expressed as % inhibition (+ve) or stimulation (−ve) of growth in the presence of 250 μg. steroid/ml. medium as compared with growth obtained in the absence of added steroid. The steroids are listed in order of decreasing inhibitory effects.

Compound	No. of expts.	Mean % inhibition or stimulation
Androstan-3,17-dione	4	96
Δ^1-androsten-3,17-dione	4	96
Nortestosterone	3	94
11-deoxycorticosterone*	4	91
Δ^4-androsten-3,17-dione	4	89
5β-androstan-3-one-17β-ol	3	88
Calciferol*	4	79
Testosterone*	4	77
7-Dehydrocholesterol*	4	77
$\Delta^{1,4}$-androstadiene-3-one-17β-ol	3	72
Progesterone*	4	70
5α-androstan-3-one-17β-ol	3	62
11α-hydroxyprogesterone*	4	59
Androsterone	3	57
Corticosterone	4	44
Lumisterol	4	42
Oestradiol-17β*	4	39
Oestradiol-17α*	4	39
Ergosterol	3	28
Epitestosterone	3	20
Oestrone	3	11
Δ^5-androsten-3β,17β-diol	3	10
17α-hydroxyprogesterone	3	10
Prednisolone	3	7
Androstan-3α,17β-diol	3	4
Aldosterone†	1	0
Pregnenolone	3	0
Hydrocortisone*	4	−1
Cortisone*	4	−3
17α-hydroxy-11-deoxycorticosterone	4	−4
Lanosterol*	4	−5
2α-methyl-9α-fluorohydrocortisone	3	−9
9α-fluorohydrocortisone	3	−12
Stigmasterol	3	−14
Cholesterol*	4	−18
Sitosterol	3	−18
17α-hydroxypregnenolone	3	−37

* Previously reported (Chattaway *et al.* 1959).
† 50 μg./ml. medium.

DISCUSSION

There has been some emphasis in the literature on the marked ability of 11-deoxycorticosterone to inhibit fungal growth; thus Lester *et al.* (1958) reported it as the most active steroid in a group of 33 which inhibited the growth of *Neurospora crassa*,

Tarbet *et al.* (1953) found a similar result with *Microsporum gypseum* and Casas-Campillo *et al.* (1960) reported that it, together with 19-norprogesterone, was the most effective inhibitor of *Trichophyton mentagrophytes*. The present study also shows this compound to have high activity against *T. rubrum*, but nor-testosterone and androstane-3,17-dione were equally potent inhibitors and compounds with such widely different biological activity as calciferol and progesterone showed activity little below that of 11-deoxycorticosterone. Thus little support is given by the present work to the concept that steroids in micro-organisms may control processes analogous to those under hormonal control in mammals. Evidence in support of this concept was advanced by Lester & Hechter (1959) in the effect of 1-deoxy-corticosterone on sodium and potassium concentration in *N. crassa* and by the same authors (1961) in the antagonistic effects displayed by oestradiol-17β and 11-deoxy-corticosterone upon the growth of *Penicillium puberulum*. The inhibitory activity of testosterone on *T. rubrum* is comparable with that reported by Reiss (1949) for dermatophytes, Lester *et al.* (1958) for *N. crassa* and Maxwell, McGuire & Tomkins (1960) for *Saccharomyces fragilis*. Tarbet *et al.* (1953), however, found it to be slightly stimulatory for *M. gypseum* when assayed by its effect on the germination of macroconidia. Similarly androstan-3,17-dione and Δ^1-androsten-3,17-dione were reported to have fungistatic effects (Maxwell *et al.* 1960) and the inactivity of 17α-hydroxy steroids has been reported by a number of workers. Thus in general it appears that growth of *T. rubrum* was optimally inhibited by C_{17} or C_{19} steroids with a C_3-carbonyl substituent associated with a variety of C_{17} substituents of the β-configuration and that such activity was decreased by modifications at other sites in the molecule and abolished by the presence of a 17α-hydroxyl group.

Of the sterols tested ergosterol has been reported as inhibitory for *Penicillium chrysogenum*; stimulatory for the growth of other moulds (Matkovics, 1957, Matkovics & Sipos, 1959) and without effect upon *Neurospora crassa* (Lester *et al.* 1958). The low inhibitory activity of ergosterol for *Trichophyton rubrum* is of interest since this compound has been found in the normal mycelium (unpublished results). The considerable inhibitory power of 7-dehydrocholesterol, calciferol and lumisterol does not appear to have been recorded previously. The low stimulatory power of cholesterol, stigmasterol and sitosterol together with the marked stimulation by 17α-hydroxypregnenolone suggests that structures may be found which have even greater powers of stimulation.

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The Cultivation of a Single Strain of *Actinomyces israelii* in a Simplified and Chemically Defined Medium

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SUMMARY

Growth curves were determined for a single strain of *Actinomyces israelii* grown in systematic modifications of the partially defined medium of Howell & Pine (1956). These determinations resulted in a progressive simplification of the Howell & Pine medium and in the elucidation of a completely defined medium containing 335 μg. total-N/ml. instead of 948 μg. total-N/ml. in the original medium; the yield of organism was increased concomitantly from 55 μg. total-N/ml. of medium to 80 μg. total-N/ml. of medium. This simplified medium contained no ammonium sulphate, purine or pyrimidine bases but twenty amino acids (of which L-cysteine and L-tryptophan were found to be essential) and sixteen growth factors.

INTRODUCTION

Actinomyces israelii is a Gram-positive slow-growing anaerobic to micro-aerophilic organism forming small (0·5–2·5 mm. diameter) white coherent 'bread-crumb' or 'cauliflower' colonies which grow at the bottom of vessels of liquid media, leaving a clear supernatant medium at all stages of growth. Microscopic observation of pure cultures shows a non-sporing rebranching filamentous organism, the filaments seldom exceeding 1 μ in diameter (Erikson, 1940, 1949; Erikson & Porteous, 1955). The organism has a reputation for being difficult to isolate in pure culture and to maintain in laboratory culture for prolonged periods (see Erikson, 1940, 1953; Holm, 1948, 1951; Thomson & Lovestedt, 1951). Rosebury, Epps & Clark (1944) recommended cultivation in alternate complex undefined media. Other references to the use of complex media are given by Erikson & Porteous (1953) who made the first successful attempt to maintain *A. israelii* in simpler media. They succeeded in obtaining good growth of 6 strains in serial subculture in a medium composed of equal volumes of broth + 1 % (w/v) casein hydrolysate + 0·5 % (w/v) glucose; 5 of these strains were trained to grow in continued subculture, though in diminished yield, in a medium composed of 99 volumes of 1 % (w/v) casein hydrolysate + 1 volume of heart broth + 0·5 % (w/v) glucose. These authors could not replace the broth by chemically defined nutrients but showed that *A. israelii* could be maintained in these media for periods up to 2 years. They concluded that the 'short life,. . . and at best lessened activity' of *A. israelii* reported by earlier workers who used more complex media might have been due to the presence in those media of growth inhibitory substances. Howell & Pine (1956) reported a medium which

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was chemically defined except for the starch and possibly the oleic acid that it contained, and which supported the growth of 13 strains of anaerobic *Actinomyces* in serial subculture; 5 of the strains were typical of *A. israelii* as defined above. The present work is in continuation of that by Erikson & Porteous (1953) and is based on the significant advance made by Howell & Pine (1956). A preliminary account of the work has been published (Christie & Porteous, 1959).

METHODS

Organism. The organism used was one of a collection of strains of *Actinomyces israelii* maintained by Dr Dagny Erikson and given to one of us (J.W.P.) in 1953. Cultures were freeze-dried at that time and maintained for various periods since 1956 in medium S or medium A of Erikson & Porteous (1953).

Freeze-drying. Cultures were routinely freeze-dried at intervals of 3–6 months by using an LB5 rotary dryer (Edwards High Vacuum, Ltd., Crawley, Sussex) and a suspension medium of equal volumes of ox-heart broth, horse serum (Burroughs Wellcome and Co., London) and 15% (w/v) glucose solution.

Reagents. All media and analytical solutions were made up in distilled water unless stated to the contrary. The following compounds were used in preparing media: KH_2PO_4, $(NH_4)_2SO_4$, $MgSO_4.7H_2O$, Na acetate.$2H_2O$, $CaCl_2.6H_2O$, $FeSO_4.7H_2O$, glucose (analytical reagent quality); $MnSO_4.2H_2O$, $NaMoO_4.2H_2O$ (reagent quality); casein hydrolysate (vitamin-free for biological assay; batch 1650; Allen and Hanburys, Ltd.); *p*-aminobenzoic acid, oleic acid, haemin (British Drug Houses, Ltd.); folic acid, pimelic acid, pyridoxal.HCl, pyridoxamine.HCl, thiamine.HCl, thioctic acid, purine and pyrimidine bases (L. Light and Co. Ltd.); biotin, calcium pantothenate, inositol, nicotinic acid, nicotinamide (Roche Products Ltd., Welwyn Garden City, Hertfordshire); glutathione (Distillers Company Ltd.); coenzyme A (70% assay; Sigma Chemical Company; agents G. T. Gurr Ltd., London); vitamin B_{12} (crystalline; Glaxo Laboratories); the amino acids were the best quality available, and, except for L-cysteine.HCl, were not further purified.

Measurement of pH values. A Model 30 pH meter (Electronic Instruments Ltd., Richmond, Surrey) was used except for measurements on 0·1 ml. samples of inoculated media which were made with a capillator indicator set (British Drug Houses Ltd., Poole, Dorset).

Sterilization. All glassware was autoclaved at 120° for 20 min. Maintenance media were heat sterilized and dispensed as described by Erikson & Porteous (1953); chemically defined media, or component parts thereof, were sterilized by filtration through a P11 porcelain filter (Baird and Tatlock Ltd., London) or through a cellulose acetate filter membrane (A. Gallenkamp and Co. Ltd., London).

Culture vessels. Unless otherwise stated, rimless Pyrex tubes (12 × 75 mm.) containing 2 ml. liquid medium were used. Small volumes of all media were dispensed as described by Erikson & Porteous (1953).

Anaerobiosis. Stock cultures were maintained under hydrogen in an anaerobic jar (Erikson & Porteous, 1953). Experimental media were sealed in individual culture vessels with a layer of sterile liquid paraffin.

Inoculation technique. All inoculations were made with Pasteur pipettes. In all quantitative growth experiments, preparation of inoculum and quantitative inoculation were carried out as described by Christie & Porteous (1960). In the

routine maintenance of stock cultures, the larger colonies in tubes of liquid medium were broken up, so far as possible, with the tip of a Pasteur pipette and four to eight of the resulting small clumps transferred to each 2 ml. volume of fresh medium. Cultures were incubated at 37°.

Tests for culture purity. All cultures were tested periodically as follows. (i) Small fragments of colonies were stained with 0·1 % (w/v) methylene blue and examined microscopically under a cover-slip with an oil-immersion objective. Cultures were rejected when they showed any signs of contamination or when they failed to accord with the description given in the introductory paragraph of this paper. (ii) Replicate tubes of medium were inoculated and incubated aerobically; cultures were rejected when growth occurred in a second aerobic subculture (Erikson & Porteous, 1955). (iii) Duplicate nutrient agar plates were inoculated by spreading fragments of colonies over the agar surfaces; both plates were incubated at 37°, one aerobically, the other under hydrogen. Cultures were rejected when the aerobic plate showed any growth which survived a second aerobic subculture or when the anaerobic plate failed to produce typical *Actinomyces israelii* colonies only, i.e. abruptly raised, nodular colonies (Erikson & Porteous, 1953, 1955). (iv) Biphasic agar-gel media were inoculated as described by Erikson & Porteous (1955); cultures were retained only when the colonies which developed in these media were discrete, compact, nodular, and free from any 'tailing'. Medium S of Erikson & Porteous (1953) was used in all the above culture tests.

Analytical techniques. (i) Growth curves were determined by inoculating replicate tubes of liquid medium with *Actinomyces israelii* in the manner already described. At appropriate times after inoculation and incubation, two pairs of tubes were withdrawn; colonies were quantitatively removed from a pair of tubes by Pasteur pipette into an 18 × 150 mm. Pyrex test tube, the colonies allowed to sediment in the tube and then washed twice by sedimentation in a total of 25 ml. distilled water. Colonies from the other pair of culture tubes were treated in the same way and the duplicate washed samples analysed for total-N content; all growth yields quoted are the average of two such determinations expressed as μg. total-N content of the colonies harvested from 1 ml. medium, after correction for the total-N content of the corresponding inoculum. The remaining medium from each pair of culture tubes was filtered through Whatman No. 1 filter paper to remove any liquid paraffin and the duplicate filtrates taken for analysis. All results quoted are the average of the duplicate determinations expressed as the quantity of the compound determined per ml. medium.

(ii) Total-N determinations on harvested colonies and on media were done in duplicate as described by Christie & Porteous (1960), using the digestion apparatus described by Porteous (1960).

(iii) α-Amino-N was determined by the method of Pope & Stevens (1939) on 1–5 ml. samples of medium.

(iv) Ammonia-N was determined by Conway's method (1950), water blanks and ammonium sulphate standards being included with each set of determinations.

RESULTS

Media; terminology. All experimental media were derived from that published by Howell & Pine (1956) by progressive elimination of certain components. The composition of the Howell & Pine medium, referred to hereafter as medium HP, is shown in Tables 1 and 2; progressive modifications of this medium are designated HP1, HP2, etc. Any component omitted from a member of this series of media was omitted from all subsequent members of the series. Inoculation into a new

Table 1. *Composition and preparation of medium HP (Howell & Pine,* 1956)

	per litre (g.)	per litre (mg.)
1. Major salts		
KH_2PO_4	15·0	—
$MgSO_4.7H_2O$	—	200·0
$CaCl_2.2H_2O$	—	20·0
$CH_3COONa.2H_2O$	—	300·0
2. Minor salts		
$FeSO_4.7H_2O$	—	4·0
$MnSO_4.2H_2O$	—	0·15
$NaMoO_4.2H_2O$	—	0·15
3. Glucose	10·0	—
4. $(NH_4)_2SO_4$	1·0	—
5. Cysteine.HCl	1·0	—
6. Glutathione	—	500·0
7. L-Asparagine	—	100·0
8. L-Tryptophan	—	40·0
9. Purines and pyrimidines		
Adenine sulphate	—	20·0
Guanine hydrochloride	—	20·0
Uracil	—	20·0
Xanthine	—	20·0
Thymine	—	20·0
10. (*a*) Casein hydrolysate (vitamin free); or (*b*) amino acids as listed in Table 4, column A	4·0	—
11. Vitamins and growth factors as listed in Table 2	—	—
12. Purified potato starch	—	500·0

A solution of the above components, less the starch, was adjusted with 2N-KOH to the required pH value (7·2 in the present work) before dilution to a known volume with water and sterilization by filtration. Heat sterilized starch solution was then added to give the desired starch concentration and to dilute other components to the required concentration.

Table 2. *Growth factor components of medium HP (Table* 1)

11. Growth factors	(mg./l.)		(μg./l.)
p-Aminobenzoic acid	2	DL-thioctic acid	100
Thiamine.HCl	2	Biotin	100
Riboflavin	2	Pimelic acid	100
Nicotinic acid	1	Haemin	200
Nicotinamide	1	Folic acid	500
Pyridoxal.HCl	1	Vitamin B_{12}	1
Pyridoxamine.HCl	1	Citrovorum factor	100
Ca pantothenate	2		
Inositol	2		
Oleic acid	1		
Coenzyme A (70% assay)	1		

member of the series of experimental media was always made from a mother culture in the simplest available medium of the series.

Selection of test organism. All eleven organisms available were isolated from human actinomycotic lesions. All strains were microscopically, macroscopically and physiologically typical of *Actinomyces israelii* when grown in medium S of Erikson & Porteous (1953). Two strains failed to grow in medium HP 1 (medium HP, less citrovorum factor and less coenzyme A; Tables 1, 2). Three other strains survived only a few serial subcultures in this medium. The remaining 6 strains survived serial subculture in medium HP1. The techniques available for preparing uniform inocula of *A. israelii* (Christie & Porteous, 1960) and for measuring the growth of the inoculum (see: Methods, Analytical techniques) restricted the number of strains which could be investigated quantitatively and exhaustively. Strain Wills was selected as apparently representative of the 6 strains available which would grow in medium HP1; all results presented below refer to strain Wills of *A. israelii*.

Optimum initial pH value of the medium. Good growth occurred when the initial pH value of medium HP1 lay between 7·0 and 7·6; outside these limits the yield of organism decreased rapidly until at an initial pH of 6·6 or 7·8 the inoculum failed to grow. In further experiments all media were adjusted initially to pH 7·2.

Change in the pH value of the medium during growth of Actinomyces israelii

Preliminary experiments showed (Fig. 1) that (*a*) the pH value of medium HP1 decreased rapidly from pH 7·1 to 5·5 during the period when the increase in cell nitrogen was rapid with respect to time (analogous to the logarithmic growth phase of simple bacteria); (*b*) after growth ceased, the pH value of the medium continued to decrease to a limiting value of pH 5·0. These observations were found to be valid in several subsequent modifications of the medium; colonies harvested during the period of most rapid growth (pH of medium = 7·1 to 6·1) continued to grow at the same rate when subcultured to a medium of the same initial composition and pH 7·2; colonies harvested during the stationary phase (pH of medium 5·5 or less) showed a lag of 30–60 hr. when subcultured to fresh medium of the same initial composition and pH 7·2. pH measurements were therefore routinely used to determine the approximate growth phase of cultures, particularly of those intended as sources of inocula for quantitative growth experiments designed to measure the response of *Actinomyces israelii* to major components of medium HP.

Progressive simplification of the Howell & Pine medium

In all experiments reported hereafter, glucose and the major and minor salts were retained in experimental media at the concentrations given in Table 1 (items 1, 2 and 3); coenzyme A and citrovorum factor were omitted from all media and the remainder of the growth factors (Table 1, item 11) retained at the concentrations shown in Table 2. Table 3 shows the contribution of the various major components (see Tables 1, 2) to the total-N content of medium HP1.

The requirements for ammonium sulphate, L-cysteine and glutathione in the growth medium for *Actinomyces israelii* Wills strain were investigated in turn; the requirement for purine and pyrimidine bases was then investigated before modifying the amino acid concentration of the resulting medium. In these investigations

growth curves were determined in the manner described. With an exception (noted below) all inocula were taken from a mother culture in the rapid growth phase. All growth curves for nutritionally adequate media were of the general form shown in Fig. 1, i.e. a lag of 20–30 hr. followed by a period of rapid growth, with the culture entering the stationary phase between 70 and 90 hr. after inoculation.

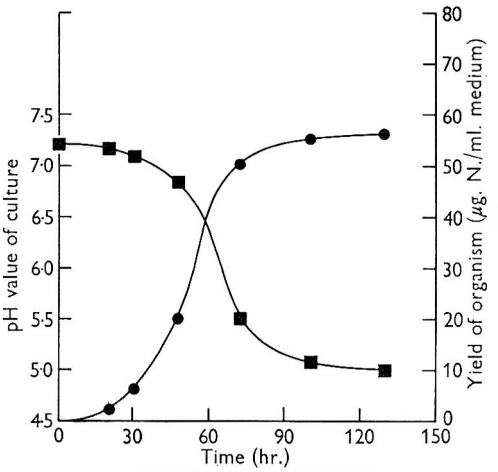

Fig. 1. Changes in the pH value of medium HP1 (Table 6) during growth of the Wills strain of *Actinomyces israelii* at 37° under liquid paraffin seals. ■—■, pH; ●—●, growth.

The successive modifications to medium HP1 and the maximum growth obtained in the modified media are shown in Table 6. Five concentrations of ammonium sulphate were tested in modifications of medium HP1. Since the presence or absence of ammonium sulphate did not affect the growth rates or yields of organism this salt was omitted from medium HP1 to give medium HP2 which was then modified to contain L-cysteine at six concentrations within the range 0–1000 μg./ml. No growth was obtained when L-cysteine was omitted; at initial concentrations between 20 and 100 μg. cysteine/ml. there was a roughly linear growth response and maximal growth was obtained with an initial L-cysteine concentration of 200 μg./ml.

Table 3. *Calculated total-N content of the major nitrogen-containing components of medium HP (Table 1)*

Component	Calculated total-nitrogen content (μg.N/ml.medium)
Casein hydrolysate	520*
$(NH_4)_2SO_4$	212
L-cysteine.HCl	90
Glutathione	68
Purine and pyrimidine bases	32
L-Asparagine	21
L-Tryptophan	5
	Total: 948

* This value determined experimentally on a separate stock solution of casein hydrolysate.

Medium HP2 modified to contain cysteine at this concentration was termed medium HP3, with a glutathione content of 500 μg./ml. In initial experiments medium HP3 was modified to contain 0, 100, 250 and 500 μg. glutathione/ml. The corresponding growth curves are shown in Fig. 2. In further experiments only the first and last of the above modifications of medium HP3 were used; normal growth curves were obtained which are also shown in Fig. 2. Both sets of results showed that the final cell yield was independent of the initial glutathione concentration. In the initial experiments there was a pronounced lag which was most marked in the absence of glutathione from the medium; the inocula in this experiment were taken from a culture in the stationary phase (pH of medium at harvest = 5·0), but the longest lag was greater than could be accounted for solely by the use of stationary phase inocula. In the second experiment there was no apparent lag whether glutathione was present or absent; the inocula in this instance were taken from a culture in its most rapid phase of growth (pH of medium at harvest = 6·6). Although there did not seem to be a specific requirement for glutathione it was retained at a concentration of 100 μg./ml. for further experiments and medium HP3 modified in this way became medium HP4.

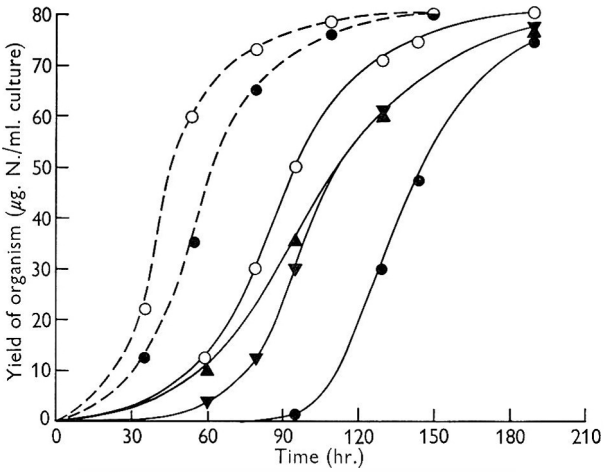

Fig. 2. Growth of the Wills strain of *Actinomyces israelii* at 37° under liquid paraffin seals in 2 ml. volumes of medium HP3 (Table 6) modified to contain various initial concentrations of glutathione (μg./ml. medium): ○—○, 500 (medium HP3); ▲—▲, 250; ▼—▼, 100 (medium HP4, Table 6); ●—●, 0. Growth of an inoculum taken from a stationary phase mother culture is indicated by a full line; growth of an inoculum taken from a rapidly growing mother culture is indicated by a broken line.

Omission of the purine and pyrimidine bases from medium HP4 did not affect the yield of organism and gave medium HP5 which became the basal medium for investigation of the amino acid requirements.

Howell & Pine (1956) supplemented their casein hydrolysate medium (Tables 1, 2) with L-asparagine and with L-tryptophan. Omission of L-asparagine from medium HP4 decreased the yield of organism by 15%. When L-tryptophan was omitted from medium HP4 no growth occurred. Both these amino acids were therefore retained at the original concentration in all subsequent media.

Howell & Pine (1956) replaced the casein hydrolysate in their medium by an

amino acid mixture (item 10*b*, Table 1) which was supposed to simulate the concentrations of amino acids supplied by the casein hydrolysate. In the experiments of Howell & Pine replacement of their casein hydrolysate by this amino acid mixture had no significant effect on the rate of growth or on the final yields of organism with the three organisms they tested, one of which was identified as *Actinomyces israelii*, one as probably *A. israelii* and one as *A. naeslundii*. Replacement of the casein hydrolysate of medium HP5 by the amino acid mixture used by Howell & Pine gave medium HP6/A in which the yield of organism was only 65% of that in the casein hydrolysate medium HP5 (Table 6). It appeared that the amino acid mixture did not simulate the casein hydrolysate used in the present work. In a comparative experiment medium HP5 was therefore modified by substituting two amino acid mixtures ('A' and 'B', Table 4) for the casein hydrolysate to give media HP6/A and HP6/B, differing in the proportions of the amino acids present but essentially identical in their total-N content. Concurrent experiments with medium HP5 had shown that starch (Table 1) was not required for the growth of *A. israelii* Wills strain. Starch was therefore omitted from media HP6/A and HP6/B. The yield of organism obtained in medium HP6/A was only 74% of that obtained in medium HP6/B, where the yield was 87% of that in the casein hydrolysate medium HP5 (Table 6). Medium HP6 containing amino acid mixture 'B' (Table 4) was therefore adopted for further experiments.

Further experiments showed that medium HP7 (medium HP6/B modified to contain glutamic acid at a concentration of 100 μg./ml. and each of the other amino acids of mixture 'B' (Table 4) at a concentration of 50 μg./ml.) would just support

Table 4. *The composition of two amino acid mixtures (A and B) used in the preparation of the completely defined media HP6/A and HP6/B (Table 6), together with the total-N contribution of the amino acids to the media. Amino acid mixture (A) was that used by Howell & Pine* (1956)

	Concentration in the medium (μg./ml.)		Total-N contribution to the medium (μg. N/ml.)	
Amino acid	(A)	(B)	(A)	(B)
Glycine	20	200	3·7	37·3
L-Alanine	220	200	34·6	31·4
DL-Serine	308	200	41·0	26·7
L-Threonine	152	200	17·9	23·5
L-Leucine	388	200	41·5	21·4
L-Isoleucine	252	200	26·9	21·4
DL-Valine	260	200	31·1	24·0
L-Glutamic acid	932	1000	88·8	95·2
L-Aspartic acid	244	200	25·7	21·0
L-Lysine	304	200	58·3	38·4
L-Arginine	172	200	55·3	64·0
L-Histidine	84	200	22·8	54·2
L-Proline	320	200	39·0	24·4
L-Hydroxyproline	12	200	1·3	21·4
L-Phenylalanine	200	200	17·0	17·0
L-Tyrosine	268	200	20·7	15·5
L-Tryptophan*	48	—	6·5	—
DL-Methionine	136	200	12·8	18·8
Total:	4320	4200	544·9	555·6

* Already present, together with L-cysteine and L-asparagine in the basal medium (Table 1).

growth of *Actinomyces israelii* strain Wills. Earlier attempts to obtain a nitrogen balance sheet for the growth of the organism in medium HP1 (Fig. 1) failed because of the low yields of organism relative to the nitrogen content of the medium (Table 6). The attempt was repeated for growth in medium HP7 with the results shown in Table 5. The ammonia-N content of the medium (arising from the ammonia used to dissolve haemin in preparing the medium) was essentially unaltered at the end of the growth period. The yield of organism balanced the decrease in the α-amino-N content of the medium and this decrease accounted for 97% of the decrease in the total-N content of the medium.

Table 5. *Nitrogen balance after growth of the Wills strain of* Actinomyces israelii *for 6 days at 37° under liquid paraffin seal in medium HP7 (Table 6)*

	Calculated 0 days	Determined 0 days	Determined 6 days	Increase or decrease
		μg. N/ml. medium		
Organism (total-N)	—	0	29	+29
Medium (total-N)	182	179	149	−30
Medium (α-amino-N)	130	122	93	−29
Medium (ammonia-N)	0	2	2·5	+ 0·5

The absolute yield of organism in medium HP7 was however low. Medium HP6/B was therefore further modified to contain half the concentration of each of the amino acids in mixture 'B' (Table 4). A yield of organism equivalent to 80 μg. total-N/ml. medium was obtained after 6 days incubation in this medium HP8 containing initially 335 μg. total-N/ml. medium. This may be compared (Table 6) with the yield equivalent to 55 μg. total-N/ml. of medium HP1 which contained initially 948 μg. total-N/ml. (Fig. 1, Table 3).

Miscellaneous experiments with modifications of medium HP

In an experiment reported above a partial requirement for L-asparagine in the nutrition of *A. israelii* strain Wills was noted. Addition of L-glutamine to medium HP5 (Table 6) at 100, 200 and 300 μg./ml. had no significant effect on growth rates or on yields of organism. Addition of ascorbic acid at 200 μg./ml. to medium HP4 (Table 6) made no significant difference to the yield of organism although a slight decrease in the length of the lag phase was noted.

DISCUSSION

The most significant recent advance in our knowledge of the nutritional requirements of *Actinomyces israelii* and related organisms was made by Howell & Pine (1956) who showed that a complex but essentially chemically defined medium, originally devised for the culture of *Histoplasma capsulatum*, also supported several Actinomyces species. By determining the growth curves for a single strain (Wills) of *A. israelii* in systematic modifications of the medium of Howell & Pine it has now been possible to assess the nutritional value of the major nitrogen-containing components of the original medium. It has been found that all the ammonium sulphate and the purine and pyrimidine bases of the original medium may be

Table 6. *Cumulative modifications to the Howell & Pine* (1956) *medium, together with the calculated total-N content of each medium and the yields of Wills strain of* Actinomyces israelii *grown in each medium*

Medium and cross-reference	Cumulative modifications to medium HP	Calculated total-nitrogen content of medium (μg. N/ml. medium)	Maximum yield of organism as cell-N (μg. N/ml.) medium)
HP (Tables 1, 2, 3)		948	—
HP1 (Tables 1, 2, 3)	Coenzyme A and citrovorum factor omitted	948	55
HP2 (Table 3)	$(NH_4)_2SO_4$ omitted	736	65
HP3 (Table 3)	L-cysteine.HCl concentration decreased to 1/5 concentration in medium HP	664	75
HP4 (Table 3, Fig. 2)	Glutathione concentration decreased to 1/5 concentration in medium HP	610	80
HP5 (Table 3)	Purine and pyrimidine bases omitted	578	80
HP6/A (Tables 1, 3, 4)	Starch omitted; casein hydrolysate replaced by amino acid mixture (A)	603	52
HP6/B (Table 4)	As HP6/A but amino acid mixture (A) replaced by amino acid mixture (B)	613	70
HP7 (Tables 4, 5)	Glutamic acid concentration decreased to 1/10 and concentrations of other amino acids of mixture (B) decreased to 1/4 the concentrations in medium HP6/B	182	29
HP8 (Table 4)	Concentration of all amino acids of mixture (B) decreased to 1/2 the concentrations in medium HP6/B	335	80

omitted. The initial concentration of cysteine in the medium was decreased to one-fifth of its original concentration without affecting the final yield of organism and under certain circumstances glutathione could be omitted. Casein hydrolysate was successfully replaced by a mixture of twenty amino acids. Of the eighteen growth factors present in the original Howell & Pine medium, coenzyme A and citrovorum factor were found to be unnecessary for the cultivation of the Wills strain of *A. israelii*. These modifications to the original medium, together with the elimination of starch from it, led to the formulation of a simplified defined medium (HP6/B, Table 6) which has supported the Wills strain of *A. israelii* in continued subculture over a period of 6 months. The medium contains approximately 65% of the total-N of the Howell & Pine medium and consistently gives a better yield of the organism (Table 6). Less extensive tests have shown that the Wills strain of *A. israelii* can survive at least five serial cultures in medium HP8 (Table 6), containing only 35% of the total-N of medium HP, without diminution in the growth yield. Of the twenty amino acids present in media HP6/B and HP8, cysteine and tryptophan are essential for the Wills strain of *A. israelii*.

The results depicted in Fig. 2 indicated that glutathione was not an essential component of the growth medium but that this compound was effective in decreasing the prolonged lag exhibited when an inoculum taken from a mother culture which had entered the stationary phase was used. Inocula taken from a rapidly developing mother culture gave no lag whether or not glutathione was present in the medium. Glutathione might fulfil either or both of two functions; it might act as a redox buffer (E'_0 = 40 mV.; pH 7) and/or as a nutrient for growth. Addition of ascorbic acid (E'_0 = 50 mV.; pH 7) to medium HP 4 (Table 6) containing 100 μg. glutathione/ml. did slightly decrease the lag in a controlled experiment. This result suggests that the redox potential of the medium might be critical especially in the initial growth phase of *Actinomyces israelii* strain Wills but does not prove that glutathione acts exclusively or even mainly as a redox buffer. Glutathione may be required for metabolism within the cell and the actively growing organism is perhaps able to synthesize glutathione at a rate adequate for continued rapid growth, while the organism taken from a stationary phase culture is perhaps unable initially to synthesize the peptide sufficiently fast for rapid growth. It may be significant that L-cysteine proved to be an essential amino acid in the nutrition of the organism and it may be recalled that Erikson & Porteous (1953) emphasized the need for frequent subcultivation in the successful maintenance of *A. israelii*.

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The Growth Factor Requirements of the Wills Strain of *Actinomyces israelii* Growing in a Chemically Defined Medium

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SUMMARY

A defined medium HP6/B used by Christie & Porteous (1962) for the cultivation of a single strain (Wills) of *Actinomyces israelii* contained sixteen growth factors. By using the quantitative inoculation technique of Christie & Porteous (1960) and a semi-quantitative method for assessing growth it has now been found that the Wills strain will grow in the presence of only five of the sixteen growth factors. These growth factors are: biotin, inositol, nicotinic acid, pyridoxal, riboflavin.

INTRODUCTION

Howell & Pine (1956) described a medium originally devised for the cultivation of *Histoplasma capsulatum*, which also supported the growth of certain strains of several actinomyces species including *Actinomyces israelii*. Christie & Porteous (1962) eliminated starch from the original medium thereby converting it to a completely defined medium. Since it was also possible to decrease the nitrogen content of the Howell & Pine medium to about 65% of the original value, with a concomitant increase of 27% in the yield of organism (Christie & Porteous, 1962) it was decided to see whether the sixteen growth factors present in the Christie & Porteous medium could be decreased in number. An account is given of experiments which elucidated the minimal growth factor requirements of the Wills strain of *A. israelii*.

METHODS

Organism. The Wills strain of *Actinomyces israelii* was again used throughout the present work.

Medium. Medium HP6/B (Christie & Porteous, 1962) was selected as the control medium containing sixteen growth factors. Experimental media were prepared by adding selected growth factors to medium HP6/B less all growth factors. Control and experimental media were prepared with ion-exchange water and were dispensed on the same day into replicate tubes and sealed with liquid paraffin (Christie & Porteous, 1962). Each growth factor was always incorporated at the concentration given by Christie & Porteous (1962).

Culture technique. The general culture techniques were those described by Christie & Porteous (1962). In assessing the ability of experimental media to support the Wills strain of *Actinomyces israelii* the quantitative determination of growth

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curves (Christie & Porteous, 1962) was abandoned for present purposes and the following semi-quantitative technique used. An inoculum was prepared and transferred by the technique of Christie & Porteous (1960) to at least four tubes of medium HP6/B and to at least four tubes of each test medium. Cultures were observed daily and growth scored visually, at the end of each incubation period of 5 days, on a scale such that the inoculum scored 1 and growth in the control medium HP6/B scored 5. Transfers were made at 5-day intervals to identical media, subculturing (so far as possible) equal inocula in each case. Visual inspection of the cultures included observation of the type of colony formed. Typical, i.e. coherent 'bread-crumb' or 'cauliflower' colonies which left a clear supernatant medium were recorded as 'T'; non-coherent, soft, slimy or 'fluffy' colonies were recorded as 'S'. Furthermore, a record was kept of the number of serial subcultures which the organism survived in any one medium. Subcultivation was continued as long as growth occurred, up to a maximum of five serial transfers. No medium was accepted as adequate unless it supported good typical growth of the organism during each of these five serial subcultures. On this method of observation a score, for example, of 1S2 indicated that no growth occurred, that the colonies were atypical and that they survived only two serial subcultures; likewise a score of 3T4 indicated that the amount of growth obtained was 3/5 of that in the control medium, that the colonies were typical and survived four serial subcultures.

RESULTS

In the absence of any published information on the growth factor requirements of *Actinomyces israelii*, initial experiments were designed in a largely arbitrary manner. The slow growth of the organism, and the difficulties involved in securing reasonably uniform inocula during any one experiment, precluded systematic screening tests in media lacking only one growth factor in turn. Table 1 records the results of the initial experiments. Pyridoxamine and *p*-aminobenzoic acid were not included in this test series on the supposition that any requirement for these compounds would be met by the inclusion of pyridoxal and folic acid, respectively, in the test media. Poor growth, atypical colony appearance and poor survival in serial subcultures went hand in hand and indicated that the combinations of growth factors represented by test media 1–8 in Table 1 did not meet the requirements of the Wills strain of *A. israelii*. Nevertheless, the organism survived two serial cultures in those media (4, 5, 6, 8) which contained nicotinamide and/or nicotinic acid. Comparison of the results obtained in these media did not indicate a preferential requirement for one or other of these two compounds. The second part of Table 1 records the results of tests of growth factors in other mixtures, most of which included nicotinic acid. Media 9, 11, 12, 14, 16, 18 and 20 provided mixtures of growth factors which satisfied the criteria for an adequate medium mentioned above. Medium 10 suggested a requirement for nicotinic acid in the nutrition of the Wills strain of *A. israelii*; similarly media 15, 17 and 19 suggested a requirement for inositol. Of the adequate media, medium 20 contained the least number of growth factors of which two (nicotinic acid, inositol) were apparently essential. These two growth factors were therefore incorporated in all subsequent test media and the remaining five growth factors of medium 20 (Table 1) incorporated in all possible mixtures of four and of three. The results of growth tests in these

media are summarized in Table 2. Medium 22 served as the control; media 23–27 inclusive contained the possible mixtures of four test-growth factors. Media 24, 25 and 23 suggested a requirement for riboflavin, pyridoxal and biotin, respectively, in the nutrition of the organism. Comparison of the results for media 22, 26 and 27 suggested, but did not prove, that neither calcium pantothenate nor folic acid was required.

Media 28–37 inclusive contained all possible mixtures of three out of five test-growth factors. Medium 28 confirmed the requirement for biotin; medium 37 confirmed the suggestion that neither calcium pantothenate nor folic acid was required, singly or together. Comparison of the results obtained in medium 37 with those obtained in all other media in Table 2 showed that the mixture of nicotinic acid, inositol, riboflavin, pyridoxal and biotin represented an apparently minimal requirement for strain Wills of *Actinomyces israelii*. Medium HP6/B modified to contain only these five growth factors was designated HP9 in conformity with the nomenclature used by Christie & Porteous (1962).

KEY TO TABLES 1 AND 2

Abbreviations for growth factors: N = nicotinic acid, Na = nicotinamide, I = inositol, R = riboflavin, P = pyridoxal, B = biotin, Pan. = Pantothenic acid, F = folic acid, T = thiamine, T.a = thioctic acid, O = oleic acid, Pim. = pimelic acid, B_{12} = cyanocobalamin (vitamin B_{12}), H = haemin.

Other symbols: Each test medium is denoted by a number in the first column.

The only growth factors present in a test medium are those indicated by a + sign.

The symbol *a* indicates that this growth factor was not added to the medium but that a requirement for it is indicated by comparison of observations on growth in this medium with observations on growth in the medium immediately above in the table.

The symbol *b* is used in the same sense as *a* except that comparison should be made with observations on growth in the medium immediately below in the table.

The symbol *c* is used in the same sense as *a* except that comparison should be made with observations on growth in the preceding medium but one in the Table.

For explanation of symbols in the 'Observations' column, see the text.

Table 1. *Elucidation of the growth factor requirements of the Wills strain of* Actinomyces israelii. *Basal medium as indicated in text; 5-day incubation period, temperature* 37°, *media sealed with liquid paraffin*

Medium	N	I	R	P	B	Pan.	F	T	T.a	O	Pim	Na	B_{12}	H	Observations
1	.	.	.	.	.	.	.	.	.		.	.	.	.	1S1
2	.	.	+	+	+	+	+	.	.	.	.	.	.	.	1S1
3	.	.	+	+	+	+	+	+	+	.	.	.	.	.	1S1
4	+	.	+	+	+	+	+	+	+	.	.	.	.	.	1S2
5	.	.	+	+	+	+	+	+	+	.	.	+	.	.	1S2
6	+	.	+	+	+	+	+	+	+	.	.	+	.	.	1S2
7	.	.	+	+	+	+	+	+	+	.	.	.	+	.	1S1
8	+	.	+	+	+	+	+	+	+	.	.	+	+	.	1S2
9	+	+	+	+	+	+	+	.	.	+	+	.	.	.	5T5
10	*a*	+	+	+	+	+	+	.	.	+	+	.	.	.	1S2
11	+	+	+	+	+	+	+	+	+	+	+	+	.	.	5T5
12	+	+	+	+	+	+	+	+	+	+	+	.	.	+	5T5
13	.	.	+	+	+	+	+	+	+	.	.	+	.	+	1S1
14	+	+	+	+	+	+	+	.	.	+	+	.	.	.	5T5
15	+	*a*	+	+	+	+	+	.	.	+	+	.	.	.	1S1
16	+	+	+	+	+	+	+	.	.	+	.	.	.	.	5T5
17	+	*a*	+	+	+	+	+	.	.	+	.	.	.	.	1S1
18	+	+	+	+	+	+	+	.	.	.	+	.	.	.	5T5
19	+	*a*	+	+	+	+	+	.	.	.	+	.	.	.	1S1
20	+	+	+	+	+	+	+	.	.	.	.	.	.	.	5T5

Table 2. *Elucidation of the growth factor requirements of the Wills strain of* Actinomyces israelii. (*For conditions of culture and explanation of symbols, see Table* 1.)

Medium	N	I	R	P	B	Pan.	F	Observations
23	+	+	+	+	*b*	+	+	1T3
22	+	+	+	+	+	+	+	5T5
24	+	+	*a*	+	+	+	+	1S1
25	+	+	+	*c*	+	+	+	1S2
26	+	+	+	+	+	.	+	5T5
27	+	+	+	+	+	+	.	5T5
28	+	+	+	+	*a*	+	.	1S2
29	+	+	+	+	.	.	+	1S2
30	+	+	+	.	+	.	+	1S1
31	+	+	.	+	.	+	+	1S1
32	+	+	+	.	.	+	+	1S2
33	+	+	+	.	+	+	.	1S2
34	+	+	.	+	+	.	+	1S1
35	+	+	.	.	+	+	+	1S1
36	+	+	.	+	+	+	.	1S1
37	+	+	+	+	+	.	.	5T5
38	+	+	+	+	.	.	.	1T3
39	+	+	+	.	.	.	.	1S1
40	+	+	.	.	.	.	.	1S1

DISCUSSION

Caution is necessary in interpreting the present results which do not exclude the possibility that inositol alleviated inhibition of growth by some other growth factor or combination of growth factors present in the test media (Table 1). Similar remarks apply to the apparent requirement for nicotinic acid (Table 1) and, with less force, to the apparent requirement for riboflavin, pyridoxal and biotin (Table 2). On the data available the five growth factors nicotinic acid, inositol, riboflavin, pyridoxal and biotin represent the apparent rather than the absolute minimal requirement for *Actinomyces israelii* strain Wills.

Of the five growth factors apparently required by the Wills strain of *Actinomyces israelii*, nicotinic acid, riboflavin, pyridoxal and biotin have established biochemical functions and call for no comment. The biochemical function of inositol as a growth factor is unknown; it is well established as a growth factor for many fungi and for many mammalian cell cultures but is not a trace nutrient for any species of the Schizomycetes so far investigated (Knight, 1955). The apparent requirement for inositol, if confirmed for the Wills and other strains of *Actinomyces israelii*, would make this species an exception to the general rule since several criteria (Waksman, 1940; Waksman & Henrici, 1943; Erikson, 1949; Cochrane, 1961) place the Actinomycetes among the Schizomycetes rather than among the Eumycetes.

The unexpectedly simple growth factor requirements of the Wills strain of *Actinomyces israelii* (Tables 1, 2) probably imply a greater biosynthetic capacity than was suggested by the view current up till 1953 that only extremely complex media would support the growth of *A. israelii* (Erikson & Porteous, 1953). Thus the fact that neither coenzyme A (Christie & Porteous, 1962) nor pantothenic acid was required for growth was surprising for a reputedly fastidious anaerobic heterotroph. In the absence of any detailed knowledge of the metabolism of *A. israelii* the present results may mean either (*a*) that the organism carries out reactions involving

coenzyme A, but is able to synthesize this compound *de novo*, or (*b*) that coenzyme A plays no part in the metabolism of *A. israelii*. In the light of present knowledge it would seem more probable that the Wills strain of *A. israelii* synthesizes coenzyme A *de novo*. In this respect, it may be significant that L-cysteine proved to be an essential amino acid in the nutrition of the Wills strain of *A. israelii* (Christie & Porteous, 1962).

Thioctic acid (α-lipoic acid), an established hydrogen- and acyl-carrier, was not required by the Wills strain of *A. israelii*. Nor was there any requirement for thiamine which, as thiamine pyrophosphate, is associated with or bound to α-lipoic acid (Reed, 1957; Reed & deBusk, 1954) as a coenzyme to certain α-oxo-acid dehydrogenases.

None of the related compounds *p*-amino-benzoic acid, folic acid and 'citrovorum factor' (N^5-formyl-tetrahydrofolic acid) was required by the Wills strain of *Actinomyces israelii* (Tables 1, 2; Christie & Porteous, 1962). McIntosh, Purko & Woods (1957) suggested that *Escherichia coli* could hydroxymethylate α-oxovaleric acid in the synthesis of pantothenic acid without the participation of folic acid derivatives; should such a mechanism be present in the Wills strain of *A. israelii* and operative for this and other 'one carbon transfer' reactions the simultaneous absence of any nutritional requirement for *p*-aminobenzoic acid, folic acid, 'citrovorum factor', purine and pyrimidine bases, pantothenic acid and coenzyme A (Table 2; Christie & Porteous, 1962) would be readily explained; the first three compounds would play no part in the economy of the organism and the 'one-carbon transfer' reactions involved in the *de novo* biosynthesis of the others would still be possible. Alternatively, it must be assumed that *A. israelii* synthesizes 'citrovorum factor' and its component, *p*-aminobenzoic acid, *de novo*. If *p*-amino-benzoic acid is synthesized by *A. israelii*, it is noteworthy that L-tryptophan is an essential nutrient for the organism (Christie & Porteous, 1962) although both compounds have a common precursor, shikimic acid (Davis, 1955).

The deductions discussed above would very likely be vitiated if the culture investigated were contaminated. Every precaution was taken in the present work to avoid contamination (Christie & Porteous, 1962) and no evidence of contamination of experimental cultures was found; Erikson & Porteous (1955) have, however, emphasized the difficulties of detecting small numbers of contaminants within the mycelial colonies of *A. israelii* and pointed out that changes in the colony form in liquid or on surface media may not be apparent in relatively lightly contaminated cultures of *A. israelii*.

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Studies on Staphylococcal Penicillinase

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SUMMARY

The induction of penicillinase in *Staphylococcus aureus* resistant to benzyl penicillin has been investigated. Inducers tested included several new penicillins derived from 6-aminopenicillanic acid, benzyl penicillin amide and cephalosporin C. Fifteen strains of *S. aureus* were examined, one in detail and the others using only two or three inducers. All strains possessed basal penicillinase and all were inducible, although maximum enzyme levels varied somewhat from one strain to another.

Hydrolysis of the penicillins by staphylococcal penicillinase was investigated by using supernatant fluid from a cephalosporin C-induced culture of *Staphylococcus aureus* 524 as the source of enzyme. Under these conditions all the penicillins except methicillin were inactivated, although at different rates. Hydrolysis of methicillin was only detectable at much higher enzyme concentrations. Hydrolysis of cephalosporin C was not observed.

The use of penicillins for the treatment of staphylococcal infections is discussed in the light of these results.

INTRODUCTION

Bondi & Dietz (1945, 1948) observed that penicillinase was produced by all penicillin-resistant strains of *Staphylococcus aureus* that occurred clinically. They concluded that penicillinase production contributed to penicillin resistance. Bondi, de Saint Phalle, Kornblum & Moat (1954) reported that enzyme formation was constitutive, but Geronimus & Cohen (1957, 1958) found that penicillinase was inducible by penicillin, although there was a fairly high level of basal enzyme. They were unable to detect its presence in culture supernatants. They showed that the increase in penicillinase activity on induction with penicillin was due not to an increase in the rate of transport of substrate to pre-existing enzyme but to an increase in concentration of the enzyme within the cells. The above work was carried out using benzyl penicillin as the inducing agent, but benzyl penicillin is so rapidly inactivated that unless repeated additions of small amounts of penicillin are made at intervals it is difficult to demonstrate induction.

Little is known of the variation in penicillinase production by different penicillinase-producing strains of staphylococci, and we used inducers which are not rapidly hydrolysed (gratuitous inducers) in order to study the maximum enzyme production of which the strains are capable. For these reasons a number of strains of penicillinase-producing staphylococci were studied, using cephalosporin C and 6-(2:6-dimethoxybenzamido)-penicillanic acid (methicillin) as inducers, and following the production of enzyme for 4 hr. Also, a limited study was made using benzyl penicillin and 6-aminopenicillanic acid as inducers and estimating the penicillinase after 2 hr.

METHODS

Organisms. The penicillinase-producing strain of *Staphylococcus aureus*, strain 524 SC (Rogers, 1953) was normally maintained in ampoules in the dried state and grown back in broth. Several nutrient agar slopes were inoculated from the broth culture, and after incubation overnight at 35° were stored at +2° until required. After 8 days a subculture was made on to fresh agar slopes and any remaining from the first batch were discarded.

We are grateful to Professor L. P. Garrod, St Bartholomew's Hospital, London, for a number of strains of staphylococci resistant to benzyl penicillin, isolated from human infections (Strains 41, 46, 50, 74, 75 and 82 to 90). The samples as obtained were freeze-dried and grown when required as above.

Media. The staphylococci were found to grow well in Hedley–Wright broth containing 2·5% (w/v) of sodium β-glycerophosphate and 0·1% (w/v) glucose (Rogers, 1954), or in casein hydrolysate medium containing 0·5% (w/v) glucose, 1 μg./ml. nicotinic acid and 1 μg./ml. thiamine (Rogers, 1945).

Induction tests

(*a*) *Staphylococcus aureus* 524 *SC.* The organisms on one of the stored agar slopes were washed off with 1 ml. of the appropriate medium and added to 50 ml. of the Hedley–Wright broth or the casein hydrolysate medium in a 250 ml. conical flask. The cells were then incubated at 35° on a graded shaker (1 foot throw, 86 cyc./min., Kantorowicz, 1951) until the opacity was equivalent to between 0·1 and 0·2 mg./ml. dry weight of cells. A 10% solution of gelatin was usually added at this stage to give a final concentration of 1% gelatin in order to reduce inactivation of the enzyme.

Conical flasks (25 ml.) containing the test substance at appropriate concentrations in 0·4 ml. of 0·01 M-phosphate buffer of pH 7 were inoculated with 2·6 ml. of the culture of *Staphylococcus aureus*. The flasks were then placed on the shaker and incubated at 35° for 2 hr., after which 0·4 ml. 0·005 M-8-hydroxyquinoline was added to each flask, and the contents chilled in ice. Since there was some variation in basal enzyme and induced enzyme from day to day, two control flasks were always used, one containing only culture and phosphate buffer and the other containing culture, phosphate buffer and 5 μg./ml. cephalosporin C.

(*b*) *Other strains.* The supplemented Hedley–Wright broth (100 ml. in a 500 ml. conical flask) was inoculated with 0·4 ml. of a fully grown broth culture and incubated at 35° on the shaker. When the optical density was approximately equivalent to 0·125 mg./ml. dry weight of cells, 10 ml. of melted 10% gelatin was added, and then the inducer. For routine tests cephalosporin C (5 μg./ml.) or methicillin (0·5 μg./ml.) were used, since these concentrations were found to have a negligible effect on growth but gave strong induction of penicillinase. Samples were taken every hour for 4 hr. The proportion of free enzyme was also estimated in the 4 hr. sample by centrifuging a sample and assaying the supernatant.

Penicillinase assay. The manometric assay of Henry & Housewright (1947) was used under the conditions employed by Pollock (1952). The enzyme activity of the samples is expressed in units/ml. (Pollock & Torriani, 1953); one unit of penicillinase is the quantity of enzyme which destroys 1 μ mole of benzyl penicillin in 1 hr. under

standard conditions. The same technique was used to measure the rate of hydrolysis of the other penicillins using a known enzyme concentration.

Compounds tested. The compounds tested were benzyl penicillin (penicillin G), phenoxymethyl penicillin (penicillin V), phenoxyethyl penicillin (phenethicillin), phenoxypropyl penicillin (PA-248), 6-aminopenicillanic acid (6-APA), 6-(2:6-dimethoxybenzamido)-penicillanic acid (methicillin), 6-(2:4:6-trinitrophenyl)-aminopenicillanic acid (picramido-PA), benzyl penicillin amide and cephalosporin C. Benzyl penicillin amide was synthesized by the method of Holysz & Stavely (1950). It was an amorphous yellow powder, and was about 80% pure by the hydroxamate assay (Ford, 1947). Picramido-PA was prepared by the reaction of equimolar proportions of 6-APA and picryl chloride in a mixture of acetone and 0·5M phosphate buffer at pH 7. It was isolated and tested as the triethylamine salt. It was orange-brown and water soluble, and was 91% pure assayed by the hydroxamate method. Penicillin G and penicillin V were obtained as the sodium and potassium salts respectively from Glaxo Ltd., Greenford, Middlesex. We are grateful to Pfizer Ltd., Folkestone, Kent, for gifts of 6-aminopenicillanic acid, phenoxyethyl penicillin and PA-248; to Beecham Research Laboratories Ltd., Brockham Park, Betchworth, Surrey, for a gift of methicillin (BRL 1241), and to Dr E. P. Abraham, F.R.S., Sir William Dunn School of Pathology, Oxford, for a gift of cephalosporin C sodium salt.

Preparation of enzyme for substrate studies

The supplemented Hedley–Wright broth (500 ml.) was inoculated with *Staphylococcus aureus* 524 SC and incubated at 35° on the shaker until the opacity was equivalent to 0·1 mg./ml. dry weight of cells. Cephalosporin C was added to give a concentration of 5μg./ml. Sterile gelatin was added to a concentration of 1% and the mixture returned to the shaker at 35° for a further 4 hr. 50 ml. 0·005M-oxine was then added and the cells centrifuged at 3000 ***g*** for 20 min. The supernatant was decanted and stored at +2°. The mean of two penicillinase assays of this preparation was 28·4 units/ml.

RESULTS

Induction tests

The supplemented Hedley–Wright broth was a somewhat more satisfactory medium (for induction of enzyme) than the casein hydrolysate medium, and was used throughout. The addition of gelatin (Manson & Pollock, 1953) had little effect on the enzyme titre in the 2 hr. period studied.

The results of typical experiments on *Staphylococcus aureus* 524 SC with the various penicillins are shown in Table 1. Abraham & Newton (1956) and Pollock (1957), using *Bacillus cereus* 569 as test organism, had noted that an intact β-lactam-thiazolidine nucleus of the type found in benzyl penicillin seemed to be essential for induction, but this criterion has now to be extended somewhat since cephalosporin C has a β-lactam-dihydrothiazine nucleus (Abraham & Newton, 1961). Benzyl penicillin and phenoxymethyl penicillin did not give clear-cut increases in penicillinase. Growth of the organisms, however, was not affected by 25μg./ml. Since both of these penicillins are rapidly inactivated by the basal enzyme present in the culture, the concentration of penicillin which arrives at the primary site of inducer action might be considerably different from that of the medium as a whole, and may

depend on a large number of variables. Phenoxyethyl penicillin behaved similarly to benzyl penicillin. Phenoxypropyl penicillin (PA-248) gave small but definite induction at 1 μg./ml. Benzyl penicillin amide was insoluble in water, and was dissolved in a small volume of acetone and diluted with an equal volume of water. On dilution with culture this gave a colloidal suspension of the amide. It gave a slight induction, and at 20 μg./ml. was somewhat inhibitory.

Table 1. *Induction of penicillinase in* Staphylococcus aureus 524 *SC by various penicillins and cephalosporin C*

The experiments were carried out in Hedley–Wright medium, as described in the text, and the density of the organism on adding inducer was about 0·1 mg./ml. dry weight. Enzyme levels were estimated after 2 hr. at 35°. Single additions of benzyl penicillin, phenoxymethyl penicillin and phenoxyethyl penicillin (25 μg./ml.) gave enzyme increases of up to twice the basal level, which was of doubtful significance, but see Table 2.

Compound	Basal enzyme (units/ml.)	Range of inducer concentration giving optimal induction (μg./ml.), and enzyme level obtained (units/ml.)		Lowest concentration of inducer giving significant inhibition of growth (μg./ml.)
		Inducer	Enzyme	
Phenoxypropyl penicillin (PA-248)	3·4	1–5	8–11	25
Benzyl penicillin amide	2·9	2–10	9–11	20
6-Aminopenicillanic acid (6-APA)	3·8	10–50	40–60	50
Methicillin	3·0	0·5–2	50–65	2
Picramido-PA	4·1	5–10	85–110	50
Cephalosporin C	1·0	10–50	30–50	100

6-Aminopenicillanic acid could be tested over a wide range of concentration since it has very low antibiotic activity compared with penicillin G (Batchelor, Doyle, Nayler & Rolinson, 1959). It is a much more powerful inducer than the preceding compounds tested. Good induction was obtained at 1 μg./ml. and maximum induction at about 50 μg./ml. Picramido-PA was a strong inducer, giving the highest titres recorded in this series, possibly because its antibacterial activity was very low, and its rate of hydrolysis by the enzyme was also low. Methicillin is a strong inducer of penicillinase, as reported by Rolinson *et al.* (1960), and maximum induction under the conditions used here is shown at low concentrations in the range 0·5–2 μg./ml. It is, however, an inducer at very low concentrations, e.g. 0·05 μg./ml. At concentrations higher than 2 μg./ml. growth of the organisms is inhibited. Cephalosporin C differs from the penicillins in that induction is hardly demonstrable at 1 μg./ml., while at 5 μg./ml. and above considerable induction occurs.

Enzymic hydrolysis of penicillins

The rates of hydrolysis of the penicillins at 30° by penicillinase from *Staphylococcus aureus* 524 SC are given in Table 2. All were tested under the same conditions using 2·8 μ moles/ml. of the penicillin and 1·0 ml. of enzyme preparation containing 9·1 units/ml. The reaction mixture was buffered at pH 7. In each case where reaction took place the rate of reaction was constant over the period of measurement.

The rates given are the average of five experiments carried out under identical conditions on each penicillin.

The most readily hydrolysed penicillins are phenoxymethyl penicillin, benzyl penicillin and phenoxyethyl penicillin. It was reported by Garrod (1960) that phenethicillin was somewhat more effective against resistant staphylococci than phenoxymethyl penicillin, and the latter was slightly more active than benzyl penicillin. Since phenoxymethyl penicillin is hydrolysed even more rapidly than benzyl penicillin, it is possible that other factors, such as its relative acid stability, may play a part in determining its effectiveness against staphylococci. The low rate of hydrolysis of benzyl penicillin amide may have been due to it being in a colloidal state rather than in true solution.

Table 2. *Inducers of penicillinase in* Staphylococcus aureus

Compound	Induction	Rate of hydrolysis by *S. aureus* penicillinase relative to benzyl penicillin (all at 2·8 mM substrate concentration)
Benzyl penicillin (Penicillin G)	+*	1
Phenoxymethyl penicillin (Penicillin V)	+*	1·27
Phenoxyethyl penicillin (Phenethicillin)	+*	0·92
Phenoxypropyl penicillin (PA-248)	+	0·60
6-(2:6-Dimethoxybenzamido)-penicillanic acid (Methicillin)	+	0·003†
6-(2:4:6-Trinitrophenyl)-aminopenicillanic acid (Picramido-PA)	+	0·11
6-Aminopenicillanic acid	+	0·15‡
Benzyl penicillin amide	+	0·19
Cephalosporin C	+	less than 0·01

* Repeated additions needed to demonstrate unequivocal induction.
† Novick (1962); V_{max} is 0·03 (iodometric assay).
‡ Novick (1962), by iodometric assay.

On hydrolysis of 6-aminopenicillanic acid the carboxyl group forms part of an α-amino acid grouping, and is thus not sufficiently acidic to release CO_2 from the bicarbonate solution (Steinman, 1961*a*). Its hydrolysis cannot therefore be measured manometrically, but it can be measured by the iodometric method (Perret, 1954).

Penicillinase production by different strains of staphylococci

All the fifteen penicillinase-producing strains behaved in much the same way. All of them were inducible with 6-aminopenicillanic acid, with cephalosporin C and with methicillin. Figure 1 shows the results of a typical experiment.

There was evidence of loss of enzyme activity in induced cultures of all strains. No way of preventing this loss of activity has yet been found, so that it is not possible to give exact figures for many of the parameters involved in enzyme induction. The following conclusions, however, seem to be fairly certain for induction with 6-APA, cephalosporin C and methicillin. Most strains had a basal enzyme activity equivalent to about 2–5 enzyme units per mg. dry weight of cells. The maximum level of activity generally occurs 2–3 hr. after adding inducer, with a drop at times later than this. Commonly by 4 hr. there has been a fall to three-

quarters or a half of the activity found at 2 or 3 hr. The maximum level of activity under the conditions used was generally 10–25 enzyme units/ml. of culture, equivalent to about 11–28 units per mg. dry weight of cells, although as noted in another section this could be increased by using higher levels of inducer than were used here.

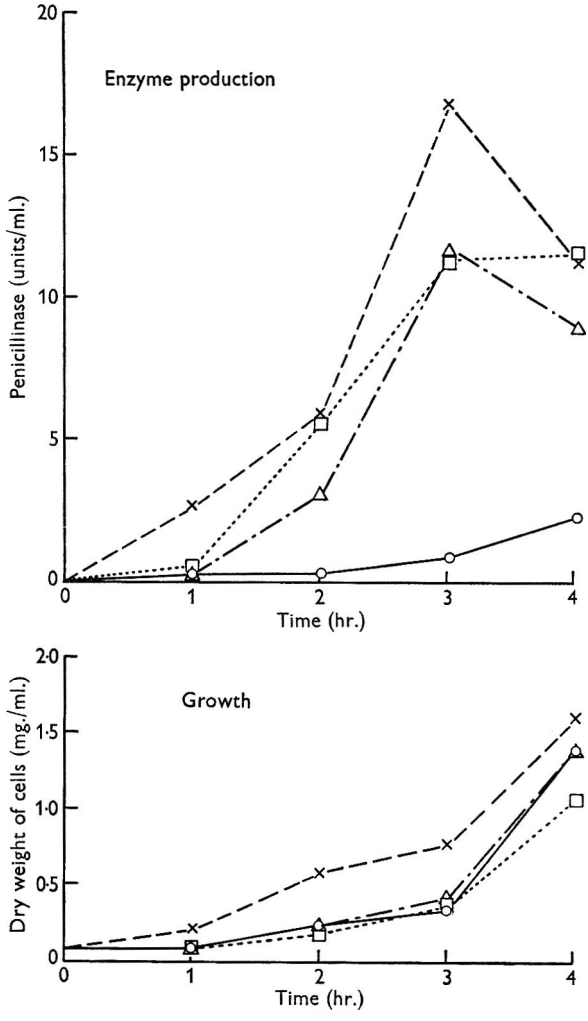

Fig. 1. Penicillinase production with various inducers in *Staphylococcus aureus* strain 46. —○— No inducer; —·△·— cephalosporin C, 5 μg./ml.; ---□--- methicillin, 0·5 μg./ml.; —×— 6-amino-penicillanic acid, 5 μg./ml.

The inducing activity of 0·5 μg./ml. of methicillin was approximately equal to that obtained with 5 μg./ml. of cephalosporin C, while 5 μg./ml. of 6-aminopenicillanic acid gave somewhat greater induction.

The level of the induced enzyme at 2–3 hr. after adding the inducer was usually 10–20 times the basal level. The lowest value for induced enzyme was four times that of the basal enzyme: it is thus clear that all the strains of penicillinase-producing staphylococci are inducible. However, high levels of enzyme are not pro-

duced by a single addition of benzyl penicillin. With all the strains the addition of 0·5μg./ml. of benzyl penicillin gave only a slight increase over the basal level of enzyme, being never more than twice that level; frequently the increase was undetectable by the technique used.

The amount of enzyme which was released from the cells was estimated 4 hr. after induction by assaying the supernatant fluid after centrifuging. It was generally found that about half the activity was cell-bound and half was extracellular. All the strains showed extracellular enzyme, but in preliminary experiments it was noted that very little cell-free enzyme was present earlier than 4 hr. after adding inducer. It may be noted, however, that the penicillinase of *Bacillus subtilis* is also mainly cell-bound in young cultures but mainly free after several hours (Pollock, 1961).

The findings above on penicillinase production in various strains of resistant staphylococci are in reasonable agreement with those of Wildhack, Ceci, Moat & Bondi (1960), Geronimus & Cohen (1957), Leitner & Cohen (1960), Rolinson *et al.* (1960), and Steinman (1961*b*).

DISCUSSION

It would appear from these results that all the strains of staphylococci resistant to benzyl penicillin can be induced to form penicillinase by treatment with the various penicillins. There does not seem to be any obvious difference in inducibility between the various resistant strains examined. Maximum induction obtained in a given time depends mainly on the initial concentration of the penicillin and on the rate at which it is hydrolysed by the enzyme as long as growth of the organism is unaffected. Other factors, such as the rate at which the inducer reaches its site of action in the bacteria, may have some slight effect, although this rate is generally assumed to be very rapid. Small amounts of benzyl penicillin were shown to be very rapidly fixed by an intracellular reaction in staphylococci by Rowley, Cooper, Roberts & Smith (1950). It is, however, possible that inducers of the cephalosporin C type may not be able to enter the cells so readily.

It is difficult with benzyl penicillin to demonstrate induction by a single addition of inducer. This is evidently because the benzyl penicillin is rapidly hydrolysed and after a short time the process of enzyme induction ceases. This is similar to most inducible enzyme systems, in which induction promptly ceases if the inducer is removed, and contrasts with the induction of penicillinase in *Bacillus cereus*, where enzyme production continues at a high rate after removal of the penicillin (Pollock, 1950). The efficacy of 6-aminopenicillanic acid, methicillin and cephalosporin C as inducers is evidently due to their resistance to inactivation by staphylococcal penicillinase. If a fairly constant concentration of benzyl penicillin is maintained by continuous addition, then induction continues and a high enzyme titre is obtained (Pollock, private communication). It follows from this that within a lesion in the animal produced by penicillin G resistant staphylococci, conditions for the induction of pencillinase may be very favourable no matter which penicillin is used for treatment. The penicillin will arrive at the site of infection continuously via the blood-stream so that even rapidly inactivated penicillins could induce considerable enzyme formation. Thus a non-inducing penicillin might therefore offer some clinical advantages. The rate of inactivation of the penicillin by the enzyme will largely

determine its effectiveness against the infection. In addition, when a penicillin is readily inactivated by the enzyme, the results of the usual *in vitro* tube-dilution sensitivity test can be very misleading as a guide to its clinical effectiveness, since the assay is commonly carried out using small and variable inocula, and the penicillin concentration at a given time will depend largely on the concentration of basal enzyme. Geronimus (1960) estimates that within a lesion the number of viable organisms could approach 10^{10} per gram of tissue, so that it is rash to predict the effectiveness of a penicillin when it is tested against inocula containing small numbers, such as 10^4 organisms/ml.

The most interesting of the true penicillins (i.e. excluding cephalosporin C) is methicillin. Its fairly low antibacterial potency compared to benzyl penicillin is offset by its resistance to staphylococcal penicillinase, making it just as effective against penicillinase-producing as against non-penicillinase-producing staphylococci. It is effective because it has an extremely low affinity for the enzyme (Novick, 1962). In clinical use the concentration of methicillin in the bloodstream, although therapeutically effective, is sufficiently low that enzymic inactivation (because of its very low affinity) is negligible. But it would not be necessary to change the enzyme affinity in order to develop clinically important resistance to methicillin; it would be sufficient if the ordinary staphylococcal penicillinase were to be produced in quantities about 1000 times greater than that found in most penicillinase-producing staphylococci. The use of penicillin has up to now selected for penicillinase-producing ability that is small but adequate from the point of view of the organism; it is possible that methicillin may be a more powerful selecting agent among the natural populations of staphylococci, either for a very high level penicillinase production or a changed penicillinase able to inactivate methicillin at low concentrations, or both. A watch should therefore be kept for the emergence of strains with a very high level of penicillinase production, as these would be clinically resistant to methicillin.

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Antibacterial Activity, Penicillinase Stability and Inducing Ability of Different Penicillins

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SUMMARY

There are now at least three main types of antibacterial activity shown by different penicillins—the ordinary penicillin G type, the type shown by methicillin active against penicillinase-producing staphylococci and the 'broad spectrum' type of activity shown by α-aminobenzylpenicillin.

The inducing capacity of different penicillins can be measured by the concentration required for maximal or half maximal induction, which gives the Michaelis (induction) constant, or by the total amount of enzyme eventually produced. But either of these methods of determining inducing capacity may give misleading results with penicillins which may be easily hydrolysed during the induction process.

Of the penicillins used methicillin is by far the best inducer—but it is uncertain how much this is due to a true 'inducing affinity' and how much a result of its resistance to hydrolysis.

INTRODUCTION

The isolation and crystallization of 6-aminopenicillanic acid from the fermentation liquor of *Penicillium chrysogenum* by Batchelor, Doyle, Nayler & Rolinson (1959) opened the way to the synthesis of large numbers of new penicillins which it had not been possible to produce previously by microbiological methods. Already several new penicillins have been produced commercially and rival claims have been made in some cases for products with not very different antibacterial activities. Two of these new penicillins, methicillin and α-aminobenzylpenicillin, have shown antibacterial activity of a quite different kind from that shown by penicillin G. It is thus no longer possible to speak of the penicillin type of antibacterial spectrum without qualification, and we can certainly no longer assume that sensitivity or resistance to one penicillin applies to others. The interpretation of laboratory sensitivity tests in the case of penicillins is complicated by the fact that some organisms have enzymes which destroy the antibiotics. It is, therefore, important to know with any new penicillin how stable it is to 'penicillinases' which may be produced by different organisms against which it is likely to be used. Some micro-organisms show 'inherent' (Knox & Smith, 1961) resistance to penicillin of a non-penicillinase type; for example many strains of *Escherichia coli* and *Proteus* spp. are inherently resistant to penicillin G, though they may be quite sensitive to the 'broad spectrum' penicillin, α-aminobenzylpenicillin (Rolinson & Stevens, 1961). In addition some penicillins are more capable of inducing penicillinase in organisms brought into contact with them than others. For example, methicillin has been found to be a better inducer of staphylococcal penicillinase than penicillin G.

(Rolinson, Stevens, Batchelor, Cameron-Wood & Chain, 1960; Knox, 1960; Steinman, 1961*a*).

We have investigated six new penicillins and 6-aminopenicillanic acid with regard to their antibacterial activity, their stability to staphylococcal penicillinase and their ability to induce penicillinase in staphylococci. The results of these experiments are here reported.

METHODS

Penicillins. Penicillin G was obtained from Glaxo Laboratories Ltd. (Greenford, England), penicillin V, phenethecillin ('Broxil'), methicillin ('Celbenin'), α-aminobenzyl penicillin ('Penbritin') and 6-aminopenicillanic acid from Beecham Research Laboratories (Brockham Park, Surrey), and phenoxypropyl penicillin from Pfizer Limited (Folkestone, Kent).

Measurement of antibacterial activity. Most of the bacterial cultures used were freshly isolated from clinical material but in some cases stock strains were used. Cultures were inoculated into nutrient broth and incubated overnight. One drop (0·02 ml.) of undiluted broth culture or of various dilutions was dropped either into liquid media or on to nutrient agar plates containing serial dilutions of the antibiotic under investigation. In some experiments, in which large numbers of plates were required, volumes of about 0·01 ml. were inoculated by means of the phage-typing machine described by Tarr (1958) as a replicating device. Inoculated tubes or plates were incubated overnight at 37° and again for a second day.

Source of staphylococcal penicillinase. The culture used was the E3 penicillinase-producing strain of *Staphylococcus aureus* previously described (Knox, 1960; Knox & Smith, 1961). This strain (of phage type 77) was originally isolated before the start of treatment from a lesion in the chest wall of one of the first patients to be treated with methicillin.

Measurement of penicillinase stability of different penicillins. For determining the stability of different penicillins to staphylococcal penicillinase a suspension of organisms grown overnight at 37° in the presence of methicillin (0·5μg./ml.) as an inducing agent was mixed in equal parts in 1 oz. bottles with the appropriate penicillin in a final concentration of 2 mg./ml. Sodium *p*-chlormercuribenzoate was added to 0·001 M to prevent further induction of penicillinase during the experiment (Steinman, 1961*a*, *b*). The reagents were pre-warmed and the reaction followed at 37° in a water bath. Samples of 2 ml. were taken at intervals and the amount of penicillin remaining determined by the hydroxylamine method of Boxer & Everett (1949) by estimation of the colour with an EEL (Evans Electroselenium Ltd., Harlow, Essex) colorimeter and comparison with a previously prepared standard curve.

As the molecular weights of the different penicillins used are about 350–400 the initial concentration of 2 mg./ml. was equivalent to M/175 to M/200. With 6-aminopenicillanic acid, however, with a molecular weight of 216, the substrate concentration was about 0·01 M.

Induction of penicillinase

To determine the optimal conditions for induction, preliminary experiments were done with methicillin because of its known high inducing ability. Short periods of induction were used in order to separate as far as possible induction from growth and

to minimize secondary changes and breakdown of the inducing agent which might occur in broth if induction were greatly prolonged.

Concentration of micro-organisms. *Staphylococcus aureus* strain E3 was inoculated into infusion broth and after overnight incubation the cocci were centrifuged down, washed with broth and resuspended at various optical densities in broth at 37°. Volumes of 19·5 ml. were placed in 25 ml. bottles and induced with methicillin (0·5 μg./ml.) contained in 0·5 ml. broth by shaking at 100 cycles/min. at 37°. After 1 hr., further induction was prevented by the addition of *p*-chlormercuribenzoate to

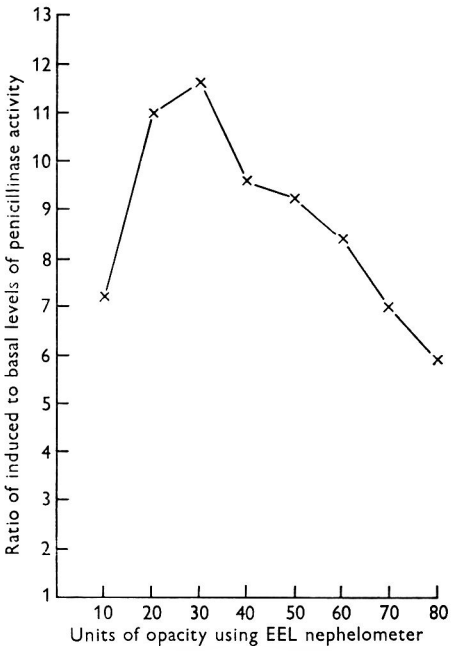

Fig. 1

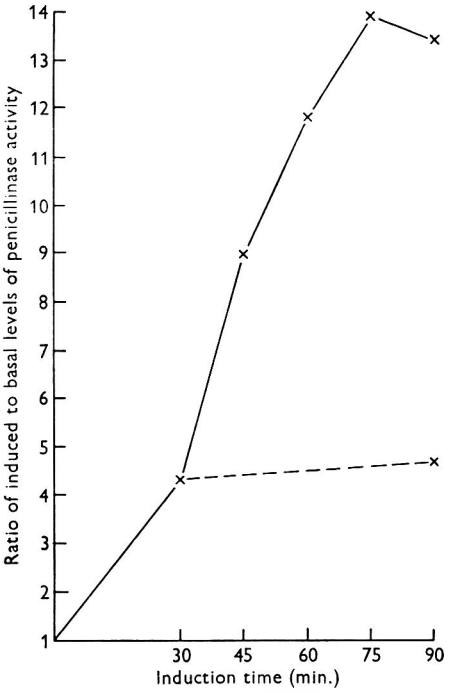

Fig. 2

Fig. 1. Effect of varying the initial concentration of organisms on the amount of penicillinase obtained during induction.

Fig. 2. Effect of varying the induction time on the amount of penicillinase obtained during induction.

0·001 M, since Henry & Housewright (1947) found that 0·001 M *p*-chlormercuribenzoate had no effect on penicillinase activity, and Steinman (1961*a*, *b*) found that it stopped any further induction of penicillinase in staphylococci. (The results of Henry & Housewright were confirmed by testing the action of 0·002 M *p*-chlormercuribenzoate for 2 hr. at 37° on the intracellular penicillinase of staphylococci defatted by the method of Gilson & Parker (1948). The defatted organisms when incubated with 0·002 M *p*-chlormercuribenzoate destroyed penicillin at the same rate as untreated defatted organisms.)

The organisms were then centrifuged and washed with 0·001 M *p*-chlormercuribenzoate in broth, centrifuged and resuspended in 0·001 M *p*-chlormercuribenzoate in broth at an optical density of 10 units measured by an EEL nephelometer. This

corresponded to $3{\cdot}3 \times 10^7$ cocci/ml. 10 ml. of a solution (4 mg./ml.) of penicillin G in 0·001 M *p*-chlormercuribenzoate in broth at 37° were then added to 10 ml. of the induced organisms at 37° and the initial rate of hydrolysis of penicillin by the induced cocci was determined as described above. The increase of penicillinase over the basal level was determined; the results are shown in Fig. 1. It can be seen that the maximum increase in penicillinase occurred when the organisms were induced at an initial optical density of 30 units which corresponds to 7×10^7 cocci/ml. (It should be noted that with the EEL nephelometer the number of 'units' is proportional to the logarithm of the viable count.)

Period of induction. To determine the optimal time for induction, a culture of *Staphylococcus aureus* E 3 grown overnight in broth was centrifuged and the deposit resuspended in broth at 37° to give an optical density of 30 units measured by the EEL nephelometer, and then induced with methicillin (0·5 μg./ml.) for different periods of time. Induction was then stopped with *p*-chlormercuribenzoate and the increase of penicillinase above the basal value determined as described above. The results are shown in Fig. 2. It can be seen that the maximal increase in penicillinase occurred when the organisms were induced for 75 min. at 37°. The dotted line shows the effect of adding *p*-chlormercuribenzoate to make a final concentration of 0·001 M at 30 min. and continuing to shake at 37° in the presence of the inducing agent until 90 min. As can be seen from Fig. 2 this concentration of *p*-chlormercuribenzoate prevented further appreciable increase of penicillinase.

Induction constants

Induction constants (Pollock, 1957) for the different penicillins were determined by finding the concentration of inducer needed to cause maximal and half maximal increase in amount of penicillinase above the basal value. The optimal conditions for induction as determined above, with methicillin as standard, were used for the various penicillins in the following way. Each concentration of the penicillin being tested in 0·5 ml. broth was added to 19·5 ml. of suspension of organisms at an optical density of 30 units measured by an EEL nephelometer. The organisms were induced for 75 min. at 37°, with shaking at 100 cycles/min. Induction was then stopped with 0·001 M *p*-chlormercuribenzoate, the organisms centrifuged down, washed and resuspended with 0·001 M *p*-chlormercuribenzoate in broth to an optical density of 10 units. Of this suspension 10 ml. were then mixed at 37° with 10 ml. of benzyl penicillin solution (4 mg./ml. in 0·001 M *p*-chlormercuribenzoate in broth) and the rate of penicillin breakdown determined by the hydroxylamine method. The maximum rate being taken as 100%, the rates due to other concentrations of inducer were calculated accordingly. The concentration of inducer necessary to cause 50% of the maximum increase in penicillinase was then calculated graphically by interpolation.

RESULTS

Antibacterial activity

The antibacterial activity of the different penicillins is shown in Table 1. Penicillin G was active against Gram-positive cocci but with penicillinase-producing staphylococci it was almost completely inactive against large inocula though highly active against small inocula. In general penicillin G showed little activity against

Gram-negative rods, though a few species were moderately susceptible (e.g. *Salmonella typhi* and some strains of *Haemophilus influenzae*). Penicillin V showed much the same type of spectrum. Against some strains of sensitive staphylococci it was even more active than penicillin G, but against some strains of *H. influenzae* and other Gram-negative organisms it was much less effective. Phenethecillin had a similar spectrum; it was slightly less effective than penicillin V or penicillin G against most Gram-positive and Gram-negative organisms, but showed greater activity than penicillin V or penicillin G against moderate inocula of penicillinase-producing staphylococci. Phenoxypropyl penicillin had slightly greater activity

Table 1. *Antibacterial activity of different penicillins*

Micro-organism	Minimum inhibitory concentration (μg./ml.)						
	G	V	Phenethecillin	Penicillin phenoxypropyl	Methicillin	α-Aminobenzyl	6APA
Staphylococcus aureus (Oxford)	0·02	0·02	0·03	0·08	1	0·2	—
S. aureus (5 penicillinase negative strains)	0·02	0·01	0·03	0·07	1	0·6	—
S. aureus E3 (penicillinase producing)							
15×10^6 cells	>1000	>100	>1000	>1000	7·5	>200	500
15×10^4 cells	250	>100	250	62·5	3·7	200	250
15×10^2 cells	7·5	3·1	3·7	1·8	3·7	12·5	250
15 cells	<0·9	0·8	1·8	<0·9	3·7	6·2	125
Streptococcus pyogenes	0·005	0·01	0·2	0·03	0·2	0·02	—
Strep. pneumoniae	0·005	0·01	0·005	0·01	0·05	0·1	—
Strep. viridans	0·02	0·05	0·1	0·4	0·9	0·1	—
Haemophilus influenzae A. 36	0·9	7·5	15	15	7·5	0·4	—
A. 37	1·8	7·5	7·5	7·5	3·7	0·4	—
A. 40	0·2	1·8	3·7	3·7	1·8	0·2	—
Escherichia coli A. 1	62	>250	>250	>250	>250	7·5	—
A. 2	31	125	250	>250	>250	31	—
A. 10	125	>250	>250	—	>250	15	—
*Salmonella typhi**	7·5	62	250	—	250	0·4	—
Proteus sp. A. 4	1·8	31	250	—	—	0·9	—
Pseudomonas sp.	>250	>250	>250	—	>250	>250	—

* Similar figures have been obtained for other salmonellae and shigellae.

than phenethecillin against penicillinase-producing staphylococci but the difference was not great and against very large inocula of these it seemed to be no more effective than phenethecillin, penicillin G or penicillin V. The two penicillins of outstandingly different activity were methicillin and α-aminobenzylpenicillin. Methicillin, although in general much less active than penicillin G against most sensitive cocci and even more inactive against Gram-negative bacilli, was almost equally active against both penicillinase-producing and penicillin-sensitive staphylococci in a concentration of 1–4μg./ml. Its effect against penicillinase-producing staphylococci was almost independent of inoculum size, a feature which distinguished it sharply from all the other penicillins. α-Aminobenzylpenicillin was rather less effective than penicillin G against the Gram-positive cocci but considerably more effective against many Gram-negative rods. Thus three main types of spectrum were

seen with the penicillins tested—the typical penicillin G spectrum showing activity mainly against Gram-positive cocci, the methicillin type of spectrum showing greatly decreased activity against most micro-organisms as compared with penicillin G but unique activity against penicillinase-producing staphylococci, and the 'broad spectrum' activity shown by α-aminobenzylpenicillin.

Stability of different penicillins to staphylococcal penicillinase

Table 2 shows the initial rates of hydrolysis of the different penicillins by staphylococcal penicillinase. It can be seen that the 'broad spectrum' α-aminobenzylpenicillin was the most easily hydrolysed; next came penicillin V, then penicillin G. Phenethecillin and phenoxypropyl penicillin were hydrolysed almost as fast as penicillin G. The most resistant of all was methicillin which was not appreciably hydrolysed by staphylococcal penicillinase in the conditions of our experiments. 6-Aminopenicillanic acid was somewhat more rapidly hydrolysed than methicillin but was much more resistant than all the others. However, Batchelor, Cameron-Wood, Chain & Rolinson (1961) pointed out that while at neutral pH values and at fairly low concentrations 6-aminopenicillanic acid is much more stable to staphylococcal penicillinase than is penicillin G, at suitable pH values and at optimal substrate concentration they can both be destroyed at similar rates.

Table 2. *Rate of destruction of different penicillins by staphylococcal penicillinase*

	Initial rate of destruction μg./ml./min.
α-Aminobenzylpenicillin	23·0
Penicillin V	12·8
Phenethecillin	11·6
Penicillin G	11·6
Phenoxypropyl penicillin	8·2
6-Aminopenicillanic acid	0·14
Methicillin	0·09

Induction of penicillinase

Using cultures of the penicillinase-producing strain of *Staphylococcus aureus* E3 induced with different concentrations of different penicillins, we determined the concentrations of each of these for half maximal and maximal induction and also the increase in the amount of penicillinase activity over the control basal value (see Methods). The results are shown in Figs. 3 and 4, and Table 3.

It can be seen that all the penicillins tested acted as inducers, but that the best inducer was methicillin giving an 'induction constant' (Pollock, 1957) of 0·27 μg./ml. for half-maximal induction. Thus, of the penicillins tested, the best inducer was the one least easily hydrolysed by staphylococcal penicillinase (see Tables 2 and 3) but 6-aminopenicillanic acid, which was not much less resistant to hydrolysis than methicillin, was less effective as an inducer. If the concentration of 6-aminopenicillanic acid required for half-maximal induction is taken as the criterion of inducing ability then 6-aminopenicillanic acid under the conditions used was not as good an inducer as the much more easily hydrolysable phenethecillin or phenoxypropyl penicillin (see also Tables 2 and 3).

Table 3. *Inducing ability of different penicillins*

	Concentration μg./ml. to give maximal induction	Concentration μg./ml. to give half maximal induction	Ratio of induced to basal penicillinase activity at maximum
Penicillin V	300	6·6	12·4
Penicillin G	100	9·6	11·1
α-Aminobenzyl penicillin	100	2·2	26·8
Phenethecillin	30	0·72	15·5
6-Aminopenicillanic acid	10	3·0	16·3
Phenoxypropyl penicillin	10	0·36	26·8
Methicillin	3	0·27	36·0

Summary of the data presented in Figs. 3 and 4 together with the maximal amount of penicillinase formed in relation to the basal level determined in the same experiments.

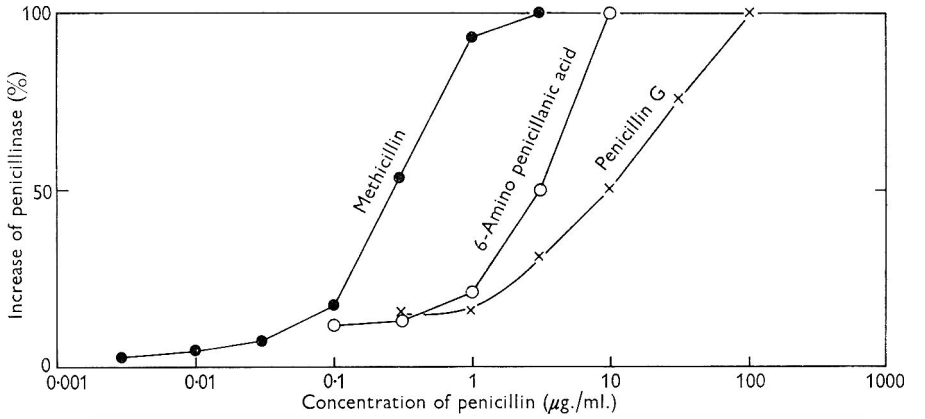

Fig. 3. Effect of concentration of antibiotic during induction on yield of penicillinase using 6-aminopenicillanic acid, methicillin and penicillin G.

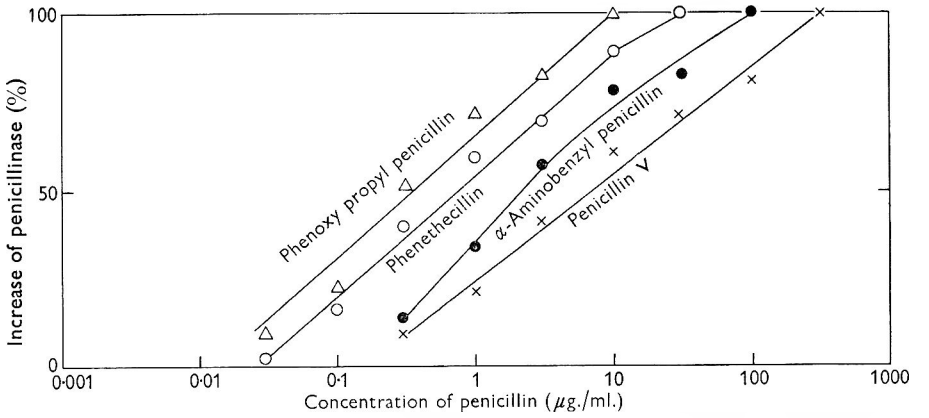

Fig. 4. Effect of concentration of antibiotic during induction on yield of penicillinase using phenethecillin, α-aminobenzyl penicillin, phenoxypropyl penicillin and penicillin V.

DISCUSSION

With an inducing agent which is not a substrate it is fairly easy to determine the concentration required to give maximal or half maximal induction. But with a penicillin rapidly hydrolysed by penicillinase it is difficult to ensure optimal conditions for induction. Steinman (1961*a*, *b*) distinguishes between the affinity of the inducer for the enzyme and the total amount of enzyme which can eventually be formed. Estimates of this affinity as measured by induction constants agree fairly well with those that Steinman obtained for methicillin, 6-aminopenicillanic acid and penicillin G. However, the total amount of enzyme formed was considerably less in our experiments. This could be expected to vary with the conditions used especially if different strains of penicillinase-producing staphylococci differ greatly in their 'constitutive' penicillinase and in their capacity for induction.

We have found that with the most easily hydrolysed penicillin, α-aminobenzylpenicillin, the yield of penicillinase in our experimental conditions was significantly increased above 'maximal' when the concentration of antibiotic known to give maximal induction (100 μg./ml.) was added both initially and halfway through the period of induction. Thus the apparent maximal concentration as determined in one set of experimental conditions does not necessarily represent the true capacity of the organisms to produce penicillinase. Even if we attempt to overcome this difficulty by repeated small additions of an easily hydrolysable penicillin its true status as an inducer in comparison with a stable inducer such as methicillin is difficult to define.

An attempt has been made to relate the antibacterial activity of different penicillins, their penicillinase stability and their ability to induce penicillinase in staphylococci by means of a diagrammatic model (Knox, 1961). The fact that methicillin is highly resistant to staphylococcal penicillinase may be due to the poorness of fit between it and active centres on the penicillinase molecule. Nevertheless, it is a good inducer of the enzyme—hence it seems that the requirements for induction may be less stringent than those for a good substrate of the enzyme. Steric hindrance may also account for the relatively poor antibacterial activity of methicillin except against penicillinase-producing staphylococci. On the other hand, the combined Gram-positive and Gram-negative activity of α-aminobenzylpenicillin may be, partly at least, due to the closeness of fit between its molecule and receptors on the surface of the bacterial cell. A similar closeness of fit between it and the penicillinase molecule may account for the rapidity with which it is hydrolysed. But with each new penicillin discovered it is important not only to know how easily it is hydrolysed by penicillinases from different sources, but also to study the possibility of breakdown by other mechanisms such as the amidases (Rolinson *et al.* 1960) which are known to be widely distributed among micro-organisms.

We are grateful to Beecham Research Laboratories for supplies of methicillin, phenethecillin, α-aminobenzylpenicillin and 6-aminopenicillanic acid, to Pfizer Limited for phenoxypropyl penicillin used in this work, and to Miss B. Whittamore for valuable technical help.

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Note added in proof

Doyle *et al.* (1961) have described a new series of isoxazolyl penicillins which are resistant to both acid and penicillinase and possess high antibacterial activity *in vivo* and *in vitro*. One of these, 5-methyl-3-phenyl-4-isoxazolylpenicillin (BRL 1400, P12) was tested for inducing ability. It was found to be a much more powerful inducer than methicillin. The maximal increase in penicillinase was 55·8 times the basal level at a concentration of 0·3 μg./ml., while the concentration required for half maximal induction was 0·019 μg./ml.

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Gene Interactions Affecting Methionine Biosynthesis and the Response to S-methylcysteine by Mutants of *Neurospora crassa*

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SUMMARY

Two non-allelic suppressors have been isolated which suppress non-allelic, leaky, methionine-requiring mutants blocked either between cysteine and cystathionine or between cystathionine and homocysteine. Strains carrying either suppressor gene in the presence of the me^+ allele were stimulated by either cysteine or methionine, whereas the suppressed methionine-requiring mutants (*me su*) although stimulated by methionine were inhibited by cysteine. S-methylcysteine supported the growth of leaky methionine-requiring mutants when it was present as the sole sulphur source and it also stimulated the growth of suppressed methionine-requiring mutants inhibited by cysteine. Sulphate or cysteine inhibited the growth response of certain methionine-requiring mutants to S-methylcysteine. The incorporation of radioactive sulphate into protein methionine was inhibited to a greater extent by S-methylcysteine than was its incorporation into protein cysteine. The results suggest that the sulphur of S-methylcysteine is converted to methionine without prior conversion to cysteine and that the suppressors act by retarding the formation of an inhibitor which accumulates as a result of the primary mutation to methionine requirement.

INTRODUCTION

It has been pointed out before (Strauss, 1955) that the phenotype of a double mutant cannot always be simply deduced from the properties of the single mutants. In the case of suppressor mutations, it has been repeatedly observed that single auxotrophic mutants can be induced to grow prototrophically by the introduction of a second mutant gene. Such suppressor genes often show a marked degree of specificity (Yanofsky & Bonner, 1955); certain suppressors are active only on particular mutant alleles but will not suppress other, closely linked, alleles of the same mutant gene. Consequently, the demonstration by Giles (1951) that a single suppressor could suppress two unlinked genes controlling different (but sequential) steps in the biosynthesis of methionine appeared of interest to us. We therefore investigated the characteristics of this type of suppressor to determine whether a new type of gene interaction was represented. Our results indicate that in methionine biosynthesis, these particular suppressors act by preventing the formation of an inhibitor accumulated as a result of the original mutation to auxotrophy, an action similar in nature to that of the suppressors of the acetate-requiring mutants (Strauss & Pierog, 1954). In the course of our studies, some observations were

made on the response of methionine-requiring mutants to S-methylcysteine. These results support the idea of Wiebers & Garner (1960) and of Maw (1961) that S-methylcysteine can act as a methionine precursor without prior conversion to cysteine.

METHODS

Organisms. The methionine-requiring mutants used in this study were isolated by the filtration–selection method of Woodward, De Zeeuw & Srb (1954). Strains M3, M4 and M17 were isolated from among conidia of wild-type *Neurospora crassa* strain 74A after treatment with ultraviolet (u.v.) radiation; strains M7 and M14 were isolated from material treated with diethyl sulphate. Although M3 and M4

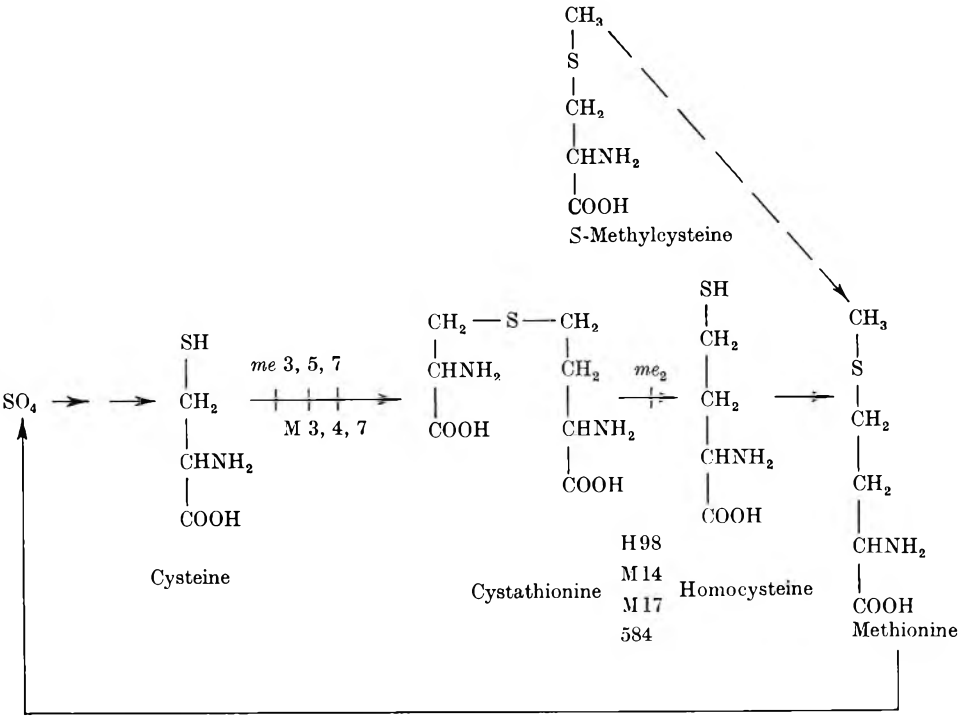

Fig. 1. Scheme of methionine biosynthesis in Neurospora.

and M7 respond to either cystathionine, homocysteine or methionine, but not to cysteine, these strains are not alleles. M3 formed heterocaryons with both M4 and M7, and in crosses of M3 × M4 and of M3 × M7 a high percentage of wild-type recombinants was recovered. Strains M14 and M17 respond to methionine and homocysteine but not to either cysteine or cystathionine. A simplified pathway of methionine biosynthesis indicating the respective nutritional blocks is shown in Fig. 1.

Strain H98 was re-isolated from a culture sent to us by Dr N. H. Horowitz. This strain has been used previously in a number of investigations (Giles, 1951; Wiebers & Garner, 1960) and responds to homocysteine but not to cystathionine in the standard growth test. The suppressor strain su^{H98} was isolated from a culture of H98 which grew on minimal medium. A further suppressor su^{M4} was isolated by

the method of Yanofsky & Bonner (1955) from M4 conidia irradiated with u.v. radiation and plated on a minimal medium. Strain 74A of *Neurospora crassa* was used as a wild-type control. In this paper we use both isolation numbers and also the designation of the allele. Strain H98 is a mutant at the *me-2* locus and is therefore written *me-2*^{H98}. Suppressor genes are identified by the strain from which they originated, i.e. *me-2*^{H98} *su*^{M4} represents the double mutant in which H98 is combined with a suppressor gene originating in strain M4. All strains responding to homocysteine but not to cystathionine are classified as *me-2* in this paper, but since three loci have been reported to yield mutants responding to cystathionine but not to cysteine (Barratt, Newmeyer, Perkins & Garnjobst, 1954), we have merely indicated such strains as *me*.

Nutritional and biochemical methods. These methods were described previously (Strauss & Minagawa, 1959). Cystathionine was used as DL allocystathionine, homocysteine as homocysteine thiolactone and S-methylcysteine as the L form. All were commercial preparations and were autoclaved in the growth medium. The minimal medium used was that described by Vogel (1956), except for those experiments in which the amount of sulphur in the medium was controlled. In such experiments, the sulphur free medium described in Strauss & Minagawa (1959) was used and supplemented as indicated.

RESULTS

All our mutants with the exception of M3 were 'leaky'; that is, were able to synthesize some methionine even though they would not initiate growth on minimal medium even after 8 days incubation at 30°. The partial character of these mutants was demonstrated in two ways: first, by the ability of all the strains except M3 to continue growth when fed a limited amount of methionine (Fig. 2); and secondly, by the ability of all the mutants except M3 to incorporate $^{35}SO_4$ into methionine (Table 1). Incorporation of radioactive sulphate was demonstrated by the method of Strauss & Minagawa (1959).

All of the mutant strains were able to utilize methionine as a sole source of sulphur with about the same efficiency as did the wild-type (Table 1). These strains therefore formed their cysteine from methionine as a sulphur source. Yet, all these strains required the addition of either cystathionine, homocysteine or methionine to minimal medium (containing sulphate) for rapid growth, indicating a nutritional block between cysteine and methionine. The rapid growth on methionine as a sole sulphur source therefore indicated that the sulphur of methionine could be converted to the sulphur of cysteine by a pathway which was not blocked. This has been formally represented as conversion to sulphate (Fig. 1), although there is no evidence that the sulphur is actually brought to this oxidation state.

Most strains were able to utilize S-methylcysteine as a source of sulphur amino acids as long as inorganic sulphate was absent from the medium. Strain M3 was the one strain unable to utilize this compound as a sulphur source (after about 10 days, even this strain produced some growth). S-methylcysteine was tested because of the report of Wiebers & Garner (1960) that a methionine-requiring strain would grow with this supplement.

Very small amounts of sulphate inhibited the response of strains M7, 14 and 17

(but not strain M4) to S-methylcysteine (Table 1, Fig. 3) and the sodium, potassium and ammonium sulphates inhibited equally well. Sulphite, thiosulphate and cysteine also inhibited the growth response to S-methylcysteine (Table 2).

Genetic studies

A reverted culture of H98 which grew on minimal medium was crossed to wild-type. On minimal medium a typical 6:2 ratio, characteristic of a suppressor

Table 1. *Growth of methionine requiring mutants at 30°*

	Three days growth in 20 ml. minimal medium (Vogel, 1956) plus			Growth on limited methionine*	Incorporation of $^{35}SO_4$ into methionine†	Growth on sulphur free medium (Strauss & Minagawa, 1959) plus			
						No addition (8 days growth)	Methionine (1 mg./20 ml.) (4 days)	S-methylcysteine (2 mg./20 ml.) (5 days)	S methylcysteine (2 mg./20 ml.) + sulphate (100 μg. S/20 ml.) (5 days)
Strain	Cysteine	Cystathionine	Homocysteine			Dry wt. mycelium (mg.)			
74A	–	–	–	62	+	2·6	73	50	125
M3	0	+	+	13	0	0	82	0	0
M4	0	+	+	47	+	0·8	NT	40	48
M7	0	+	+	77	+	0	52	12	0·2
M14	0	0	+	54	+	0	73	39	1
M17	0	0	+	73	+	0	62	53	22
H98	0	0	+	NT	+	NT	NT	NT	NT

* Dry weight of mycelium per 20 ml. after 10 days in medium containing 0·2 mg. methionine/20 ml.

† The incorporation of $^{35}SO_4$ into methionine was determined as described in the legend to Fig. 4 and in Strauss & Minagawa (1959).

NT = not tested.

Table 2. *Effect of additional sulphur compounds on the response of mutant M14 to S-methylcysteine*

Twenty ml. of sulphur free medium supplemented with 2 mg. of S-methylcysteine/20 ml. were added to all flasks. Incubation was in static culture at 30°.

		M14 (7 days growth)	Wild-type 74A (3 days growth)
Additional compound	Amount added (μg S)	Dry wt. mycelium (mg.)	
None	—	36	20
Na_2SO_4	50	2	.
	500	.	72
$(NH_4)_2SO_4$	50	2	.
	500	.	71
Na_2SO_3	100	18	.
	500	.	73
$Na_2S_2O_3$	50	6	.
	500	.	62
Cysteine	260	0·8	.
	260	.	54

mutation, was obtained (Table 3). The identity of the presumptive suppressor (*me*$^+$ *su*^{H98}) was verified by back crosses to H98. This suppressor mutant, when present with the *me*$^+$ allele, produced a partial deficiency for sulphur amino acids. It was stimulated by either cysteine or methionine as well as by cystathionine. This *me*$^+$ *su*^{H98} strain was inhibited by threonine which is itself evidence for a deficiency of sulphur amino acids in this strain, since threonine inhibition in Neurospora is counteracted by methionine (Doudney & Wagner, 1952) and since wild-type is not threonine inhibited. In contrast to the behaviour of the *me*$^+$ *su*^{H98} strain,

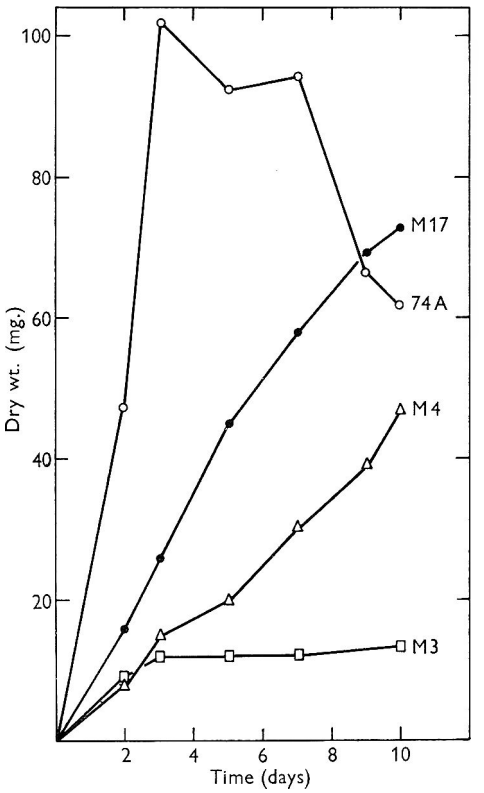

Fig. 2

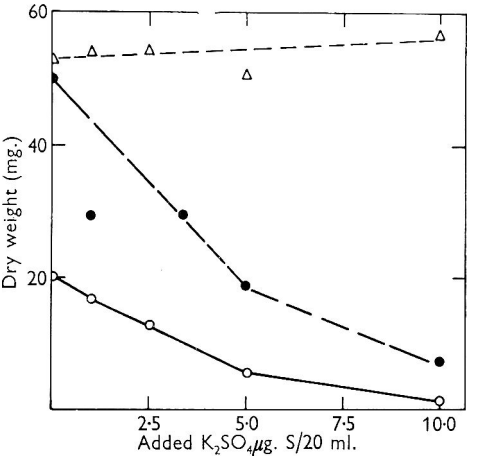

Fig. 3

Fig. 2. Growth response of a series of methionine-requiring mutants to limiting amounts of methionine. A series of flasks containing 0·2 mg. L-methionine in 20 ml. minimal medium was inoculated and incubated in static culture at 30°. The top curve represents a wild-type control (○) and the next curves represent the growth of M17 (●), M4 (△) and M3 (□) in descending order.

Fig. 3. The effect on the extent of growth of methionine-less mutant M14 of adding K_2SO_4 to sulphur-free medium containing S-methylcysteine. △ represents growth of wild-type 74A after 3 days at 30° with 2 mg. S-methylcysteine/20 ml. ●, ○ represent growth of M14 after 5 days at 30° with 4 and 1 mg. of S-methylcysteine/20 ml., respectively.

me-2^{H98} *su*^{H98} was *inhibited* by both cysteine and cystathionine. We have been able to distinguish *me*$^+$ *su* strains from *me su* strains by their different response to cysteine.

The suppressor isolated from H98 was crossed to strains M4 and M3 in order to determine whether the pattern of suppression reported by Giles (1951) represented

a general finding. In confirmation of Giles' report (1951), we found that strain M4 was suppressed by su^{H98} (Table 4A). The same pattern of stimulation by cysteine of the me^+ *su* strain and inhibition of the *me su* strain was apparent in this segregation. The cross $me^+ su^{H98} \times me^{M3} su^+$ gave an ascus in which two of the four spore pairs were obvious wild-types, indicating that the other spore pairs were of the $me^{M3} su^{H98}$ constitution. These cultures would not grow on minimal, indicating that they were not suppressed (but cf. below).

Table 3. *Segregation of the suppressor of H98*

Growth for 3 days at 30° with 2 mg. supplement added/20 ml. minimal medium.

Cross: H98 (revertant) × wild-type
$me\text{-}2^{H98}\ su^{H98} \times me^+\ su^+$

Spore pair	Genetic constitution	Supplement: None	Cysteine	Cystathionine	Homocysteine	Methionine	Threonine
		Dry wt. mycelium (mg.)					
1	$me^+\ su^+$	92	97	98	61	87	109
2	$me^+\ su^{H98}$	55	77	90	51	80	0
3	$me\text{-}2^{H98}\ su^{H98}$	21	9	0·9	18	70	0
4	$me\text{-}2^{H98}\ su^+$	0	0	0	5	40	0

Table 4. *Suppression of methionine requiring mutants by suppressors from non-homologous strains*

Recorded dry weights produced after 3 days at 30° in 20 ml. of minimal medium with 2 mg. of the supplement shown.

Spore pair	Supplement: None	Methionine	Cysteine	Genetic constitution
	Dry wt. mycelium (mg.)			
	A. Cross: $me^+\ su^{H98} \times me^{M4}\ su^+$			
1	124	113	98	$me^+\ su^+$
2	31	79	76	$me^+\ su$
4	31	80	0	*me su*
3	0	79	0	$me\ su^+$
	B. Cross: $me^+\ su^{M4} \times me\text{-}2^{H98}\ su^+$			
3	94	111	92	$me^+\ su^+$
2	36	100	82	$me^+\ su$
1	16	74	0·2	*me su*
4	0	69	0	$me\ su^+$

We were also interested in determining whether a suppressor isolated from M4 (blocked between cysteine and cystathionine) would suppress strain H98 (blocked between cystathionine and homocysteine). Such a suppressor was isolated by the method of Yanofsky & Bonner (1955) and its identity was established by appropriate segregation tests. This $me^+ su^{M4}$ strain was also stimulated by sulphur amino acids and inhibited by threonine in a manner similar to the su^{H98} strain (Table 5). The $me^{M4} su^{M4}$ strain was inhibited by cysteine but stimulated by cystathionine.

The suppressor isolated from M4 suppressed H98 (Table 4B) just as the suppressor isolated from H98 suppressed M4, but these two suppressors were not alleles. The cross $me^+ su^{H98} \times me^+ su^{M4}$ gave 25 wild-type cultures not inhibited by threonine out of the 96 single ascospore cultures isolated, indicating that these two factors were not alleles, notwithstanding their similar phenotypic effects.

The $me^{M3} su^{M4}$ strain was not suppressed, which also indicated the similarity in action of the su^{H98} and su^{M4} genes. However, M3 strains carrying either *su* gene were different from the $me^{M3} su^+$ strain since they did grow with S-methylcysteine as a supplement, in contrast to M3 strains not carrying a suppressor gene.

Table 5. *Response of strains carrying* su^{M4} *to amino acids*

Growth after 3 days at 30° on minimal medium.

Genetic constitution	Supplement (2 mg./20 ml.) None	Cysteine	Cysta-thionine	Homo-cysteine	Methio-nine	Threonine
	Dry wt. mycelium (mg.)/20 ml. medium					
$me^+ su^{M4}$	62	108	92	104	112	7·2
$me^{M4} su^{M4}$	20	0	59	60	92	0

Table 6. *Stimulation by S-methylcysteine of suppressed mutants inhibited by cysteine*

Growth for 3 days at 30° in minimal medium containing sulphate (Vogel, 1956).

Strain	Supplement per 20 ml. medium None	Cysteine 0·5 mg.	Cysteine 2·0 mg.	*S*-Methylcysteine 0·5 mg.	*S*-Methylcysteine 2·0 mg.
	Dry wt. mycelium (mg.)/20 ml. medium				
$me^{M4} su^{M4}$	18	0	0	18	31
$me^{M4} su^{H98}$	8	0	0	21	25

Studies with S-methylcysteine

Methionine will serve as a source of sulphur for all the methionine-requiring mutants tested. It seemed likely that S-methylcysteine served as a source of methionine without passing through cysteine and that the methionine formed could then act as a source of cysteine for growth.

We have been able to show that S-methylcysteine added to medium containing radioactive sulphate and limiting amounts of methionine lowered the incorporation of $^{35}SO_4$ into methionine by methionine-requiring mutants more than into cysteine (Fig. 4). The ratio of total counts in the areas methionine + methionine sulphoxide to cysteine + cysteic acid was determined after elution and gave the following values: H98, 2·32; H98 + S-methylcysteine, 1·20; M4, 2·14; M4 + S-methylcysteine, 1·03. These results indicate that the sulphur of methylcysteine is incorporated more efficiently into methionine than into cysteine. Roberts *et al.* (1955) reported that *S*-methylcysteine could lower the uptake of radioactive sulphate to

17% of the control and that it lowered the uptake of ^{35}S cystine to 71% of the control.

S-methylcysteine stimulates the growth of *me*^{M4} *su* strains which show inhibition by cysteine (Table 6). In addition, and as described above (Table 2), cysteine inhibits the response of M14 to S-methylcysteine.

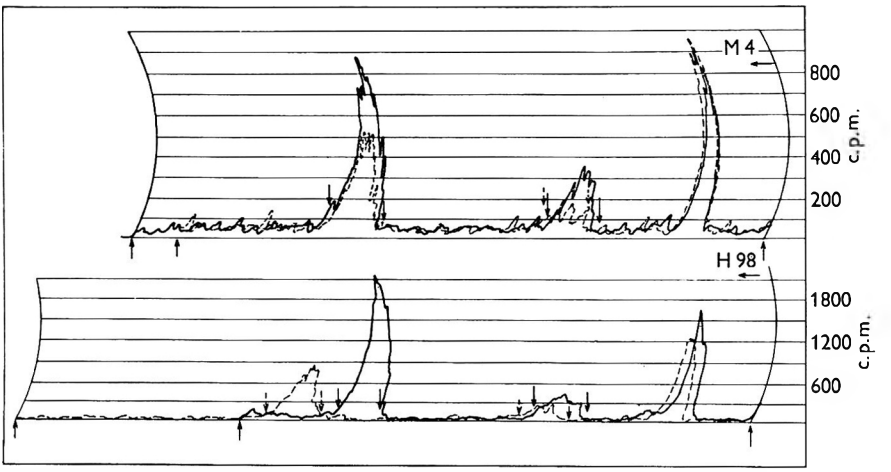

Fig. 4. Effect of S-methylcysteine on the incorporation of $^{35}SO_4$ into sulphur amino acids by leaky methionine-requiring mutants of Neurospora. Strains H98 and M4 were grown for 5 days on 20 ml. of a sulphur-free medium supplemented with 500 μg. of inorganic sulphur (as K_2SO_4) per 20 ml. and containing 0·2 mg. of L-methionine and 15 μc. of $^{35}SO_4$. S-methyl-L-cysteine was added at a concentration of 2 mg./20 ml. where indicated. The mycelium was harvested, washed with hot 50% (v/v) ethanol, then with hot water, 0·5 M-$HClO_4$ (70°), ethanol and ether. After drying, the mycelium was autoclaved at 15 PS1 with 6 N-HCl for 2 hr. in a sealed glass tube. Excess HCl was removed by repeated evaporation *in vacuo* and the hydrolysate was taken up in water and chromatographed by the descending technique on Whatman no. 1 paper with butanol + acetic acid + water (2 + 1 + 1). Typical R_F values with this solvent are: cysteine, 0·2; cysteic acid, 0·2; methionine sulphoxide, 0·42; S-methylcysteine, 0·57; and methionine, 0·68–0·7. Both methionine and methionine sulphoxide were chromatographed along with the hydrolysates and their position is indicated by arrows on the tracings as are the boundaries of the chromatograms. The position of the radioactive spots was located and traced as previously described (Strauss & Minagawa, 1959). Solid line = material grown with no S-methylcysteine. Dotted line = material grown with S-methylcysteine. The horizontal arrows indicate the direction of solvent flow and of the scanning. The vertical arrows pointing upward indicate the origin and final solvent fronts respectively. The vertical arrows pointing downwards indicate the positions of non-radioactive methionine sulphoxide (lower R_F) and methionine (higher R_F) co-chromatographed with the radioactive material. Solid arrows show the position of the compounds co-chromatographed with material grown in the absence of S-methylcysteine, dotted arrows indicate the location of compounds co-chromatographed with material grown in the presence of S-methylcysteine.

DISCUSSION

The response of suppressed strains to the sulphur amino acids is summarized in Table 7. Two strains were studied in detail, strain M4 blocked between cysteine and cystathionine, and strain H98 blocked between cystathionine and homocysteine. In addition, two non-allelic suppressors were isolated, *su*^{M4} and *su*^{H98}. Both suppressors suppress both mutants M4 and H98 and neither suppressor will

suppress M3, a non-leaky mutant. Both *me*$^+$ *su* strains grew less well on minimal medium than the unsuppressed wild-type but were stimulated in their growth by the sulphur amino acids. The *me su* mutants, on the other hand, were inhibited by certain of the sulphur amino acids which in the case of both *me* mutants were restricted to those amino acids located in the metabolic scheme before the point of the original nutritional block. Sulphur amino acids located after the point of the block either stimulated or had no noticeable effect. The same pattern of inhibition was obtained regardless of the origin of the suppressor. All the strains listed in Table 7 were inhibited by threonine.

Table 7. *Response of suppressed strains to additions to the medium*

Both *me*$^+$ *su* strains were stimulated by the compounds indicated. All strains were stimulated by homocysteine or methionine.

Genetic constitution	Addition	
	Cysteine	Cystathionine
me-2^{H98} *su*$^+$	—	—
me^{M4} *su*$^+$	—	S
me-2^{H98} *su*^{H98}	I	I
me^{M4} *su*^{H98}	I	S
me-2^{H98} *su*^{M4}	I	I
me^{M4} *su*^{M4}	I	S

I = inhibited; S = stimulated.

The fact that *me*$^+$ *su* strains are stimulated by sulphur amino acids indicates that they are partially deficient in their ability to make these substances. The methionine mutants which can be suppressed are themselves leaky. A possible explanation of the suppression is that, as a result of the *me* mutation, an inhibitor related to cysteine accumulates which reinforces the block to methionine synthesis. The combination of the leaky *me* mutation with the *su* mutations lowers the amount of inhibitor which can be formed since, as demonstrated nutritionally, the *su* strains are unable to make cysteine and other sulphur amino acids at a normal rate since these compounds stimulate growth. The hypothesis accounts for the behaviour of the *me su* strains when confronted with potential inhibitors. Compounds coming after the point of the block stimulate, since they can be readily converted to methionine; compounds coming before the point of the block inhibit, since they can be converted to the inhibitor even in the presence of the *su* gene. According to this hypothesis, only leaky mutants should be suppressible by these suppressors, but it is not necessary that *all* leaky mutants be suppressed. Non-leaky mutants appear to be relatively rare, although they can be obtained (no *me-2* mutant which is not leaky has been obtained by us or is available from the Neurospora Stock Centre at Dartmouth).

We suppose that a block between cystathionine and homocysteine or between cysteine and cystathionine brings about equivalent internal inhibition and that the production of this state of internal inhibition is prevented by either *su* gene. Such an action by the *su* gene might account for the restoration of cystathionase I and II activities observed in *me* mutants by Fischer (1957). This scheme is analogous to that devised to account for the behaviour of the suppressors of the acetate-requiring

mutants of Neurospora (Strauss & Pierog, 1954). It was supposed at that time that the acetate suppressors lowered the production of an inhibitor (acetaldehyde) formed in excess as a result of the first mutation. We suppose that genetic suppression by the interaction of the cytoplasmic reactions of intermediary metabolism is not a particularly uncommon mechanism. However, as Yanofsky (1961) has pointed out, selection of a suppressor on minimal medium eliminates any genes which suppress one mutation at the cost of introducing a new requirement. It is not possible to make any statements about the quantitative prevalence of particular types of gene interaction as a result of the compilation of cases of suppressors picked by a particular selective method.

The data presented in this paper make it very likely that the sulphur of S-methylcysteine can be converted to methionine without passing through cysteine. Cysteine inhibits *me su* double mutants, S-methylcysteine stimulates them. A number of methionine-requiring mutants respond to *S*-methylcysteine as a sole nutritional supplement; both sulphate and cysteine inhibit this response. S-methylcysteine lowers the incorporation of $^{35}SO_4$ into methionine proportionally more than cysteine. These data are most easily explained by supposing that S-methylcysteine may be more or less directly converted into methionine without passing through cysteine. A possible scheme for this conversion has been suggested by Wiebers & Garner (1960).

Some time ago we supposed (Strauss & Minagawa, 1959) that the methionine synthesized by a leaky methionine-requiring mutant was formed via the pathway cysteine–cystathione–homocysteine–methionine. We based our conclusion upon the accumulation and then gradual disappearance of cystathionine when our strain (584) was grown with limiting amounts of sulphur. However, S-methylcysteine has been reported as a natural metabolite in Neurospora (Ragland & Liverman, 1956) and it is now apparent that the methionine formed by leaky mutants could come via an alternate pathway based on this compound. In fact, the existence of two pathways to methionine could help to account for the surprisingly high proportion of leaky methionine-requiring mutants. An alternate pathway based on S-methylcysteine might be inhibited or repressed in the ordinary course of events and the suppressor genes might act by removing a feedback inhibitor or repressor, thereby permitting the alternate pathway to function. Those mutants which are non-leaky might then be supposed to be blocked before the point at which the alternate pathway diverges from the standard, although it is possible that mutants exist, blocked at this position, which are leaky for other reasons. The two absolute mutants now available are blocked between cysteine and cystathionine. It has always been puzzling to note that three non-allelic genes are located at what has been presumed to be a single metabolic step reaction (Barrat *et al.* 1954); the proposed alternate pathway is presumably not operative in all three. Our conclusion is, therefore, similar to that of Wiebers & Garner (1960) and of Maw (1961).

Although we find the sulphate inhibition of S-methylcysteine utilization to be an interesting phenomenon and have used it in our argument above, its metabolic basis is at present completely unknown. Some strains are resistant to sulphate inhibition while others, blocked in what is apparently the same position, are susceptible. It is likely that an explanation must wait for an enzymic description of these phenomena.

Since submitting this paper we have completed additional genetic tests on the suppression of known *me* mutants obtained from the Neurospora stock centre. Mutants blocked at the *me-2*, *me-3* and *me-7* loci were suppressed by both su^{H98} and su^{M4}. Two mutants at the *me-5* locus, one leaky and one non-leaky, were not suppressed. We do not yet have sufficient data to assert that our me^{M3} is an *me-5* allele.

Dr Noreen Murray (personal communication) has tested the majority of her *me-2* mutants and finds them all leaky, only one of six *me-5* mutants was leaky, whereas the *me-3* and *me-7* strains tested were leaky.

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Uptake of ^{14}C-streptomycin by some Micro-organisms and its Relation to their Streptomycin Sensitivity

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SUMMARY

The uptake of radioactivity from ^{14}C-streptomycin, in a form which cannot be displaced from the organisms by unlabelled streptomycin, was determined in several species of bacteria and one yeast growing under standard conditions. This uptake showed a good correlation with the sensitivity of the organism to streptomycin, and in streptomycin-resistant organisms was about 1 % of that in a highly sensitive organism, *Bacillus megaterium*. The quantity of streptomycin taken up when growth ceased represented an intracellular concentration 10- to 50-fold higher than that in the growth medium. Environmental factors, such as anaerobiosis or presence of carbon monoxide, which increased the resistance of *Staphylococcus aureus* to streptomycin also resulted in decreased rates of uptake of ^{14}C-streptomycin. When *Bacillus subtilis* grew in medium containing the streptomycin antagonist 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide, the rate of uptake of radioactivity was reduced to about 30 % of that in absence of the antagonist; the rate at which harvested organisms oxidized succinate and malate was reduced to a similar extent.

INTRODUCTION

The uptake of streptomycin by bacterial cells has been investigated on a number of occasions with organisms under different experimental conditions and by various methods for estimating the amount of streptomycin taken up. In early work, Linz (1949) estimated biologically the antibiotic activity released on lysing streptomycin-treated *Micrococcus lysodeikticus* with lysozyme; Linz & Arnaud (1950) studied some other organisms using extraction with warm salt solutions, and Berkman, Henry, Housewright & Henry (1948) studied uptake by dense suspensions of *Staphylococcus aureus*. These investigations showed that, under the conditions used, only about 1–3 % of the total streptomycin present was taken up, and that cells of streptomycin-resistant and sensitive strains took up similar amounts of streptomycin. Since it is now known that streptomycin is not bactericidal to *Escherichia coli* or *Staphylococcus aureus* when the organisms are prevented from growing (Anand & Davis, 1960; Hancock, 1960), the results of these earlier studies using non-growing organisms may not be relevant to the processes leading to death of the organisms.

More detailed studies of streptomycin uptake have been made possible recently by the use of ^{14}C-labelled streptomycin. Szybalski & Mashima (1959) showed that *Escherichia coli* K 12 took up radioactivity during the period during which viability was lost following addition of ^{14}C-streptomycin, whereas streptomycin-resistant

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organisms took up a negligible amount; in these experiments, only that streptomycin which was not removed by washing with solutions containing unlabelled streptomycin was determined. The kinetics of uptake of streptomycin by *E. coli* W were studied by Anand, Davis & Armitage (1960) and by Plotz, Dubin & Davis (1961); an initial rapid uptake, similar in both sensitive and resistant organisms, was followed by a second slower uptake which occurred only in sensitive organisms and depended on growth of the organisms. A rapid uptake of streptomycin, by a process similar to adsorption and resulting in a change in the charge of the cell surface, had been observed earlier in *E. coli* H and in *Staphylococcus aureus* (McQuillen, 1951).

The experiments described in the present paper had the particular object of investigating the relationship between sensitivity to, and uptake of, streptomycin, in organisms of different sensitivity and in environments which affect the activity of streptomycin on particular organisms. Some of these results have been published in abstract (Hancock, 1961*a*).

METHODS

Organisms and media. The following organisms were used: *Bacillus subtilis*, originally National Collection of Type Cultures (NCTC) no. 8236 but now of different streptomycin-sensitivity; *B. megaterium* KM; *B. pumilis* NCTC 8241; *Escherichia coli* B; *Streptococcus faecalis* NCTC 8213; *Clostridum welchii* SR 12; *Cl. sporogenes* NCTC 532; *Staphylococcus aureus* Duncan and a streptomycin-resistant strain derived from it; *S. aureus* strains 57 and 60, clinical isolates obtained from Dr R. M. Stirland of this Department; *Candida utilis* IMI 23311 (Commonwealth Mycological Institute, Kew).

Candida utilis and *Bacillus megaterium* were grown in C medium (McQuillen & Roberts, 1954) + glucose (1 mg./ml.) + Difco Yeast extract (0·1 mg./ml.) (named here CGY medium). The other organisms were grown in nutrient broth (Difco No. 2; pH 7·5), with addition of glucose (1 mg./ml.) for the staphylococci. Cultures were grown in 100 ml. quantities of medium in 250 ml. Erlenmeyer flasks fitted with side arms to fit the Unicam SP 600 spectrophotometer. The prewarmed medium was inoculated from an overnight culture to give an initial population density equivalent to about 50 μg. dry wt. organism/ml., estimated from the optical density (700 mμ) and a calibration curve for the appropriate organism. Flasks containing cultures of clostridia were evacuated and gassed with N_2 and incubated statically at 37°; cultures of other organisms were shaken in a constant temperature water bath at 37°. For growth of *Staphylococcus aureus* in a gas phase containing carbon monoxide, the flasks were successively evacuated and filled three times with the appropriate gas mixture, which was prepared in an aspirator from cylinder carbon monoxide and air. Organisms were harvested by centrifugation (6000 *g*; 5 min.) and, unless otherwise stated, washed once with cold growth medium. When ^{14}C-streptomycin was used, the washing medium contained unlabelled streptomycin (100 μg./ml.).

Sensitivity tests. The minimum inhibitory concentrations of streptomycin for the organisms used were determined by a conventional technique. Tubes containing 2·5 ml. of appropriate medium, with streptomycin added when required, were inoculated with 1 drop of a similar culture grown for 24 hr., and were incubated for 24 hr. at 37°.

Radioactivity determinations. The relatively low specific activity of the ^{14}C-strepto-

mycin preparation necessitated the use of a windowless gas-flow Geiger counter, made in the workshops of this department and based on the design of Banks, Blow & Francis (1956); the flushing gas was 1·5 % (v/v) isobutane in argon. Since the observed radioactivities were low by usual standards, it was important to know their limits of accuracy. It was repeatedly found that the standard deviation of ten estimates of background and of experimental samples, counted for 2 min. periods, was not more than that expected statistically. At least 400 counts were recorded from all samples, which were prepared and counted in duplicate, so that the standard deviation of the mean value of the difference (sample–background) was not more than 40 counts, or 5 %. Unless specifically stated, all the results in this and the following paper are from samples which contained between 20 and 200 counts/min. above background, which varied over long periods between 23 and 27 counts/min.

Samples were mounted on aluminium planchets of 2 cm. diameter, as described elsewhere (Park & Hancock, 1960); these were weighed before and after mounting and drying the samples, which usually contained less than 20 mg. dry wt. of material. The observed radioactivity was corrected to infinite thinness, when necessary, by using a self-absorption curve determined experimentally by preparing planchets containing different quantities of *Bacillus megaterium* and a standard amount of ^{14}C-streptomycin. It was assumed that this curve was applicable to all the organisms used.

Manometric experiments with *Bacillus subtilis* were carried out under conditions described previously (Hancock, 1961*b*); the manometer flasks contained the equiv. of about 4 mg. dry wt. organisms.

Streptomycin. ^{14}C-streptomycin $CaCl_2$ complex was preparation L 561034-1-4 of Merck, Sharpe and Dohme Research Laboratories. The specific activity was such that 47 counts/min. were recorded from 1 μg. streptomycin base. Concentrations of streptomycin are given throughout in terms of the free base. 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide was kept as a solution containing 50 μg./ml. in 0·01 N-NaOH.

RESULTS

Effects of washing. It was not practicable to carry out detailed investigations of the effects of washing on each organism. However, some radioactivity was removed from all those organisms investigated, with the exception of *Bacillus megaterium*, by washing in growth medium containing unlabelled streptomycin (Table 1). The radioactivity removed in this way may represent streptomycin bound in a manner similar to that bound in the initial phase of streptomycin action in *Escherichia coli* W, which can be removed by washing with buffers but not with distilled water (Anand *et al.* 1960). In addition, in the organisms studied here some streptomycin may be bound to negatively charged cell-wall constituents, such as teichoic or teichuronic acids. It was also not practicable to show that the radioactivity taken up by each organism represented unaltered streptomycin; it is expressed in terms of streptomycin in this paper, but its identity has only been shown in the case of *B. megaterium* (Hancock 1962).

Rate of uptake of streptomycin by growing organisms

To compare the rates of uptake of streptomycin by different organisms, standard experimental conditions were used in which streptomycin was added to 10 μg./ml

to an exponentially growing culture when the population density was about equiv. to 100 μg. dry wt. organisms/ml. There was a good correlation between the rate of uptake of streptomycin under these conditions and the sensitivity to streptomycin, within the group of organisms investigated (Fig. 1). Both naturally resistant organisms (e.g. the clostridia), and resistant strains of normally sensitive organisms, showed rates of uptake of the order of 1 % of that in the most sensitive organism used, *Bacillus megaterium*. The quantity of streptomycin finally taken up by some of these organisms was also determined, when using streptomycin at concentrations which inhibit growth completely after about 1 hr. (Table 2).

Table 1. *Retention of ^{14}C-streptomycin on washing*

Several identical samples of each culture were harvested when inibition of growth was just complete, except in the experiments in the first and last columns. With the exception of *Bacillus megaterium* which was harvested and washed on membrane filters, the organisms were harvested by centrifugation.

	Organism				
	Bacillus megaterium		*Staphylococcus aureus*	*Bacillus subtilis*	*Clostridium welchii*
	Concentration of streptomycin (μg./ml.)				
	1	1	12	40	10
	Time of exposure (min.)				
	30	60	120	120	120
	Counts/min./sample				
Control (unwashed)	39	74	479	213	79
Washed × 3 with growth medium	40	73	—	—	—
Washed with growth medium containing unlabelled streptomycin (100 μg./ml.) × 1	38	70	210	138	28
Washed with growth medium containing unlabelled streptomycin (100 μg./ml.) × 3	39	65	201	143	27
Washed × 2 with M-NaCl	34	68	—	—	—

Table 2. *Quantity of streptomycin taken up when growth is fully inhibited*

Organisms were harvested when growth had just ceased, and washed once in growth medium containing unlabelled streptomycin (100 μg./ml.). The results are from three separate experiments for *Bacillus megaterium*.

Bacillus megaterium	*Staphylococcus aureus*	*Bacillus subtilis*	*Escherichia coli*
Concentration of streptomycin (μg./ml.)			
1	10	40	10
Time of exposure (min.)			
125	130	120	70
Final number of viable organisms, % initial value			
0·2	0·08	0·7	0·8
Final uptake of streptomycin (μg./g. dry wt.)			
38	1150	1230	320
51			
46			

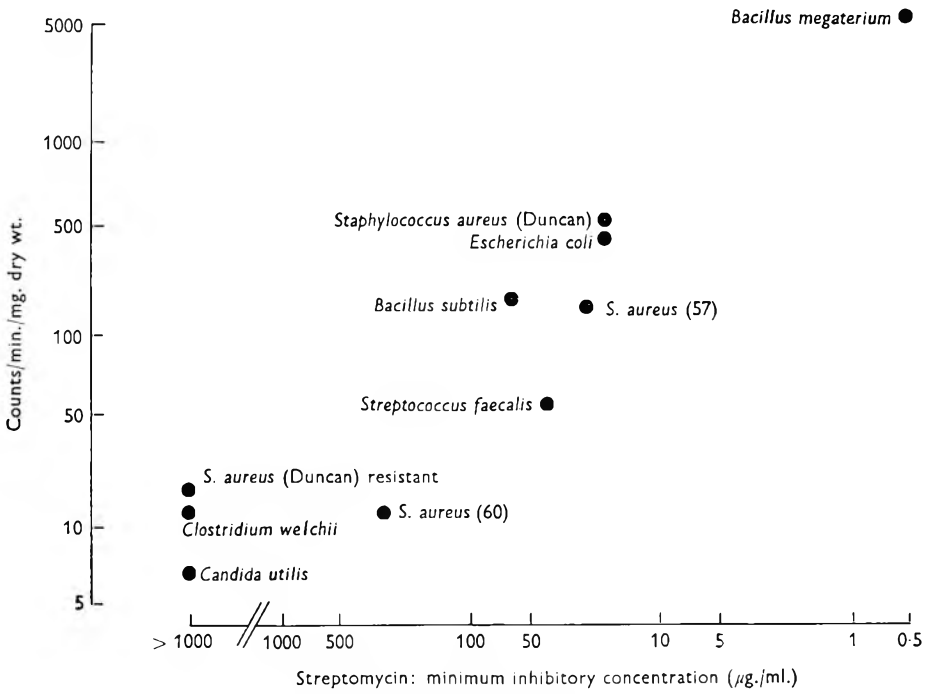

Fig. 1. Minimum inhibitory concentrations of streptomycin for some micro-organisms and uptake of ^{14}C-streptomycin during growth under standard conditions. Uptake was determined with streptomycin at 10 μg./ml. after growth for 60 min., except for *Bacillus megaterium* where the rate is calculated from that for the first 15 min. period, after which loss of viability began.

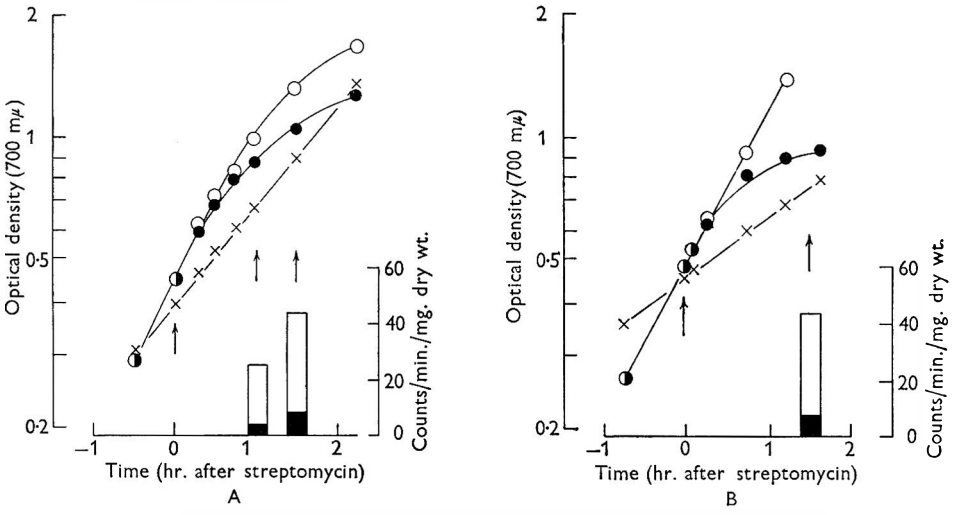

Fig. 2. Uptake of ^{14}C-streptomycin by *Staphylococcus aureus* following addition of streptomycin (8 μg./ml.) at first arrow. Points represent optical density of cultures without streptomycin (○); with streptomycin in air (●); and with or without streptomycin (×) in nitrogen (A) or in 80 % (v/v) carbon monoxide in air (B). Columns represent radioactivity of organisms, at times indicated by arrows, in air (open columns) or in other gases (solid columns).

Uptake of streptomycin by Staphylococcus aureus *during anaerobic growth*

Facultatively anaerobic organisms such as *Staphylococcus aureus* are more resistant to streptomycin during anaerobic growth, and also during growth in the presence of carbon monoxide (Lightbown, 1957). When *S. aureus* was grown anaerobically the uptake of streptomycin was about 20 % of that during aerobic growth (Fig. 2A), although the anaerobic growth rate was about 65 % of the aerobic rate. Similar results were obtained with cultures growing in a gas mixture of 80 % (v/v) carbon monoxide + 20 % (v/v) air (Fig. 2B).

Effect of streptomycin antagonists on uptake of streptomycin by Bacillus subtilis

The inhibitory action of streptomycin on *Bacillus subtilis* and some other organisms can be antagonized by certain alkyl-hydroxyquinoline *N*-oxides (Lightbown, 1954) at concentration ratios of antagonist:streptomycin of the order

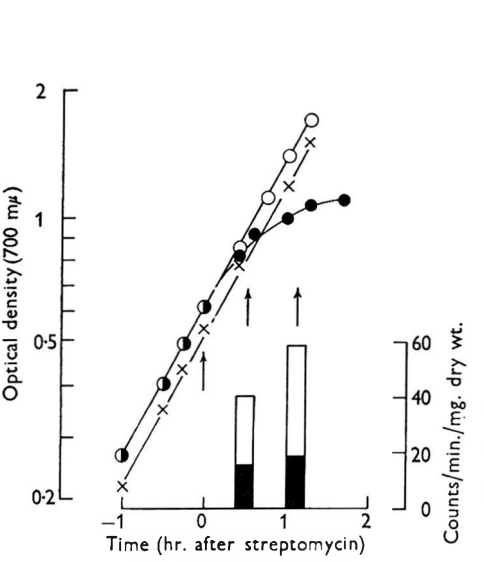

Fig. 3

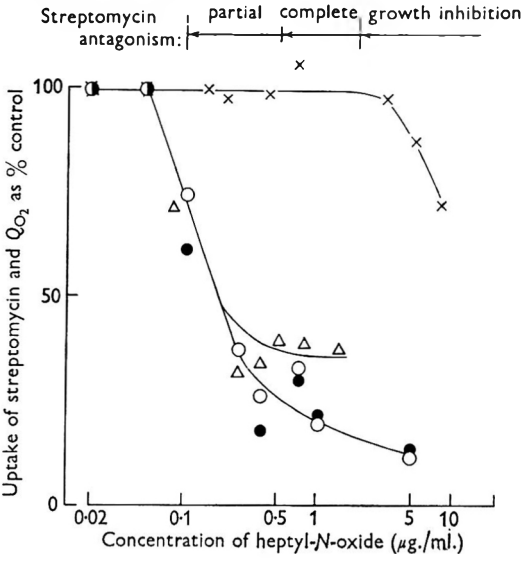

Fig. 4

Fig. 3. Effect of heptyl-*N*-oxide on growth of *Bacillus subtilis* and uptake of ^{14}C-streptomycin (30 μg./ml.) added at the first arrow. Points represent optical density of cultures without streptomycin (○), with streptomycin alone (●), and with both streptomycin and heptyl-*N*-oxide (0·35 μg./ml.) (×). Columns represent radioactivity of organisms, at times indicated by arrows, with streptomycin alone (open columns) or with both streptomycin and heptyl-*N*-oxide (solid columns).

Fig. 4. Uptake of ^{14}C-streptomycin by *Bacillus subtilis* in cultures containing heptyl-*N*-oxide (△), and ability of organisms to oxidize glucose (×), succinate (●) and malate (○), relative to values in control cultures without heptyl-*N*-oxide. ^{14}C-streptomycin was added at subinhibitory concentration (18 μg./ml.) to cultures when the optical density (700 mμ) was 0·3; the organisms were harvested after 90 min., when control organisms contained between 31 and 44 counts/min./mg. dry weight. Q_{O_2} values were determined using organisms harvested after growth for 60 min. with heptyl-*N*-oxide, which was added to cultures when the optical density was 0·15. Antagonism of streptomycin, or inhibition of growth, by heptyl-*N*-oxide was determined from the optical density of growing cultures when heptyl-*N*-oxide was added at an optical density of 0·15 and streptomycin (50 μg./ml.) at an optical density of 0·3. The growth rate was not affected by heptyl-*N*-oxide at concentrations below 2 μg./ml., but at concentrations greater than this the growth rate was reduced.

of 1:100. At low concentrations, 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide (heptyl-*N*-oxide) did not affect the growth rate of *B. subtilis* but conferred complete resistance to subsequent addition of streptomycin. The uptake of streptomycin in such cultures was between 30 and 40 % of the rate in a control culture without heptyl-*N*-oxide (Fig. 3).

Since heptyl-*N*-oxide is known to be an inhibitor of certain respiratory systems in resting bacteria and cell-free preparations (Lightbown & Jackson, 1956), we examined whether the respiratory activities of organisms growing in its presence were similarly affected. When organisms were harvested from cultures containing heptyl-*N*-oxide at concentrations which antagonize streptomycin but did not affect the growth rate, their ability to oxidize succinate and malate was below the control values although oxidation of glucose was not affected (Fig. 4). At higher concentrations, heptyl-*N*-oxide has itself growth-inhibitory activity, and in cultures containing the antagonist at these concentrations the ability of harvested organisms to oxidize glucose was also impaired. The rate of uptake of streptomycin, determined in similar cultures, was depressed during growth in the presence of heptyl-*N*-oxide to an extent very similar to that of oxidation of succinate and malate (Fig. 4).

DISCUSSION

Many types of process can be envisaged by which micro-organisms can become resistant to an antibacterial agent (Davis & Maas, 1952); among these are alterations in the metabolic pathways used by the organisms, an alteration in the rate of uptake of the agent into the organism or to its site of action, or an enzymic inactivation of the antibiotic. In all the cases investigated here, and within the limits of the assumptions made about the use of the same washing procedure for each organism (which may have to be modified in the light of further experience), resistance to streptomycin, determined either genetically or by environmental factors, was associated with a decreased uptake of streptomycin. This relationship is similar to that in *Escherichia coli* K 12 (Szybalski & Mashima, 1959) and in the secondary phase of uptake in *E. coli* W (Anand *et al.* 1960); however, in the latter organism the initial uptake of streptomycin is the same in sensitive and resistant organisms. It differs from earlier findings of Linz (1949) and of Berkman *et al.* (1948), and from those of Sager (1961) in *Chlamydomonas reinhardi*. A similar relationship is found between sensitivity to, and rate of uptake of, penicillin in naturally occurring strains, though not in laboratory strains, of staphylococci (Rowley, Cooper, Roberts & Lester Smith, 1950; Eagle, 1954). Such a correlation between sensitivity and rate of uptake of streptomycin makes it unnecessary to postulate differences in, or absence of, the streptomycin-sensitive metabolic processes in streptomycin-resistant organisms. However, such differences cannot be excluded because if uptake of streptomycin were caused directly by its interference in a metabolic system, for example, by combination with an enzyme, metabolite, or structural unit, then organisms which lacked this system would be resistant to streptomycin and also would not take up streptomycin.

The quantity of streptomycin taken up when growth has ceased differed considerably in each organism examined here, but in every case it represented a concentration of streptomycin within the cell higher than that outside if the streptomycin is distributed throughout the water space of the organisms. Thus, using the

values of Mitchell & Moyle (1956) for the water spaces of *Escherichia coli* and *Staphylococcus aureus*, and of Weibull (1955) for *Bacillus megaterium*, the intracellular concentrations finally attained, calculated from the results in Table 2, are 110, 480, and 11 μg./ml., at external concentrations of 10, 10, and 1 μg./ml., respectively.

Sensitivity of bacteria to streptomycin shows some general correlation with the degree of dependence on, or use of, aerobic respiratory processes, and the rate of uptake of the antibiotic also shows some correlation with those factors. The suppression of ability to oxidize succinate and malate by organisms growing in the presence of the streptomycin antagonist heptyl *N*-oxide shows that the inhibition of these reactions in resting organisms (suggested as the basis for antagonism of streptomycin, Lightbown & Jackson, 1956), also occurs in growing organisms precisely at those concentrations which confer resistance to streptomycin. It may perhaps be re-emphasized that these alterations occur without any effect on the growth rate of the organisms. It remains to be investigated whether the suppression of these oxidative activities is due to a reduction in enzyme formation or merely in activity. It is interesting to speculate that the residual oxidation of succinate and malate in the presence of heptyl *N*-oxide, and also the reactions involved in glucose oxidation, may be mediated through the flavoprotein oxidative systems which occur in *Bacillus subtilis* (Lightbown & Kogut, 1959). The close correlation in *B. subtilis* between the ability to oxidize succinate and malate and the rate of uptake of streptomycin suggests that streptomycin uptake may be closely related to the rate of operation of either the electron transport systems for these substrates or of the terminal oxidation pathways, such as the tricarboxylic acid cycle, which depend on them.

I am grateful to Dr J. W. Lightbown for a gift of 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide.

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Uptake of ^{14}C-Streptomycin by *Bacillus megaterium*

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SUMMARY

More than 90 % of the radioactivity taken up by *Bacillus megaterium* growing in medium containing inhibitory concentrations of ^{14}C-streptomycin appeared to be identical with streptomycin, as shown by its behaviour on an ion-exchange resin and by recrystallization with streptomycin derivatives. Streptomycin was not removed from the organisms by washing in growth medium with or without addition of unlabelled streptomycin, or in distilled water. It could be extracted with inorganic acids and trichloroacetic acid, but not by some other commonly used extraction procedures. When the organisms were converted to protoplasts and these lysed and fractionated, up to 99 % of the streptomycin in the organisms was recovered in the material sedimented from the cytoplasmic fraction by centrifugation at 105,000 *g*. Very small amounts of streptomycin were found in the fraction containing protoplast 'ghosts', but these may have represented contaminating cytoplasmic material. This distribution may not, however, be a true indication of the location of streptomycin before fractionation, since a similar distribution was found when streptomycin was added to lysed protoplasts immediately before fractionation. The uptake of streptomycin was decreased at pH values below 7. Uptake depended on continued synthesis of cell material, and environmental conditions which prevented growth and bactericidal action also considerably decreased uptake of streptomycin. The quantity of streptomycin finally taken up was proportional to the concentration of streptomycin in the growth medium, and at the lowest growth-inhibitory concentrations was about 5×10^4 molecules of streptomycin per bacterium.

INTRODUCTION

The previous paper (Hancock, 1962) described investigations of the uptake of ^{14}C-streptomycin by several micro-organisms, and some factors which affected it. *Bacillus megaterium* was chosen for a more detailed investigation since its high sensitivity to streptomycin allowed the use of relatively small quantities of ^{14}C-streptomycin, and because well-established methods for fractionating cells of the organism into morphological constituents (e.g. Weibull, Beckmann & Bergström, 1959) might enable localization of the antibiotic in the cells.

METHODS

Organism, growth and harvesting. Bacillus megaterium KM was grown in CGY medium (Hancock, 1962) adjusted to pH 7·2, except in one experiment where the pH value was deliberately changed. The conditions of growth were the same as

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those used earlier for this organism (Hancock, 1961). Streptomycin was added to cultures in the exponential phase of growth when the optical density (at 700 mμ) was 0·6–0·7. The organisms were usually harvested from culture samples of more than 40 ml. volume by centrifugation (6000 *g*; 5 min.). When rapidity of sampling was necessary, smaller volumes of culture (40 ml. or less) were filtered and washed on Oxoid membrane filters (effective filter diameter 2 cm.); for the equivalent of 10–15 mg. dry wt. organisms this usually took about 30 sec. Except in preliminary experiments (specified in detail) the organisms were washed once in cold CGY medium containing unlabelled streptomycin (100 μg./ml.).

Radioactivity determinations, mounting of samples and self-absorption corrections were carried out as described by Hancock (1962). Membrane filters were fixed to aluminium planchets with 'Durofix' (The Rawplug Co. Ltd., London, S.W. 7). It was not practicable to determine directly the dry weight of organisms present in filtered samples, but this was calculated when necessary from the optical density (700 mμ) of the culture by using a calibration curve for the organisms. The use of filters is specified in the experimental protocols. Self-absorption corrections for materials in subcellular fractions were assumed to be the same as those for intact organisms.

Isolation of intracellular radioactive compounds. Large quantities (about equiv. to 2 g. dry wt.) of organisms which had grown in the presence of ^{14}C-streptomycin until growth had almost ceased, were harvested and washed in CGY medium; since addition of unlabelled streptomycin during washing did not remove any more radioactivity than CGY medium alone (Table 1), unlabelled streptomycin was not added to the washing medium in these experiments, to avoid any possible dilution of intracellular ^{14}C-streptomycin. The organisms were suspended in 5% (w/v) trichloroacetic acid (TCA) at 5° for 20 min., by which time all the ^{14}C was extracted (Fig. 4, Table 6); the cell residue was centrifuged and washed once with TCA, and the pooled extracts (about 40 ml.) were shaken five times with 50 ml. ether to remove TCA. The remaining extract was carefully neutralized to pH 7 and freeze dried. The total time of exposure to acid conditions was about 90 min. To provide a control on recovery during the isolation procedure, a similar quantity of normal organisms were harvested and a known quantity of ^{14}C-streptomycin added immediately after suspension of the organisms in TCA; the following procedures were carried out identically in experimental and control samples. The dried extracts were taken up in water, and a known quantity (about 10 mg.) of unlabelled carrier streptomycin was added to locate the streptomycin peak in the fractions eluted from an ion-exchange column.

Preparation and fractionation of protoplasts. The procedures were used as described by Hancock (1961). High-speed centrifugations were carried out in the No. 40 rotor of the Spinco centrifuge; values for the centrifugal force are the average values as given in the centrifuge manual.

Streptomycin reineckate was prepared and recrystallized by the procedure of Fried & Wintersteiner (1945) after addition of carrier unlabelled streptomycin. The radioactivity of reineckate samples was corrected for self-absorption from an experimentally determined self-absorption curve. Melting points are uncorrected.

Hydrolysis of streptomycin. A small quantity (about 1 mg.) of ^{14}C-streptomycin

was hydrolysed in 6N-HCl at room temperature for 8 hr.; the solution was freeze dried and redried from a small volume of water. Ribonuclease, deoxyribonuclease and trypsin were recrystallized preparations from L. Light and Co., Colnbrook, Bucks. All other materials and methods were as decribed by Hancock (1962). Concentrations of streptomycin are expressed throughout in terms of the free base.

Separation of streptomycin on ion-exchange columns. The procedure was based on that of Doery, Mason & Weiss (1950). Amberlite IRC-50 (H) (Analytical grade) was washed with water and converted to the Na form by suspending three times in saturated $NaHCO_3$ solution. Columns 10×1 cm. were prepared and washed with 500 ml. distilled water, and the experimental sample was run through the column at a rate of about 10 ml./hr. The column was washed with 50 ml. distilled water, and eluted with 50 ml. of 0·25N-acetic acid followed by 100 ml. of 0·5N-acetic acid at a flow rate of about 20 ml./hr.; 2·5 ml. fractions were collected. Streptomycin was assayed in the effluent fractions by the maltol method (Boxer, Jelinek & Leghorn, 1947), but with one-quarter of the recommended volumes of sample and reagents.

Streptomycin helianthate was prepared by the procedure used by Karow, Peck, Rosenblum & Woodbury (1952) and recrystallized from warm 30 % (v/v) methanol in water. Precipitates of streptomycin helianthate were separated on Oxoid membrane filters. A self-absorption curve was determined experimentally for streptomycin helianthate and used to correct the observed radioactivity to infinite thinness.

RESULTS

Retention of streptomycin on washing

Under the experimental conditions used, addition of streptomycin to 1 μg./ml. inhibited growth fully after a period of about 1 hr.; at this time the number of viable organisms had fallen to less than 1 % of the initial number. It was found (Table 1) that at various times during this period no significant quantities of

Table 1. *Retention of* ^{14}C-*streptomycin on washing*

A number of identical samples were taken at each time after addition of streptomycin (1 μg./ml.), and from the same culture except for the 15 min. samples; the organisms were harvested on membrane filters.

	Period of growth with streptomycin (min.)			
	15	30	45	60
Washing medium	Counts/min./sample			
–(control)	85	39	76	74
Growth medium ×1	—	36	72	—
Growth medium ×3	89	40	71	73
Growth medium + unlabelled streptomycin (100 μg./ml.) ×1	—	38	—	—
Growth medium + unlabelled streptomycin (100 μg./ml.) ×3	84	39	63	65
NaCl (M) ×1	—	36	71	—
NaCl (M) ×2	—	34	—	68
Distilled water	—	—	75	75

streptomycin were removed from the organisms by washing in growth medium, with or without addition of excess unlabelled streptomycin, or by washing in distilled water or in M-NaCl. In subsequent experiments, the organisms were routinely washed once in growth medium containing unlabelled streptomycin.

Uptake by growing organisms

At sub-inhibitory concentrations of streptomycin, uptake of streptomycin was parallel to growth of a culture (Fig. 1 A) but at a slightly higher rate. At concentrations which inhibited growth, the uptake followed closely the residual growth of the culture and ceased when growth ceased (Fig. 1 B). It was not possible to detect any

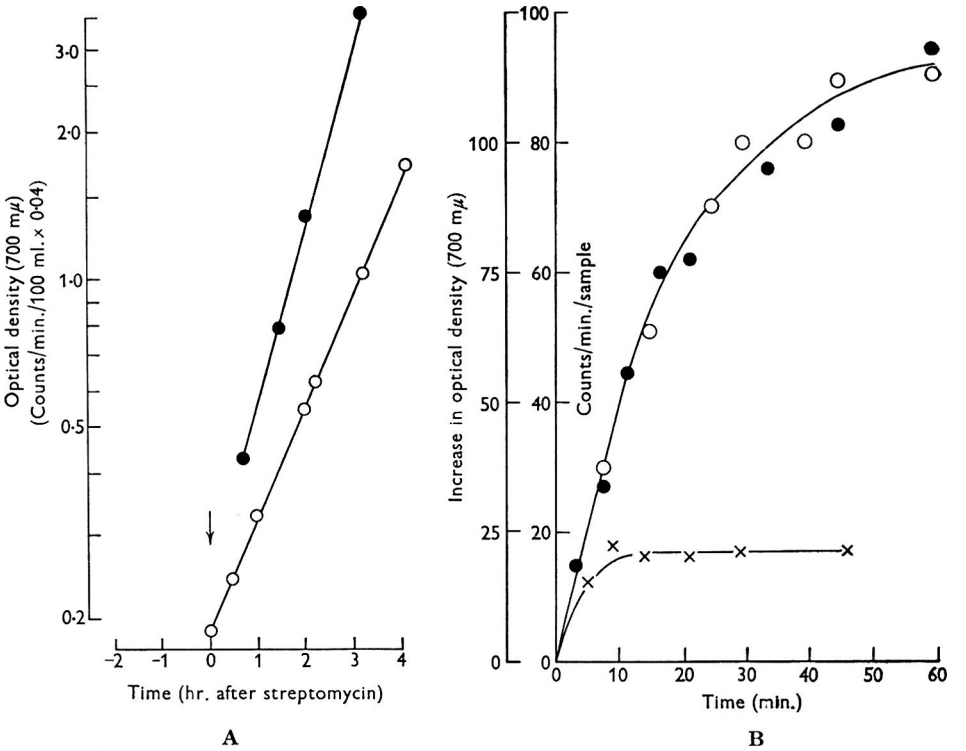

Fig. 1. Uptake of ^{14}C-streptomycin by *Bacillus megaterium* (●) at a sub-inhibitory concentration (0·05 μg./ml.) (A) and at a growth-inhibitory concentration (1 μg./ml.) (B), and optical density (○). Filtered samples. In one experiment (×), chloramphenicol (200 μg./ml.) was added to a culture at the same time as streptomycin.

separate phases in the uptake curve, such as occur in *Escherichia coli* W (Anand, Davis & Armitage, 1960). When streptomycin was added at concentrations below or above 1 μg./ml., growth was inhibited after a longer or shorter period; when the organisms were harvested as soon as growth had ceased it was found that less streptomycin had been taken up at the lower concentrations of streptomycin (Fig. 2).

In two experiments, radioactivity was taken up from ^{14}C-streptomycin, which had

been hydrolysed under conditions for liberating the streptidine moiety, at less than 3 % of the rate of uptake of the intact antibiotic.

Streptomycin does not cause loss of viability when added to cultures of *Escherichia coli* or *Staphylococcus aureus* under conditions in which the organisms cannot grow (Anand & Davis, 1960; Hancock, 1960). Similar results have been obtained with *Bacillus megaterium* (Hancock, unpublished results), and under these conditions the uptake of streptomycin is much lower than that by growing organisms (Table 2). As in *Escherichia coli* W (Anand *et al.* 1960) a major part of the uptake process therefore depends on growth of the organisms in the presence of streptomycin, as does loss of viability.

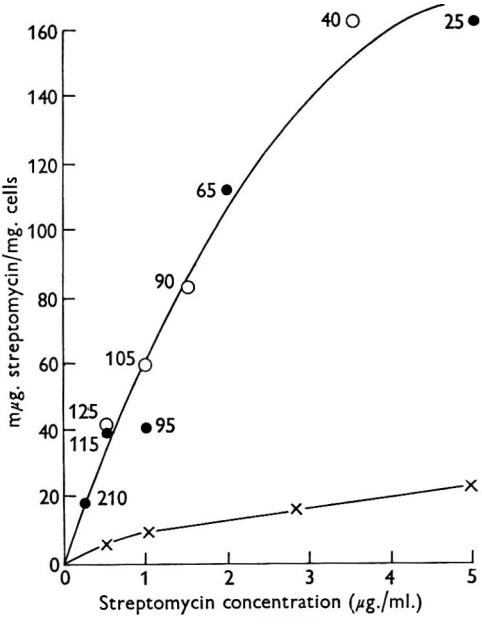

Fig. 2

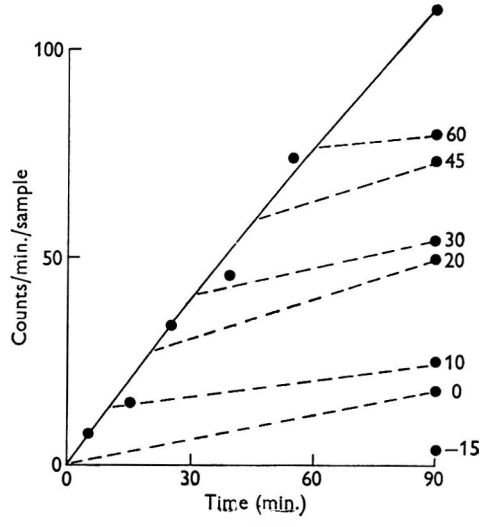

Fig. 3

Fig. 2. Amount of ^{14}C-streptomycin finally taken up by *B. megaterium* when growth ceases, from 2 experiments (● and ○). Figures by points indicate the time of harvesting (min.) after addition of streptomycin. Chloramphenicol (100 μg./ml.) was added to one set of cultures (×) 10 min. before streptomycin and these were harvested after 30 min.

Fig. 3. Uptake of ^{14}C-streptomycin by *B. megaterium* (1 μg./ml.) following addition of chloramphenicol (100 μg./ml.) to samples of a culture at the times indicated by each final point (min. after addition of streptomycin).

Table 2. *Effect of inhibition of growth on uptake of* ^{14}C-*streptomycin*

Organisms incubated for 45 min. in growth medium under the conditions indicated, and harvested on membrane filters.

Conditions of incubation	Counts/min./ sample
37°, air	71
5°, air	7
37°, N_2	13
37°, air, +chloramphenicol (200 μg./ml.)	16
37°, air, without glucose	3

Effect of chloramphenicol on uptake of streptomycin

At growth-inhibitory concentrations of chloramphenicol (CAP), uptake of streptomycin, measured after long periods, was considerably decreased (Table 2). Kinetic experiments showed that under these conditions the uptake of streptomycin rapidly reached a plateau and thereafter remained constant (Fig. 1), in a similar way to that with *Escherichia coli* W (Anand *et al.* 1960). The quantity of streptomycin taken up in the presence of CAP also depended on the concentration of streptomycin in the medium (Fig. 2). When CAP was added at successive times during inhibition of growth to samples of a culture containing streptomycin, the uptake of streptomycin was arrested almost completely (Fig. 3).

Effect of pH *value*

The uptake of streptomycin at subinhibitory concentrations showed a marked dependence on the pH value of the growth medium, under conditions in which the rate of growth was affected only to a small extent (Table 3). This would suggest that the streptomycin cation competed with hydrogen ions at some stage in the uptake process, and is probably the basis of the decrease in activity of streptomycin when the pH values of the growth medium is lowered (Abraham & Duthie, 1946).

Table 3. *Effect of* pH *on rate of uptake of* ^{14}C-*streptomycin*

^{14}C-streptomycin was added at sub-inhibitory concentration (0·2 μg./ml.) to each culture when the population density was equiv. to 150 μg. dry wt./ml. After 75 min. the organisms were harvested on membrane filters and washed once with growth medium (pH 7·2) containing unlabelled streptomycin (100 μg./ml.).

pH value of growth medium	Final population density (μg. dry wt./ml.)	Counts/min./g. dry wt. organism
5·0	305	250
6·0	405	240
7·0	345	940
7·2	340	1270

Uptake of streptomycin after breakage of the osmotic barrier

Addition of toluene to a culture of *Escherichia coli* W in the presence of CAP results in an increased uptake of streptomycin (Anand *et al.* 1960). The effects on uptake of streptomycin of a number of procedures which break the osmotic barrier of *Bacillus megaterium* was investigated (see Table 4). To avoid complications due to different responses of treated organisms to washing, the organisms or cell residues were not washed in these experiments.

In the presence of CAP, addition of toluene or butanol to a growing culture resulted in increased uptake of streptomycin to values approaching that reached in the absence of CAP; uptake was not, however, increased when the suspension was heated. However, when the organisms were incubated at a higher bacterial concentration, all the ^{14}C-streptomycin added to the incubation medium was taken up in the presence of cetyltrimethylammonium bromide (CTAB) or toluene, or in boiled suspensions.

Table 4. *Effect of osmotic barrier damage on uptake of* ^{14}C-*streptomycin*

^{14}C-streptomycin (1·5 μg./ml.) was added directly to a growing culture (columns 1, 2 and 3) or after harvesting the organisms and resuspending in growth medium at ten times the original population density (column 4); other additions were then made to the samples as indicated. Each sample (1·8 ml.) in column 4 contained streptomycin at 1·5 μg./ml. (total radioactivity 126 counts/min./sample).

Time with streptomycin (min.) ...	50		15	
Cell density (μg. dry wt. organisms/ml.)	300	300	300	3000
Chloramphenicol (100 μg./ml.) present	−	+	−	−
Additions	Counts/min./sample			
−(control)	82	21	27	25
Cetyltrimethylammonium bromide (200 μg./ml.)	3	5	8	126
Toluene (4 %, v/v)	53	69	42	123
Butanol (25 %, v/v)	50	60	48	76
Boiled (15 min.)	5	11	7	130

Extraction of streptomycin

The release of streptomycin from washed organisms was investigated by several procedures commonly used to liberate intracellular constituents (Table 5). Breakage of the osmotic barrier alone did not appear to be sufficient to effect release, and the only agents which released streptomycin were acidic solutions; the rate of release by several different acids was found to be similar (Fig. 4). It was later found that there was no detectable breakdown of streptomycin by trichloroacetic acid (TCA) under these conditions, but it is possible that the mineral acids may have caused a small degree of hydrolysis.

Table 5. *Extraction of* ^{14}C-*streptomycin*

Organisms were harvested by centrifugation, washed once in growth medium + unlabelled streptomycin (100 μg./ml.) and resuspended in the appropriate medium at a concentration equiv. to about 4 mg. dry wt./ml. The residues were recovered by centrifugation after extraction. Results of two separate experiments.

Extracting agent		^{14}C-streptomycin in residue (counts/min.)	
−(control)		38	21
5 % (w/v) trichloroacetic acid	5°, 30 min.	2	3
	100°, 20 min.	0	2
0·5 N-perchloric acid	5°, 30 min.	—	0
	100°, 20 min.	—	0
Hydrochloric acid at 5° for 30 min.	0·1 N	—	14
	N	0	—
−(control)		47	24
Water, 100°, 20 min.		46	25
Ethanol, 75 %, v/v, 60°, 30 min.		45	20
Butanol, 10 %, v/v, 25°, 30 min.		43	—
Toluene, 4 %, v/v, 25°, 30 min.		44	24
Cetyltrimethylammonium bromide 250 μg./ml., 25°, 30 min.		42	25

Identity of intracellular radioactive compounds

All the radioactivity in TCA-extracts of organisms grown with ^{14}C-streptomycin was eluted from an ion-exchange column in precise coincidence with the peak containing streptomycin (Table 6; Fig. 5). A control sample of ^{14}C-streptomycin which had been treated in the same way was also recovered completely, showing that the TCA treatment had not resulted in any breakdown of streptomycin (Table 6). Carrier unlabelled streptomycin was added to samples of the material in the streptomycin peaks from each column, and streptomycin helianthate and reineckate prepared and recrystallized. From the specific acitivities of the products after recrystallization, it appeared that, within experimental error, all the radioactivity present in the experimental sample was in a form which would recrystallize with these streptomycin derivatives (Table 7).

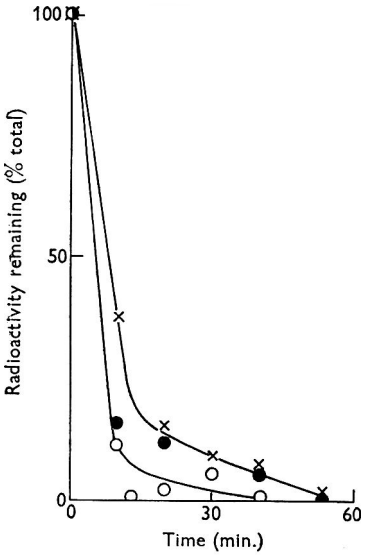

Fig. 4

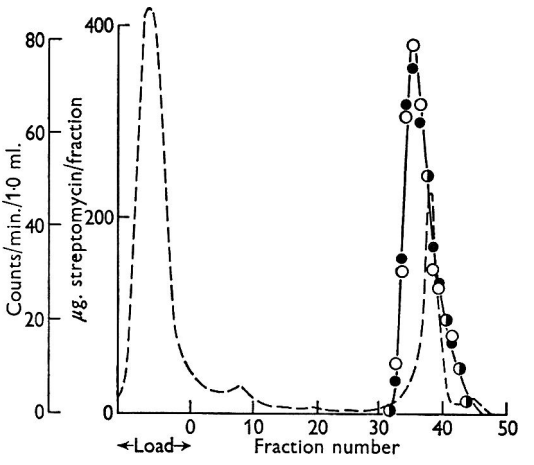

Fig. 5

Fig. 4. Release of ^{14}C-streptomycin from washed *Bacillus megaterium* at 5° by 0·5 N-HCl (×), 0·25 N-perchloric acid (●), and 5 % (w/v) TCA (○). Suspension density equiv. to about 6 mg. dry wt. organism/ml.

Fig. 5. Elution of radioactivity, extracted with 5 % (w/v) TCA, from a cation-exchange column. Radioactivity (●) was determined using 1·0 ml. from each eluted fraction; carrier unlabelled streptomycin was estimated as maltol (○). The broken line represents the elution of radioactivity from a column loaded with acid-hydrolysed ^{14}C-streptomycin; the smaller major peak is streptidine.

Location of streptomycin in cell fractions

The fractionation procedure used resulted in separation of the cell wall, 'ghost' and cytoplasmic fractions (Weibull *et al.* 1959). When organisms which had taken up ^{14}C-streptomycin for short or long periods were fractionated, almost all the radioactivity was recovered in the protoplasts (Table 8). In a few preparations, some radioactivity was lost during conversion of intact organisms to protoplasts, but since this did not occur in most preparations it is thought likely that it was due to a small

Table 6. *Extraction of radioactivity from washed organisms by* TCA *and recovery with streptomycin in column eluates*

Bacillus megaterium grown with ^{14}C-streptomycin until growth had just ceased, and normal organisms, were extracted with 5 % (w/v) TCA. ^{14}C-streptomycin was added to the control extract immediately on addition of TCA. Each extract was from the equivalent of about 2 g. dry wt. organism, and was loaded onto and eluted from a column of Amberlite IRC-50 as described under Methods.

	Total counts/min. in			
	Control		Experimental	
Cell suspension	2800	4670	2420	2190
Cell residue	0	600	0	0
Loaded onto column	2700	5100	2030	2000
Eluted in streptomycin peak	2890	5120	2270	2160

degree of lysis of protoplasts which sometimes occurred during preparation, rather than indicating location of some streptomycin in the cell-wall fraction which is removed at this stage.

When the 'ghost' fraction, which contains the cytoplasmic membrane of the organisms (Weibull *et al.* 1959) was first separated, about 20 % of the total radioactivity in the organisms was associated with it. On washing in 'lysing medium' (Storck & Wachsman, 1957), almost all of this radioactivity was removed but could be recovered from the washing medium by centrifugation at 105,000 *g* for 4 hr. For this reason, it is thought to represent contamination of the 'ghosts' with cytoplasmic material, since the radioactive material in the cytoplasm can also be sedi-

Table 7. *Recovery of intracellular* ^{14}C *as streptomycin derivatives*

Unlabelled streptomycin was added to samples of the eluate from an ion-exchange column and the derivatives were formed as described under Methods. Values for the streptomycin content of streptomycin helianthate and reineckate are taken from Kuehl, Peck, Walti & Folkers (1945) and Fried & Wintersteiner (1945), respectively.

	Streptomycin helianthate			Streptomycin reineckate	
Times crystallized	mg. used	Counts/ min.	Melting point (°)	mg. used	Counts/ min.
1	—	—	—	21·2	66
2	22·8	44	215–220 decomp.	—	—
3	10·8	22	—	5·7	21
4	23·0	46	218–220 decomp.	9·0	28
Final specific activity (counts/min./g. derivative)		2000			3100
Assumed streptomycin content of derivative (mg./g.)		350			390
Specific activity, (counts/min./g. streptomycin)		5700			7900
Carrier streptomycin added (mg.)		73·0			89·5
Radioactivity in original sample (counts/min.) due to streptomycin, calculated from specific activity of derivative		415			710
Total radioactivity present in original sample (counts/min.)		450			630

Table 8. *Distribution of* ^{14}C-*streptomycin in cell fractions of* Bacillus megaterium

Organisms were harvested from cultures (500–1500 ml.) when inhibition of growth was just complete, at 60–120 min. after addition of ^{14}C-streptomycin (1 or 2 μg./ml.), except in one experiment (column 1) when harvesting was 10 min. after addition of streptomycin. Results in the last column represent the distribution of radioactivity in one experiment in which ^{14}C-streptomycin was added to protoplasts from normal organisms immediately before lysing.

Time of growth with streptomycin (min.) ...	10	60–120			0
		No. of experiments	Mean	Range	
Total radioactivity in cells (counts/min.)	145	—	—	210–1580	—
	% total radioactivity in fraction				
Protoplasts	97	9	87	76–103	100
'Ghosts':					
Unwashed	—	1	22	—	—
Washed in lysing medium	9	5	6	4–8	9
Supernatant cytoplasm	77	5	91	87–96	—
Pellet from cytoplasm 105,000 *g*, 4 hr.	42	4	79	49–99	87

mented in this way. In all preparations a very small amount of radioactivity remained in the washed 'ghost' fraction; on some occasions the labelling of this fraction was so small that its significance was doubtful, but it was observed repeatedly. It was not removed by washing, with or without unlabelled streptomycin, but could be removed by repeated washing with M-NaCl or by treatment with ribonuclease (Table 9).

Between 87 and 96 % of the radioactivity in the organisms remained in the cytoplasmic fraction after removal of cell-wall fragments and 'ghost' fraction.

Table 9. *Removal of* ^{14}C-*streptomycin from cell fractions of* Bacillus megaterium *by washing and enzymic treatments*

Each preparation was from a culture harvested when inhibition of growth by streptomycin (1 or 2 μg./ml.) was just complete. The 'ghosts' had been washed once in lysing medium before further washing. Enzyme treatments were carried out for 15 hr. at room temperature in 0·05 M-phosphate buffer (pH 7·2) using 100 μg. of each enzyme/ml.

	Counts/min./remaining in					
	Ghosts				Pellet after 4 hr. 105,000 *g*	
Washing medium						
−(control)	136	174	86	73	490	460
Distilled water	55	99	85	67	260	—
Distilled water + unlabelled streptomycin (100 μg./ml.)	65	126	—	—	290	—
NaCl (M)	0	0	—	—	—	—
Enzyme treatment						
Trypsin	—	—	—	—	160	22
Ribonuclease	—	—	16	12	74	140

Almost all of this radioactivity could be sedimented by centrifugation at 105,000 *g* in some preparations, but in others only about 50 % was sedimented in this way (Table 8); possible reasons for this variation are discussed later. The labelled material in the sedimentable fraction could be washed, with or without unlabelled streptomycin, with only partial loss of radioactivity; but after digestion with trypsin or ribonuclease most of the radioactivity became non-sedimentable (Table 9). It is known that such high-speed centrifugation sediments most of the ribonucleic acid (RNA) of *Bacillus megaterium* (Weibull, 1953), probably in particulate form as with other organisms (Schachman, Pardee & Stanier, 1952). Since streptomycin forms complexes with nucleic acids (Cohen, 1947), the possibility had to be considered that association of streptomycin with this fraction might be due to fortuitous adsorption following breakage of the organisms, and might not reflect the location of streptomycin before breakage. To investigate this, ^{14}C-streptomycin was added to a suspension of protoplasts from normal organisms immediately after these had been lysed, and the preparation was fractionated in the usual way. In this preparation, the distribution of streptomycin was identical with that in lysates of organisms which had grown with streptomycin (Table 8).

DISCUSSION

All the streptomycin taken up by growing cells of *Bacillus megaterium* at bactericidal concentrations of the antibiotic is bound in such a way that it is not removed by washing the organisms in growth medium, water or sodium chloride, or displaced by unlabelled streptomycin. This situation contrasts with that in resting *Staphylococcus aureus* (Berkman, Henry, Housewright & Henry, 1948), where all streptomycin taken up could be recovered by washing in M-NaCl. With *Escherichia coli*, Szybalski & Mashima (1959) found indications that some streptomycin taken up could be removed by washing in saline; and Anand *et al.* (1960) found that almost all the streptomycin taken up could be removed by washing in buffer. Since streptomycin is strongly basic, and the cell surface of many organisms is negatively charged at the pH value of normal growth media (for example, Dubos, 1949), it would perhaps not be surprising if streptomycin were non-specifically adsorbed to some organisms in certain situations, and were displaced competitively by other cations. This would seem to be an important hazard in interpreting any observations about uptake of streptomycin. Breakage of the osmotic barrier is necessary but not sufficient to release bound streptomycin; with the exception of CTAB, only agents having low pH values are effective. This suggests that after breakage of the osmotic barrier, streptomycin must be displaced from its binding site by H^+ ions or by positively charged CTAB ions. Breakage of the osmotic barrier by butanol or toluene can apparently lead to greater accessibility of intracellular binding sites to streptomycin, with consequent increased binding.

The kinetics of uptake of streptomycin in growing and non-growing *Bacillus megaterium* (inhibited by CAP) appear superficially similar to those of the uptake of penicillin by *Staphylococcus aureus* (Rowley, Cooper, Roberts & Lester Smith, 1950), and suggest that a certain quantity of streptomycin-receptor may exist initially in the organisms, more being formed during further growth. Although we were unable to detect two separate phases in the uptake of streptomycin by *B. megaterium*, such

as occur in *Escherichia coli* W (Anand *et al.* 1960), the accuracy of estimation at low values of uptake makes it impossible to exclude such a process completely. The finding that addition of CAP during the later stages of uptake of streptomycin by *B. megaterium* prevents or slows further uptake, suggests that even at these later stages the uptake process is not due solely to diffusion of streptomycin into the organisms, but depends on continued synthesis of some cell constituents.

Death of *Bacillus megaterium* does not appear to follow inevitably the attainment of a certain intracellular concentration of streptomycin, since at lower streptomycin concentrations the organisms were killed after a smaller amount of streptomycin has been taken up, and *vice versa*. Low concentrations of streptomycin within the organism did not, however, necessarily lead to inhibition of growth; the antibiotic was still taken up at sub-inhibitory concentrations. In the presence of CAP and high concentrations of streptomycin, the organisms may take up streptomycin to values approaching those reached in growing organisms at lower concentrations of streptomycin, although in the former case they remain viable whereas in the latter they do not. It is clear therefore that the dependence of killing by streptomycin on the growth of *B. megaterium* cannot be due solely to the dependence of uptake of streptomycin on growth; other factors must also be involved. The maximum amount of streptomycin taken up by *B. megaterium* depends on the concentration of streptomycin in the growth medium, as it does for *Escherichia coli* (Szybalski & Mashima, 1959). Counts showed that 1 mg. dry wt. of *Bacillus megaterium* contained approximately 4×10^8 organisms; the presence of more than one organism per rod made counting difficult and this value is therefore a minimal one. The uptake of streptomycin at the lowest bactericidal concentrations used (0·25 μg./ml.) corresponds to not more than about 5×10^4 molecules/bacterium; this value is somewhat lower than the uptake associated with loss of viability in *Escherichia coli* K 12 (Szybalski & Mashima, 1959). It would seem important in considering possible mechanisms of action of streptomycin that at growth-inhibitory concentrations the quantities of streptomycin taken up, between 20 and 200 μg. streptomycin/g. dry wt. organisms, are much lower relative to the amount of cellular DNA than the concentrations at which streptomycin forms cross-linked complexes with DNA *in vitro*, for which approximately equal concentrations of streptomycin and DNA are necessary (Cohen, 1947).

The identity of the intracellular radioactive compounds has not been demonstrated in other work with ^{14}C-streptomycin; in the case of penicillin, this antibiotic can only be recovered from cells in an altered form (Schepartz & Johnson, 1956). There seems little doubt that the radioactivity within *Bacillus megaterium* represents unaltered streptomycin; its behaviour on an ion-exchange resin rules out the possibility of any major breakdown of the molecule or of alterations affecting the charge of the guanido or other groups, and it would seem unlikely that the products of any small modifications or substitutions of the molecule would form derivatives crystallizing with streptomycin derivatives in the manner observed.

The variation in the proportion of the total streptomycin found in the fraction containing the ribosomes may reflect the instability of these particles caused by activation of latent hydrolytic enzymes, such as ribonuclease, which they may contain (Elson, 1958). Attempts were made to investigate the distribution of streptomycin among the different sizes of ribosomes by centrifugation in a sucrose density

gradient, but it was not possible to obtain preparations of sufficiently high specific activity. Since the streptomycin in the cytoplasmic fraction was associated with the RNA-containing fraction, that in the 'ghost' fraction may also be associated with cytoplasmic RNA-containing material adhering strongly to the ghosts; its removal by ribonuclease would agree with such a process. Since ^{14}C-streptomycin added to lysed protoplasts was distributed among the cell fractions in the same way as that taken up during growth, at the moment it cannot be excluded that the observed distribution of streptomycin was to some extent an artifact following lysis of protoplasts, and does not represent the state of affairs before breakage. For example, streptomycin might be free in the cytoplasm of the intact organism but prevented from combining with the acidic groups of RNA because these are neutralized with other cations, which are free to diffuse away when the ionic enviroment is changed by lysis of the protoplast, leaving the RNA free to combine with streptomycin.

For help throughout the work in this and the previous paper, I am greatly indebted to Drs H. B. Woodruff and C. Rosenblum (Merck, Sharpe and Dohme Research Laboratories, Rahway, New Jersey, U.S.A.) for a supply of ^{14}C-streptomycin, and to Drs A. Gowenlock and A. Hartley (Department of Chemical Pathology, University of Manchester) for advice on, and facilities for, the extensive use of a windowless gas-flow counter. I am also grateful to Mr F. McManus for valuable technical assistance.

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The Morphology of *Bacillus cereus* Bacteriophages

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SUMMARY

The 'negative contrast method' was used to study by electron microscopy four previously described *Bacillus cereus* bacteriophages. The main features of the structure of these phages are described and the observations correlated with similar work on phages associated with other genera.

INTRODUCTION

The application of the 'negative contrast' technique to the electron microscopy of phage particles by Brenner *et al.* (1959) and its use in the examination of a variety of different phages by Bradley & Kay (1960) considerably extended our knowledge about the fine structure of phage particles. Observations made by using this technique have so far been concerned with phages of a small number of bacterial genera and there is a need for information relating to other types. Little is known about the morphology of the phages of aerobic spore-forming bacteria and only one electron micrograph showing a phage associated with protein crystal-forming *Bacillus cereus* strains appears to have been published, namely, a shadowed preparation showing the gross morphology (Afrikian, 1960). Roslycky & Hannay (1961) in a brief note referred to a phage derived from 'Fowler's bacillus', which closely resembles *B. cereus*, stating that it has 'heads approximately 60×60 mμ and tails 200 mμ in length'. The present communication records observations made when the phosphotungstate negative contrast technique was applied to the study of four phages isolated from *B. cereus* and from protein crystal-forming bacteria related to *B. cereus* (Norris, 1961).

METHODS

Bacteriophages. Phages A and B were isolated from *Bacillus entomocidus* var. *entomocidus*, phage C from *B. entomocidus* var. *subtoxicus* and phage D from *B. cereus* strain 826. The details of these bacterial strains and of the methods of isolation and propagation of the phages were described by Norris (1961).

Preparation of specimens for electron microscopy. Phages for electron microscopy were propagated on cultures of the appropriate bacteria grown on the surface of 1% (w/v) agar containing 1% (w/v) peptone in Petri dishes. Two drops of sterile filtered stock suspension of phage in nutrient broth containing about 10^8 infective units/ml. were spread over the dry surface of plates previously inoculated with the propagating bacterium by flooding with an overnight broth culture. After incuba-

tion at 30° overnight each plate was flooded with 2 ml. of a 2% (w/v) solution of ammonium acetate in distilled water and the areas which showed confluent phage lysis were gently rubbed with a smooth glass rod. The liquid was removed with a Pasteur pipette and the yield from similar plates pooled. The resulting suspension was centrifuged at 2000 rev./min. for 10 min. to deposit bacteria and the supernatant phage suspension was used for electron microscopy with as little delay as possible. Potassium phosphotungstate was prepared from a 2% (w/v) solution of phosphotungstic acid in distilled water. Undissolved material was spun down and the solution adjusted to pH 7·4 by dropwise addition of 40% (w/v) KOH solution. Phage suspension and potassium phosphotungstate solution were mixed in equal volumes and the mixture sprayed by an 'atomizer' spray on to specimen grids previously coated with carbon films and washed free from grease. The prepared specimens were studied immediately in the Siemens electron microscope 1 at a magnification of $\times 40{,}000$.

RESULTS

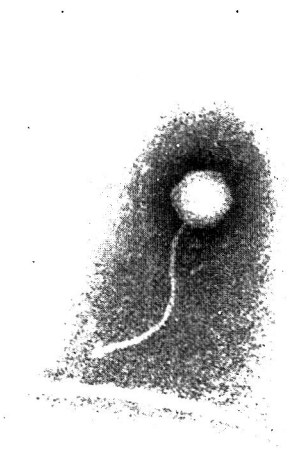
The general features of the preparations resembled those described by Bradley & Kay (1960). The phage particles appeared as electron-transparent objects surrounded by an electron-dense background of phosphotungstate and showed the usual differentiation into head and tail components. The preparations consisted of mixtures of active and discharged phage particles, the empty head membranes of the latter being easily distinguished from the electron-opaque deoxyribonucleic acid (DNA)-filled heads of the former (Pl. 1, figs. 1–4).

The four phages are remarkably similar in appearance. The head shows in each case a hexagonal outline, the diameter usually a little greater than the apex to tail dimension. It is not possible to determine the three-dimensional shape of the heads from pictures of this kind, the choice apparently lying between an octahedron and an icosahedron (see Bradley & Kay, 1960; Horne, Brenner, Waterson & Wildy, 1959). Occasional heads show signs of internal membranous structures which may possibly be associated with the mechanism for DNA ejection. The tails are relatively long and flexuous and sometimes appear hollow when attached to empty heads. Terminal knobs of indefinite shape are often seen with each of the phages. Contractile tail sheaths were not noted and there was no clear indication of fine structure in the tails or in the head membranes. These phages appear to fall into the morphological group 2 of Bradley & Kay (1960). Table 1 shows the dimensions of the four phages.

Table 1. *Dimensions of four* Bacillus cereus *phages. Phosphotungstate preparations*

Phage	Head (Å.)*	Tail (Å.)†	Illustration
A	$640 \pm 56 \times 640 \pm 40$	$2000 \pm 20 \times 95 \pm 10$	Pl. 1, fig. 1
B	$630 \pm 30 \times 680 \pm 30$	$2000 \pm 50 \times 80 \pm 10$	Pl. 1, fig. 2
C	$620 \pm 30 \times 590 \pm 40$	$2000 \pm 50 \times 75 \pm 5$	Pl. 1, fig. 3
D	$620 \pm 40 \times 580 \pm 30$	$2155 \pm 90 \times 75 \pm 5$	Pl. 1, fig. 4

* The apex–tail dimension is given first then the width.
† The length is given first then the width.



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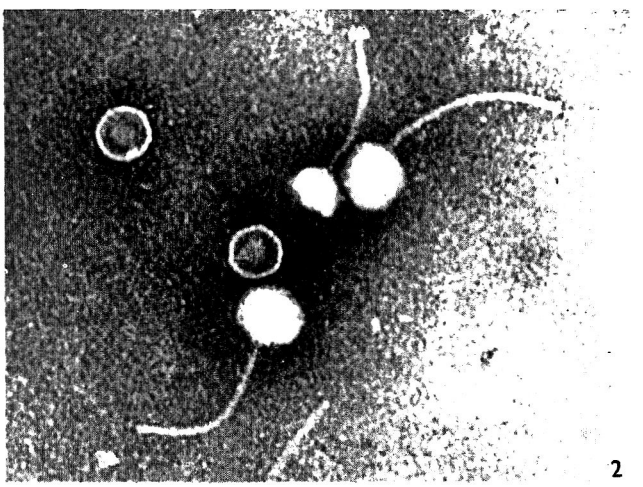

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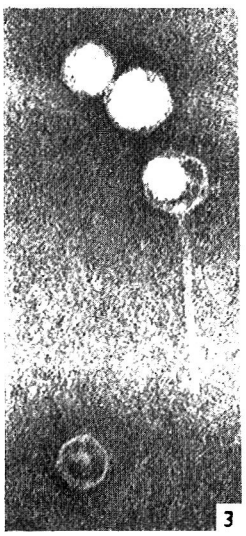

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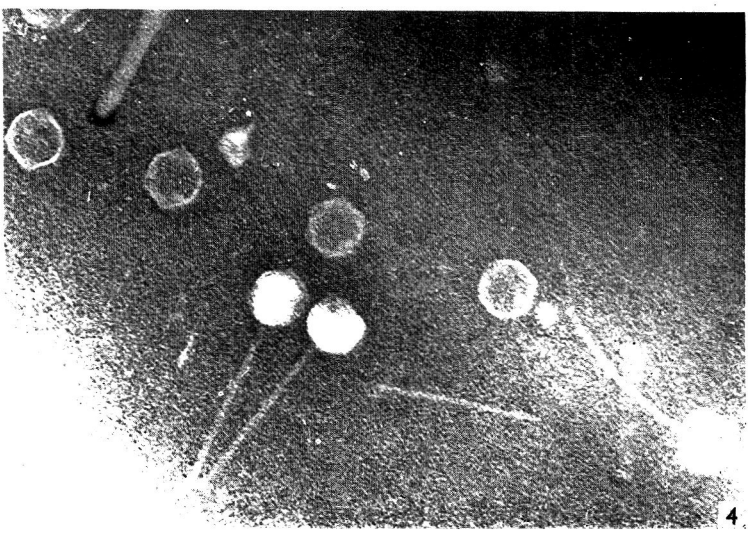

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DISCUSSION

Little is known about the general characteristics of the phages of the *Bacillus cereus* group and virtually nothing about their morphology. The results presented here are primarily of morphological interest and demonstrate that these phages have the 'conventional' structure associated with phages of several other genera. The similarity between these *Bacillus cereus* phages and some of the phages of *Pseudomonas*, *Staphylococcus*, *Escherichia coli* and *Salmonella typhi* described by Bradley & Kay (1960) is striking and emphasizes that a study of morphology is unlikely to be of great assistance in developing a system of classification of bacteriophages. The absence of contractile sheaths in these phages would appear to preclude the DNA ejection system which operates in some of the coli-typhoid phages. An alternative as yet unknown mechanism, possibly associated with the membrane structures sometimes seen inside the phage heads, must be assumed.

One of us (J.R.N.) is indebted to the Agricultural Research Council for a grant in aid of this work.

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EXPLANATION OF PLATE

Plate 1

Electron micrographs of *Bacillus cereus* bacteriophages. Phosphotungstate preparations.

Fig. 1. Phage A. ×122,000.

Fig. 2. Phage B. ×120,000.

Fig. 3. Phage C. ×123,000.

Fig. 4. Phage D. ×120,000.

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Studies on the Microflora of Soil under Chronic Irradiation

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SUMMARY

The number of micro-organisms present in soil located at a distance of 4 cm. from a ^{60}Co-source 1 m. above ground declined considerably after 24 hr. of exposure. Respirometric determination showed no decrease in oxygen uptake at this time. The distance at which the effect of irradiation could be demonstrated increased with increasing duration of exposure; after 396 days a reduction of the oxygen uptake as well as of the number of microbes could be detected at a distance of more than 3 m. and at a depth of 10 cm. as well as at the surface.

There appeared to be no significant difference between the effects of chronic irradiation on spore-forming and on non-spore-forming bacteria, respectively.

The plot of microbial count showed a better fit to a double-logarithmic than to a semi-logarithmic diagram according to Lea's formula. Possible explanations of this fact are discussed.

INTRODUCTION

Few investigations have been conducted on the effect of extended periods of irradiation on micro-organisms in soil, although some contributions deal with the possibility of soil sterilization by irradiation. Dunn, Campbell, Fram & Hutchins (1948) used X-rays and found that 100% inactivation of bacteria required between 5×10^5 and 10^6 r. McLaren, Reshetko & Huber (1957) studied enzymic activity in the soil under the influence of β-rays which, at the required levels of irradiation, seemed to cause less changes than the conventional methods of sterilization. Doses between 0·9 and $1{\cdot}1 \times 10^6$ r.e.p. inactivated all fungi and 1·1 to $2{\cdot}2 \times 10^6$ r.e.p. the bacteria of the soil as well. Stotzky & Mortensen (1959) found no definite effect of γ-rays from a ^{60}Co-source in doses of up to 250 krad. on the bacterial flora, while 8 krad. did reduce the number of fungi; this latter effect persisted after 54 days, probably due to competition from the rapidly multiplying bacteria rather than to the irradiation. Nitrification, ammonium formation and carbon dioxide production were not affected by irradiation. None of the authors make any statements concerning possible rules governing the inactivation of the populations studied, and it is doubtful whether sterility in the usual sense of the word was achieved. Platt & Mohrbacher (1959) have planned an extensive programme of research concerned with the effects of radiation from an unprotected reactor on the macro- and microflora and -fauna in an area of 10,000 acres.

* Present address.

The present study was designed to obtain a general picture of the effects of a ^{60}Co-source on the microflora in adjacent soils, as expressed by oxygen uptake of soil samples and by counts of bacteria and fungi.

METHODS

Irradiation took place in an open-air gamma-field at Risö (Brynjolfsson & Holm, 1960). The source consisted of ^{60}Co of a strength of 76 curies on 1 April 1959, producing 91 rad./hr. at a distance of 1 m. at the beginning of the experiments. The final intensity at the conclusion of the experiments on 1 September 1960, was 76 rad./hr. at a distance of 1 m. The calculated dose rate was checked by ferrous/ferric dosimetry at varying distances.

An ordinary clayey soil was used for the experiments, the pH being 7·4, the humus content 2 % and the water capacity 17 %. The soil was carefully mixed prior to laying out the experiments.

The present part of the study comprised the following two series.

Series 1. Aluminium cups were used; diameter 6 cm., height 7 cm., capacity 250 g. soil. The samples were placed on a table at the following distances from the source: 0·04, 0·125, 0·25, 0·50, 1·00 and 32·25 m. The distances were measured from the centre of the cups. The total amount of soil was analysed.

Series 2. Plastic tubes were used, diameter 35 cm., depth 50 cm., placed under the surface of the soil at the following distances from the source: 1·12, 1·28, 1·57, 2·24, 3·15 and 32·25 m. The distances were measured as in series 1, but the sampling was made over an area of approximately 200 cm.^{2} of the soil surface and at a depth of 10 cm. The 'surface samples' were collected after removal of the upper 1–2 cm. of soil which are continuously contaminated with micro-organisms from the surroundings.

Each dose measured refers to a point, and hence the unilateral radiation used for the present experiments makes it impossible to state the actual total dose. The relation between the calculated total doses, on the other hand, represents the true value. The results for the samples at a depth of 10 cm. were referred to the doses calculated for the surface samples, since it proved impossible to determine the total doses at this depth due to the variations in the water content of the soil over extended periods as used here. In actual fact the curves for a depth of 10 cm. should be transposed to a dose level which is lower by 25–40 %.

For series 1 samples were taken after 1, 2, 5 and 10 'days' and for series 2 after 71, 148, 266 and 396 'days'. A 'day' in this context is not synonymous with 24 hr. of radiation since for a few hours a day the gamma-field must be available for other studies. Thus the daily period of irradiation varied from 19·6 to 23·6 hr. in series 1 and from 19·5 to 19·9 hr. in series 2.

On the basis of the above the total doses for individual samplings at different distances were calculated as listed in Table 1.

The microbiological activity was measured quantitatively by means of the oxygen uptake of the soil samples in a Warburg apparatus (Dixon, 1951; Umbreit, Burris & Stauffer, 1957) and by counting of colonies of bacteria and fungi on agar plates, and qualitatively by examination of pure cultures at various distances from the source.

All respiration measurements were made in duplicate using accurately weighed

samples each of about 2 g. of soil. An addition of 1 ml. of 1% glucose was made at all measurements in series 1 and after 396 days of exposure in series 2, whereas the addition to the remaining samples was only 0·5 ml. of 1% glucose.

Table 1. *Total doses in krad. received by the soil samples at different distances over different periods*

Duration of exposure		Distance in m. from ^{60}Co-source					
'Days'	Hours	0·04	0·125	0·25	0·50	1·00	32·25
		Series 1					
1	19·6	931	95	24	6	1·5	0
2	47·3	2246	230	58	14	3·6	0
5	106·2	5044	517	129	32	8·1	0
10	213·3	10140	1038	259	65	16·2	0
		Distance in m. from ^{60}Co-source					
		1·12	1·28	1·57	2·24	3·15	32·25
		Series 2					
71	1412	101	77	51	25	12·7	0·13
148	2919	205	157	104	51	26·0	0·25
266	5220	359	275	183	90	45·6	0·43
396	7719	519	397	264	130	65·8	0·63

The bacteria were counted after plating on Difco nutrient agar + 0·5% glucose, and the fungi were counted after spreading on Czapek-Dox agar, pH 4·5–5·0, to which had been added 2 p.p.m. of aureomycin. The soil was used directly as sampled from the gamma-field without drying and remoistening for the respiration measurements as well as for the counts; but in the case of each individual sample a dry-matter analysis was made on which was based the calculation of the results.

The suspension for the counts were prepared in the following way. After thorough mixing 10 g. of soil was transferred to 0·5 l. Erlenmayer flasks containing 250 ml. of sterile salt solution according to Thornton (5·0 g. NaCl, 1·0 g. $MgSO_4.7H_2O$, 1000 ml. H_2O), and the mixture was shaken mechanically for a period of 30 min. Part of the resulting suspension was boiled for 5 min. in order to destroy vegetative cells. The various dilutions used for the experiments with boiled and non-boiled soils were prepared from the suspensions which had been thus treated. The samples were incubated at 25°. Twelve repeats were made of each count.

RESULTS

The chief results of the respiration experiments are found in Table 2, in which the samples below the dotted lines, i.e. the samples which are approximately 10% lower than the control, are considered to show the effect with certainty.

A 1-day exposure produced only a very faint effect on the oxygen uptake at a distance of 4 cm. After 10 days of exposure it was not yet possible to demonstrate any effect at a distance of 50 cm. The table shows the spreading effect for 396 days when it could be detected at a distance of a good 3 m. from the source at the surface as well as at a depth of 10 cm. In the case of the shortest period of exposure the respiration curves are close together, but with increasing duration of the

exposure the samples closest to the source branch off and spread downwards, fan-shaped, the control remaining virtually constant. The first hours of respiration after 10 days of exposure in the series 1 samples may be seen from Fig. 1.

The results of the counts of bacteria and fungi may be seen in Figs. 2 and 3. The number of micro-organisms was not particularly high compared to that often found in soil, and there was no particular difference between the number found at the surface of the soil and that at a depth of 10 cm. There was a strong seasonal variation in the number of bacteria. Hence in the case of series 2 only a comparison of the course of the curves is possible.

Table 2. *Total uptake of oxygen per g. dry soil after 10 hr. respiration*

The samples below the dotted lines drawn are considered to show the effect of irradiation with certainty.

Duration of irradiation 'days'	μl. O_2	Relative value	μl. O_2	Relative value	μl. O_2	Relative value	μl. O_2	Relative value	μl. O_2	Relative value	μl. O_2	Relative value
	Distance from ^{60}Co-source (m.)											
	0·04		0·125		0·25		0·50		1·00		32·25	
	Series 1											
1	67	92	78	107	81	111	71	98	79	109	72	100
2	66	76	73	84	76	87	80	92	82	94	87	100
5	54	77	59	85	66	94	72	103	73	104	70	100
10	39	52	49	65	51	68	72	95	71	94	75	100
	Distance from ^{60}Co-source (m.)											
	1·12		1·28		1·57		2·24		3·15		32·25	
	Series 2, soil 'surface'											
71	36	84	34	80	38	89	43	100	—	—	43	100
148	27	75	24	66	23	64	25	71	34	96	36	100
266	32	79	30	73	31	77	34	84	37	89	41	100
396	57	71	58	73	55	69	74	93	72	91	80	100
	Series 2, depth 10 cm.											
71	38	76	35	70	44	89	48	97	48	96	49	100
148	21	62	19	58	32	97	32	97	29	88	33	100
266	25	80	26	82	26	84	32	100	32	100	32	100
396	40	52	46	60	48	63	60	78	64	83	77	100

In Fig. 2 the results of series 1 are recorded on a semilogarithmic scale, the numbers of bacteria and fungi being related to the time of exposure which is used as an indication of the dose. After 10 days of exposure the bacteria were affected at a distance of up to 1·0 m., and the fungi up to 0·5 m. At a distance of 4 cm. the number of bacteria was so low and uncertain at exposures of 5 and 10 days that the data do not lend themselves to statistical treatment, whereas a certain amount of fungus growth remained at these durations.

Figure 3 shows the results from series 2. After a period of 71 days the effect could be detected at the surface closest to the source (1·12 m.) in boiled as well as in non-boiled soil samples. The effect could be traced at increasing distances with increasing duration of the exposure; after 396 days it could be detected at a distance of a good 3 m. in all cases, which is in good agreement with the respiration studies.

Although the 5 min. of boiling reduced the number of bacteria in the soil to approximately 1% of its initial value, the course of the curves remains the same. Hence surprisingly the spore-forming bacteria appear to be no more resistant to the chronic irradiation than are the non-spore-forming ones.

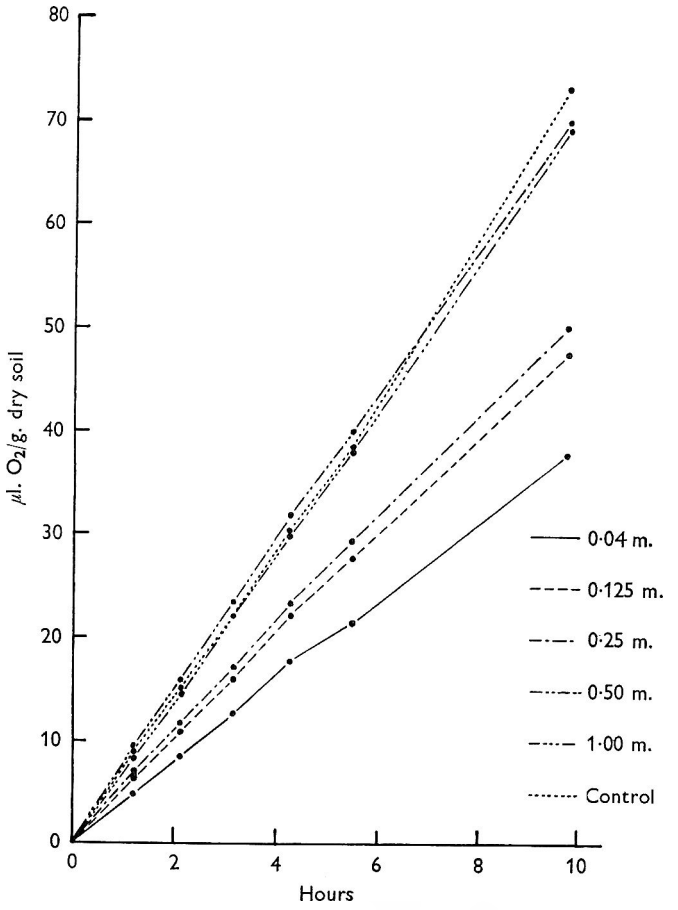

Fig. 1. The first hours of oxygen uptake at different distances from the source after 10 days of exposure in series 1.

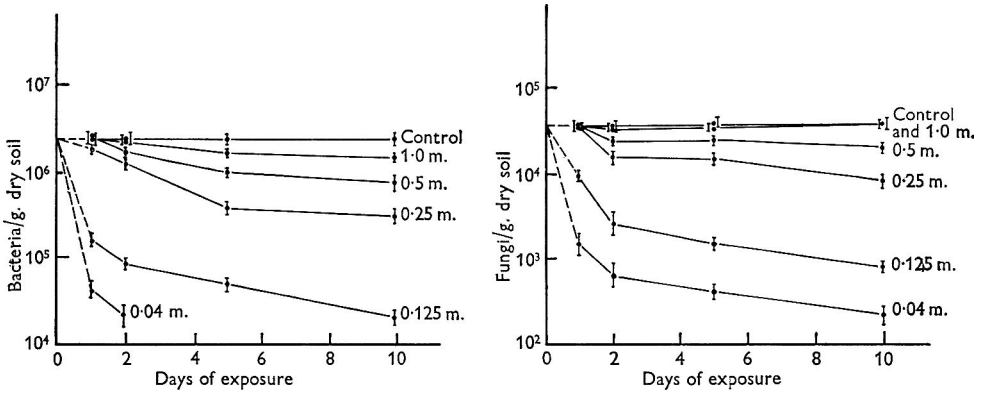

Fig. 2. Numbers of bacteria and fungi at different distances from the source in series 1.

The curves also show that the difference between the effect at the surface and at a depth of 10 cm. is but slight.

In connexion with series 2 a study was made of the following 15 pure cultures in

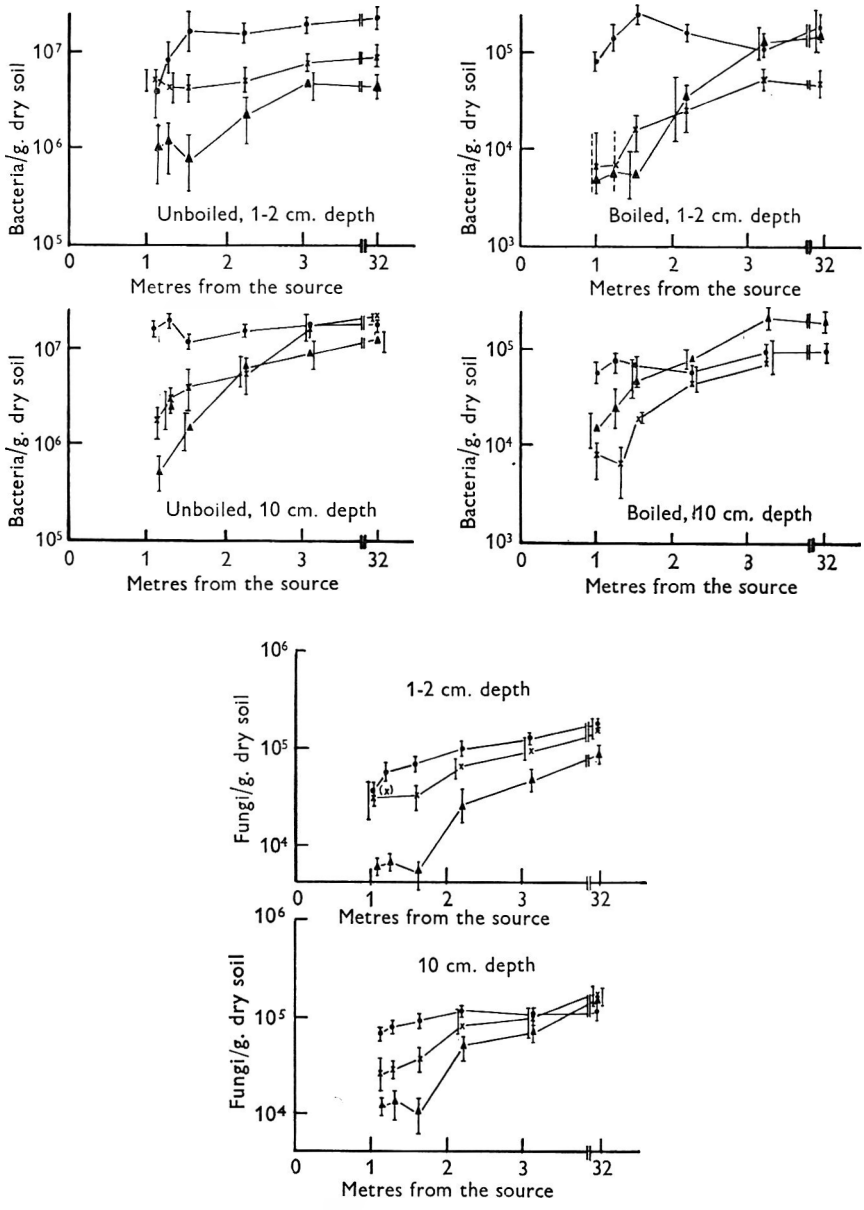

Fig. 3. Number of bacteria and fungi at different distances from the source in series 2. ●, 71 days of exposure; ×, 266 days of exposure, ▲, 396 days of exposure.

sterile garden soil: *Pseudomonas fluorescens* Migula, *Bacillus subtilis* Cohn, *B. megaterium* de Bary, *Clostridium bifermentans* (Weinberg & Seguin) Bergey, *Azotobacter chroococcum* Beijerinck (3 cultures), *Rhizobium meliloti* Dangeard (contaminated

after 266 days), *Corynebacterium* sp. (isolated from soil), *Mycobacterium* sp. (isolated from soil), *Nocardia corallina* (Bergey) Waksman & Henrici, *Streptomyces griseus* (Krainsky) Waksman & Henrici, *Rhizopus nigricans* Ehrenberg, *Penicillium notatum* Westling, *Cladosporium herbarum* (Pers.) Link. Twelve of these 15 cultures remained alive at all distances after 396 days of exposure; only the 3 cultures of *Azotobacter chroococcum* had died at distances from 1·28 to 1·57 m.

The spore-forming bacteria, the Phycomycetes and the yeasts proved to be the most resistant species at brief exposures closest to the source, whereas the non-spore-forming bacteria and the *Aspergillus* and *Penicillium* species were obviously more sensitive.

DISCUSSION

For both series, the reduction in uptake of oxygen is considerably lower than the reduction which might be expected to result from the reduction in the number of micro-organisms. The difference is most pronounced in the case of series 1. This is probably due to the enzymic activity in the soil, since the enzymes in general are not affected nearly as much as the micro-organisms (see, for example, Billen, Stapleton & Hollaender (1953), Siu (1957), and McLaren *et al.* (1957)).

A number of authors have studied the laws governing the inactivation of pure cultures by irradiation, but only few have related these problems to natural populations. Davis, Sheldon & Auerbach (1956) studied a natural population from a hollow in a beech tree and found it impossible to compute LD 50 values or regression lines for the curves because the population caused too great deviations from Lea's formula of $N = N_0 e^{-kD}$ (Lea, 1955) which is valid in a number of cases of irradiation of pure cultures. The authors consider this to be due to the fact that each segment of the population is inactivated at its own specific rate.

Halkier (1959, 1960), working with various pure cultures of bacteria isolated from fish, found his results to agree better with the following formula than with that of Lea's:

$$\frac{N}{N_0} = \left(\frac{D}{D_0}\right)^{-a},$$

which converted to the logarithmic form becomes $\log N = k - a \log D'$, N_0 being the number of organisms in untreated cultures, D_0 being the highest dose at which the number of organisms is still N_0, N being the number of organisms producing visible colonies at the dose D. a is a non-dimensional gradient. $D' = D/D_0$; defined for $D' \geqslant 1$. For $D' < 1$, $N/N_0 = 1$. From $D' = 1$ the formula gives a straight line on a double logarithmic plot.

This formula gave much better agreement than did Lea's when used on the present data. The results from series 1 are seen in Fig. 4 in which each dotted line refers to a specific distance and each plot represents a sampling.

For bacteria as well as for fungi there is a minimum effective dose corresponding to D_0, and for each distance the inactivation appears to follow a straight line. However, the author dare not postulate that the lines are straight as there is frequently significance at the 95% level. In spite of the variations the corresponding curves from series 2 give the same picture. Examples may be seen in Fig. 5.

When used for the same formula the data of McLaren *et al.* (1957), and of Stotzky & Mortensen (1959) prove to be excellently suited for double-logarithmic plotting,

whereas the data of Dunn *et al.* (1948) show a better fit to plotting according to Lea's formula.

Recently Etchells, Costilon, Bell & Rutherford (1961) have published a work on irradiation of microbes from cucumber fruits and blossoms. For all the seven groups

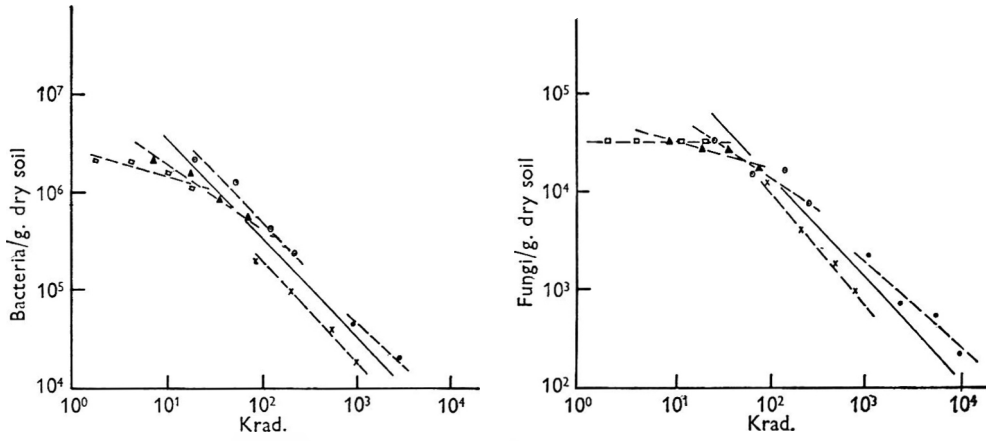

Fig. 4. The number of bacteria and fungi from series 1 in double-logarithmic diagram. ●, 0·04 m. (47·5 krad./hr.); ×, 0·125 m. (4·86 krad./hr.); ⊙, 0·25 m. (1·22 krad./hr.); ▲, 0·50 m. (0·30 krad./hr.); □, 1·00 m. (0·076 krad./hr.).

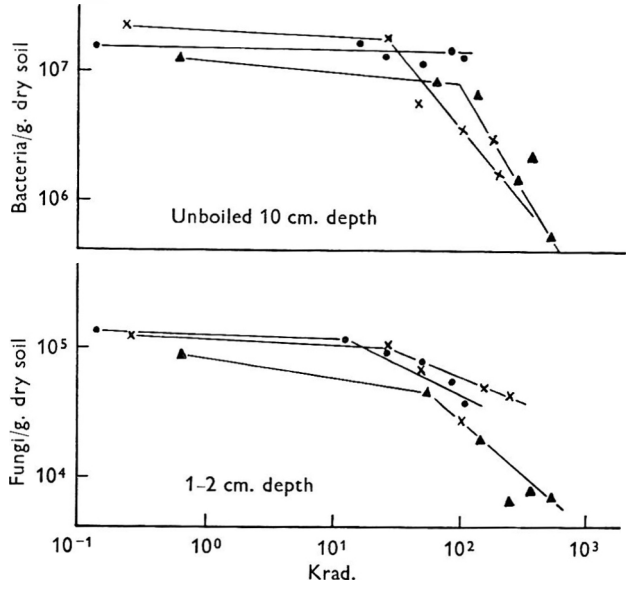

Fig. 5. Examples from series 2 of the number of bacteria and fungi in double-logarithmic diagram. ●, 71 days of exposure; ×, 266 days of exposure; ▲ 396 days of exposure.

of organisms tested the results will give a straight or nearly straight line in a double-logarithmic plot.

These results support Davis's theory of each organism (segment) of the population

producing a characteristic curve of its own. Highly sensitive organisms exercise their greatest influence on the sum-curve of the population during its initial course; subsequently the more resistant species grow in influence, and finally the most resistant species become those which chiefly determine the shape of the sum-curve.

If Lea's formula is suitable for a majority of the species in a natural population, then the shape of the sum-curve for a natural population of wide spectrum such as found in the soil approaches or gives a straight line on a double-logarithmic plot, but if the spectrum of the population is not sufficiently wide, curves with one or more bends may result, and if a few species of different sensitivities constitute the majority of the population, the curve will not fit either of the formulae.

Lea's formula produces no D_0-value, but in many cases sigmoid curves have resulted from the use of this formula (the multi target theory, Lea, 1955, and extrapolation number, Alper, Gilles & Elkind, 1960), thus increasing the probability of a direct connexion between the two formulae.

Hence the conclusion is drawn that the same laws apply to irradiation of natural populations and to irradiation of pure cultures, only the results are depicted differently, because in the case of the natural populations the curve is a sum-curve.

As the two series are in accordance on a double-logarithmic plot (i.e. identical at high and low dose rates), it appears to be probable that the effect of the microorganisms may be accumulated in such a way that the effect becomes apparent only after a few generations. An inhibition of the reproduction of 0·1–1·0 % of the value required to maintain the population may not be noticeable in one individual generation, but nevertheless it may cause a considerable reduction of the number in the course of a few generations.

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Kinetics of Diphtheria Toxin Formation

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SUMMARY

Studies on the kinetics of diphtheria toxin formation in iron-free culture media by variants of the PW no. 8 strain of *Corynebacterium diphtheriae* labelled with ^{14}C-phenylalanine or ^{35}S-methionine, showed that the toxin protein was synthesized *de novo* from amino acids by growing organisms. Release of toxin into the extracellular medium occurred without lysis of more than a minor proportion of the bacterial population.

INTRODUCTION

Diphtheria toxin is only synthesized by strains of *Corynebacterium diphtheriae* which are lysogenic for a particular bacteriophage or one of its mutants (Freeman, 1951; Groman, 1953; Barksdale, 1955) and which are growing under conditions of decreasing bacterial iron content (Pappenheimer, 1955). The toxin is released into the external culture medium as it is formed during the terminal stages of growth, and at any given time, only traces can be extracted from the bacteria themselves (Raynaud, Turpin, Mangalo & Bizzini, 1954). While there seems to be general agreement that the genetic information which controls toxin synthesis is carried by the prophage, the exact relationship between phage and toxin formation has not been established. Barksdale and his co-workers (Barksdale, 1959; Barksdale, Garmise & Horibata, 1960; Barksdale, Garmise & Rivera; 1961) presented evidence which suggests that under certain conditions at least, induction of prophage to the vegetative state by ultraviolet (u.v.) radiation may accelerate the release of toxin and enhance its yield several fold. These authors suggested that under the usual conditions for producing toxin, its formation may be a consequence of 'autoinduction' and phage multiplication resulting from a decreased bacterial iron content. Barksdale *et al.* (1961) observed a steady decline in viable count during toxin production in cultures of a variant of the PW no. 8 strain of *C. diphtheriae*. Unfortunately, this strain has been reported to carry a 'defective' prophage which does not give rise to plaque-forming particles following induction (Barksdale, 1959) so that it was not possible for them to establish a direct relationship between the decrease in viable count and phage multiplication.

From the earlier work by Barksdale & Pappenheimer (1954), Hatano (1956), Yoneda & Pappenheimer (1957), Yoneda (1957*a*), Edwards (1960) and others, it seems most unlikely that the liberation of toxin is associated with the lysis of any significant fraction of the bacterial population. Nevertheless, because of the recent suggestion that phage multiplication may be directly involved in toxin production,

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it seemed to us worthwhile to re-investigate the kinetics of toxin formation as a function of bacterial growth and lysis. In the present work we have followed toxin production by organisms of *C. diphtheriae* PW no. 8 strains labelled with ^{35}S-methionine and ^{14}C-phenylalanine growing in an iron-free unlabelled medium. The results have shown conclusively that toxin is synthesized *de novo* from amino acids and that its liberation into the extracellular medium is not associated to any significant degree with bacterial lysis.

METHODS

Organisms. Variants of the classic Park–Williams strain of *Corynebacterium diphtheriae* were used including the SM-1 strain of Yoneda (1957*b*) and the rough and smooth strains, PW no. 8_r (Pd) and PW no. 8_s (Pd), kindly sent us by Dr W. L. Barksdale.

Culture media and toxin production. The PW no. 8 (Pd) strains were grown on the casein hydrolysate medium of Mueller & Miller (1941). SM-1 was grown on the AMC medium of Yoneda (1957*b*). For toxin production the organisms were grown overnight on media supplemented with 0·1 μg./ml. $FeSO_4.7H_2O$ and 2% (w/v) maltose to an optical density (OD) at (590 mμ) of 2–2·5. The culture was centrifuged and the bacteria washed with iron-free medium and resuspended to OD = 5–6 in iron-free medium containing 4% (w/v) iron-free maltose as described by Yoneda (1957*b*). In all experiments the organisms were grown on a rotating shaking machine at 33–35°.

Growth was followed by measuring optical density at 590mμ, of samples diluted in distilled water, in a Bausch and Lomb Spectronic 20 and by dry-weight determinations. Dry weight was determined by filtration of suitable samples (5 or 10 ml.) on weighed Millipore filters. The bacteria were washed thoroughly on the filter with distilled water and then dried to constant weight. An OD of 1·0 was found to correspond to 0·47 $\pm$ 0·02 mg. dry weight bacteria/ml.

Amino acids. ^{35}S-L-methionine was obtained from Abbot Laboratories; ^{14}C-L-phenylalanine from Oak Ridge Laboratories. Radioactivity was measured in a low background (2·7 counts/min.) windowless gas counter (Nuclear Chicago).

Antitoxin. The rapidly flocculating pepsin-treated horse antitoxin no. 5353A was used. This antitoxin precipitates toxin completely over a broad zone (Pappenheimer & Yoneda, 1957) and has been shown to give only one band of precipitate on immuno-electrophoresis (Raynaud & Relyveld, 1959).

Fumarase was determined spectrophotometrically at 24° by following the rate of fumarate formation from malate at 240 mμ according to the method of Racker (1950).

Coproporphyrin III was determined by adsorption on alumina at pH 5 followed by elution with N-HCl as described by Yoneda & Pappenheimer (1957). Porphyrin concentration was calculated from the extinction at 403 mμ, assuming a molar extinction coefficient of $5{\cdot}3 \times 10^5$ (Jope & O'Brien, 1945).

RESULTS

Toxin production and growth

A 500 ml. culture of *Corynebacterium diphtheriae* PW no. 8_r (Pd) was grown overnight on casein hydrolysate medium to OD = 2·5. It was centrifuged, washed twice with iron-free medium and resuspended in 200 ml. iron-free medium containing 4% (w/v) maltose. After a homogeneous suspension had been obtained by shaking at 35° for 45 min., 100 ml. portions were placed in two 1 l. flasks. Samples were removed immediately from each flask and at intervals thereafter for determination of optical density, pH value and dry weight of organisms. Porphyrin and toxin were determined in the bacteria free filtrate. The final yields of toxin in the two flasks after 30 hr. shaking at 35° were 116 and 108 Lf/ml., respectively. Table 1 shows that optical density and bacterial mass, as measured by dry weight, were strictly proportional over the entire 30 hr. period. Coproporphyrin release at a linear rate began almost immediately after the bacteria were suspended in the iron-free medium. Toxin appeared in the filtrate soon afterward and increased at a maximum rate over a 15 hr. period during which the bacterial mass nearly doubled. A net increase in bacterial dry weight of 4·7 mg. was associated with the liberation of approximately 250 μg. toxin protein (about 5% by weight).

Table 1. *Relation of optical density to dry weight during toxin production by* Corynebacterium diphtheriae *strain PW no.* 8_r (Pd)

Time (hr.)	Optical* density at 540 mμ	Dry weight* (mg./ml.)	Porphyrin* (m-mole $\times 10^{-2}$/ml.)	Toxin* (Lf/ml.)
0·75	6·0	3·10	0·019	Not done
3·75	7·6	4·50	0·132	Not done
6·25	11·2	5·89	0·381	5–7·5
10·5	—	7·32	0·587	35
22·25	21·0	10·55	1·21	108
28·25	25·5	11·6	1·13	100
30·75	22·0	12·5	1·38	116

* Figures given average of determinations on duplicate flasks.

Fumarase synthesis during growth on iron-deficient medium

An overnight culture of *Corynebacterium diphtheriae* PW no. 8_s (Pd) was centrifuged, washed with iron-free medium and resuspended in the same medium containing 4% (w/v) maltose to OD = 5·0. The culture was incubated with shaking at 35°, samples were removed at intervals over a 22 hr. period and their OD determined. Samples (3 ml.) were diluted to exactly 15 ml. in 0·02 M-phosphate buffer (pH 7·4) and disrupted for 10 min. in the 10 kc Raytheon sonic oscillator. It was observed that as the bacterial iron content decreased in successive samples, the organisms became progressively more fragile and more easily broken by ultrasonic disintegration. After 22 hr. the culture filtrate contained 70 Lf toxin/ml. Figure 1 shows that fumarase, an enzyme which does not contain iron, increased about fivefold during the 22 hr. period in proportion to the increase in bacterial mass as measured by OD. Thus the bacteria were able to form new cellular protein at a

constant differential rate of synthesis during toxin production. This result would hardly be expected if toxin were released either by autolysis or by phage lysis of a major proportion of the bacterial population.

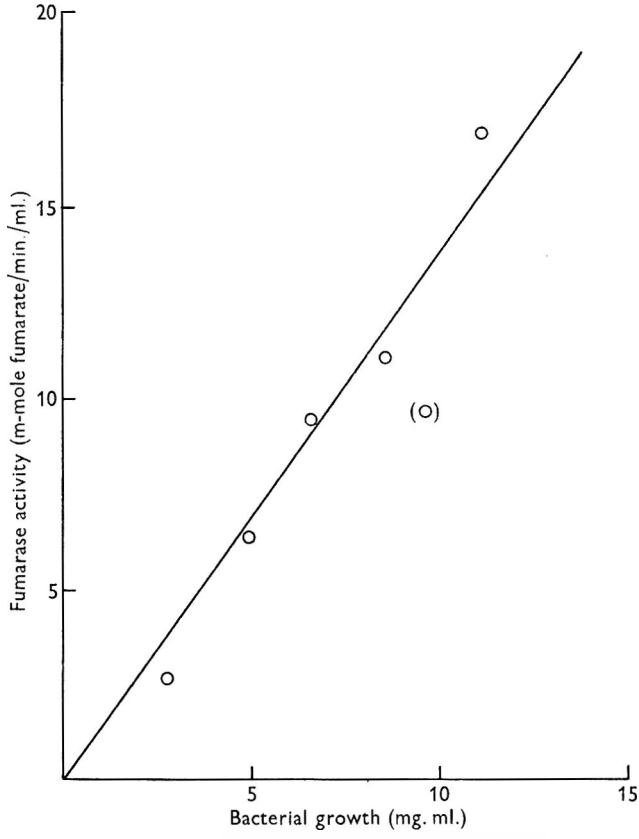

Fig. 1. Fumarase formation in Fe-free medium by *Corynebacterium diphtheriae* PW no. 8_s strain. The final yield of toxin in this experiment was 70 Lf/ml.

Toxin production from ^{35}S-methionine-labelled bacteria

The SM-1 variant of *Corynebacterium diphtheriae* PW no. 8 is exacting for L-methionine. Organisms washed with methionine-free AMC medium (Yoneda, 1957*b*) were inoculated to OD = 0·1 in 200 ml. AMC medium containing 2% (w/v) maltose, 0·1 μg. $FeSO_4.7H_2O$/ml. and 50 μg. ^{35}S-L-Methionine (specific activity = 2520 counts/min./μg.) per ml. After growth for 15 hr. at 35°, the culture was harvested, centrifuged in the cold and washed twice with chilled iron-free medium containing no methionine. The labelled bacteria were then thoroughly resuspended in 125 ml. iron-free AMC medium containing 4% (w/v) maltose, and 200 μg. unlabelled L-methionine/ml. The homogeneous bacterial suspension (OD = 4·3) giving 48,000 counts/min./ml. was distributed in 30 ml. amounts into four 300 ml. Erlenmeyer flasks and incubated at 33°. After 1, 2, 4 and 6 hr. of incubation a flask was removed from the shaking machine, the OD measured and the bacteria removed by centrifugation at 5000 rev./min. in the Servall SS-2 for 30 min. After determining

the flocculation titre of the supernatant fluid, the toxin was quantitatively precipitated by antitoxin, from each of two 10 ml. samples. To the first sample the calculated amount of antitoxin was added. To the second sample an amount of purified unlabelled toxin was added sufficient to bring the total to 400 Lf, followed by the addition of 1 ml. antitoxin 5353 AD (440 units/ml.). The flocculation mixtures were placed in the water bath at 40° for 1 hr. and then left overnight in the cold. The specific precipitates were collected by centrifugation and washed with chilled saline until no radioactivity could be detected in the supernatant fluids. The precipitates were then dissolved and made up to exactly 2 ml. in 0·25 N-acetic acid and 0·1 ml. samples were placed on planchets, dried and counted.

Table 2. *Toxin production by ^{35}S-methionine-labelled* Corynebacterium diphtheriae *strain PW no. 8 SM-1 variant*

Time (hr.)	Growth* (OD 590)	Total toxin in 10 ml. sample (Lf)	Unlabelled toxin added (Lf)	Specifically precipitable methionine (counts/min.)	^{35}S- (μg.)	Total methionine precipitated† (μg.)
1	5·9	50	350	412	0·16	1·77
		50	0	412	0·16	1·77
2	8·3	100	300	800	0·32	3·54
		100	0	368	0·15	3·54
4	10·0	200	200	595	0·24	7·08
		200	0	440	0·17	7·08
6	12·5	350	50	595	0·24	13·2
		350	0	453	0·18	13·2

* Washed bacteria inoculated at time zero to OD = 4·3 and 48,000 counts ^{35}S-methionine/min./ml. into Fe-free medium containing 200 μg. unlabelled L-methionine.

† Calculated, assuming 2·44 μg. toxin protein/Lf and a methionine content of 1·45 %.

The results are given in Table 2. It will be noted that from 10 ml. of culture of ^{35}S-labelled bacteria giving a total of 480,000 counts/min. (190 μg. methionine) only 400–600 counts/min., equivalent to 0·16–0·24 μg. methionine, were specifically precipitable from the supernatant by antitoxin. The methionine content of diphtheria toxin is approximately 1·45 % (Pappenheimer & Yoneda, 1957). Assuming 2·44 μg. protein /Lf, it can be calculated that of the 5 Lf toxin/ml. liberated during the first hour, only 0·45 Lf/ml. or 9 % was labelled. This amount of toxin might well have been preformed and carried over with the inoculum, or formed from an ^{35}S-methionine pool carried over with the inoculum. Over the next few hours, there was little or no increase in the specifically precipitable ^{35}S and of the 35 Lf toxin/ml. released during the entire 6 hr. period only 2 % or less contained the label.

Toxin production from ^{14}C-labelled bacteria

The SM-1 variant of *Corynebacterium diphtheriae* PW no. 8 strain does not require phenylalanine for its growth. However, in the presence of an excess of ^{14}C-phenylalanine, bacterial synthesis of this amino acid is repressed and the organisms take up, during growth, an amount of ^{14}C equivalent to a phenylalanine content of about 2·5 %.

A culture of the SM-1 strain was inoculated to OD = 0·1 in 400 ml. AMC medium containing 0·1 μg. $FeSO_4.7H_2O$/ml., 2 % (w/v) maltose and 24 μg. ^{14}C-L-phenylalanine

(specific activity = 350 counts/min./μg.)/ml. After shaking for 16 hr. at 34°, the culture had reached OD = 1·9. It was centrifuged and washed once with 100 ml. iron-free AMC medium containing no phenylalanine. The washed organisms were homogenized and resuspended in chilled iron-free medium containing 4% (w/v) maltose and divided into two equal portions, A and B, of 86 ml. each. To A was added 3 ml. of 0·8% (w/v) ^{14}C-L-phenylalanine (350 counts/min./μg.) and the culture distributed into four 125 ml. Erlenmeyer flasks: 25 ml. in A1 and A2, 20 ml. in A3 and A4. To B was added 3 ml. of 1% (w/v) unlabelled phenylalanine and the culture distributed as in series A. All eight flasks were placed on the shaking machine and incubated at 34°. At 1, 2, 4 and 7 hr., one flask from each series was removed. After determining the OD, duplicate 0·5 ml. samples were filtered through Millipore filters and the bacteria washed thoroughly on the filter with ice-cold 5% (w/v) trichloroacetic acid containing 150μg. unlabelled phenylalanine/ml. The filters were glued to planchets, dried and counted. The remainder of each culture was centrifuged at 5000 rev./min. for 20 min. in the cold and the supernatant fluid filtered through a Millipore filter. Duplicate 0·1 ml. samples of filtrates from series B were placed on planchets, dried and counted. Flocculation tests on the 4 hr. filtrates showed 10 Lf/ml. (A3 and B3) and at 7 hr., 22 Lf/ml. (A4 and B4).

Table 3. *Growth and lysis of bacteria labelled with ^{14}C-phenylalanine in Fe-free medium containing phenylalanine*

Organisms: *Corynebacterium diphtheriae*, PW no. 8 strain, SM-1 variant

Flask no.*	Time (hr.)	Bacterial growth (mg. dry wt./ml.)†	Bacterial ^{14}C (Counts/min./ml.)	Bacterial ^{14}C (Counts/min./mg. dry wt.)	Supernate‡ (Counts/min./ml.)
A_0	0	(2·0)§	(7400)	(3700)	94,000
B_0		(2·0)	(7400)	(3700)	(59)
A_1	1	2·40	9970	4170	—
B_1		2·40	7225	3000	107
A_2	2	2·86	11,000	3850	—
B_2		2·86	7030	2460	151
A_3	4	3·41	15,000	4400	—
B_3		3·41	6630	1940	261
A_4	7	4·14	19,300	4650	—
B_4		4·14	6630	1600	392

* A, 270μg. ^{14}C-L-phenylalanine (9·4 × 10^4 counts/min./ml.)/ml. B, 340μg. unlabelled L-phenylalanine/ml.

† Calculated from OD assuming 0·47 mg. dry wt./OD unit.

‡ Corrected for self absorption; actual count 32% lower.

§ Zero time values in parentheses by extrapolation.

Table 3 shows that from a bacterial inoculum containing 7400 counts /min./ml., only 285 counts/min./ml. (corrected for self absorption) were released into the supernatant fluid during the 6 hr. which elapsed between the first and last samples in series B, equivalent to the lysis of less than 4% of the initial bacterial suspension. In other words, if toxin were released by lysis of the initial bacterial suspension, the 55μg. unlabelled toxin released into the external medium during the course of the experiment (see Table 4) must have been derived from only 80μg. *labelled* organisms. Toxin, therefore, is not a product of cell lysis.

Toxin was precipitated quantitatively and specifically from duplicate 10 ml. samples of culture filtrates from flasks incubated for 1 and 2 hr. and from duplicate 5 ml. samples from the 4 and 7 hr. filtrates. An amount of purified unlabelled toxin (370 Lf/ml.; 2·5 μg. protein/ml.) was added to each sample so as to bring the total Lf content to approximately 200 Lf, and then 200 units of antitoxin added. In the case of the 7 hr. filtrates (A 4, B 4) unlabelled toxin was added only to one sample: the other 5 ml. sample was precipitated by 100 units of antitoxin without blending. The flocculation mixtures were placed in the water bath at 40° for 1 hr. and then left overnight in the cold. They were centrifuged and washed 3 times with chilled saline containing 0·1 % (w/v) unlabelled phenylalanine. The washed floccules were suspended in 1 ml. saline and the precipitate completely dissolved by addition of

Table 4. *Toxin production by bacteria labelled with ^{14}C-phenylalanine*

Organism: *Corynebacterium diphtheriae*, strain PW, no. 8, SM-1 variant. The inoculum was equiv. 2·1 mg. dry wt. ^{14}C-bacteria/ml. giving 7400 counts/min./ml. in Fe-free medium. A, 270 μg./ml. ^{14}C-phenylalanine ($9{\cdot}4 \times 10^4$ counts/min./ml.) B, 340 μg./ml. unlabelled phenylalanine.

Time (hr.)	Bacterial growth (mg. dry wt. organism/ml.)	Total toxin (Lf/ml.)	Specifically precipitable ^{14}C (counts/min./ml.) A	Specifically precipitable ^{14}C (counts/min./ml.) B	^{14}C-Toxin* (Lf/ml.) A	^{14}C-Toxin* (Lf/ml.) B
1	2·40	—	19	2·3	0·9	0·11
2	2·86	—	78	3·5	3·7	0·17
4	3·41	10	217	6·9	10·3	0·33
7	4·14	22	458	6·9	21·8	0·33

* Calculated assuming 21 counts/min./Lf.

2·5 ml. cold 5 % (w/v) trichloroacetic acid containing 150 μg. unlabelled phenylalanine/ml. After a few minutes at room temperature, the precipitate reformed and after a further 30–60 min. was collected on Millipore filters, washed with 5 % (w/v) trichloroacetic acid, dried and counted. The results given in Table 4 are averages of duplicate determinations agreeing within 5 % in every case. From the radioactivity specifically precipitable by antitoxin from the 4 and 7 hr. samples, it is easily calculated that the toxin formed in the A series contained 21 counts/min./Lf. Assuming that phenylalanine synthesis was completely repressed by the excess labelled phenylalanine, a specific activity of 350 counts/min./μg. phenylalanine and 2·44 μg. protein/Lf, the phenylalanine content of diphtheria toxin is calculated to be 2·5 %. (The concentration of ^{14}C-phenylalanine (24 μg./ml.) used to prepare the labelled inoculum was sufficient to repress bacterial synthesis of this amino acid by only 40–50 %. The presence of a large excess of phenylalanine in the iron-free medium, giving almost complete repression, accounts for the increasing ^{14}C content of the bacteria in series A during the course of toxin production; column 5, Table 3). From the counts specifically precipitated from A 1 and A 2 filtrates, the toxin synthesized after growth for 1 and 2 hr. is calculated to be 0·9 and 3·7 Lf/ml., respectively.

Turning to the B series, we find that from a bacterial inoculum containing 7400 counts phenylalanine/min./ml., only 2·3 counts specifically precipitable ^{14}C/min./ml. was released into the culture filtrate during the first hour of growth. This small

amount of labelled toxin (equivalent to only 0·1 Lf in series A) might well have been transferred with the inoculum. After 7 hr. the culture filtrate still contained only 6·9 counts specifically precipitable ^{14}C/min./ml. Thus, at most, 1·5–3 % of the toxin released during the experiment was labelled, and the remainder must have been synthesized from unlabelled phenylalanine by bacteria actively growing on the iron-free medium. This result agrees well with that obtained in the previous experiment with bacteria labelled with ^{35}S-methionine.

DISCUSSION

It is clear from the results presented that in an iron-free culture medium, diphtheria toxin is produced by growing bacteria. During the period when toxin is being released, the bacterial mass increases several fold, whether measured by optical density, dry weight or by the synthesis of a protein enzyme, fumarase. Whether or not the fourfold to fivefold increase in cell mass observed in these experiments or in those of others (Pope & Healy, 1933; Pappenheimer, 1947; Raynaud, Alouf & Mangalo, 1959; Edwards, 1960) is accompanied by actual multiplication of bacteria remains uncertain. Nishida (1954) and Barksdale *et al.* (1961) reported that the viable count decreased to 20 % or less while toxin was being formed. Edwards (1960), on the other hand, reported an experiment in which the number of viable bacteria increased fivefold during toxin formation, although in other experiments he was unable to obtain any consistent relationship between viable count and toxin yield. It should be stressed that the tendency of *Corynebacterium diphtheriae* strain PW no. 8_r (Pd) to form clumps at the high population densities required (about 10^{10} organisms/ml.) makes reproducible and reliable viable counting difficult.

Barksdale *et al.* (1961) showed that when u.v.-irradiated cultures of toxigenic strains were placed in media of relatively low iron content, toxin release began somewhat earlier than in unirradiated cultures. They suggested that under the usual conditions for toxin production, as the bacterial iron content is decreased, phage multiplication is induced (autoinduction) and lysis of a proportion of the bacteria takes place. Whether or not a low bacterial iron content induced the change from prophage to vegetative phage, the present studies show conclusively that lysis occurs in too small a fraction of the bacteria to account for the amount of toxin released. In an experiment with bacteria labelled with ^{14}C-phenylalanine, producing toxin in a medium containing unlabelled phenylalanine, less than 4 % of the label, equivalent to only 80 μg. dry wt. bacteria, was found in the supernatant fluid at a time when 55 μg. unlabelled toxin had been liberated. It should be recalled that Yoneda & Pappenheimer (1957) were unable to detect appreciable amounts of nucleic acid in culture filtrates of *Corynebacterium diphtheriae* strain C7 (β) until *after* toxin production was maximal and bacterial growth had ceased. When bacteria labelled with either ^{35}S-methionine or ^{14}C-phenylalanine are placed in iron-free media containing unlabelled amino acids, the toxin formed is unlabelled except for a small fraction (probably preformed) released during the first hour. Thus diphtheria toxin is synthesized *de novo* from amino acids as has been shown to be the case with the β-galactosidase of *Escherichia coli* following addition of inducer (Hogness, Cohn & Monod, 1955).

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Mitotic Cycle of the Kala-Azar Parasite, *Leishmania donovani*

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SUMMARY

The growth rate and the time schedule of the mitotic cycle of the leptomonads of *Leishmania donovani*, the Kala-azar parasite, were studied. The numbers of cells in the different division stages were estimated by photomicroscopy after Feulgen staining. The observed time intervals of resting, prophase, metaphase, anaphase, telophase and binucleate condition were 15·2, 1·1, 3·9, 1·1, 0·9 and 1·8 hr., respectively; the total generation time was 24 hr.

INTRODUCTION

The fine structure organization of the protozoon *Leishmania donovani*, the Kala-azar parasite, has been the subject of several recent investigations (Chang, 1956; Inoki, Nakanishi, Nakabayashi & Ohno, 1957; Inoki, Nakanishi & Nakabayashi, 1958; Pyne, 1958; Pyne & Chakraborty, 1958; Chakraborty & Das Gupta, 1960), but no study has so far been made on the time schedule of the mitotic cycle of this parasite. A precise measurement of the time schedule of the different stages of the mitotic cycle has now been made from photomicroscopic and electron microscopic investigations, combined with a measurement of the mean generation time.

METHODS

The leptomonad form of *Leishmania donovani* was cultured in NNN medium (Nicolle 1908) in this laboratory. NNN medium is a solid medium with a little water of condensation on the top of the slant. The organisms crowd together in this water of condensation.

Determination of the mean generation time. The water of condensation from a stock culture tube of NNN medium containing the organisms was mixed with about 2 ml. normal saline. This mixture was equally distributed into each of 30 culture tubes, which were then incubated at $22° \pm 1°$. At the end of 24 hr., the water of condensation containing the organism was carefully pipetted into a centrifuge tube. Some organisms remained attached to the agar surface which were washed with a little normal saline and collected in the centrifuge tube. The saline solution containing the organisms was centrifuged at about 3000 rev./min. The organisms settled down, and the supernatant fluid was discarded. The sediment containing only the organisms was diluted with a measured volume of normal saline, then a loop of organism was put on the coverslip on which a drop of procaine solution was previously dried for immobilization of the organisms. The coverslip was then inverted and placed over the haemocytometer slide and the total number determined by the usual procedure. Estimation of cell viability was made by direct observations with optical microscopy.

In exponentially growing cultures young organisms are more numerous than old ones owing to the division of one organism into two daughters. On account of this fact the fraction of the average life span of a cell, spent in any stage, is not simply equal to the proportion of cells observed in that phase; a correction has to be made. It has been shown by Powell (1956) and Edwards *et al.* (1960) that if C.P.I. is the cumulative phase index, i.e. fraction of all cells between division and a particular phase of growth, and X the cumulative fraction of time between division up to and including the particular phase in question then

$$\text{C.P.I.} = 2(1-2^{-x}),$$

or

$$x = 1 - \frac{\log(2-\text{C.P.I.})}{\log_2}.$$

With the help of this equation the durations of the different phases of the mitotic cycle were estimated. The variation in the number of counts with days was recorded. From a logarithmic plot of the number of the organisms against time after inoculation, the mean doubling time could be deduced in the usual manner as shown in Fig. 1.

Identification of the phases of the mitotic cycle. Specimens were examined by the optical microscope and by the electron microscope. For optical microscope studies leptomonads of *Leishmania donovani* from 4- to 7-day cultures were fixed for 2 hr. in the following fixative: chromic acid, 1 g.; $HgCl_2$, 2 g.; glacial acetic acid, 2 ml.; water to 100 ml. After fixation, the specimens were hydrolysed in N-HCl at 60° for 7 min. and stained with Feulgen solution for 45 min. Counterstaining was done in 0·2 % (w/v) Light Green (Gurr's; George T. Gurr Ltd., London, S.W. 6) dissolved in absolute ethanol for 15 min. The specimens were then photomicrographed at a magnification of about × 1000. For electron microscope study the organisms were first washed thoroughly in normal saline to remove impurities and then fixed in 1 % (w/v) osmium tetroxide, pH 7·4, for 30 min. (Palade, 1952; Sjostrand, 1956). After fixation the organisms were dehydrated through the 30, 50, 70, 90 and 100 % ethanol and then embedded in a mixture of 4 parts of butyl methacrylate monomer, 1 part of methyl methacrylate monomer (v/v) and 2 % (w/v) Luperco CDB. After polymerization (24 hr. at 48°), sections were cut with a Porter Blum microtome and the specimens were examined with a Siemens Elmiskop I at 60 kV.

RESULTS

Characteristics of the growth curve

Changes in the total number of the organisms at different times following inoculation are shown in Fig. 1. The organism multiplied exponentially from the second day onwards. The observed doubling time during the exponential growth phase, as shown in Fig. 1, was estimated to be 24 hr. This is equivalent to the average life span of the cells in the culture. The mean generation time found here agrees with that observed by Chang & Negherbon (1947) who used a different medium. All Leishmania were found to remain viable throughout the 10 days of the growth curve. The counting, however, could not be continued beyond the tenth day since most of the organisms then formed a rosette and it was very difficult to count them. Also it was observed that after this period some organisms were dead or dying.

Duration of the phases of the mitotic cycle

Observations with the optical microscope. About 400 specimens in which the nuclear division stage could be clearly established were observed with the optical microscope after Feulgen staining. Most of these specimens were taken from cultures from the middle of the log phase, i.e. 6 days after inoculation. A few typical photomicrographs obtained after the Feulgen staining are shown in Plates 1 and 2,

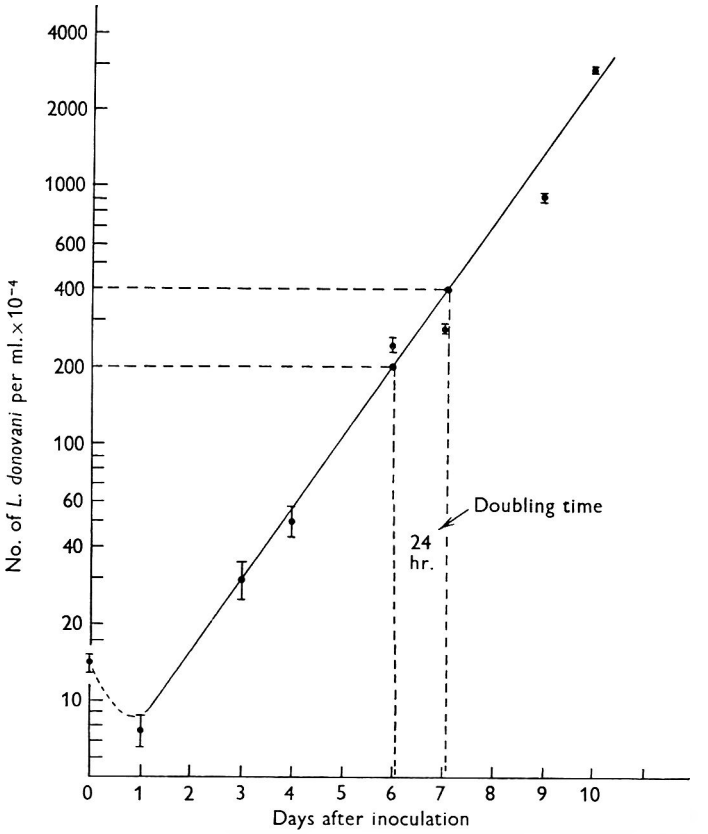

Fig. 1. Growth curve of *Leishmania donovani* in NNN medium at 22° ± 1°.

Table 1. *Duration of the phases in the mitotic cycle of the leptomonad form of* Leishmania donovani *during log phase of growth*

Nuclear phase	Phase incidence (%)	Cumulative phase index (C.P.I., %)	Duration of each phase (hr.)	Time from beginning of cycle (hr.)
Interphase	71	71	15·2	15·2
Prophase	4	75	1·1	16·3
Metaphase	13·5	88·5	3·9	20·2
Anaphase	3·5	92	1·1	21·3
Telophase	2·5	94·5	0·9	22·2
Binucleate	5·5	100	1·8	24·0

figs. 1A to 6A. Mean values for the numbers of organisms and the duration of the different phases of the division cycle are shown in Table 1.

Observation with the electron microscope. The nuclear division stages observed with the optical microscope after Feulgen staining were strikingly confirmed by the electron micrographs of the ultra-thin sections. The size of the nucleus of this parasite is small and the individual chromosomes at metaphase are almost at the limit of resolution of the optical microscope. The electron micrographs show the details of the division stages more clearly.

Plate 1, fig. 1, is the resting nucleus within which a single prominent nucleolus is visible. Plate 1, fig. 2, represents a prophase nucleus in which the nucleolus has disappeared and the chromosomes have just become visible. Plate 1, fig. 3, is the polar view of the metaphase stage, with the 8 chromosomes arranged in the equatorial plane. Plate 2, fig. 4, is the anaphase stage showing the separated chromosomes moving towards the opposite poles. Plate 2, fig. 5, shows the telophase nucleus with a slight constriction in the nuclear wall at the equator and Plate 2, fig. 6, represents the binucleate condition where the nuclear division is completed with the formation of two daughter nuclei, but the organism is not yet divided. Such electron micrographs confirm the division stages observed with the optical microscope and represented in Pls. 1 and 2, figs. 1A to 6A. About 70 good electron micrographs were obtained which show the organism in different stages of nuclear division. The number of organisms in the different stages were counted from these electron micrographs. Within the limitations of the sectioning technique, the frequency of occurrence of the different stages agreed with those derived from optical microscopy.

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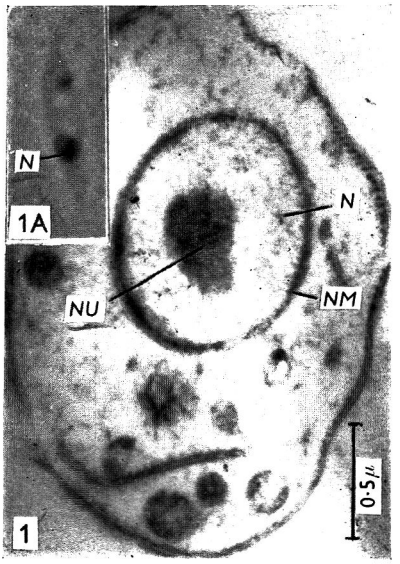
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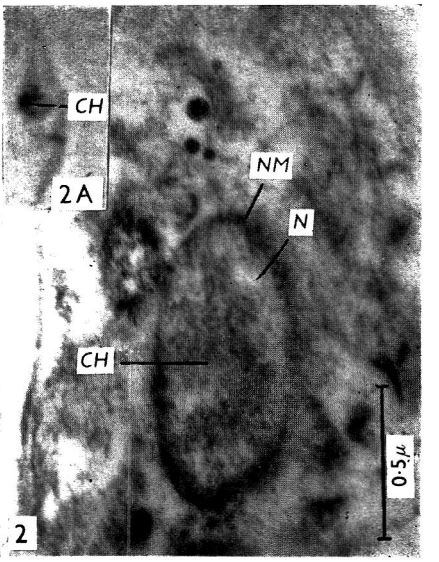
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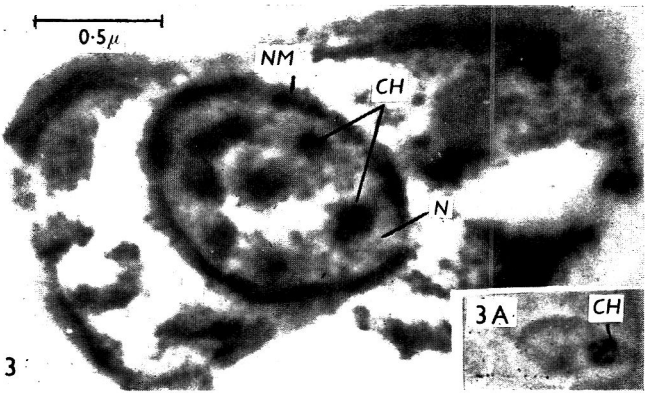
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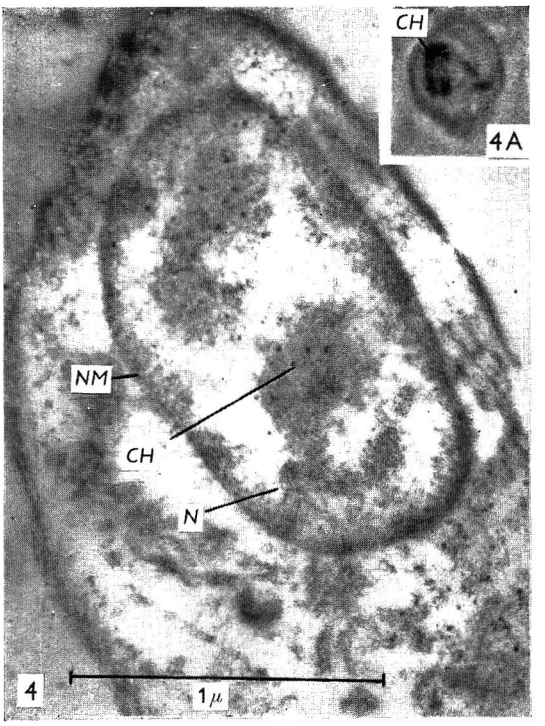
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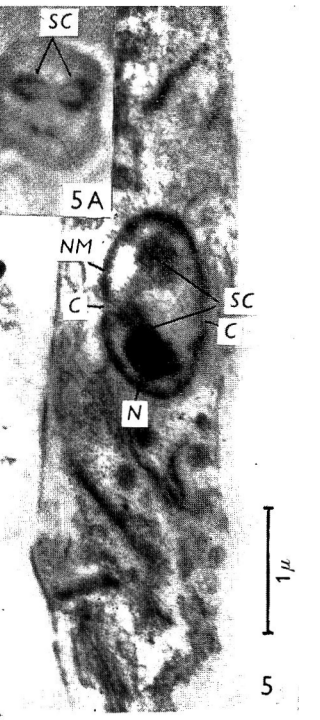
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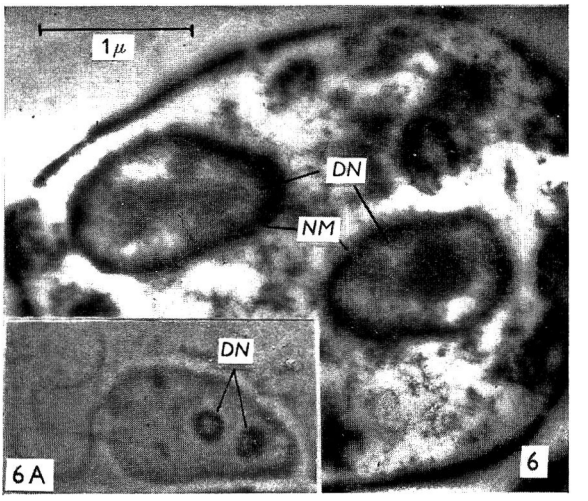
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EXPLANATION OF PLATES

Plates 1 and 2. Figs. 1–6, electron micrographs of the different division stages; magnifications indicated by a μ mark. Figs. 1A–6A, photomicrographs (light microscope) of the corresponding stages after Feulgen staining. × 3000.

PLATE 1

Figs. 1 and 1A. Resting stage of the nucleus (*N*) of *L. donovani*, showing the prominent nuclear membrane (*NM*) and a single irregularly shaped nucleolus (*NU*).

Figs. 2 and 2A. The nucleus in prophase stage. The chromosomes (*CH*) are just visible but the nucleolus is absent.

Figs. 3 and 3A. The nucleus in the metaphase stage. The chromosomes (*CH*) are arranged at the equatorial plate (polar view).

PLATE 2

Figs. 4 and 4A. Anaphase nucleus with separated daughter chromosomes (*CH*) moved away from the equator.

Figs. 5 and 5A. Telophase nucleus with separated chromosomes (*SC*) at the opposite poles. A slight constriction (*C*) at the equatorial region of the nuclear membrane (*NM*) is also visible.

Figs. 6 and 6A. Binucleate condition of the organisms, with two daughter nuclei (*DN*) in each body.

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An Appraisal of *Caryophanon latum*

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SUMMARY

Cultures of the giant bacterium *Caryophanon latum* Peshkoff, among them our own isolates from cow dung, were studied. A method for isolation is described. A fluid medium containing acid-hydrolysed casein, thiamine, biotin, sodium acetate, sodium butyrate and dipotassium phosphate, at pH 7·8, was devised which, without added colloids, allowed luxuriant growth of *C. latum* when sufficient aeration was provided. *C. latum* has been found to utilize acetic, butyric and β-hydroxybutyric acids. A variety of other carbon sources did not appear to be readily utilized by *C. latum*. The organism produced a carotenoid-like yellow pigment, the synthesis of which was inhibited by diphenylamine. A large amount of poly-β-hydroxybutyric acid (PHB) was synthesized by *C. latum* growing in the fluid medium described above. Much of the PHB was liberated as free granules. *C. latum* was found to be Gram-positive, in contrast to previously published reports.

INTRODUCTION

The giant bacterium *Caryophanon latum* is 3μ wide $\times$ 15μ long, as an average. Although a number of papers concerning its cytology have appeared (Peshkoff, 1940, 1946; Pringsheim & Robinow, 1947; DeLamater, 1952; Bisset, 1953; Sall & Mudd, 1955; Tomcsik & Grace, 1955; Murray, 1957), there is little information about the ecology, nutrition and basic physiology. *C. latum* was first described by Peshkoff (1940); it was isolated from cow dung and was cultivated on manure extract agar but not on 'ordinary' media. Pringsheim & Robinow (1947) re-isolated *C. latum* from cow dung, presented a definitive description of its structure, and contributed materially toward an understanding of its ecology and nutrition. The organism did not appear to be an inhabitant of the bovine intestine. It could be grown on an agar medium (PYA) containing 0·5% (w/v) Bacto peptone, 0·5% (w/v) Bacto-yeast extract, and 0·01% (w/v) sodium acetate, at pH 7·4 to 7·6, but grew poorly, if at all, in fluid media unless these were supplemented with small amounts of agar. The first report of an effort to grow *C. latum* with defined nutrients was published by May & Gershenfeld (1955). These workers used a medium containing soluble sodium silicate, hydrolysed cellulose, salts, asparagine and pantothenic acid. Weeks & Kelley (1958) were not able to grow *C. latum* well in fluid media (PYA less agar) even with the incorporation of various types of particulate matter.

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Somewhat better growth was obtained in cow-dung extract fluid medium. The present work reappraises some parts of previous studies and supplies new information about the nutrition and physiology of *Caryophanon latum*.

METHODS

Isolation of Caryophanon latum *cultures.* Intensive efforts to isolate *C. latum* from nature were made only with cow dung as source material. Relatively fresh (from a few hours to one day old) cow dung samples were collected from pastures, barns and pens. These samples upon return to the laboratory were incubated for one day at room temperature (about 20°) in glass bottles with loose screw tops. Wet mount preparations were then examined microscopically (×440) for typical *C. latum* trichomes. Most samples thus examined did not show *C. latum*. However, some positive samples were found; isolations of *C. latum* were made from dung samples found in close association with hay and straw, and from a pasture dung sample.

For isolation, a positive sample was divided into two parts; one part was autoclaved (121°, 15 min.) and inoculated with several loopsful of the remaining portion. After incubation for 24 hr. at 25°, the inoculated dung teemed with *Caryophanon latum* trichomes; a cultural enrichment was thus obtained. *C. latum* appeared to grow somewhat better in cow dung than most other bacteria present in the inoculum. The enriched sample was suspended in water (about 2 g. in 10 ml.), and the suspension forced through several layers of gauze to remove large particles. The filtrate was centrifuged at 50 *g* for 8 min., the sediment resuspended in water and recentrifuged as many as 11 times, with discard of the supernatant fluid each time. A mechanical enrichment of the relative numbers of *C. latum* was achieved because the large trichomes sedimented with this low-speed processing. The resulting sediment was streaked on plates of PYA medium (Pringsheim & Robinow, 1947). After 24 hr. at 25°, the plates were examined microscopically for minute colonies of the granular greenish appearance typical of *C. latum*; twelve cultures were isolated and purified by continued streaking.

Cultures were obtained from the American Type Culture Collection (Washington, D.C., U.S.A.; ATCC 12871 and ATCC 13872), Dr E. G. Pringsheim (Göttingen University, Germany), and Dr T. Gibson (University of Edinburgh); these cultures, with our own isolates, formed the collection on which the work reported here was based. The cultures were maintained on PYA slopes at 4° and transferred at monthly intervals.

Standard procedures. All fluid cultures were incubated at 25° on a rotary shaker (about 200 rev./min.) to provide vigorous aeration. For tube cultures, racks were constructed which held up to 50 tubes (16 × 150 mm.) at an angle of about 40° from horizontal and which could be clamped to the shaker platform.

Inocula were prepared by suspending organisms from PYA slopes grown for 24 hr. at 25°, in 5 ml. water or fluid medium. Four ml. were removed and the organisms washed (3 times in vitamin requirement studies, once or twice in most other experiments), with centrifugation periods of 5 min. at 500 *g*. The washed organisms were finally resuspended in a volume of 4 ml. (optical density at 500 mμ about 0·2). Inocula were added to fluid media at 1% (v/v) of the medium volume.

Turbidimetric measurements were made with a Bausch & Lomb ('Spectronic 20' colorimeter, at 625 mμ in coloured media and at 500 mμ in colourless media. Optically matched 16 × 150 mm. Pyrex culture tubes were used in growth experiments.

Dry-weight determinations were made with 3 ml. samples in tared glass cups held at 90–100° for 24 hr. and cooled over $CaCl_2$ before weighing.

Sterilization of fluid media was usually done with 0·45 μ pore diameter membrane filters (Millipore Filter Corp., Bedford, Mass., U.S.A.).

The following staining procedures were used: the Hucker modification of the Gram stain and the Burdon lipid stain (*Manual of Microbiological Methods*, 1957), and a negative stain with an aqueous 7% (w/v) filtered suspension of nigrosin.

Nutritional studies. A fluid medium was developed containing: 1% (w/v) 'salt-free' acid-hydrolysed casein (Hy-Case SF, Sheffield Chem. Co., Norwich, N.Y., U.S.A.); 0·5% (w/v) sodium acetate, anhydrous; 0·5% (w/v) sodium butyrate; 0·1% (w/v) K_2HPO_4; pH 7·8–7·9. When supplemented with a mixture of B-vitamins, this medium supported good growth of *Caryophanon latum*. Aqueous solutions of the B-vitamins were prepared at × 100 the final desired medium concentrations (see Table 1 for final concentrations) and added to or individually deleted from the above basal medium. Growth responses were measured turbidimetrically. PYA medium, without added agar, and the defined medium of May & Gershenfeld (1955) were also examined for ability to suport the growth of *C. latum*.

Utilization of carbon and energy sources. The ability of *Caryophanon latum* to utilize a variety of carbon compounds was tested by measuring growth responses when these compounds were added to a basal medium incapable of supporting growth unless supplemented with a utilizable carbon and energy source. The basal medium contained: 1% (w/v) Hy-Case SF; 0·1% (w/v) K_2HPO_4; thiamine HCl 0·2 mg./l.; biotin 0·04 mg./l.; pH 7·8–7·9. This basal medium is referred to as CHB.

Sodium acetate and sodium butyrate, sterilized in aqueous solution by membrane filtration, were tested at various concentrations. Carbohydrates, sterilized with ethylene oxide, were added to CHB medium at 0·5% (w/v). The C_5–C_9 saturated fatty acids (as sodium salts, sterilized by autoclaving) were tested at final concentrations of 0·2% (w/v). Tricarboxylic acid cycle and related compounds (formate, pyruvate, propionate, oxaloacetate, malate, α-ketoglutarate, fumarate, succinate; as sodium salts, sterilized both by membrane filtration or autoclaving) were added to CHB medium at 0·02 M to detect utilization. In later work, 'sparker' concentrations of acetate and butyrate (0·025% w/v) were incorporated into CHB medium for retesting the ability of *Caryophanon latum* strain 1.8 to utilize glucose, pyruvate, caproate and α-ketoglutarate.

To detect oxygen uptake on the sodium salts of the C_2–C_9 saturated fatty acids by resting suspensions of *Caryophanon latum*, conventional Warburg equipment was used. Each flask contained 3·1 ml. total fluid, consisting of: 2 μmole of fatty acid (Na salt) in 0·1 ml. water, 1 ml. 0·067 M-phosphate buffer (pH 7·5), 1 ml. of washed suspension of organism (equivalent to about 1·3 mg. dry wt.); 0·9 ml. water; 0·1 ml. of 20% (w/v) KOH in the centre well containing a piece of fluted filter paper. The temperature of the water bath was maintained at 24·9°. The resting organisms were used as soon as prepared, with no starvation period.

Pigment studies. Four-day cultures in CHM medium (100 ml.) were harvested

and washed twice in 0·85% (w/v) NaCl. A concentrated suspension (2 ml.) was spread on filter paper and dried over $CaCl_2$. (Extraction with acetone removed most of the pigment.) The dried organisms were extracted with 50 ml. boiling methanol for 5 min., the extracts filtered (Whatman no. 50 paper) and their optical density values from 230 to 700 mμ at 10 mμ intervals determined in a Beckman DU spectrophotometer (1 cm. silica cells, 3 ml. volume) with a methanol blank. Diphenylamine was incorporated into CHM medium at 0·02–0·10 mg./ml. medium to determine whether inhibition of pigment synthesis would occur. Methanol extracts were prepared and examined, as described above, from the amine-containing cultures.

Poly-β-hydroxybutyric acid (PHB) studies. Washed CHM culture harvests were dried either *in vacuo* over $CaCl_2$ or by lyophilization. The dried material was first extracted for several hours in a Soxhlet apparatus with anhydrous di-ethyl ether which removed yellow cell pigment and much lipid material, and then extracted to exhaustion with chloroform; the latter removed the PHB. Dry weights of chloroform extracts were determined by boiling away the chloroform from measured samples. Samples of crude extracts were tested for melting point and solubilities (see Williamson & Wilkinson, 1958) and for absorption spectrum when dissolved in hot (100°) concentrated H_2SO_4 (Slepecky & Law, 1960). An assay for the PHB in the extracts, similar to that of Law & Slepecky (1961), but with the use of a crotonic acid standard curve, was performed. Crotonic acid used as the standard was purified by recrystallization from water three times.

The utilization of sodium-β-hydroxybutyrate by *Caryophanon latum* was determined by incorporating this compound (0·02 M and 0·5%, w/v, membrane-filtered) into CHB medium and measuring growth responses turbidimetrically.

General studies. A solid medium containing acid-hydrolysed casein, yeast extract and sodium acetate (CYA, see Results) was used in determining the biochemical capacities of *Caryophanon latum* as concerns the production of catalase, reduction of nitrate (0·1%, w/v, KNO_3 added), production of indole (0·1%, w/v, DL-tryptophan added), and liquefaction of gelatin (agar replaced with 12% w/v, gelatin). These tests were done as outlined in the *Manual of Microbiological Methods* (1957). Studies of the relation to oxygen (in deep shake tubes of CYA medium) and to culture survival on PYA slopes were also done.

The effect of lysozyme was tested on organisms suspended in an aqueous solution containing 7·5% (w/v) polyethylene glycol, 0·01 M-NaCl, 0·005 M-$MgSO_4$, and lysozyme 375 μg./ml. at pH 7·6.

The effects of antibiotics and sulfa drugs ('Sensi-Discs', Baltimore Biological Laboratory, Inc., Baltimore, Md., U.S.A.) were tested on plates of PYA medium. The effects of crystal violet (1/100,000) and sodium azide (0·03%, w/v) were determined by incorporation of these compounds into PYA medium.

RESULTS

Growth of Caryophanon latum *in fluid media*

Table 1 shows typical results obtained in the vitamin-omission studies. Lessened growth of both *Caryophanon latum* cultures tested occurred only upon the omission of thiamine HCl and biotin. Thiamine HCl was required for growth. The requirement

for biotin was not so clear-cut; some growth occurred when it was omitted, but this was accompanied by heavy clumping. Further testing, with thiamine HCl as the only vitamin added to the basal medium, showed (lower part of Table 1) that it alone could not satisfy the vitamin requirements of *C. latum*. The addition of biotin allowed luxuriant growth of both cultures, thus indicating a requirement for both thiamine HCL and biotin.

Table 1. *Vitamin requirements of* Caryophanon latum

Growth responses are expressed as optical density values at 625 mμ. The basal medium contained 1 % (w/v) Hy-Case SF, 0·5 % (w/v) each of sodium acetate and sodium butyrate, 0·1 % (w/v) K_2HPO_4; pH 7·8. Vitamins and concentrations were (mg./l.): *p*-aminobenzoic acid, 0·2; biotin, 0·04; folic acid, 0·04; cyanocobalamine, 0·015; Ca-D-pantothenate, 0·04; nicotinic acid, 0·2; pyridoxal HCl, 1·0; riboflavin, 0·2; thiamine HCl, 0·2.

	Culture			
	Isolate 1.8		ATCC 12871	
Vitamins omitted	48 hr.	96 hr.	48 hr.	96 hr.
All	0·12	0·08	0·01	0·02
None	1·03	1·82	1·15	1·22
Thiamine HCl	0·14	0·12	0·01	0·02
Biotin	0·48	0·50	0·80	0·88

	Culture			
	Isolate 1.8		ATCC 12871	
Vitamins added	48 hr.	120 hr.	48 hr.	120 hr.
None	0·07	0·05	0·01	0·03
Thiamine HCl	0·06	0·08	0·02	0·06
Thiamine HCl + biotin	1·18	1·96	0·01	1·66

With the addition of these two growth factors, a fluid medium (CHM) for the growth of *Caryophanon latum* was developed. It consisted of (in g./l.) Hy-Case, 10·0; sodium acetate, anhydrous, 5·0; sodium butyrate, 5·0; K_2HPO_4, 1·0; and (in mg./l.) thiamine HCl, 0·20; and biotin 0·04; pH 7·8–7·9. This medium was usually sterilized by filtration through an 0·45μ pore diameter membrane filter, but it could be autoclaved (121°, 10 min.). Removal of acetate and butyrate from CHM medium resulted in a basal medium (CHB) which did not allow extensive growth of *C. latum*; CHB medium was used in the carbon and energy source utilization studies (see above).

Large crops of organism (2·0–2·5 g. dry wt./l. in 72 hr.) were produced in CHM medium with adequate aeration. Typical growth responses, of representative *Caryophanon latum* cultures in CHM medium, with and without aeration, are shown in Table 2. All cultures, except isolate 2.7, grew in the medium on continued transfer. Culture ATCC 12871 showed a rather long lag period which was characteristic of this culture when grown in CHM medium. The lag seemed to be overcome to some extent on continued transfer in CHM medium (Table 2). The addition of 0·1 % (w/v) Bacto yeast extract to CHM medium allowed the growth of culture isolate 2·7 and overcame the lag period of culture ATCC 12871. The addition of salts ($MgSO_4.7H_2O$, 0·25 mg./ml.; $MnSO_4.H_2O$, 0·01 mg./ml.; $FeSO_4.7H_2O$, 0·01 mg./ml.)

allowed the production of a more intense yellow pigmentation. The acid-hydrolysed casein in CHM medium could not be replaced with 0·5% (w/v) $(NH_4)_2SO_4$; no attempts were made to replace it with known amino acids and peptides.

Without vigorous aeration, none of the *Caryophanon latum* cultures grew well, even in CHM medium. Table 2 shows typical growth responses of still (non-shaken) cultures (5 ml. amounts in 16 × 150 mm. culture tubes).

Table 2. *Growth of representative* Caryophanon latum *cultures upon serial transfer with vigorous aeration in CHM medium and growth in CHM medium without aeration*

Figures in parentheses indicate day on which optical density shown was attained and also day when one loopful of culture was transferred to next set.

		Aerated			Non-aerated	
		Set no. 1	Set no. 2	Set no.3	72 hr.	144 hr.
		Optical density at 625 mμ				
Culture	Source					
P	E. G. Pringsheim	1·82 (2)	1·70 (2)	1·92 (2)	0·03	0·08
PG	T. Gibson	1·52 (3)	1·70 (3)	1·52 (2)	0·03	0·075
12871	ATCC	1·26 (4)	2·0 (2)	2·0 (3)	0·02	0·18
13872	ATCC	1·70 (2)	1·70 (2)	1·52 (2)	0·02	0·06
1.8	Present isolate	1·70 (1)	1·70 (2)	1·70 (1)	0·11	0·19
1.2	Present isolate	1·70 (1)	1·52 (1)	1·70 (1)	0·14	0·20
2.7	Present isolate	0·02	—	—	0·02	0·03
2.9	Present isolate	1·70 (3)	1·70 (2)	1·60 (2)	0·02	0·085
3.1	Present isolate	1·52 (2)	1·52 (3)	1·52 (3)	0·05	0·12

A solid medium (CYA) was developed for the routine culture of *Caryophanon latum*. This medium consisted of (in g./l.) Hy-Case SF, 10·0; Bacto yeast extract, 5·0; sodium acetate, anhydrous, 5·0; and agar, 15·0; pH 7·8–7·9.

Considerably greater crops of organism were produced on this medium than on the PYA solid medium of Pringsheim & Robinow (1947). The higher concentration of acetate (0·5%, w/v) in the CYA medium (PYA contains only 0·01%, w/v) was probably responsible for the enhanced growth.

PYA medium, in a fluid form without added agar, did not yield large crops even with vigorous aeration. Maximum optical density values at 625 mμ produced in this medium were in the range of 0·30–0·40 with isolate 1.8 and ATCC 12871. Omission of the acetate 0·01 %, (w/v) did not markedly affect the amount of growth, suggesting that there was too little acetate in PYA medium to have much effect as an energy source.

A study of the effects of the nutrients in the defined medium of May & Gershenfeld (1955) revealed the following. Asparagine (0·5%, w/v) could not replace the Hy-Case SF of CHM medium. Asparagine + pantothenate (0·04 mg./l.) + riboflavin (0·2 mg./l.) + salts (mg./ml.): $MgSO_4.7H_2O$, 0·25; KCl, 0·25; $NnSO_4.H_2O$, 0·01; $FeSO_4.7H_2O$, 0·01 could not replace the Hy-Case SF. Glucose (0·5%, w/v) could not substitute for the acetate and butyrate in CHM medium. It would appear that the defined medium of May & Gershenfeld was not able to yield large crops of *Caryophanon latum*. Sodium silicate was not tested since the present work showed that *C. latum* did not require colloidal material for growth in fluid media.

Carbon and energy sources for Caryophanon latum

Manometric studies (Table 3) showed that *Caryophanon latum* oxidized acetic and butyric acids, but not other saturated fatty acids. *C. latum* isolate 1.8 resting organisms, from growth in CHM medium supplemented with 0·3% (w/v) each of sodium caproate and heptanoate, showed no enhanced ability to oxidize the C_5–C_9 acids.

Table 3. *Oxidation of fatty acids by resting suspensions of* Caryophanon latum *isolate* 1.8

Flasks contained equiv. 1·3 mg. dry wt. organisms harvested at 24 hr. from CHM medium, washed three times in 0·85% (w/v) NaCl in water. The fatty acids were used as sodium salts.

Substrate (2 μmole)	μl. O_2 (5 hr.)	Q_{O_2} (average μl. O_2/mg. dry wt./hr.)
None	67·5	9·4
Acetate (C_2)	138·0	19·3
Propionate (C_3)	60·5	8·5
Butyrate (C_4)	136·0	19·1
Valerate (C_5)	74·0	10·3
Caproate (C_6)	81·5	11·5
Heptanoate (C_7)	81·0	11·3
Octanoate (C_8)	74·5	10·4
Pelargonate (C_9)	67·0	9·4

Table 4. *Stimulation of growth of* Caryophanon latum *by acetate and butyrate*

Figures in parentheses indicate day on which optical density shown was reached.

Addition to CHB medium*	Organism: Isolate 1.8	Organism: ATCC 12871
	Max. turbidity (optical density at 625 mμ)	
None	0·005	0·005
0·05 M-sodium acetate	0·62 (2)	1·21 (4)
0·05 M-sodium butyrate	1·70 (6)	2·0 (5)

* See Results for composition of CHB.

Of a variety of fatty acids, tricarboxylic acid cycle acids, carbohydrates and related compounds tested, only acetate and butyrate allowed substantial growth of *Caryophanon latum* when added to CHB medium. Table 4 shows typical responses of *C. latum* to acetate and butyrate. The concentrations (0·05 M) are far in excess of the minimum amounts of these acids necessary for growth stimulation; namely, about 0·01 M. A stimulatory effect on butyrate utilization in the presence of acetate (Table 5) in isolate 1.8 was observed.

No substantial growth stimulation of isolate 1·8 or ATCC 12871 was provided by any of the following, added singly to CHB medium: C_5–C_9 saturated fatty acids at 0·2% (w/v) (some growth on the C_5 and C_6 acids with both cultures after 8–11-day lag periods); glycerol, arabinose, glucose, galactose, maltose, cellobiose, soluble starch at 0·5% (w/v) each: formate, pyruvate, propionate, oxaloacetate, malate, α-ketoglutarate, fumarate, succinate, all as sodium salts at 0·02 M each (slight

turbidities, OD = 0·02–0·04 at 625 mμ, were produced on malate, α-ketoglutarate, fumarate). The addition of small amounts of acetate and butyrate (0·025%, w/v, each) to CHB medium and the subsequent testing of isolate 1.8 for growth on glucose, pyruvate, caproate, or α-ketoglutarate (concentrations as above) showed apparent utilization of caproate and α-ketoglutarate, but not glucose and pyruvate; this was deduced from the greater turbidities in the former than were produced in the low acetate + butyrate medium without supplementation.

Table 5. *Stimulation of butyrate utilization by acetate with* Caryophanon latum 1.8

Addition to CHB	Optical density at 625 mμ		
	24 hr.	48 hr.	144 hr.
None	0·01	0·01	0·01
0·5% (w/v) sodium acetate	0·37	0·80	0·42
0·5% (w/v) sodium butyrate	0·02	0·02	1·73
0·5% (w/v) of both acetate and butyrate	1·24	1·50	1·87

Caryophanon latum *pigment*

A yellow pigment was prominent with all organisms of our collection of *Caryophanon latum* when large crops were obtained. Diphenylamine inhibited the production of the pigment, but not growth of isolate 1.8, at 0·02, 0·04 and 0·06 mg./ml. in CHM medium. Growth of culture ATCC 12871 was inhibited by lower concentrations of diphenylamine (0·04 mg./ml.). Methanol extracts of 4-day CHM cultures of *C. latum* isolate 1.8, grown with and without 0·02 or 0·04 mg. diphenylamine/ml., showed an absorption peak at 430 mμ in the non-inhibited cultures but no strong absorption in this range in the inhibited cultures. From the absorption peak of the *C. latum* pigment and previous work (Ellinghausen & Pelczar, 1957) which indicated diphenylamine to be an inhibitor of carotenoid pigment synthesis, it seems likely that the *C. latum* pigment is carotenoid.

Poly-β-hydroxybutyric acid (PHB) in Caryophanon latum

Caryophanon latum cultures grown in CHM medium presented a cloudy opalescent supernatant portion on standing for several hours after removal from the shaker. Staining with basic dyes revealed no particulate matter in these supernatant fluids, but negative staining with nigrosin showed small spherical refractile granules (Pl. 1, fig. 1). These free granules did not stain well with the Burdon lipid material stain (Pl. 1, fig. 2). Intact *C. latum* trichomes settled to the bottom in still cultures, and thus were not responsible for the supernatant turbidity. The supernatant portion (mostly granules) of settled cultures could account for a large percentage (Table 6) of the particulate dry weight of CHM cultures. The free granule material (supernatant) increased rapidly about one day after the initial rise in culture turbidity. Analysis of chloroform extracts of CHM culture harvests revealed large amounts of a material with the properties of PHB as described by Williamson & Wilkinson (1958) and with a peak absorption at 235 mμ when dissolved in hot (100°) concentrated H_2SO_4 (Slepecky & Law, 1960). Thus, it appeared that the supernatant granules were mainly PHB granules, probably liberated on lysis of the organisms. Table 6 also shows an assay for the PHB content of a 6-day CHM culture.

The percentage PHB is higher than any previously reported; but it should be borne in mind that a considerable degree of lysis had occurred, thus decreasing the particulate dry weight due to organisms. Analysis of a 4-day CHM culture showed a PHB content of about 50%.

The PHB appeared to be synthesized mainly from butyrate; but organisms grown only with acetate in CHB medium also showed some, though lesser, accumulation of lipid granules. The large synthesis of PHB in CHM medium may have reflected a high C:N ratio in this medium. Increasing the Hy-Case SF content of CHM to 3% (w/v) did not diminish the production of PHB. When tested manometrically, no utilization of PHB itself was found, but isolate 1.8 and ATCC 13872, the only strains investigated in this regard, did grow in CHB medium supplemented with either 0·25% or 0·5% (w/v) sodium-β-hydroxybutyrate.

Table 6. *Data on free lipid granule and poly-β-hydroxybutyric acid (PHB) content of 6-day CHM culture of* Caryophanon latum *isolate* 1.8

Two 15 ml. portions were removed from the 6-day culture and allowed to settle for about 24 hr. The supernatant granule sample was taken from one; the other was resuspended to provide a sample representative of the whole culture. The clarified medium was prepared by centrifugation at 11,000 *g* for 15 min.

Culture fraction	g. dry wt./ 3 ml. sample	Percentage of particulate dry weight
Supernatant granules	0·0043	60·5
Suspended culture	0·0071	100·0
Clarified medium	0·0428	—
Total culture crop dry wt.	1·2371 g.	—
Chloroform extract dry wt.	0·94 g.	(76% of total crop dry wt.)

Poly-β-hydroxybutyrate: 80% of chloroform extract, 61% of total crop dry wt.

General properties of Caryophanon latum

In contrast to the reports of previous workers (with the possible exception of Tuffery, 1953), in the present work *Caryophanon latum* was found to be Gram-positive. PYA slope cultures, stained at 13, 15, 19, 24 and 48 hr., were uniformly Gram-positive when the Hucker modification of the Gram stain, or a further modification with the crystal violet concentration decreased from 2 to 0·5% (w/v) and an increase in decolorization time to 75 sec. were used.

Caryophanon latum produced catalase and did not reduce nitrate to nitrite, produce indole, or liquefy gelatin. CYA shake tubes showed growth of *C. latum* only at the surface, with the exception of culture isolates 1·2 and 1·8 which grew to a depth of about 1 mm. PYA slope cultures grew at 10°, 19°, 25°, and 37°, none grew at 45° and 25° seemed optimal. Cultures of *C.latum* ATCC 12871 survived for 9 weeks (not tested further) on PYA slopes at −20°, 4°, 25° and 37°. *C. latum* cultures survived for 9 months (not tested further) at room temperature (about 20°) on tightly enclosed PYA slopes. Cultures have also survived lyophilization for one year.

Spheroplasts (protoplasts?) were produced with lysozyme from culture ATCC 12871. Continuing work (F. Tyeryar, private communication) has indicated a general susceptibility of *Caryophanon latum* to the action of lysozyme. *C. latum*

cultures were resistant to a variety of sulfa drugs but not antibiotics such as penicillin, bacitracin, tetracycline and others. Crystal violet and sodium azide also were inhibitory.

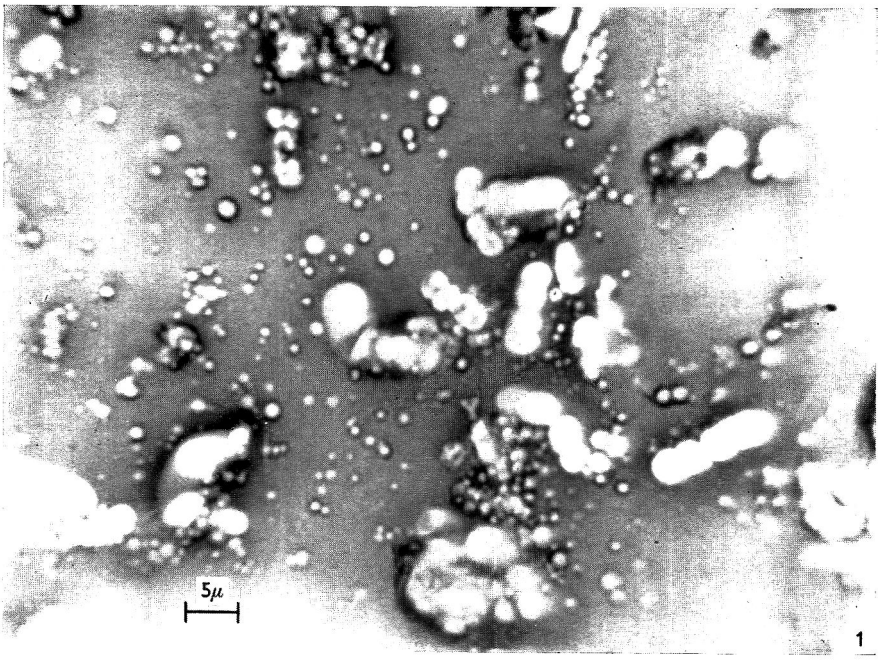
DISCUSSION

Although isolated to date only from cow dung, *Caryophanon latum* does not appear to be an intestinal inhabitant. The obligate aerobic nature of *C. latum* provides perhaps the most convincing reason why it could not be an inhabitant of the anaerobic intestinal tract. Like Pringsheim & Robinow (1947) and Weeks & Kelley (1958) we were not able to isolate *C. latum* from freshly voided dung, i.e. dung which had not come in contact with soil, hay, grass, dust or insects. It would seem likely that these materials are natural harbourers of *C. latum* and that only by chance does the organism reach the cow dung wherein it rapidly proliferates. The fatty acid content (e.g. acetic and butyric acids) and slightly alkaline pH value, about 7·5, of cow dung are probably major factors responsible for allowing the proliferation of *C. latum*; medium CHM is similar to cow dung in these characters.

A greater oxygen uptake (about 2·5 times) on butyric acid might be expected than on acetic acid. The equal oxygen uptakes obtained in the present work might be explained either of two ways; it might be argued that a larger percentage of butyrate than of acetate was assimilated into storage material. Measurements of O_2 uptake with butyrate (up to 24 hr.) did not exceed that observed with acetate at any time. In addition, O_2 uptake on fatty acids C2 through C4 was linear throughout the experimental period. A second and more interesting possibility is that butyrate might have protected the stored poly-β-hydroxybutyric acid in the cells from being metabolized (compare Doudoroff & Stanier, 1959), while acetate conferred no such protection; thus the endogenous metabolism on butyrate might have been far less than on acetate. The high endogenous activity obtained in the present experiments serves as the only indication that *C. latum* can utilize poly-β-hydroxybutyrate.

This compound has been demonstrated in a variety of bacteria (Lemoigne, 1927; Forsyth, Hayward & Roberts, 1958; Levine & Wolochow, 1959; Doudoroff & Stanier, 1959). The amount of poly-β-hydroxybutyrate shown in *Caryophanon latum* in the present work is somewhat greater than that reported for any other bacterium. However, it should be considered that the amount of accumulation of a storage product such as poly-β-hydroxybutyrate is a reflexion of the chemical environment of the organism, as discussed by Herbert (1961). Our fluid medium (CHM) has a high carbon:nitrogen ratio, providing conditions under which heavy accumulation of carbon storage products (such as poly-β-hydroxybutyrate) would be expected.

The nutritional and physiological characters of *Caryophanon latum* revealed thus far do not indicate that this giant bacterium differs in any basic way from other heterotrophic eubacteria. The present work has not been concerned with the cytology of *C. latum* but, considering the structure as elucidated by Pringsheim & Robinow (1947), it seems to us that this structure differs from that observed in the multicellular forms of bacilli only in regard to the frequency of septation, rather in the nature or order of formation. If this be so, it seems that a rather convincing argument could be presented for classifying *C. latum* among the eubacteria rather than in a separate order.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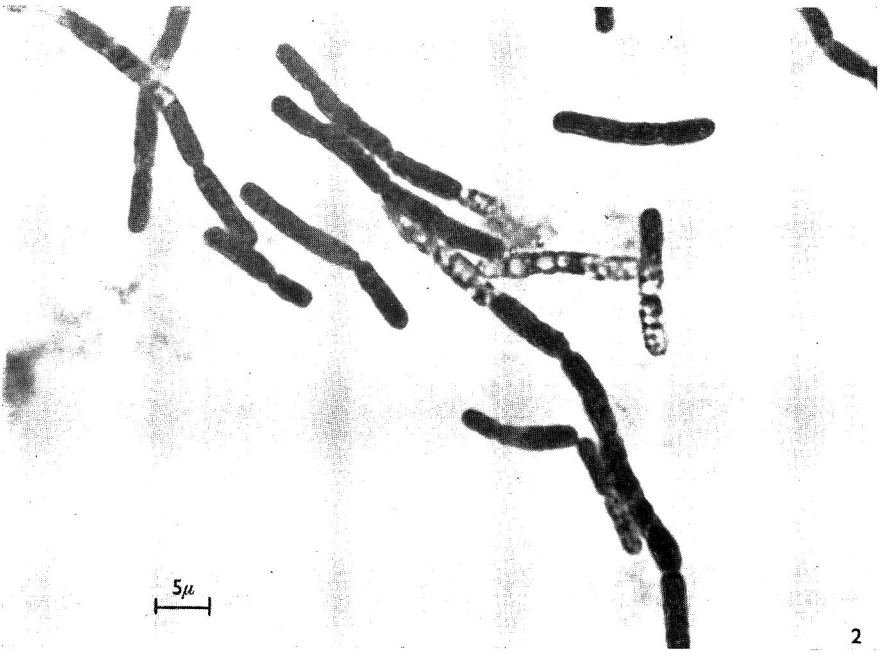
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EXPLANATION OF PLATE 1

Fig. 1. Nigrosin preparation of the sediment (24 hr. settling) in a 6-day CHM culture of *Caryophanon latum* isolate 1.8. Note the free poly-β-hydroxybutyrate granules and the disintegrating and misshapen trichomes. $\times$ 1330.

Fig. 2. Burdon lipid stain of a 24 hr. CHM culture of *Caryophanon latum* isolate 1.8. The dark areas represent lipid. $\times$ 1330.

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Mechanisms of Antibacterial Action of Bacitracin

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SUMMARY

At minimal bacteriostatic concentrations, bacitracin had four distinct actions on *Staphylococcus aureus*: (*a*) prevention of growth; (*b*) induction of lysis; (*c*) suppression of induced enzyme synthesis; (*d*) stimulation of the reduction of 2,3,5-triphenyltetrazolium chloride. In contrast, penicillin and cycloserine had activities (*a*) and (*b*) but neither (*c*) nor (*d*), and chloramphenicol had activities (*a*) and (*c*) but neither (*b*) nor (*d*). With bacitracin, properties (*a*) and (*b*) but neither (*c*) nor (*d*) were enhanced by Zn^{2+}; other metal ions were inactive. Sequential inactivation of bacitracin by autoclaving revealed that the bacteriostatic property was quite labile whereas the other three activities were more heat resistant. Thus, although several distinct mechanisms have been demonstrated whereby bacitracin can injure bacterial cells, the present data do not indicate which of these, if any, is associated with the primary biochemical lesion that results in suppression of growth.

INTRODUCTION

The literature on the biochemical mechanisms of drug action indicates that, of the antibiotics in clinical use, bacitracin is unique: at minimal bacteriostatic concentrations it apparently possesses a dual mode of action. On the one hand, in common with penicillin and cycloserine, bacitracin suppresses the synthesis of bacterial cell walls. For example, the drug causes *Staphylococcus aureus* to lyse (Crawford & Abraham, 1957), to form protoplasts (Abraham, 1957), to accumulate uridine nucleotides (Abraham & Newton, 1958; Park, 1958, 1960), and to be unable to incorporate radioactive amino acids into cell wall mucopeptides (Park, 1958; Mandelstam & Rogers, 1959). On the other hand, in common with chloramphenicol, bacitracin prevents *S. aureus* from synthesizing such proteins as β-galactosidase (Creaser, 1955; Gale & Folkes, 1955) and α-haemolysin Hinton & Orr, 1960). However, bacitracin does not prevent *S. aureus* from incorporating radioactive lysine into cell protein (Park, 1960) nor does the drug inhibit the biosynthesis of M protein by streptococci (Dr T. D. Brock, personal communication).

The thermal and storage stability of bacitracin is enhanced by the presence of an equimolar concentration of Zn^{2+} (Gross, 1954) and the antibacterial action of the drug is potentiated by an excess concentration of Zn^{2+} (Weinberg, 1959). Unfortunately, in published studies on the biochemical mechanisms of action of the drug, the role of Zn^{2+} has not been considered and the divergent results obtained in the studies on suppression of protein synthesis might have been caused in part by the lack of control of the metallic environment. The experiments reported in the present

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paper were designed to: (*a*) confirm the existence of the dual mechanism of action; (*b*) determine whether either of the two mechanisms is primarily responsible for the antibiotic action of the drug; (*c*) learn whether Zn^{2+} is required for either mechanism.

METHODS

Organism, culture medium, and antibiotics. A coagulase positive strain of *Staphylococcus aureus*, designated S-14 in our Departmental stock culture collection, was used. The components of the culture medium are listed in Table 1. The first nine ingredients were dissolved in de-ionized water, the mixture adjusted to pH 7·4 with NaOH, and the medium was sterilized by autoclaving at 121° for 15 min. Solutions of glucose and vitamin mixture were autoclaved separately and added aseptically to the medium before inoculation. Solidified medium, when desired, was obtained by the inclusion of 2 % (w/v) agar (Difco, Noble) in the liquid medium; the agar medium was autoclaved and then poured into Petri plates. The antibiotics used were bacitracin and potassium penicillin G (California Corporation for Biochemical Research), D-cycloserine (Commercial Solvents Corporation), and chloramphenicol (Parke, Davis and Company); these were dissolved in de-ionized water and sterilized by passage through ultra-fine sintered glass filters. Fresh solutions were prepared for each experiment.

Table 1. *Composition of the basal medium*

Compound	Concentration	
	μg./ml.	M × 10^{-6}
Casamino acids*	10,000	—
Uracil	1·12	10
K_2HPO_4	3,140	18,100
KH_2PO_4	950	6,900
$MgSO_4.7H_2O$	616	2,500
$CaCl_2$	278	2,500
$FeSO_4.7H_2O$	28	100
$MnSO_4.H_2O$	3·4	20
Citric acid.H_2O	2,100	10,000
D-Glucose†	1,800	10,000
Nicotinic acid	1·23	10
Thiamine.HCl	0·337	1·0
Biotin	0·001	0·004

* Bacto (Difco certified) Casamino acids.

† Lactose (3600 μg./ml.; 10,000 × 10^{-6} M) was substituted for glucose to obtain cocci for the mannitol induction tests because glucose-grown cocci contained mannitol dehydrogenase.

Determination of bacteriostatic and bacteriolytic activities of the antibiotics. The bacteriostatic activities of the antibiotics were determined by inoculating a series of liquid cultures containing different quantities of the respective antibiotics and incubated at 37° on a rotary shaker. Erlenmeyer flasks (125 ml.) containing 25 ml. of medium inoculated with about 5×10^5 viable cocci/ml. culture medium were used. At intervals of time, 1 ml. of culture fluid was removed from each flask and diluted to 5 ml. with de-ionized water for measurement of optical density (O.D.) in a Bausch & Lomb Model Spectronic 20 Spectrophotometer at a wavelength of 625 mμ.

The ability of the antibiotics to suppress cell wall synthesis was determined by exposing growing organisms to the drugs and measuring the extent of lysis. Cultures grown on solid medium and in liquid medium were used. The tests on solid medium were made on agar surfaces that had been inoculated by spreading about 5×10^6 viable cocci and incubated for 4 hr. at 37°. At that time, a thin film of visible growth had developed. Filter-paper disks containing 100 μg. antibiotic were placed on the agar surfaces and the plates incubated overnight at 37°. The diameters of the zones in which visible growth disappeared were measured and the areas of lysis calculated from these data.

The lytic tests on cocci grown in liquid medium were made by incubating shaken cultures for 7 hr.; at that time the cocci were harvested by centrifugation and suspended in 10 ml. (in 50 ml. Erlenmeyer flasks) of fresh medium lacking Casamino acids. The suspension of cocci was adjusted so that a 1/5 dilution gave an O.D. of 0·25 at wavelength 625 mμ. Different quantities of the respective antibiotics were added to the flasks which were then incubated at 37° on a rotary shaker for 16 hr. The extent of lysis at that time was determined by obtaining the O.D. of a 1/5 dilution of each culture.

Determination of the ability of the antibiotics to suppress induced enzyme synthesis. The ability of the antibiotics to suppress protein synthesis was determined by exposing the cocci to an inducing substrate in the presence of antibiotic and observing the extent of inhibition of induced enzyme formation. Portions (100 ml.) of liquid medium contained in Erlenmeyer flasks (500 ml.) were inoculated with the test organism (so that the culture medium contained about 5×10^7 viable cocci/ml.) and incubated at 37° on a rotary shaker for 16 hr. The cocci were harvested by centrifugation, washed once in 0·025 M-phosphate buffer (pH 7·5) and then inoculated into 25 ml. portions of the modified liquid medium contained in 125 ml. Erlenmeyer flasks. Sufficient cocci were used to obtain a concentration of 5 to 10×10^9 viable cocci/ml. culture medium. The modification of the medium consisted in the omission of glucose and the decrease of the Casamino acids concentration from 10,000 to 1000 μg./ml. In earlier tests, the latter quantity of Casamino acids had been shown to contain enough nitrogenous material for enzyme synthesis, and plate counts indicated that under these conditions the number of viable cocci was not altered during the 5 hr. period of induction. Different quantities of the respective antibiotics were included in the experimental flasks. In the tests for suppression of the synthesis of β-galactosidase, 10 mM (1800 μg./ml.) glucose was included in the control non-induced cultures and 10 mM (3600 μg./ml.) lactose was included in the flasks in which synthesis of induced enzyme was desired. In tests for the suppression of the synthesis of mannitol dehydrogenase, 10 mM (3600 μg./ml.) lactose was included in the control (non-induced) flasks and 10 mM (1820 μg./ml.) mannitol in flasks in which induction was desired. The flasks were incubated at 37° on a rotary shaker for 5 hr. to allow induction to occur. The cocci were then harvested by centrifugation and washed once in 0·025 M-phosphate buffer (pH 7·5) in the β-galactosidase tests and at pH 7·0 in the mannitol dehydrogenase tests.

The activity of β-galactosidase was determined spectrophotometrically at a wavelength of 425 mμ (Lederberg, 1950). The protein content of the cocci used in the assay was 10 mg./sample as determined by the method of Robinson & Hogden (1940). The assay of mannitol dehydrogenase was indirect inasmuch as the forma-

tion of organic acids rather than dehydrogenation was measured. Into each 125 ml. Erlenmeyer flask was placed 1·0 ml. coccal suspension (about 10 mg. protein) obtained from various experimental flasks and 10 mg. of mannitol. The total volume was adjusted to 10 ml. with de-ionized water and the flasks incubated at 37° on a rotary shaker for 1 hr., at which time the experiment was stopped by the addition of 2·5 ml. of 95 % (v/v) ethanol in water. The quantity of acids that formed was determined by titration with 0·005 N-NaOH.

Determination of the effects of antibiotics on the reduction of 2,3,5-*triphenyltetrazolium chloride* (*TTC*). Portions (100 ml.) of the liquid medium contained in Erlenmeyer flasks (500 ml.) were inoculated with the organisms and incubated at 37° on a rotary shaker for 16 hr. The cocci were harvested by centrifugation and washed once in 0·025 M-phosphate buffer (pH 7·0). The following ingredients were placed into 25 × 200 mm. test tubes contained in an ice bath: 1·0 ml. of 0·025 M-phosphate buffer (pH 7·0); 100 μg. TTC; 100 μg. of glucose, sodium formate or glycerol, or 10 mg. of 95 % (v/v) ethanol in water; different quantities of antibiotics; enough cocci to give 2 mg. protein. The concentrations of antibiotics tested were per ml. 0–50 μg. bacitracin or chloramphenicol, 0–10 μg. potassium penicillin G, 0–100 μg. of cycloserine. The total volume was adjusted to 5 ml. with de-ionized water. Control tubes that lacked substrate or antibiotic, or both, were included in each experiment. The tubes were incubated statically for 30 min. in a water bath at 37°; they were then returned to the ice bath and 5 ml. acetone added to dissolve the reduced TTC. The cocci were removed by centrifugation and the O.D. of the reduced TTC was determined at wavelength 525 mμ.

RESULTS

The ability of bacitracin, chloramphenicol, penicillin, and cycloserine to inhibit growth of *Staphylococcus aureus* is shown in Table 2. The presence of added Zn^{2+} at 1×10^{-5} M increased the bacteriostatic activity of bacitracin twofold and at 1×10^{-4} M 10-fold. The presence of added 5×10^{-4} M-disodium ethylenediaminetetra-acetic acid (EDTA) decreased the growth inhibitory action of bacitracin by a factor of ten. Neither the bacteriostatic activity of the three other antibiotics nor the rate of growth of cocci cultured in the absence of the antibiotics was affected by Zn^{2+} or by EDTA.

The ability of bacitracin, penicillin and cycloserine to induce lysis of *Staphylococcus aureus* grown on solid medium is indicated in Table 3. Lysis was obtained consistently when the antibiotics were added to 4 hr. cultures; but when the addition was delayed beyond 6 hr., no lytic activity was detected. Chloramphenicol was unable to induce lysis of cultures of any age. In Table 3, it may be noted that EDTA suppressed the lytic activity of bacitracin and penicillin and had a lesser depressing effect on cycloserine. The presence of added Zn^{2+} enhanced considerably the lytic action of bacitracin but did not influence penicillin or cycloserine. The concentration used of EDTA and Zn^{2+} had no effect on the growth of the test organisms. Despite previous reports that in some experimental systems the antibacterial action of penicillin and cycloserine is affected moderately by Co^{2+}, and Cu^{2+} and Fe^{2+}, respectively (Weinberg, 1957), in the present study the lytic action of penicillin, cycloserine and bacitracin was not altered by added Co^{2+}, Cu^{2+}, Fe^{2+}, or Mn^{2+}.

Bacitracin, penicillin and cycloserine were each able to induce lysis of *Staphylococcus aureus* grown in liquid medium (Fig.1) provided that the procedure described in Methods was used; i.e. resuspension in fresh medium lacking Casamino acids. When the cocci were allowed either to remain in the staled culture medium or when they were transferred to fresh medium containing per ml. more than 100 μg. Casamino acids, lysis by the three antibiotics was suppressed. For example, the

Table 2. *Comparison of the growth-inhibitory concentrations of certain antibiotics with the concentrations necessary to inhibit enzyme synthesis in* Staphylococcus aureus

Compound	Concentration of antibiotic that prevented half-maximum growth at			Concentration of antibiotic that inhibited 50 % of the biosynthesis of inducible enzymes	
	20 hr.	40 hr.	60 hr.	β-galactosidase	Mannitol dehydrogenase
	Antibiotic concentration (μg./ml.)				
Bacitracin	6·0	36·5	71·0	5·2	10·8
Bacitracin + 10^{-4} M-Zn^{2+}	0·5	3·5	7·5	2·2	10·0
Chloramphenicol	2·2	4·8	7·7	8·4	12·0
Penicillin G; K salt	0·005	0·015	0·043	> 2·0	> 2·0
Cycloserine	4·5	16·5	18·2	> 90	> 90

Table 3. *Effect of EDTA and Zn^{2+} on the ability of certain antibiotics to lyse* Staphylococcus aureus *grown on solid medium*

Antibiotic	Addition to basal medium									
	EDTA (M)				Nothing added	Zn^{2+} (M)				
	1×10^{-3}	1×10^{-4}	1×10^{-5}	1×10^{-6}		1×10^{-6}	1×10^{-5}	1×10^{-4}	1×10^{-3}	1×10^{-2}
Bacitracin	2·6*	2·6	3·3	3·0	4·3	3·8	4·1	4·9	6·6	8·7
Penicillin G; K salt	13·8	17·3	16·8	17·3	20·8	20·8	20·8	20·8	20·8	20·8
Cycloserine	6·6	7·1	7·6	7·1	7·6	7·6	7·6	7·6	7·6	7·6

* Area of the zone of lysis in cm.^{2} including the area of the disk which is 1·3 cm.^{2}. The antibiotic impregnated disks were placed on the agar surfaces after visible growth appeared; the extent of lysis was observed 16 hr. later.

percentage decreases in turbidity caused by 100 μg. bacitracin per ml. in the presence of 0, 100, 250, or 500 μg. Casamino acids were 43·5, 40·0, 30·5 and 26·0, respectively. That this concentration of bacitracin possessed antibacterial action in cultures in which lysis was suppressed was shown by the fact that a 95 % decrease in the viable count occurred in such cultures during the 16 hr. of incubation. Later experiments showed that the initial period of growth could be extended to at least 16 hr. without interfering with the subsequent lytic process; however, the time of contact between the antibiotics and cocci could not be reduced if maximum lysis was to be obtained. It may be observed in Fig. 1 that 5×10^{-4} M added Zn^{2+} enhanced strongly the lytic action of bacitracin. The addition of 1×10^{-4} M-EDTA in the place of Zn^{2+} markedly depressed the activity of the drug.

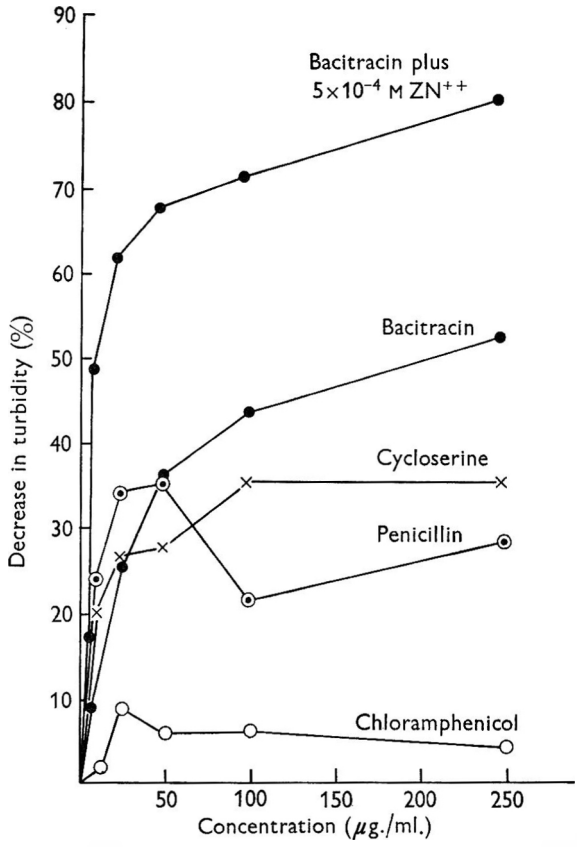

Fig. 1. Ability of antibiotics to lyse *Staphylococcus aureus* grown in liquid medium. The compounds were added to 7 hr. cocci and the extent of lysis observed after a 16 hr. period of incubation.

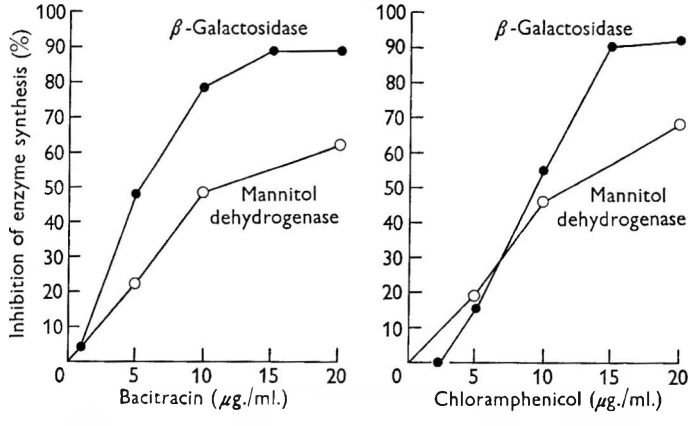

Fig. 2. Effect of bacitracin and chloramphenicol on the ability of *Staphylococcus aureus* to synthesize inducible enzymes. The antibiotics were added to 16 hr. cocci and the activity of the enzymes measured after a 5 hr. period of induction.

For example, the percentage decrease in turbidity caused by 25 μg. bacitracin/ml. in the presence of (*a*) 1×10^{-4} M-EDTA, (*b*) neither EDTA nor added Zn^{2+}, or (*c*) added 5×10^{-4} M-Zn^{2+} was 5, 26, and 62, respectively. In the absence of bacitracin, the cocci were neither killed nor lysed by EDTA or Zn^{2+}.

The content of β-galactosidase in non-multiplying cocci which had been incubated in the presence of lactose or galactose was fifteen or twenty times more, respectively, than that of non-induced cocci. Non-multiplying cocci which had been incubated in the presence of mannitol contained thirty times more mannitol dehydrogenase than did non-induced cocci. The ability of minimal bacteriostatic concentrations of bacitracin and chloramphenicol to suppress induced enzyme synthesis by *Staphylococcus aureus* is shown in Fig. 2 and in Table 2. Unexpectedly, added Zn^{2+} had essentially no effect on the activity of bacitracin in this test system (Table 2). It also may be noted in Table 2 that concentrations of penicillin and cycloserine much greater than the minimal bacteriostatic concentrations were unable to suppress enzyme formation.

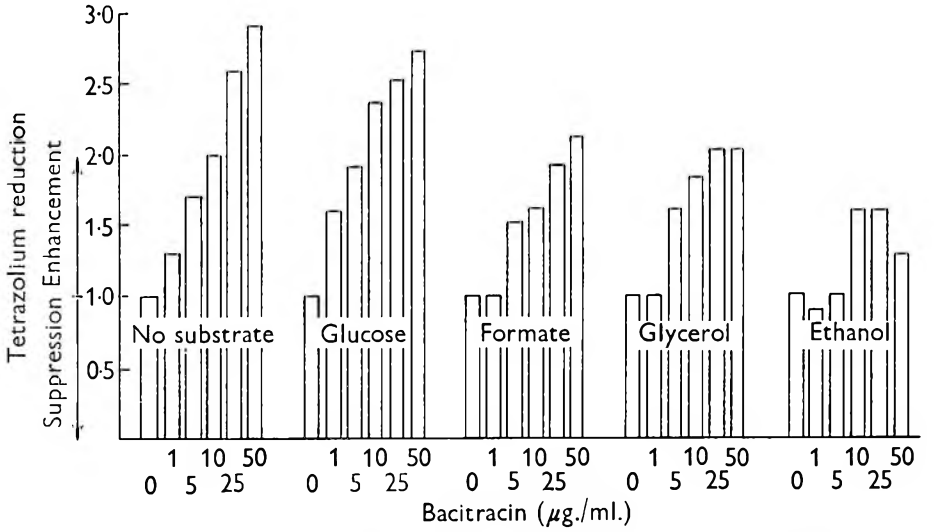

Fig. 3. Effect of bacitracin on the ability of *Staphylococcus aureus* to reduce 2,3,5-triphenyltetrazolium chloride (TTC) in the presence of various substrates. The values on the ordinate scale represent the ratios of the O.D. of the tubes containing bacitracin to those lacking bacitracin. The antibiotic was added to 16 hr. cocci and the quantity of reduced TTC measured after a 30 min. period of incubation.

The ability of minimal bacteriostatic concentrations of bacitracin to stimulate the reduction of 2,3,5-triphenyltetrazolium chloride (TTC) by washed whole cocci of *Staphylococcus aureus* is indicated in Fig. 3. Added Zn^{2+} was unable to enhance this activity of bacitracin; indeed, the test system was remarkably sensitive to Zn^{2+} since as little as 1×10^{-5} M completely suppressed the reduction of TTC. Other metallic ions (Cu^{2+}, Co^{2+}, Fe^{2+}, Mn^{2+}) had no effect either on the test system or on bacitracin activity. The influence of EDTA on bacitracin activity could not be tested since, as with Zn^{2+}, EDTA alone inhibited reduction of TTC by the cocci. Reduction of TTC was neither stimulated nor inhibited by chloramphenicol, penicillin or cycloserine.

Sequential inactivation by heat of the four biological properties of bacitracin described above is shown in Fig. 4. Samples of bacitracin at 0·1 % (w/v) in deionized water (pH 6·3) were autoclaved for 15, 30, and 60 min. at 121° and then assayed to determine the extent of inactivation of each property. The bacteriostatic activity of bacitracin was eliminated by autoclaving for 15 min.; longer periods were necessary to destroy the ability of bacitracin to suppress induced enzyme synthesis, stimulate reduction of TTC, or induce lysis.

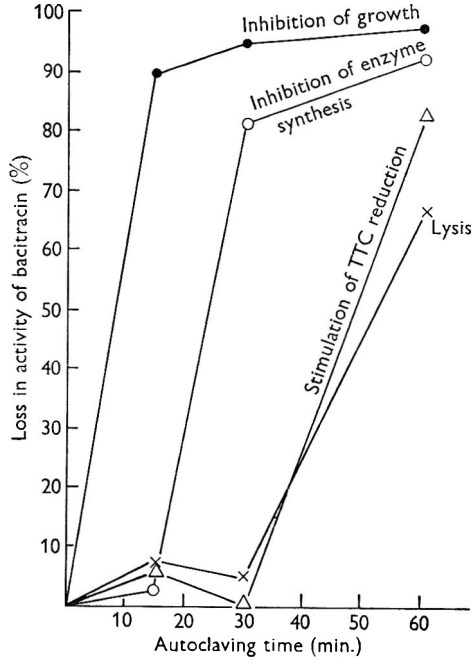

Fig. 4. Effect of autoclaving on the biological properties of bacitracin.

DISCUSSION

It is currently the fashion to assign antibiotics to one of three groups with respect to their primary biochemical mechanism of action: (*a*) suppression of cell wall synthesis, (*b*) suppression of protein synthesis, (*c*) disruption of lipoprotein membranes. In group (*a*) have been placed penicillin, cycloserine and bacitracin (Park, 1960); in group (*b*) chloramphenicol, the tetracyclines, erythromycin, puromycin, streptomycin (Park, 1960; Brock & Brock, 1959; Creaser, 1955; Yarmolinsky & de la Haba, 1959); in group (*c*) gramicidin S, polymyxin, subtilin, tyrocidin (Newton, 1958). At concentrations in excess of those needed to inhibit growth, chlortetracycline (but not oxytetracycline) possesses activity (*a*) as well as (*b*) (Park, 1960), and penicillin possesses activity (*b*) as well as (*a*) (Creaser, 1955). No antibiotic, however, has been stated thus far to be active at minimal growth inhibitory concentrations in more than one of the three categories. Nevertheless, the results described in the present study together with those obtained by Gale & Folkes (1955) by Creaser (1955), and by Park (1958, 1960) indicate clearly that bacitracin, unlike

penicillin, cycloserine and chloramphenicol, possesses at minimal bacteriostatic concentrations both activities (*a*) and (*b*), i.e. suppression of cell wall synthesis and protein synthesis.

Bacitracin is also unique in that, of the four antibiotics tested, it alone can stimulate the reduction of TTC by *Staphylococcus aureus*. This observation has been confirmed by Dr S. C. Lippincott (VA Center, Bay Pines, Florida, U.S.A.; personal communication). The stimulation of reduction of TTC may be a manifestation of enhancement by the bacitracin of cellular permeability. However, in subsequent studies (to be published) we have observed that bacitracin is considerably less active than such cationic surfactants as cetyltrimethylammonium bromide with respect to ability to lower the surface tension of water and to induce the release of nitrogenous constituents from non-multiplying cocci of *S. aureus*.

In the present study, bacitracin-induced protoplasts of *Staphylococcus aureus* could not be obtained. Similar tests by K. Crawford were stated to have yielded protoplasts (Abraham, 1957) but the details of these experiments have not yet been published. Likewise, it is quite difficult to obtain bacitracin-resistant variants in either *in vitro* (Stone, 1949; Gezon, Fasan & Collins, 1950) or *in vivo* (Lowbury, 1960) systems. The instability of protoplasts and the lack of emergence of drug-resistant variants may be a consequence of the fact that, at minimal growth inhibitory concentrations, multiple rather than single biochemical lesions are produced by bacitracin.

Molecules of bacitracin A probably exist as resonating and tautomeric structures (Craig, Konigsberg & Hill, 1958) and when heated, stored, or placed in solution, may undergo a stepwise series of changes to progressively less active forms. The thermal and storage stability of the most biologically active form of bacitracin is preserved by allowing the molecule to form a complex with Zn^{2+} (Gross, 1954); possibly the dependence on Zn^{2+} of the bacteriostatic and bacteriolytic properties of bacitracin indicate that these two activities require a single stabilized molecular configuration. The precise binding site for Zn^{2+} on the bacitracin molecule is unknown; however, Garbutt, Morehouse & Hanson (1961) postulated that Zn^{2+} (as well as Cu^{2+}, Ni^{2+}, and Co^{2+}) is bound by the imidazole group of the histidine moiety of bacitracin A. It is possible that the formation of a metal bridge between the α-amino group of isoleucine and the imidazole moiety would stabilize the molecule and prevent transformation to an inactive form. Indeed, since there is a 100-fold difference in bacteriostatic action depending on whether the medium is enriched with Zn^{2+} or with a zinc-binding agent (Weinberg, 1959, and this paper), it is probable that in the complete absence of available Zn^{2+} bacitracin would possess no bacteriostatic property whatsoever.

Alternately, Zn^{2+} may be required for the attachment of bacitracin to sensitive enzymes involved in the bacteriostatic and bacteriolytic actions but not those that are operative in induced enzyme synthesis or in the stimulation of TTC reduction. Additional evidence that the bacteriostatic property is distinct from the three other activities of bacitracin was obtained by exposing solutions of the drug to 121° for different periods of time and observing the differential rates at which each property was inactivated. It must be concluded that the actual primary mechanism of the antibacterial action of bacitracin remains unknown. The results of previous and present investigations have shown only that (1) at minimal growth inhibitory

concentrations, bacitracin can prevent cell wall synthesis, and also enzyme synthesis and perhaps enhance permeability, and (2) at subinhibitory concentrations, the compound can stimulate growth of various microbes (Novosel, 1961), germ-free plants (Nickell & Finlay, 1954), birds and mammals (Goldberg, 1959). Clearly, a substance that possesses such an array of fascinating activities merits further investigation.

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