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A Method for the Control of E_h and pH during Bacterial Growth

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SUMMARY

A method is described whereby pH and E_h of a medium can be closely controlled during the growth of *Clostridium welchii* type A for long enough to study the bacteriostatic effects of horse antiserum. The pH value was controlled manually to about $\pm 0{\cdot}01$ unit (S.D.), by adjustments in the CO_2 tension of the gas above the medium. The E_h was controlled by the addition of oxygen to a system with an inherent tendency to become reduced. With manual control the E_h was maintained within ± 2 mV (S.D.); automatic control gave better regulation than this.

INTRODUCTION

Specific antiserum inhibits the growth of *Clostridium welchii* type A *in vitro* provided that the medium has a suitable pH and E_h (Bullen & Dobson, 1962, 1963; Bullen, Dobson & Wilson, 1964). The study of this property of serum required an apparatus which would allow the E_h and pH of a medium to be set at predetermined values and then closely controlled for long enough for an effect on bacterial growth to show itself. The apparatus described here gave a better reproducibility and closer control of E_h and pH than had previously been attained.

Knight (1930, 1931) described an apparatus for maintaining a low oxygen tension in the gas phase above the medium. This method was intended for E_h control of a medium before the germination of spores of *Clostridium tetani* (Knight & Fildes, 1930). Vennesland & Hanke (1940) extended its use to growing cultures of *Bacteroides vulgatus*, but they did not attempt pH control. Hanke & Katz (1943), in a study of the growth of *C. sporogenes*, used an electrolytic method for controlling the E_h to within 10–20 mV and the addition of alkali for pH control to within 0·2 unit. The electrolytic method was not used in later work partly because of the large pH changes induced by electrolysis, and partly because it was suspected that the products of electrolysis were toxic. Hanke & Bailey (1945), in a study of several species of Clostridium, preferred the original method of Knight for controlling the E_h, but it is not clear how closely the E_h and pH were controlled. The authors considered that their results were consistent with an error of 10 mV with respect to E_h.

The apparatus we have used controls the pH value of a medium containing bicarbonate by the continuous control of the carbon dioxide tension in the gas above the liquid. The E_h is controlled by the intermittent addition of oxygen to offset a drift towards reducing conditions which is inherent in the medium used.

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METHODS

Media. The media for bacterial growth were: (1) allantoic fluid from 11-day embryonated eggs (Bullen & Dobson, 1962); (2) a medium consisting of Bacto Casitone (Difco) 1 % (w/v), Bacto yeast extract 0·3 % (w/v), $NaHCO_3$ 0·25 % (w/v), glucose 0·05 % (w/v); (3) a chemically defined tissue culture medium (Bullen *et al.* 1964).

Buffers. Borate and phthalate buffers for primary pH standards were prepared according to the specification in British Standards 1647: 1950. Sterile phosphate

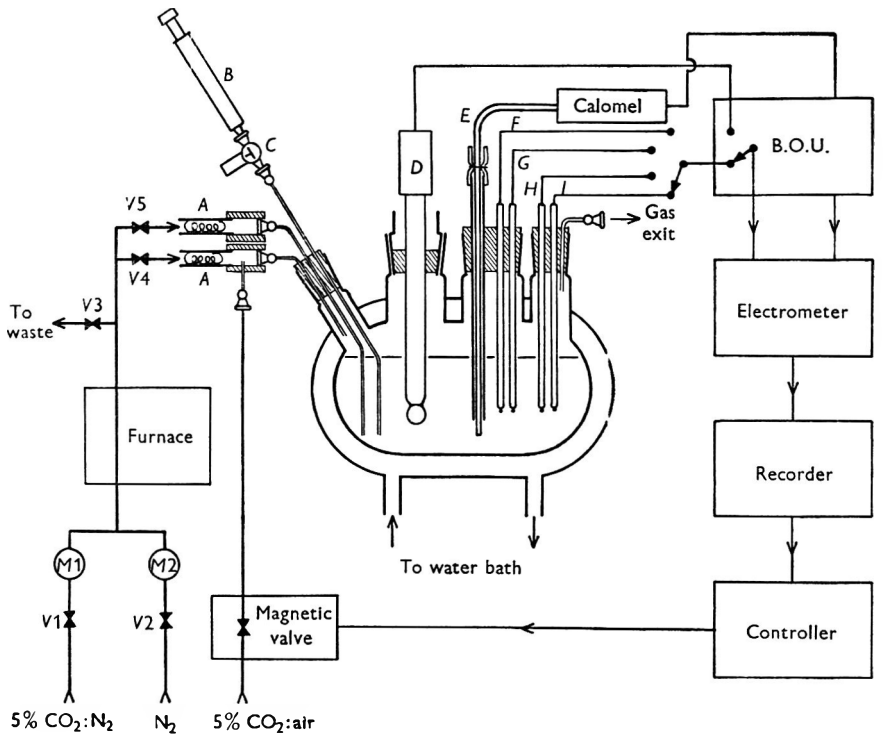

Fig. 1. Culture vessel and associated equipment. (Not to scale.) *A*, Sterile cotton-wool plug; *B*, tuberculin syringe; *C*, three-way tap; *D*, glass electrode; *E*, KCl-agar bridge in P.V.C. tubing (this is tied into a P.V.C. collar on a glass tube); *F*, *G*, *H*, *I*, platinum electrodes; B.O.U., backing-off unit; *V* 1–5, valves controlling gas flow (*V* 1–3 fine control needle valves); *M* 1, *M* 2, Gas flowmeters.

buffer pH 6·85 used as a secondary standard was prepared by autoclaving 0·5 M-Na_2HPO_4, 0·5 M-KH_2PO_4 in small bottles. This was diluted 1 in 20 with sterile distilled water before use. For standardizing the E_h electrodes, phthalate buffer saturated with quinhydrone was used at 37°, E_h = +443 mV.

Culture vessel. The glass culture vessel consisted of an inner horizontal cylinder surrounded by an outer jacket through which water at 37·0 ± 0·1° was circulated. The total volume of medium was 70 ml., leaving a gas space of 30 ml. (Fig. 1). As sunlight appeared to abolish the inhibitory effect of serum on bacterial growth in the presence of redox dyes, light was excluded by shrouding the vessel with a rubber

and velvet jacket, and manipulating it within a large dark box. The medium was stirred magnetically.

A glass electrode, four platinum electrodes and a 3·5 M-KCl agar bridge in polyvinyl chloride tubing passed through gas-tight seals into the medium. Provision was made for passing gas over the surface of the medium or through it. A tuberculin syringe was used to remove samples of medium through a three-way tap without admitting air.

Electrodes. The 3·5 M-KCl calomel reference electrode and buffer solutions were kept in the water bath at 37·0 ± 0·1°. The shielded glass electrode (G.G. 33, Electronic Instruments, Ltd, Richmond, Surrey) was also kept at the same temperature as this appeared to improve its stability. The electrode was sterilized by standing overnight in '20 vol.' H_2O_2, rinsed with sterile distilled water and standardized with sterile phosphate buffer before immersion in the medium. At the end of each experiment the drift was checked with phosphate buffer and the electrode system accurately calibrated with phthalate and borate buffers. Even after discarding electrodes with more erratic readings, the drift over a 5–8 hr incubation period varied considerably between individual electrodes. Thus in 42 experiments with three electrodes, the mean drift, disregarding the sign was 0·006 pH unit. Three less satisfactory electrodes had an average drift of 0·024 unit in 16 experiments. However, the error due to drift was decreased to about 0·01 pH unit by calibration of the electrode during the experiment. When the electrode was removed from the vessel, the admission of air was prevented by increasing the flow of gas.

The E_h electrodes were made by fusing 26 S.W.G. platinum wire to give a sphere 1 mm. diameter at the end. This was sealed in soda glass tubing leaving only the sphere projecting. In earlier experiments with a spiral of wire as the electrode there was a tendency for aggregation of the organisms around the spiral when growth in the presence of serum was heavy; the small spherical electrodes appeared to circumvent this difficulty. E_h electrodes were cleaned in hot chromic acid, sterilized in steam, and standardized after each experiment. The KCl agar bridge, sealed with clips, was sterilized by boiling in distilled water.

Poising. Electrodes immersed in unpoised allantoic fluid differed by 50 mV or more. It is conceivable that the disagreement between electrodes commonly found in an unpoised situation reflects the absence of a unique E_h, as electron transfer reactions can be very slow and an homogeneous mixture of redox systems does not possess a unique E_h until equilibrium between them has been reached. To avoid this difficulty it was decided to work only in a situation where three E_h-electrodes agreed to within 1 mV. For this criterion to be satisfied it was necessary to work near the E_h° of a suitable poising agent. The poising agents (British Drug Houses Ltd) were used at 30 or 60 μM concentration for the following approximate E_h: thionine, +60 mV; methylene blue, 0 mV; indigo carmine, −140 mV; 1-naphthol-2-sodium sulphonate-indo-3′,5′-dichlorophenol, +60 mV. In a well-poised medium the electrodes generally agreed to within 0·2 mV.

Electrical apparatus. Electrode potentials were measured with a Vibron electrometer (Electronic Instruments Ltd., Richmond, Surrey) which had a sensitivity of 0·1 mV on the lowest voltage range. The full sensitivity of the electrometer was exploited by using a backing-off unit which could switch a potential of ± 500·0 mV in steps of 5·0 mV in series with the calomel electrode. The backing-off voltage

could be standardized by a comparison with a Weston cadmium cell with the electrometer as a null point detector. The backing-off unit also had a switch which allowed a choice of input from either the glass electrode or an E_h electrode with suitable precautions for maintaining the high impedance input characteristics of the electrometer.

The output of the electrometer was monitored by a 0–10 mV potentiometer recorder, accurate to 0·1 mV (George Kent Ltd, Luton, Beds.; 6 point Multelec run as a single channel recorder) which sensed the output every 2 sec. and printed 18 times in 10 min. For automatic E_h control a 4 in. diameter cam was fitted to the robust potentiometer movement. This cam could be preset to operate a microswitch between a reading of 2·5 and 7·5 mV so that when the reading was between 0 and the preset voltage, the switch was open, and when between the preset voltage and 10 mV, the switch was closed. The differential of the switch was 0·1 mV.

The E_h controller, which was actuated by the closing of the microswitch in the recorder, consisted of a switching device with suitable delays and a power supply to operate a magnetic valve (Radiometer, Copenhagen).

pH control. The pH value of the medium was controlled by adjusting the tension of CO_2 in the gas passing over the medium by mixing different proportions of 5 % (v/v) CO_2+95 % (v/v) N_2, with either N_2, or 25 % (v/v) CO_2+75 % (v/v) N_2. This usually required altering only once or twice during the course of an experiment once it was correctly set. Gas from each cylinder passed through a pressure reduction valve, a needle valve and a flowmeter. After mixing, the gases passed over copper heated in an electric furnace, then either to waste through a needle valve, or over the medium through a sterile cotton-wool plug. An alternative path through the medium was provided for rapid initial pH adjustment. Gas left the culture vessel through a wide bore needle and was bubbled through 2 cm. water. The gas flow through the vessel was normally about 80 ml./min. but could be increased to about 500 ml./min. whenever the incubation chamber was unsealed, e.g. for inoculation.

E_h control. The medium was reduced to the desired E_h by adding from a micrometer syringe burette freshly made 5 % (w/v) $Na_2S_2O_4$ solution. After a rapid initial fall, a slow secondary downward drift of E_h occurred. The E_h was controlled manually by the cautious addition of air bubbles through the sampling tap so that the downward drift was arrested. The automatic control of the E_h was developed initially to cope with the inconveniently frequent additions of oxygen necessary to maintain control when working more than 20 mV away from the E_h° of a redox dye.

The controller (Fig. 2) operated when the E_h drifted below a value preset within the recorder. *S*7 in the recorder applied the mains voltage to the controller motor. When the motor started, the cam *A* closed *S*4, locking the mains supply onto the motor for 1 revolution. Then cam *B* closed *S*5, energizing the magnetic valve until the same cam opened *S*6. When the magnetic valve was operated, expired air from a football bladder passed at about 20 ml./min. into the stream of gas passing over the surface of the medium. One minute after the cycle started, *S*4 opened. If the addition of oxygen had raised the E_h so that *S*7 was now an open circuit, the motor stopped; but if *S*7 was still closed another switching cycle was begun. The duration of the addition of air during the cycle could be varied continuously from 0–20 sec. by rotating *S*6 in an arc about the motor spindle. Usually it was adjusted below

3 sec. so that the maximum mean flow was insufficient to disturb the pH by altering the CO_2 tension appreciably.

Further information on the organisms, sera and experimental procedure are given by Bullen *et al.* (1964).

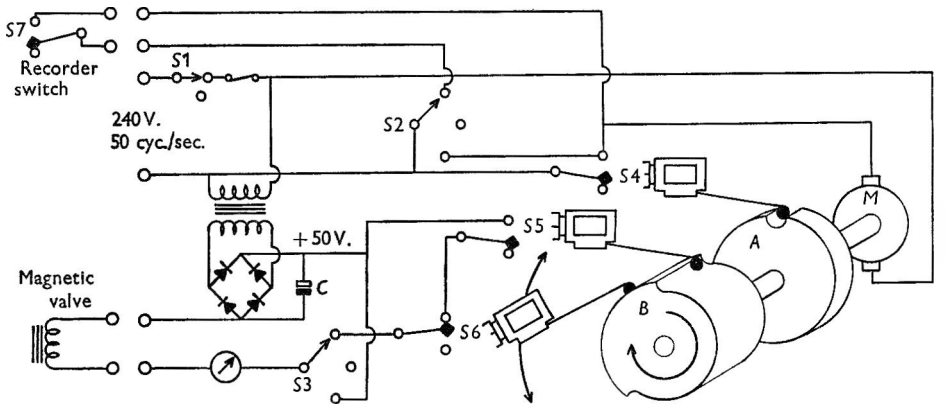

Fig. 2. Circuit of E_h controller. (Not to scale.) *M*, Electric clock motor, 1 r.p.m.; *A*, *B*, cams shown in 'motor off' position; *S*1, on/off switch; *S*2, *S*3, operation switches shown in 'control' position; *S*4–*S*6, cam-operated micro-switches; *S*7, micro-switch in recorder; *C*, 8μF condenser. *S*6 is mounted on a plate which can rotate about the axis of the motor spindle. The position of this plate can be finely adjusted with a worm gear.

RESULTS

Similar control was observed with the three media used. Good agreement between readings with different E_h electrodes was noted with thionine and methylene blue as poising agents in the presence or absence of antiserum. With indigo carmine, agreement was good in the presence of antiserum, but not in its absence. This was not serious, as a little uncertainty could be tolerated about conditions during growth in the absence of antiserum at low E_h. With 1-naphthol-2-sodium sulphonate-indo-3',5'-dichlorophenol, however, good agreement was obtained in the absence of antiserum but not in its presence. It appeared that an interaction of a redox dye with antiserum might affect its poising properties.

Control of E_h was lost when the organisms in the medium grew beyond a concentration of about 10^6/ml. Starting from an initial population of 4×10^3/ml. control was usually possible for at least 3 hr of normal growth. Since antiserum started to inhibit growth after about 2 hr, there was sufficient latitude to allow the inhibition to be studied. Control of pH value could be maintained until much higher numbers of organisms were present.

Some caution may be necessary in using large amounts of air to keep a high E_h when bacteria are present in large numbers. In one experiment in the absence of antiserum when control was prolonged by about 30 min. by injecting large amounts of air with the automatic controller, the readings with different E_h electrodes started to disagree. Soon after control was abandoned, a temporary inhibition of growth was observed.

Since evaluation of the results was possible only when the E_h was controlled, the

conditions for each experiment are described by the mean E_h taken every 15 min., together with the standard deviation from this mean and the period this control was maintained. Readings of pH value taken over the same time are expressed similarly.

In 30 arbitrarily selected experiments controlled from 3 to 8 hr with manual E_h control, the difference between the intended and observed mean E_h, disregarding the sign, averaged 1·0 mV (maximum 2·0 mV). The mean of the standard deviations of E_h was $\pm$2·0 mV (range 0·3–3·6 mV). The mean difference between the intended and observed pH value, disregarding the sign, was 0·009 (maximum 0·019). The mean of the standard deviation of pH value was 0·007 (range 0·002–0·014).

Experience with the automatic E_h controller was more limited, but it clearly gave closer and more convenient E_h control than the manual method under the same circumstances. For example, in two experiments the E_h stayed within ranges of 1·4 and 0·8 mV, respectively, over a period of 5 hr. Preliminary experiments indicate that satisfactory E_h control is possible 50 mV from the E_h° of a poising agent, where manual control would be either too laborious or too wide. Doubtless a systematic exploitation of its full capability of controlling the E_h would give a closer control, but further improvement would not be justified without a corresponding improvement in pH control. The limitation in this respect is probably the stability of the glass electrode.

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Bacteriostatic Effects of Specific Antiserum on *Clostridium welchii* type A. The Role of E_h and pH of the Medium

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SUMMARY

The bacteriostatic effect of specific antiserum on *Clostridium welchii* type A was profoundly influenced by the pH and E_h of the medium. With suitable concentrations of specific antiserum relatively high pH and E_h values led to a well-marked inhibition of growth accompanied by destruction of the bacteria. A relatively low pH or E_h led to a decrease or abolition of the inhibitory power of the specific antiserum. Neither complement nor properdin appeared to be involved in the bacteriostatic effect.

INTRODUCTION

Specific antiserum provides complete protection against many lethal doses of *Clostridium welchii* type A introduced into the allantoic cavity of embryonated eggs. The protective effect appears to reside in the ability of the antiserum to inhibit the growth of the bacteria. However, antiserum does not inhibit bacterial growth in killed eggs (Bullen, Wilson & Cordiner, 1961). Since dead tissue is generally considered to be more reducing and more acid than living tissue we decided to investigate the effects of E_h and pH on the bacteriostatic properties of *C. welchii* antiserum. Preliminary experiments with allantoic fluid *in vitro* showed quite clearly that the degree of bacteriostasis was profoundly influenced by the E_h of the medium (Bullen & Dobson, 1962). Allantoic fluid was considered unsuitable for a more detailed investigation, since its composition is variable and not entirely known, (Needham, 1931; Festenstein, 1957). The experiments described in this paper were done with a chemically defined tissue culture medium in which *C. welchii* grew very well.

METHODS

Organisms. Clostridium welchii type A strain CN 2726 was used for all the experiments *in vitro*. Strain CN 2726 and type A strain CN 1491 were both used for immunization in preparing the antiserum. Both strains were obtained from the Wellcome Research Laboratories (Beckenham, Kent).

Specific Clostridium welchii antiserum. One antiserum (P 10) was used for all the experiments; this was obtained from a pony after eight courses of immunization against *Clostridium welchii* type A. Formolized whole cultures of strain CN 2726 were used for the first seven courses; for the eighth course the pony was injected intramuscularly with a mixture of concentrated toxin from strains CN 2726 and CN 1491, and subcutaneously with washed and formalized organisms of strain CN 2726. The antitoxin content of the antiserum in units/ml. was as follows;

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α-antitoxin 130 (international units); θ-antitoxin 90 (provisional units); κ-antitoxin 330 (provisional units); μ-antitoxin 11 (provisional units). The antiserum was stored at $-20°$.

Culture medium. Sufficient tissue culture medium TC199 (without phenol red; Difco) was used to give a final volume of 70 ml. after the addition of poising agent, bacterial inoculum and antiserum. The bicarbonate concentration was increased by adding 0·1–0·15 g. dry $NaHCO_3$ sterilized by heating to 160° for 2 hr.

Viable counts. Viable counts were made on blood agar plates (Bullen *et al.* 1961).

Control of E_h and pH. As described by Dobson & Bullen (1964).

Broth saline. 10 % (v/v) of papain digest broth in 0·85 % (w/v) NaCl.

Experimental procedure. TC199 medium was placed in the culture vessel. The stirrer was switched on and after a gas mixture of 5 % (v/v) CO_2 + 95 % (v/v) N_2 had bubbled through the medium for about 15 min., the $NaHCO_3$ was added. The glass electrode, after standardization in phosphate buffer, together with the four platinum E_h electrodes, was inserted in the culture vessel. The KCl agar bridge was cut at both ends with sterile scissors and one end was placed in the medium and the other in the calomel electrode. After adding 0·5–1·0 ml. of a solution of the appropriate redox dye to give a final concentration of 30 μM the pH value was adjusted by varying the concentration of CO_2 in the gas bubbling through the medium. When the pH value was approximately correct the gas stream was switched to pass over the surface of the medium. When required, warmed sterile antiserum was added at this stage. The E_h was adjusted by cautious addition of sterile 5 % (w/v) $Na_2S_2O_4$.

An actively growing culture of *Clostridium welchii* ($1\frac{1}{2}$ hr old) in papain digest broth, was centrifuged and the supernatant fluid removed. The bacteria were resuspended in broth saline and adjusted to an opacity corresponding to tube No. 8 on the Brown's scale (Burroughs Wellcome and Co.). Suitable dilutions were prepared in broth saline and the bacteria added to the medium in a volume of 0·6 ml. After adding the bacteria the culture was well mixed with the aid of the syringe, and a sample withdrawn for a viable count. Final adjustments of pH and E_h were made as quickly as possible. When antiserum was used, all the samples, after the first hour, were homogenized in an MSE (London) (5 ml.) homogenizer for 8 min. before the viable counts were made; this broke up the chains of organisms which invariably formed in the presence of antiserum.

RESULTS

In the absence of antiserum the organisms grew well (Figs. 1–4; Table 1). The lag phase varied from approximately 1 hr at E_h +60 mV, pH 7·5, to 30 min. at E_h −140 mV, pH 7·5. The culture consisted largely of well separated individual bacteria (Pl. 1, fig. 1).

The effect of E_h and pH on the generation time. At pH 7·5 faster growth in the logarithmic phase occurred under more reduced conditions. The effect of changing from pH 7·5 to 6·5 was slight (Table 1).

The effect of different concentrations of antiserum on bacterial growth. In the five experiments shown in Fig. 1 the E_h was controlled at +60 mV and the pH value at 7·5. The presence of 3 % (v/v) *Clostridium welchii* antiserum (P 10) had a stimulating effect on bacterial growth. The lag phase was decreased to 30 min. and the

Table 1. *Effect of E_h and pH on generation time of C. welchii during the logarithmic phase of growth without serum*

Redox dye	pH	E_h	Time controlled (hr)	Generation time (min.)
thionine	7·48 ± 0·007	+59 ± 1·1	3·5	27
1-naphthol-2-sodium sulphonate-indo-3′5′-dichlorophenol	7·49 ± 0·003	+60 ± 2·1	3·5	27
methylene blue	7·51 ± 0·005	0 ± 0·7	3·0	21
indigo carmine	7·51 ± 0·014	−141 ± 1·0	3·0	19·5
thionine	6·51 ± 0·006	+60 ± 1·5	3·5	24

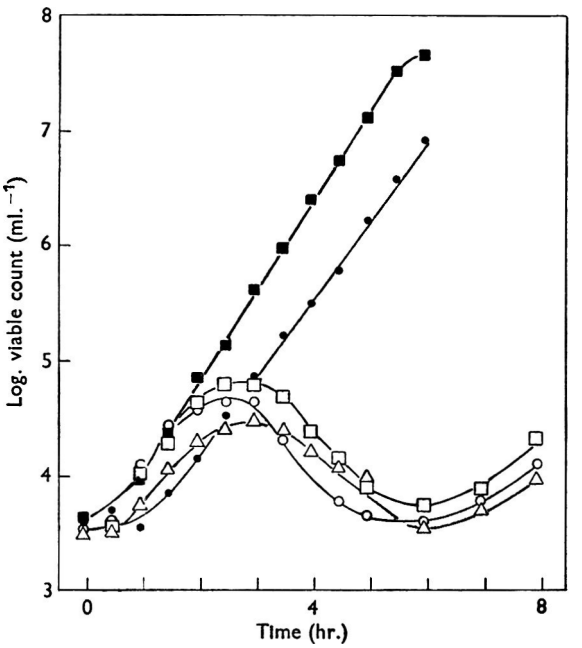

Fig. 1. The effect of specific antiserum concentration on the growth of C. welchii at pH 7·5; E_h + 60 mV.

Symbol	Antiserum P 10 (%, v/v)	pH (mean ± S.D.)	E_h (mV) (mean ± S.D.)	Time controlled (hr)
●	0	7·48 ± 0·007	+59 ± 1·1	3·5
■	3	7·50 ± 0·005	+60 ± 1·7	3·25
△	6	7·50 ± 0·010	+58 ± 1·6	8·0+
○	12	7·50 ± 0·004	+59 ± 2·0	8·0+
□	24	7·52 ± 0·012	+61 ± 0·5	8·0+

organisms grew slightly faster than in the absence of specific antiserum. Antiserum at 6 % (v/v) had a well-marked bacteriostatic effect. The organisms grew well for the first 2 hr, but by 3 hr multiplication had stopped. This was followed by a steady decline in the number of viable organisms until the 6th hr. Thereafter the number of viable organisms slowly increased. Similar patterns of growth and destruction occurred with 12 % (v/v) and 24 % (v/v) antiserum.

The effect of changes in E_h. Figure 2 shows the effect of changes in E_h on the inhibitory power of 12 % (v/v) antiserum P 10 when kept at pH 7·5. At E_h +60 mV the number of viable organisms declined rapidly between the 3rd and 6th hr; at E_h 0 mV the peak of bacterial growth was slightly greater than at +60 mV and there was less destruction between the 3rd and 6th hr; at E_h −140 mV there was no apparent destruction but the growth rate was decreased by 7–8 times compared with the control. Figure 3 shows similar experiments with 6 % (v/v) P 10 antiserum. Good inhibition of bacterial growth occurred at 60 mV; at 0 mV and −140 mV, there was no inhibitory effect.

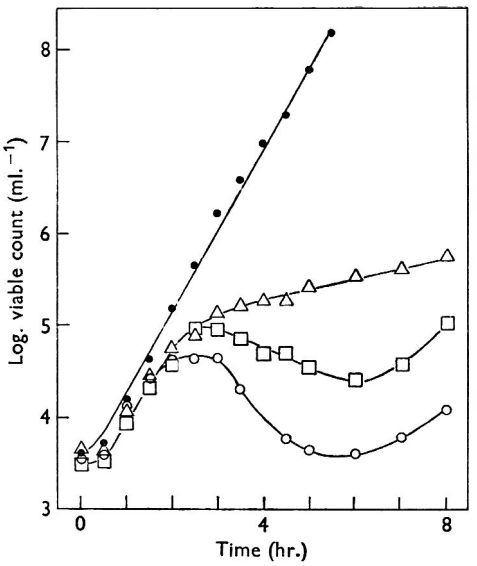

Fig. 2

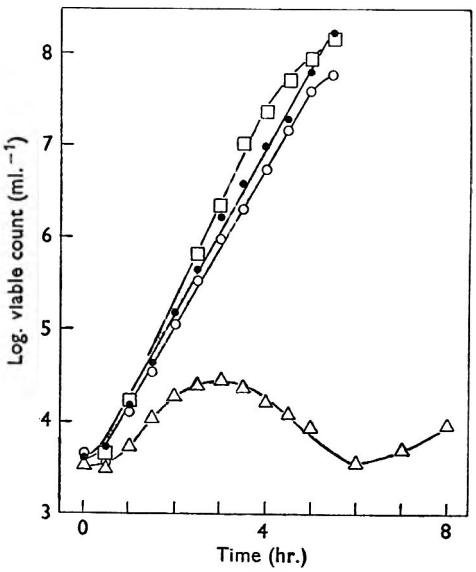

Fig. 3

Fig. 2. The effect of E_h on the growth of C. welchii with 12 % (v/v) antiserum P 10 at pH 7·5.

Symbol	Antiserum (%, v/v)	pH (mean ± S.D.)	E_h (mV) (mean ± S.D.)	Time controlled (hr)
●	0	7·51 ± 0·014	−141 ± 1·0	3·0
△	12	7·51 ± 0·011	−141 ± 2·3	8·0+
□	12	7·52 ± 0·003	−3 ± 1·9	8·0+
○	12	7·50 ± 0·004	+59 ± 2·0	8·0+

Fig. 3. The effect of E_h on the growth of C. welchii with 6 % (v/v) antiserum P 10 at pH 7·5.

Symbol	Antiserum (%, v/v)	pH value (mean ± S.D.)	E_h (mV) (mean ± S.D.)	Time controlled (hr)
●	0	7·51 ± 0·014	−141 ± 1·0	3·0
□	6	7·52 ± 0·011	−142 ± 1·0	3·0
○	6	7·51 ± 0·005	0 ± 1·0	3·0
△	6	7·50 ± 0·010	+58 ± 1·6	8·0+

The effect of changes in pH value. During the experiments shown in Fig. 4 the E_h was controlled at +60 mV. With 12 % (v/v) antiserum P 10 at pH 7·5 there was well-marked inhibition of bacterial growth. At pH 6·8 the degree of inhibition was

far less than at pH 7·5; at pH 6·5 there was no inhibition, and growth was identical to that observed in the absence of antiserum. When the antiserum concentration was decreased to 6 % (v/v) the inhibitory effect though well marked at pH 7·5 (Fig. 1) was completely abolished at pH 6·8.

The effect of changes in pH and E_h. The inhibition produced by 12 % (v/v) antiserum P 10 at pH 6·8, E_h 0 mV was so slight that it was doubtful whether it was within the experimental error of the method. A slight decrease in growth rate was noted after the 3rd hr, but E_h control was lost after 3 hr 45 min., after which growth was somewhat faster. This can be compared with the definite inhibition obtained with 12 % (v/v) antiserum P 10 at pH 6·8, E_h 60 mV (Fig. 4) and pH 7·5 E_h 0 mV (Fig. 2).

The effect of heating antiserum P 10. The antiserum was heated to 56° for 30 min. in a water bath. This had no effect on the bacteriostatic effect (Fig. 5).

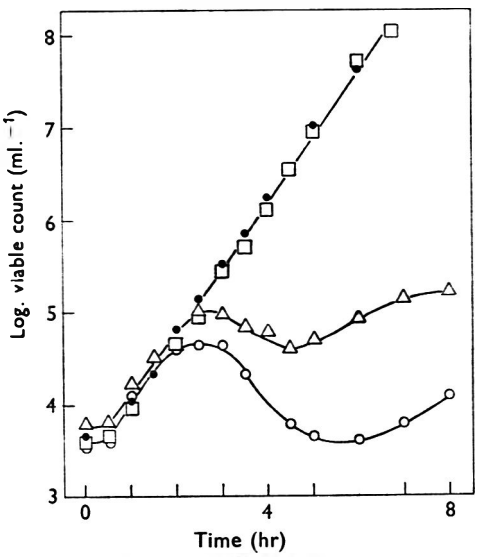

Fig. 4

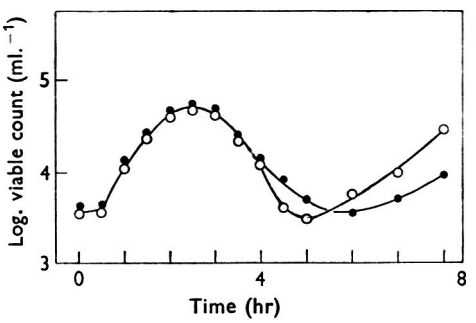

Fig. 5

Fig. 4. The effect of pH value on the growth of C. welchii with 12 % (v/v) antiserum P 10 at E_h +60 mV.

Symbol	Antiserum (%, v/v)	pH value (mean ± S.D.)	E_h (mV) (mean ± S.D.)	Time controlled (hr)
●	0	6·51 ± 0·006	+60 ± 1·5	3·5
□	12	6·50 ± 0·008	+59 ± 1·6	3·5
△	12	6·80 ± 0·003	+59 ± 1·3	8·0+
○	12	7·50 ± 0·004	+59 ± 2·0	8·0+

Fig. 5. The effect of heated antiserum P 10 on the growth of C. welchii at pH 7·5; E_h +45 mV; 12 % (v/v) antiserum.

Symbol	Antiserum treatment	pH value (mean ± S.D.)	E_h (mV) (mean ± S.D.)	Time controlled (hr)
○	Unheated	7·51 ± 0·005	45 ± 1·7	8·0+
●	Heated	7·52 ± 0·007	46 ± 1·2	8·0+

Morphology of Clostridium welchii. Actively growing organisms in tissue culture medium without specific antiserum P 10 consisted of well separated Gram-positive rods, about 1–3 μ long and 0·5–0·8 μ wide (Pl. 1, fig. 1). A similar morphology was seen under widely different E_h and pH values. In the presence of specific antiserum P 10 when conditions were unsuitable for inhibition of growth the organisms occurred in long chains, sometimes of 50 individuals or more. All these organisms were Gram-positive and were much larger than those seen in control cultures (Pl. 1, fig. 2).

In the presence of specific antiserum P 10 when conditions were suitable for inhibition of growth the organisms were much larger than those seen in control cultures and occurred in short chains. The first visible signs of damage consisted of small Gram-negative patches in otherwise intact bacteria. Some individuals became entirely Gram-negative and others showed various degrees of disintegration, leading eventually to complete lysis (Pl. 1, figs. 3–10). All stages of damage, from apparently normal individuals, to the faint outlines of 'ghosts' were sometimes seen in a single chain of bacteria. Visible signs of damage were most numerous after incubation for 5–6 hr.

DISCUSSION

In the absence of specific antiserum *Clostridium welchii* type A grew well in the tissue culture medium. At the highest E_h tested, +60 mV, pH 7·5, the organisms grew logarithmically, with loss of E_h control after 3 hr and a viable count of several million organisms/ml. after 5 hr.

The presence of 3 % (v/v) antiserum P 10 slightly stimulated growth; under the same conditions 6 % (v/v) antiserum inhibited growth. After 6 hr the viable count was similar to the initial count and E_h control was maintained for at least 8 hr. Rather to our surprise, no greater inhibition occurred when the concentration of the antiserum was increased to 24 % (v/v).

In every case where inhibition occurred there was an initial period of vigorous growth similar to or faster than the rate of growth in control cultures. This was usually followed by a rapid decrease in the viable count, accompanied by the appearance of Gram-negative organisms some of which appeared to be lysing (Pl. 1, figs. 3–10). The decrease in viable count must be attributed to death of the bacteria and not to the formation of chains, since all the samples which showed chain formation were homogenized before counting. This could be seen to break up the chains satisfactorily. However, the most striking evidence that chain formation did not affect the viable count was provided by experiments with specific antiserum where conditions were suitable for chain formation but unsuitable for inhibition of growth. In these circumstances the growth curves were identical with the controls (Fig. 4; Pl. 1, fig. 2).

The inhibition of growth produced by antiserum depends profoundly on the E_h and pH values of the medium. A decrease in E_h or pH value or in antiserum concentration decreased or abolished the inhibitory effect. However, these three variables interacted, and it was therefore not possible to define values where the inhibitory effect was lost. Thus at pH 7·5 there was still some inhibitory effect with 12 % (v/v) antiserum P 10 at E_h −140 mV, but with 6 % (v/v) antiserum there was no inhibition at 0 mV or below. Similarly, at E_h +60 mV and pH 6·8, inhibition

occurred with 12 % (v/v) antiserum but not with 6 %. This can be contrasted with the results obtained at $E_h + 60$ mV and pH 7·5 where good inhibition was obtained with both 6 % and 12 % (v/v) of antiserum. Following the period of bacterial destruction the viable count started to increase again, although much more slowly than at the beginning of the experiment. The reasons for this are unknown.

Comparatively little is known of the systems involved in the bacteriostatic effect. It seems clear that neither properdin nor complement are necessary since the antiserum can be heated to 56° without loss of activity (Fig. 5). It is suspected that proteins are involved as the antisera can be dialysed without loss of activity in embryonated eggs and *in vitro* (Bullen *et al.* 1961; Bullen & Dobson, 1962). There is also evidence that substances present in normal sera may be concerned since these sera have similar but not such powerful effects (Bullen & Dobson, 1962). It seems quite possible that the bacteriostatic effects of antisera observed *in vitro* have some relevance to the problem of immunity to *Clostridium welchii* infections. It is known that protective antisera have a bacteriostatic effect in the allantoic cavity of living embryonated eggs. In dead eggs, where the conditions should be more reducing, antisera do not inhibit growth (Bullen *et al.* 1961). In severely damaged muscle of mice and guinea-pigs where both the E_h and pH are known to be low, the presence of specific antiserum has no effect on the growth of *C. welchii*. However, in relatively undamaged muscle where the E_h and pH would be expected to be high, the presence of specific antiserum leads to an abrupt cessation of bacterial growth 4 hr after infection (Bullen & Cushnie, 1962). Whether this is due directly to bacteriostatic effects of the specific antisera is a question that cannot be answered at the moment. Nevertheless, there are no reasons for supposing that this could not be the explanation, and it is encouraging that the pattern of growth and inhibition in embryonated eggs, mice or guinea-pigs, is similar to that seen *in vitro*.

We wish to thank Mr R. J. Cook, of the Wellcome Research Laboratories (Beckenham, Kent), for measurement of the antitoxins in the serum used.

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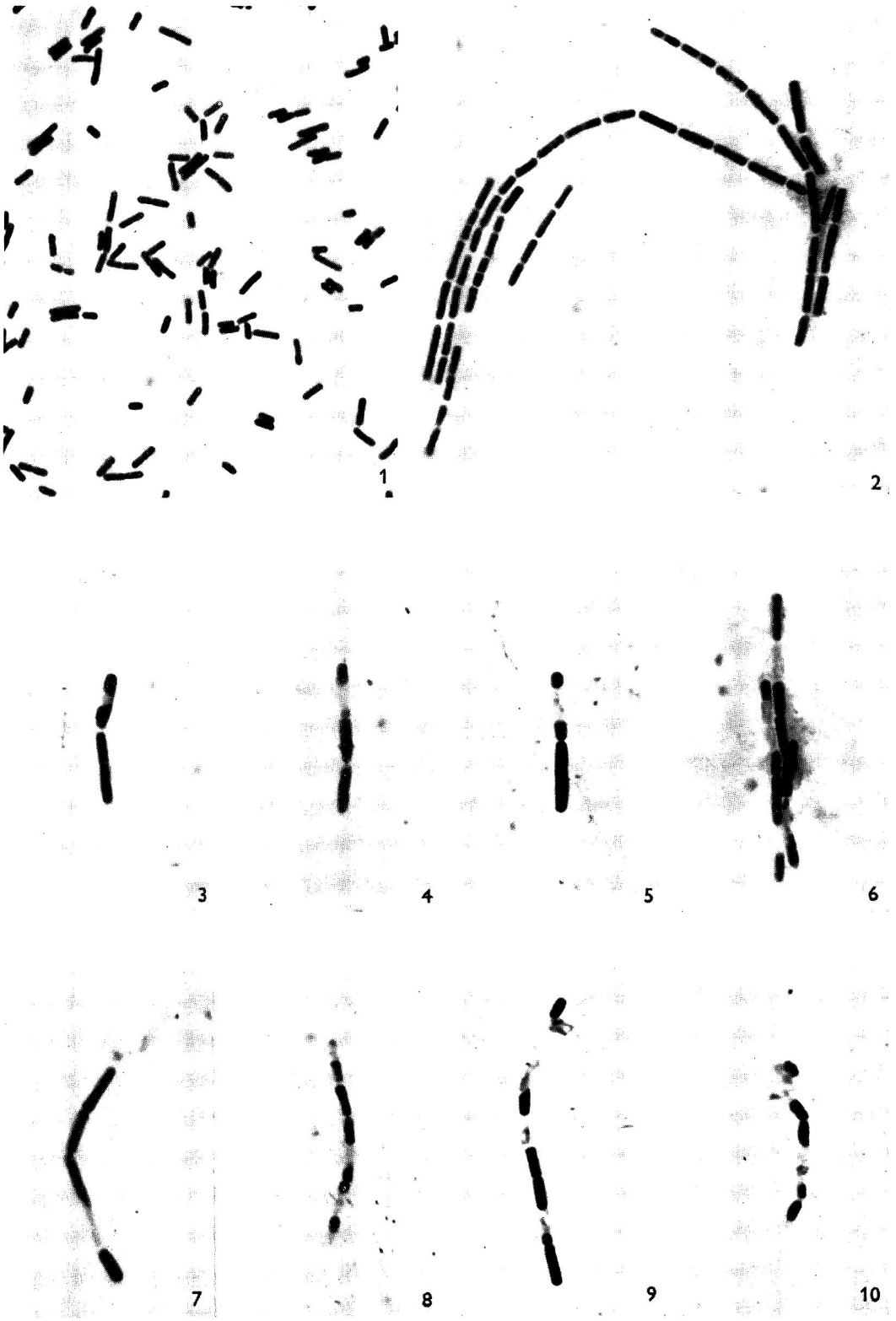
EXPLANATION OF PLATE 1

Clostridium welchii type A strain CN 2726. Growth in tissue culture medium. Gram stain. The magnification of all figures is 1450.

Fig. 1. Without specific antiserum. pH 7·2. E_h uncontrolled.

Fig. 2. 12 % (v/v) antiserum P 10. pH 6·5. $E_h + 59$ mV. No inhibition of growth.

Figs. 3–10. 12 % (v/v) antiserum P 10. pH 7·5, $E_h + 59$ mV. Inhibition of growth. Note Gram-negative patches and disintegrated rods.



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Naturally Occurring Methicillin-Resistant Staphylococci

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SUMMARY

Naturally occurring methicillin-resistant strains of *Staphylococcus pyogenes* isolated in hospitals in Britain, France and Denmark were studied. All strains belonged to a few closely related bacteriophage types and all behaved similarly in the presence of methicillin. The minimum inhibitory concentration of methicillin for the strains ranged from 5 to 100 μg./ml., but on ordinary nutrient agar growth in the presence of concentrations well below this was much less luxuriant than on control plates without antibiotic and tended to be confined to the site of heavy inoculum. Gram-stained films from these cultures showed great irregularity in the size and staining of the cocci and many swollen forms were seen, suggesting that methicillin might be inhibiting cell wall synthesis without preventing multiplication. Further work showed that with addition of an excess of electrolytes (5 % NaCl or 7·5 % $(NH_4)_2SO_4$) or decrease in agar concentration, growth in the presence of methicillin was almost equal to that on control plates. The addition of uracil to a partially defined medium had no significant effect. Initial incubation under anaerobic conditions also improved growth of these strains in the presence of methicillin.

INTRODUCTION

Naturally occurring methicillin-resistant strains of *Staphylococcus pyogenes* have now been isolated from patients in hospitals in Britain (Jevons, 1961; Knox & Smith, 1961; Barber, 1961, 1962; Stewart & Holt, 1963), France (Chabbert & Baudens, 1962) and Denmark (Eriksen & Erichsen, 1963). In spite of their diversity of origin all the recorded strains share similar rather peculiar features and, as was the case with the first benzylpenicillin-resistant staphylococci to be isolated from patients, all belong to a few closely related bacteriophage types. The present investigation was undertaken to study more carefully the characteristics of some of these strains isolated from different parts of the world.

METHODS

Source of strains. Preliminary studies were made on 59 strains; 49 came from five different hospitals in Britain, 6 from Paris and 4 from Copenhagen. Their bacteriophage types are given in Table 1. More detailed investigations were made on the following 10 strains; A. 34, A. 44, H.M.R. 4, H.M.R. 6, H.M.R. 7, H.M.R. 37, Q.M. 3 (isolated in Britain); P. 6, P. 10 (from Paris); E. 1 (from Copenhagen). The bacteriophage and antibiotic-sensitivity patterns of these strains are given in Table 2.

Culture media. For most tests the medium used consisted of (%, w/v): Lab-Lemco, 1; peptone, 1; sodium chloride, 0·5; with or without Oxoid No. 3 agar, 1·2. This medium is referred to as Lab-Lemco broth or agar. For testing the effect of electrolytes the base was the same without the sodium chloride.

Table 1. *Bacteriophage patterns of strains of Staphylococcus*

Phage pattern	No. of strains	Phage pattern	No. of strains
7.47.53.54.75.77	3	53.77	5
7.47.54.75.77	1	75.77	4
47.53.75.77	10	53.75	1
53.54.75.77	2	47.54	1
47.53.54.77	1	77	6
7.47.53.54	1	42E.53.54.75.77	1
53.75.77	4	80.47.53.83.77	3
53.54.77	2	83	6
54.75.77	1	Non-typeable	7

Table 2. *Bacteriophage and antibiotic sensitivity patterns of staphylococcal strains selected for further study*

	Antibiotic					
	Penicillin	Streptomycin	Tetracycline	Chloramphenicol	Erythromycin	
	Sensitivity on ditch plates					Bacteriophage pattern
A. 34	R	R	R	S	S	7.47.53.54.77
A. 44	R	R	R	R	S	53.75.77
H.M.R. 4	R	R	R	R	S	47.53.75.77
H.M.R. 6	R	R	R	S	S	47.53.75.77
H.M.R. 7	R	R	R	R	S	47.53.75.77
H.M.R. 37	R	R	R	S	S	80.47.53.83.77
Q.M. 3	R	R	R	S	S	75.77
P. 6	R	R	R	S	R	53.77
P. 10	R	R	R	S	R	7.47.53.54
E. 1	R	R	R	S	S	n.t.

R = resistant; S = sensitive; n.t. = non-typeable.

The partially defined medium had the following composition (per l.): a salt agar base consisting of K_2HPO_4, 0·7 g., KH_2PO_4, 0·2 g., agar, 1·2 g.; the other ingredients were sterilized separately by pasteurization and added aseptically to give final concentration as follows: glucose, 0·2–0·5 g.; acid casein hydrolysate, 10·0 g.; tryptophane, 0·5–5·0 mg.; nicotinic acid, 0·5–1 mg.; thiamine, 0·5–1 mg. For some purposes uracil was added to 50 mg./l.

RESULTS

When tested by a serial dilution technique using a fairly large inoculum (a 2–3 mm. loopful of an overnight broth culture) the minimum inhibitory concentration of methicillin for the strains studied ranged from 5 to 100 μg./ml., but when tests were made on solid medium it was apparent that in the presence of concentrations of methicillin well below the minimum inhibitory concentration only a pro-

portion of the inoculum grew and the growth was quite unlike that on control plates. Cross-resistance of a similar order was noted with cloxacillin, nafcillin, and ancillin. Typical results are given in Table 3 and illustrated in Pl. 1. It will be seen that with methicillin 5 μg./ml., only two of the six naturally occurring resistant strains (A. 34, Q.M. 3) gave typical staphylococcal growth; at higher concentrations growth of all strains was much less luxuriant than that of control cultures, and this was either completely confined to the site of heavy inoculum, or when single colonies were present elsewhere they were very small and semi-transparent. These appearances were essentially similar with methicillin and cloxacillin, except that the effects were seen with the latter in a concentration about one-quarter to one-fifth that of methicillin. The laboratory-induced methicillin-resistant strains (18 R in table 3 and Pl. 1, fig. 1) grew uniformly whether in presence or absence of the concentrations of methicillin used.

Table 3. *Growth of staphylococcal strains on Lab-Lemco agar + methicillin*

Resistant strain	Concentration of methicillin (μg./ml.) Growth effect 5	20	50	Minimum inhibitory concentration
Naturally occurring				
A 2	± v	± v	−	25
A. 34	+ + + N	+ v	± s & t	Not tested
A. 39	+ s & t	−	−	20
A. 44	+ + + v	−	−	20
P. 6	+ + + m	+ + + s	+ s	128
Q.M. 3	+ + + N	+ + + s	+ s	256
Laboratory induced				
18. R	+ + + N	+ + + N	+ + + N	128
Control strain	*0·5*	*1·0*	*2·5*	.
Oxford staphylococcus	−	+ +	+ + + N	.

− = no growth; ± to + + +, degrees of growth (+ + + growth on control plates); N = typical staphylococcal growth with colonies about 2 mm. diam.; m = colonies about 1 mm. diam.; s = colonies about 0·5 mm. diam.; t = pin-point colonies; v = colonies all sizes.

The appearance of massed growth at the site of heavy inoculum (see Pl. 1, fig. 2, 4, 5 & 6) suggested that in the presence of methicillin the organisms could only multiply when close together, rather than that only a proportion of the organisms in the culture were resistant to methicillin. This was borne out by the fact that when colonies were picked from the plates containing methicillin 20 μg./ml. and retested, similar appearances were again seen. With repeated passages strains emerged which showed an increased minimum inhibitory concentration, but the growth remained atypical in appearance in relatively low concentrations. Within the limits pH 6·0–8·0 no significant differences were noted, either in the minimum inhibitory concentration or the appearance of growth. The addition of 5 % (w/v) horse blood or serum was without significant effect.

When films were made from cultures of the methicillin-resistant strains grown in the presence of methicillin or cloxacillin it was found that the cocci were irregular in shape, size and staining and large swollen organisms, similar to those seen when

staphylococci are grown in the presence of subinhibitory concentrations of benzylpenicillin, were frequent (see Pl. 2). This suggested that the methicillin was affecting cell-wall synthesis without inhibiting multiplication. It was decided, therefore, to see whether growth in presence of methicillin could be improved if the organisms were protected from lysis by increasing the electrolyte concentration. Another notable feature was the appearance of massed growth only at the site of heavy inoculum. This seemed reminiscent of the growth of some strains of *Pasteurella* when cultivated under ordinary aerobic conditions, and for this reason it was decided to test the effect of changing the gaseous conditions.

Table 4. *Effect of concentrations of NaCl and agar*

Staphylococcus strain	NaCl (%)	Agar (%)	Methicillin (μg./ml.) 5	Methicillin (μg./ml.) 20	Cloxacillin (μg./ml.) 1	Cloxacillin (μg./ml.) 5
			Growth effect			
H.M.R. 37	0	1·2	+++s	±M	+M	−
	0·5	1·2	+++s	+M	+M	±M
	5·0	1·2	+++v	+++v	++v	+t
	0·5	0·6	+++m	++v	n.t.	n.t.
P. 6	0	1·2	+++m & s	+M	+M	−
	0·5	1·2	+++m & s	+M	+M	+s
	5·0	1·2	+++m	+++m & s	+++s	+++s & t
	0·5	0·6	+++m	+++m & s	n.t.	n.t.
Q.M. 3	0	1·2	+++s	±M	+M	±M
	0·5	1·2	+++s	+M	+M	+M
	5·0	1·2	+++m	+++m	+++t	++t
	0·5	0·6	+++m	+++m	n.t.	n.t.

Methicillin-sensitive controls	NaCl (%)	Agar (%)	Methicillin (μg./ml.) 0·5	Methicillin (μg./ml.) 1·0	Methicillin (μg./ml.) 2·5	Methicillin (μg./ml.) 5·0
			Growth effect			
1	0·5	1·2	+++N	±	−	−
	5·0	1·2	+++N	±	−	−
2	0·5	1·2	+++N	+++N	±	−
	5·0	1·2	+++N	++N	++	−
3*	0·5	1·2	+++N	++s	−	−
	5·0	1·2	+++N	++s	−	−

n.t. = not tested; − = no growth; ± to +++, degrees of growth (+++ = growth on control plates); N = typical staphylococcal growth with colonies about 2 mm. diam.; m = colonies about 1 mm. diam.; s = colonies about 0·5 mm. diam.; t = pin-point colonies; v = colonies all sizes; M = massed growth at site of heavy inoculum only.

* Oxford staphylococci.

Effect of electrolyte concentration

Sodium chloride. Tests were made with nutrient agar containing no added NaCl, 0·5 %, or 5·0 % NaCl. Typical results are given in Table 4 and illustrated in Pl. 3. All strains tested gave a heavy growth in the presence of methicillin 20 μg./ml. when 5·0 % NaCl was added, although in the absence of added NaCl the growth on this concentration of methicillin was small and present only at the site of heavy

inoculum. Decrease in the concentration of agar by one-half gave similar results. The same effect in both cases was noted with cloxacillin at appropriately lower concentrations. An increase in the concentration of NaCl did not affect the methicillin sensitivity of methicillin-sensitive strains.

Other electrolytes. The effect of 5·0 % NaCl was reproduced by adding an isotonic concentration of KCl or $(NH_4)_2SO_4$. In the presence of methicillin 20 μg./ml. good growth occurred with the addition of 5 % NaCl or 7·5 % $(NH_4)_2SO_4$, whereas with 0·5 % NaCl or 0·75 % $(NH_4)_2SO_4$ it was poor and occurred only at the site of heavy inoculum. Intermediate results were obtained with 2 % NaCl or 3 % $(NH_4)_2SO_4$. The results obtained with two strains are illustrated in Pl. 3. Gram-stained films showed that with methicillin + excess NaCl the cocci remained abnormal, whereas with $(NH_4)_2SO_4$ appearances were nearer to those of typical staphylococci (see Pl. 2, fig. 11, 12).

Bacterial counts in presence of methicillin, with and without excess electrolytes

Serial dilutions of overnight cultures were spotted on plates containing various concentrations of methicillin between 2·5 and 20 μg./ml., with and without added electrolytes; typical results are shown in Table 5 and Pl. 4. In the absence of any electrolytes the undiluted culture gave a fair degree of growth which varied

Table 5. *Bacterial counts on medium containing methicillin, with or without excess electrolytes*

Methicillin (μg./ml.)	0·5 % NaCl				7 % $(NH_4)_2SO_4$			
	Dilution of culture							
Staphylococcus strain P. 6	Neat	10^{-2}	10^{-6}	10^{-7}	Neat	10^{-2}	10^{-6}	10^{-7}
0	+ + +	+ +	±	46N	.	.	.	.
2·5	+ + +	+	73s	52s	+ + +	+ +	±	31N
5·0	+ + +	+	63s	31s	+ + +	+ +	±	26N
10·0	+ +	+	15t	–	+ + +	+ +	±	27N
20·0	+ +	+	–	–	+ + +	+	23	31N
Strain H.M.R. 37								
0	+ + +	+ + +	+ +	74N	.	.	.	.
2·5	+ + +	+ + +	+	90N	+ + +	+ + +	56N	41N
5·0	+ + +	+ + +	75	32N	+ + +	+ + +	62N	23N
10·0	+ + +	+ + +	62m	34m	+ + +	+ + +	41N	20N
20·0	+ + +	+ +	7t	–	+ + +	+ +	20N	4N

n.t. = not rested; – = no growth; – to + + +, degrees of growth; N = typical staphylococcal growth with colonies about 2 mm. diam.; m = colonies about 1 mm. diam.; s = colonies about 0·5 mm. diam.; t = pin-point colonies.

only slightly with the concentrations of methicillin used, but from the first 10-fold dilution onwards little or no growth occurred. With some strains a similar picture was seen with 0·5 % NaCl, but with others (e.g. strain Q.M. 3) numerous small semi-transparent colonies appeared on the plates containing methicillin to the same dilution as colonies appeared on control plates. In the presence of 7 % $(NH_4)_2SO_4$ growth was similar in appearance to that on control plates, although with the higher concentrations of methicillin the actual counts were somewhat lower.

Tests on a partially defined medium and effect of uracil

The naturally occurring methicillin-resistant organisms grew quite readily on the partially defined casein acid-hydrolysate medium. Although colonies were not quite as large or as pigmented as on Lab-Lemco agar, rapid colony counts gave similar figures on the two media. When the strains were tested for sensitivity to methicillin on the casein acid-hydrolysate medium the results were similar to those obtained on Lab-Lemco agar with the usual concentration (0·5 %) of NaCl. Addition of uracil to 50 mg./l. did not significantly affect the results.

Changes in gaseous conditions

Anaerobiosis. Duplicate cultures were incubated for 18 hr at 37° aerobically and anaerobically (McIntosh & Fildes jar) and then left on the bench for a further 24 hr. It was found that on plates incubated anaerobically a heavy growth occurred in the presence of concentrations of methicillin which with aerobic incubation permitted feeble growth only at the site of the heavy inoculum. This was not due to inactivation of methicillin since the antibiotic showed a similar minimum inhibitory concentration for control methicillin-sensitive cultures, whether initial incubation was aerobic or anaerobic. Cultivation in the presence of an excess of carbon dioxide was without significant effect.

DISCUSSION

In spite of the diversity of origin, all the naturally occurring methicillin-resistant strains of *Staphylococcus pyogenes* studied in this work showed a similar and rather unusual type of resistance. On Lab-Lemco agar in the presence of concentrations of methicillin ten to twenty times less than those which completely inhibited multiplication, the usual picture was poor growth at the site of heavy inoculum only. Films of this growth showed organisms of irregular size, shape and staining properties, similar to penicillin-sensitive staphylococci grown in the presence of 'sub-inhibitory' concentrations of benzyl-penicillin; but, of course, in the latter case such changes are only seen with a concentration of benzylpenicillin near that of complete growth inhibition. Rogers & Jeljaszewicz (1961) showed that methicillin, like benzylpenicillin, inhibits the synthesis of cell-wall mucopeptide. The appearances just described are consistent with the view that methicillin inhibits cell-wall synthesis of naturally occurring methicillin-resistant staphylococci, at a concentration not much greater than the minimum growth inhibitory concentration for methicillin-sensitive strains. When, however, the methicillin-resistant organisms are protected from lysis by increasing the osmotic pressure of the medium, growth in the presence of methicillin is almost equal to that on antibiotic-free medium. When a high concentration (5 %) of sodium chloride is used to increase the osmotic pressure the morphology of the organisms remains abnormal, probably because this concentration of sodium chloride is not well tolerated by staphylococci; with an isotonic concentration of ammonium sulphate the morphology more nearly resembles that of a typical staphylococcus (see Pl. 2, 11, 12).

As recorded elsewhere (Ayliffe & Barber, 1963) naturally occurring methicillin-resistant staphylococci do not show an increased capacity to inactive methicillin. So far all the naturally occurring methicillin-resistant staphylococci recorded have

been penicillinase-producing strains. This may simply reflect the fact that hospital staphylococci are usually penicillin-destroying. But it was noted by Barber (1961) that *in vitro*, stable methicillin-resistant strains could only be isolated from penicillinase-producing staphylococci. Mandelstam & Rogers (1959) suggested that penicillinase production might not be the sole cause of resistance of such strains to benzylpenicillin.

Several investigators have shown that naturally occurring methicillin-resistant strains of Staphylococcus isolated in hospitals are fully virulent in the sense that they are capable of spreading in surgical wards and causing severe disease in some of the patients infected (Stewart & Holt, 1963; Ayliffe & Barber, 1963), but from the results of the present work it is clear that the resistance to methicillin is only a partial one, and to what extent it would operate *in vivo* remains to be determined. Certainly the picture is quite different from that obtaining with benzylpenicillin.

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EXPLANATION OF PLATES

Plate 1

Appearance of growth with presence of methicillin and cloxacillin

		Strains	
	Antibiotic (μg./ml.)	Upper half	Lower half
Fig. 1.	Methicillin 5	A. 44	Laboratory induced methicillin-resistant strain
Fig. 2.	Methicillin 10	H.M.R. 4	P. 10
Fig. 3.	0	H.M.R. 4	P. 6
Fig. 4.	Methicillin 5		
Fig 5	Cloxacillin 1	H.M.R. 4	P. 6
Fig. 6.	Cloxacillin 1	H.M.R. 4	P. 10

PLATE 2

Morphological appearances. Gram-stained films. Magnification 970.

Fig. 7. No antibiotic.

Figs. 8–10. Characteristic appearances in presence of methicillin or cloxacillin.

Fig. 11. Methicillin plus 5 % NaCl.

Fig. 12. Methicillin plus 7·5 % $(NH_4)_2SO_4$.

PLATE 3

Effect of NaCl concentration. All plates contain 20 μg./ml. methicillin and were inoculated with strains H.M.R. 7 (upper half) and P. 6 (lower half).

Fig. 13. 0 % NaCl. Fig. 14. 0·5 % NaCl. Fig. 15. 5·0 % NaCl.

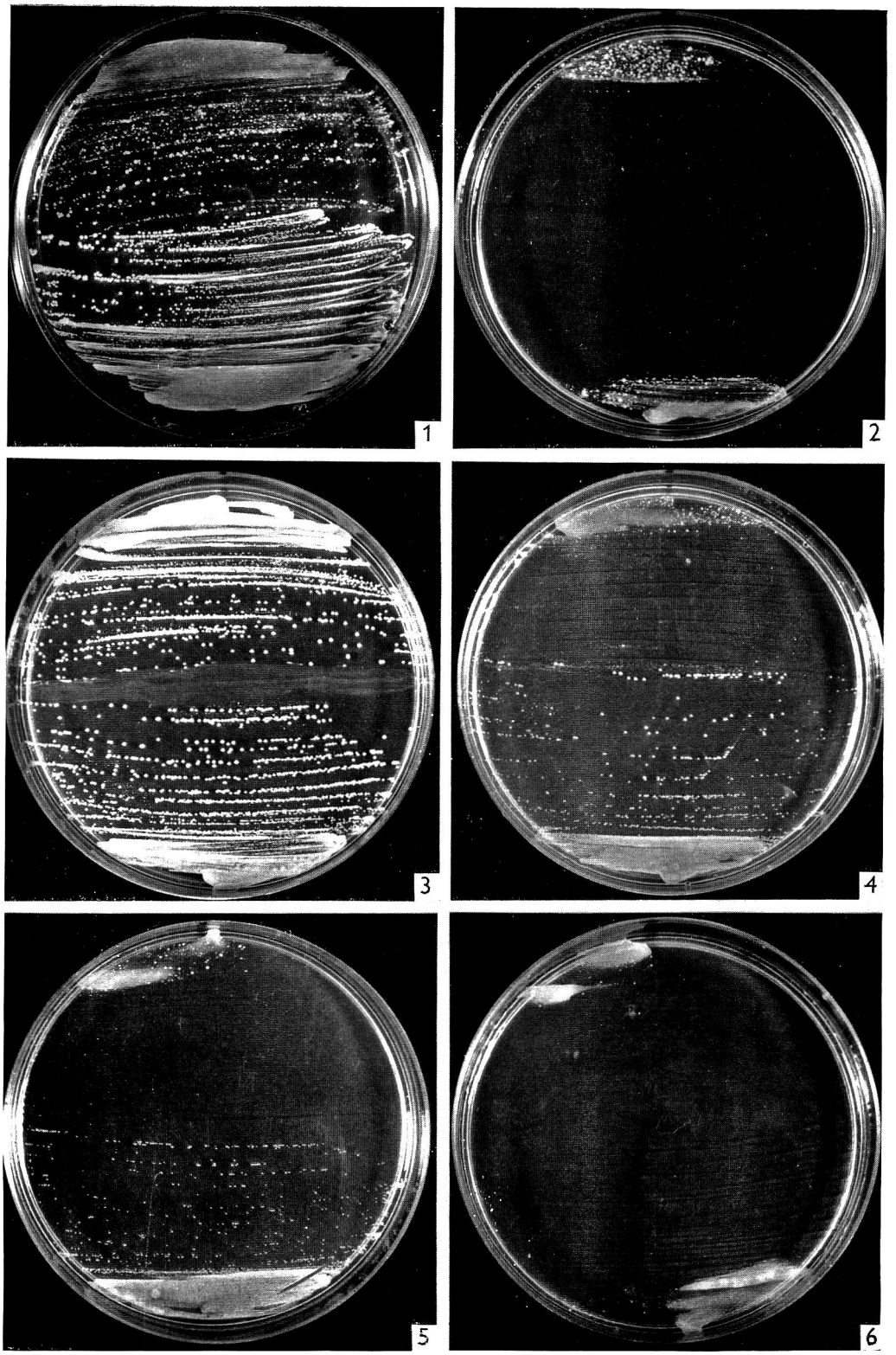
Effect of $(NH_4)_2SO_4$ instead of NaCl. All plates contain 20 μg./ml. methicillin and were inoculated with strains Q.M. 3 (upper half) and E. 1 (lower half).

Fig. 16. 0·75 % $(NH_4)_2SO_4$. Fig. 17. 3·0 % $(NH_4)_2SO_4$. Fig. 18. 7·5 % $(NH_4)_2SO_4$.

PLATE 4

Bacterial counts in presence of methicillin with and without added electrolytes. Both plates contain 20 μg./ml. methicillin and were inoculated with six serial tenfold dilutions of an overnight broth culture of strain Q.M. 3.

Fig. 19. No electrolytes. Fig. 20. 7·5 % $(NH_4)_2SO_4$.



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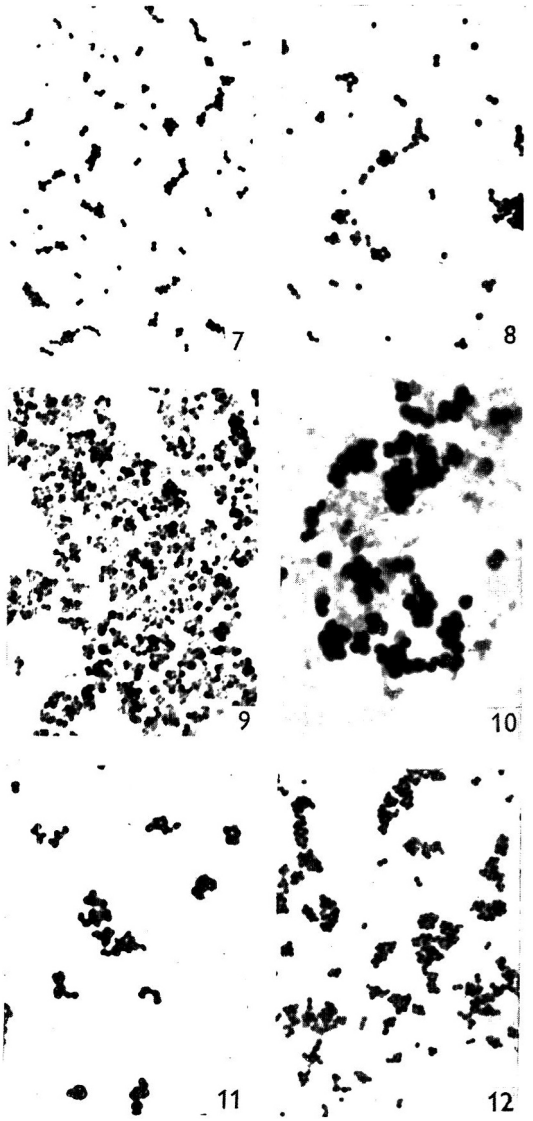
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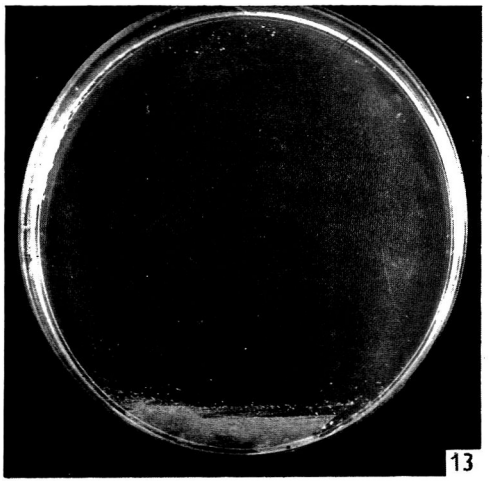
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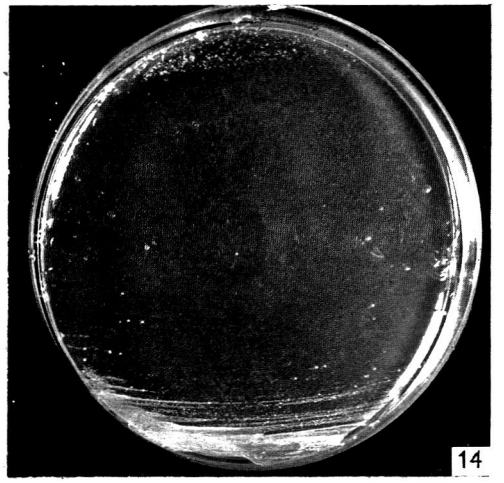
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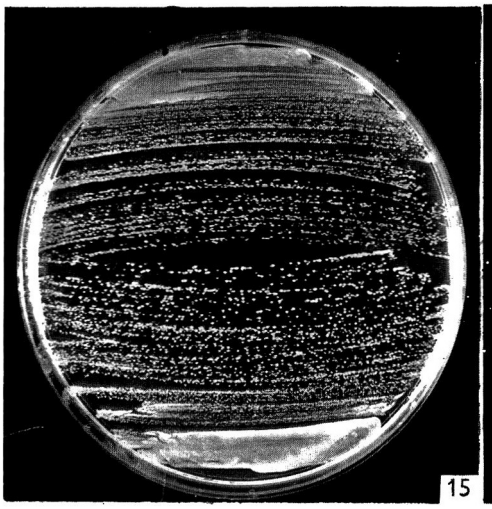
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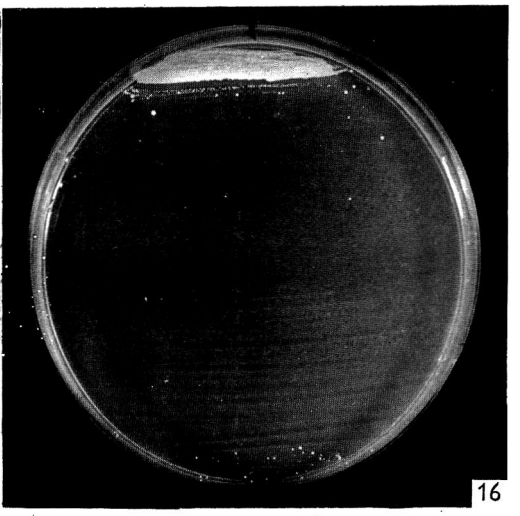
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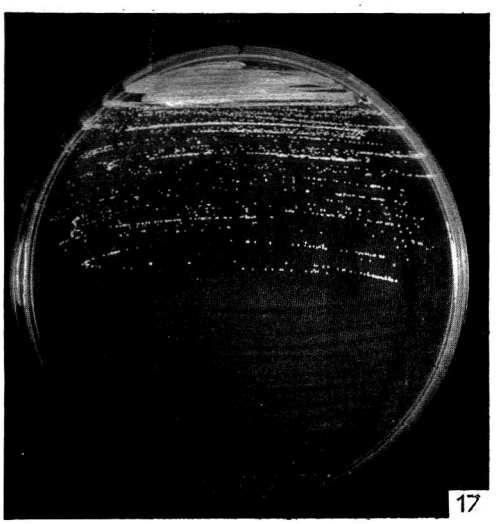
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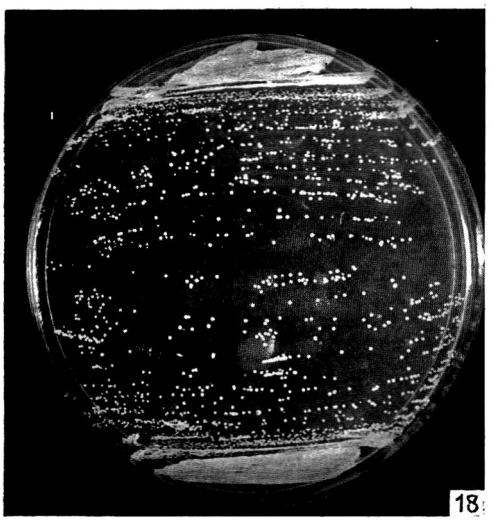
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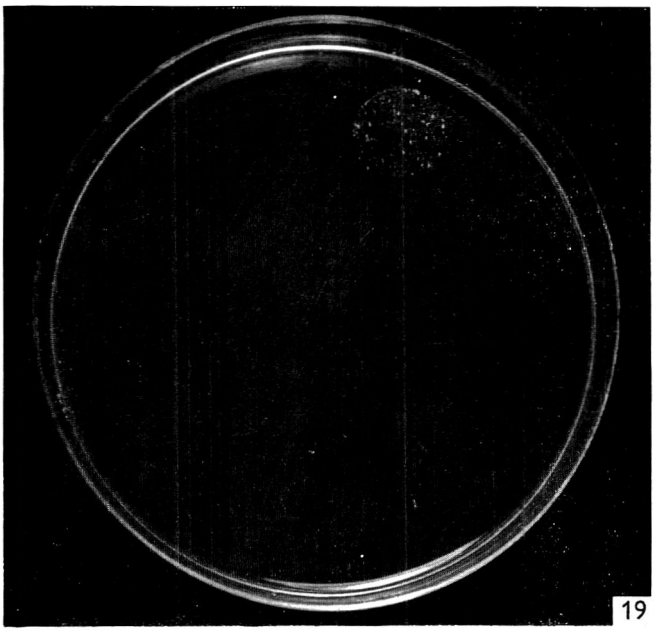
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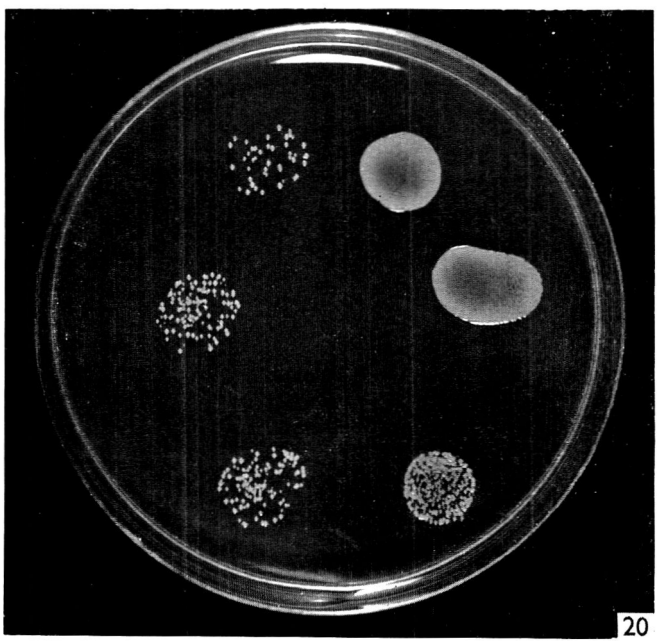
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Reversion Rate in Continuous Cultures of an *Escherichia coli* Auxotroph Exposed to Gamma Rays

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SUMMARY

Continuous cultures of the tryptophan-requiring strain of *Escherichia coli* known as WP2 were continuously exposed to gamma radiation for periods of several generation times. The frequency of prototrophic revertants increased steadily during irradiation at a rate which was proportional to the radiation dose rate. The change of revertant frequency per unit dose (rad) was equal to the induced reversion rate per mutable unit. This mutation rate (ρ) was independent of population density but slightly dependent upon the presence of supplements, such as nutrient broth, to the normal minimal medium with tryptophan. There was a marked dependence of ρ upon culture temperature, the values at 16°, 22° and 37° being $1{\cdot}2 \times 10^{-11}$, $2{\cdot}4 \times 10^{-11}$ and $3{\cdot}9 \times 10^{-11}$ per mutable unit per rad. It is probable that only a fraction of radiation-induced changes in the genetic material which could give rise to phenotypic reversions are actually expressed, this fraction being dependent on the post-irradiation temperature. The proportion of the population inactivated by radiation was intentionally kept at 10% or less in order to avoid difficulties in the interpretation of results. Other possible sources of error in ρ have been reviewed.

INTRODUCTION

Mutations which arise in continuous cultures of bacteria were first studied by Novick & Szilard (1950). By following the progressive accumulation of mutants induced in such cultures by mutagenic agents one can estimate the mutation rate and the delay in mutation expression (Kubitschek & Bendigkeit, 1958). The main purpose of the present experiments was to determine whether or not mutations were induced in growing bacteria with the same efficiency by gamma radiation at different dose rates and under different environmental conditions, e.g. different temperatures, population densities and growth media.

When the rate of growth of a culture is limited by restricting the supply of an essential component of the nutrient medium, as in the 'chemostat' of Novick & Szilard (1950) the population density and average generation time tend to constant values which are characteristic of the late log or early stationary phases of a normal batch culture. Other systems have been devised in which the optical density of a culture is automatically maintained at any desired value within the range of normal logarithmic growth, and the generation time then reaches a minimum value which is independent of the population density. Such a system has been termed a 'turbidostat' (Bryson, 1952).

Most previous genetic studies with continuous cultures known to the writers have been made with chemostats (e.g. Novick & Szilard, 1950, 1951; Fox, 1955; Kubitschek & Bendigkeit, 1961) and, although the theoretical behaviour of chemostatic populations has been examined very thoroughly, particularly by Moser (1958), selection pressures which exist under the prevailing conditions of extreme competition make the interpretation of experimental findings hazardous without other evidence. With turbidostatic cultures these particular selection pressures are eliminated and most of the others which remain can be investigated individually. In practice such cultures remain in dynamic equilibrium over many generation times and one can follow the small shifts from one steady state to another during periods of irradiation even with radiation levels which are so low that only a few per cent of the population are inactivated.

The usual staining procedures (Robinow, 1944) show two or more nuclei per bacterium in most strains of *Escherichia coli* in exponential growth, whilst X-ray survival data (Munson & Maclean, 1961) indicate that each bacterium is composed of a number of autonomous units, all of which must be inactivated to prevent colony formation. The numbers of nuclei and autonomous units are approximately equal so it may be reasonably assumed that each bacterium contains at least two complete genomes. If attention is restricted to mutations at a particular locus, a cell containing say four genomes can undergo mutations at any one of the four loci. From the radiation viewpoint each of the four loci can be provisionally regarded as a mutable unit.

Suppose that a growing population of bacteria is exposed to ionizing radiation at a dose rate d for a time Δ and the frequency of mutant bacteria is measured after a period sufficiently long to allow full expression of the mutation and complete segregation of the mutable units. It can be shown (Appendix) that the increase in mutant frequency would be proportional to $d\Delta\rho$ where ρ is the mutation rate per mutable unit per unit dose (rad). This increase would thus be a measure of ρ if the total radiation dose $d\Delta$ were known. Alternatively one could measure the mutant frequency at intervals during an exposure lasting many generation times and deduce ρ from the rate of increase of frequency which is finally reached, namely $d\rho$ (Appendix). Both methods would give the same value of ρ if there were no complicating factors such as selection. One possible selective factor could arise from a difference in the bactericidal efficiency of radiation for wild-type and mutant organisms and this would become more important with increasing radiation dose per generation time. For this reason the lowest levels of radiation which gave satisfactory yields of mutants were used in the present experiments.

METHODS

A cylindrical lead castle with walls, floor and roof 8 cm. thick housed the bacterial cultures and two 10c ^{137}Cs gamma ray sources. Each source was fixed to the lower end of a sliding vertical rod and could be lowered from the 'safe' position where it was recessed within the roof of the castle to any desired position for irradiation of the cultures. When both sources were in their safe positions the castle roof could be rolled away on horizontal rails to a position clear of the culture space. In this storage position the stray radiation within and around the culture space was

negligible (less than 5 m-rad/hr). A mechanical interlock prevented the sources being lowered unless the roof was accurately centred over the castle, so that accidental exposure of the operator was impossible.

Spaces were provided for six culture vessels C_1, C_2, ... (Fig. 1) of the type described previously (Maclean & Munson, 1961) at 60° intervals on a horizontal circle of 10 cm. diam. centred on the vertical axis of the castle. One source A was located on this axis, the other B was on a parallel line 2·8 cm. away. The average dose rate within a culture was measured by an ionization chamber having a gas space of the same shape and size as the culture and which could be accurately located in the same position. The combination of the two sources gave considerable flexibility in the choice of dose rates within the range 45–1000 rad/hr.

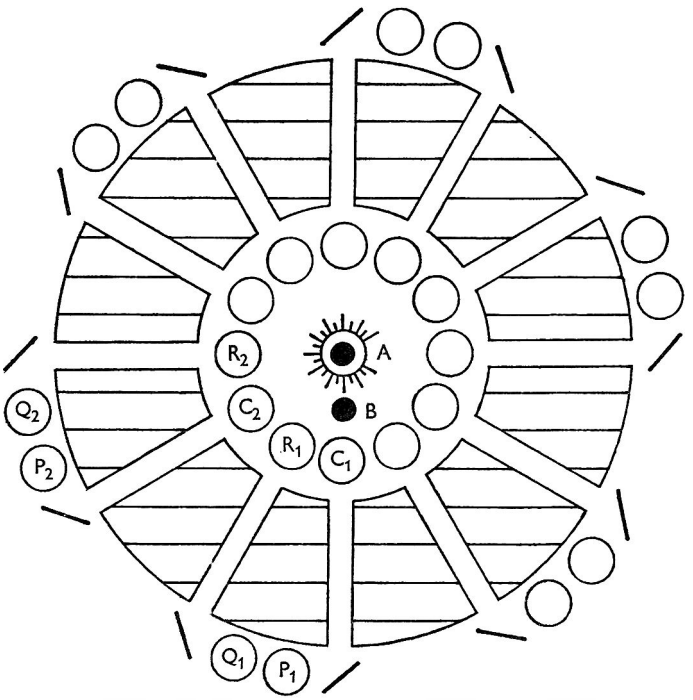

Fig. 1. Schematic sectional view of the lead castle with the 10c ^{137}Cs sources A and B, the culture vessels C_1, C_2, ..., and their corresponding reference tubes R_1, R_2, ... The system controlling the opacity of each culture, e.g. C_1, was based on a 'balance' of the currents in the associated photocells, e.g. P_1, Q_1, which received light transmitted by the culture and the reference tube respectively.

Light from a 30 W. lamp at the centre of the six culture array passed through each culture and thence through holes in the castle wall to blue-sensitive vacuum photo cells. In order to increase the amount of light transmitted by the culture and to minimize the amount of scattered light, lenses and suitably designed stops were included in the light channels. Possible gamma radiation damage to the photosensitive cathodes was prevented by turning the light away from the beams of stray gamma radiation with plane mirrors. Adjacent to each culture vessel, e.g. C_1, there was a glass tube, e.g. R_1, of similar size and shape filled with a solution of

ceric sulphate at a concentration dependent upon the desired optical density of the culture. The light channels for these reference tubes were similar in all respects to those for the cultures.

Each culture was maintained at an optical density close to the desired value by its own control system which functioned as follows. When a culture growing for example in C_1 reached the desired opacity the current through P_1 became equal to the current through the photocell Q_1 in the corresponding light channel. This equality was recognized by an electronic amplifier and a valve controlling the flow of nutrient medium which had previously been closed then opened. The optical density of the culture started to fall and when it had changed by an amount which could be detected by the amplifier the valve closed again. The number of drops passed at one opening was usually between 5 and 10 (0·33–0·66 ml.) depending upon the optical density of the culture and upon the number of drops at any one time in the supply tube to the culture. The valve opened and closed at regular intervals of a few minutes at normal growth temperatures. The two photocells associated with each culture e.g. P_1 and Q_1 were arranged in series and 50 V. was applied across them. Changes in the current through P_1 gave rise to changes in the potential of their common point and this was the signal detected by the amplifier.

Pieces of heat-absorbing glass (Chance ON 20) placed close to the central lamp prevented most of the radiant heat from reaching the cultures and this absorbed heat, together with the 30 W. generated locally, was removed by water circulating through a jacket surrounding the lamp. The water in this closed system also circulated through a parallel branch consisting of large copper coils in good thermal contact with the outside of the castle. The castle and its contents were thereby maintained at a uniform temperature. Temperature control in the range 10–40° was effected by a combination of a thermostatically controlled heater immersed in the circulating water and a refrigerating unit which maintained a constant temperature in the air space surrounding the castle.

The rate at which medium was supplied to a culture was estimated from the volume of overflow received in a given time and also by counting individual drops in the supply line using an automatic counting and timing device. A drop of medium after falling 1 cm. interrupted a light beam between a lamp and photocell (Mullard ORP 60). The resulting current pulse after amplification actuated a ratchet motor mechanically linked with a pen so that each drop caused the pen to move 0·5 mm. vertically over a recording chart. The chart was driven horizontally at a uniform speed so that the gradient of the trace indicated the flow rate. From the flow rate and the volume of the culture vessel the growth rate could be estimated (Maclean & Munson, 1961).

Organism and culture media. A sample of a tryptophan-requiring strain of *Escherichia coli* known as WP2 was kindly supplied by Dr B. A. Bridges and from this a large batch of freeze-dried samples was prepared. For a single group of experiments a stock suspension was grown from one ampoule following the method of Kada, Brun & Marcovich (1961) and this was used for not more than 1 month.

Cultures were grown in a glucose salts medium (medium 'M'; Haas & Doudney, 1957) supplemented usually with 6 μg. DL-tryptophan/ml. Other concentrations of tryptophan were sometimes used as indicated and in some experiments an amino acids pool (Kada, Doudney & Haas, 1961) or nutrient broth was added.

Continuous cultures were normally sampled by collecting a few ml. of outflow in a tube held at a constant temperature, usually 0°.

Scoring of try⁺ revertants. The number of *try*⁺ revertants/ml. was estimated in one or more of the following ways. (1) By retaining all bacteria, usually 5×10^8, from a measured volume of a sample on a 'Millipore' filter (0·46 μ pore size, 40 mm. diam. useful area), washing three times with 5 ml. amounts of 0·85 % (w/v) NaCl, placing the filter on minimal agar (M medium in 0·75 % agar) and incubating for 48 hr. at 37°. (2) By proceeding as in (1) but omitting the saline washes and either (*a*) incubating for 48 hr at 37° on minimal agar supplemented with 0·2 μg. tryptophan/ml., or (*b*) incubating for 2·5 hr at 37° (three generation times) on minimal agar supplemented with 20 μg. tryptophan/ml. and then transferring to minimal agar for 45 hr at 37°. (3) By collecting bacteria on a filter as in (1), washing them off and adding the suspension to molten agar containing M medium and tryptophan to give a final concentration in a pour plate of 0·8 μg. tryptophan/ml.

Experience showed that the number of *try*⁺ mutant colonies appearing on a Millipore filter on M agar was proportional to the total number of bacteria retained on the filter provided this did not exceed 7×10^8. On enriched plates (2(*a*) and 2(*b*)) and pour plates (3) more colonies always appeared, the excess compared with M plates being usually between 3 and 12 depending on the number of cell divisions of the *try*⁻ bacteria which occurred on the filter (Glover, 1956). Allowance was made for these plate mutants in calculating mutant frequencies on enriched plates.

Total and viable counts. The total population density was estimated by microscopic counts in a Thoma–Hawksley chamber and the viable count by spreading 0·1 ml. of a suitably diluted sample on nutrient agar and incubating for 24 hr at 37°.

Statistical errors in mutant frequency. The mutant frequency in a sample is given by the ratio of number of *try*⁺ colonies and the number of viable bacteria from which they arose. This ratio is subject to errors which are mainly statistical unless very large samples are used or mutant frequencies are high. Thus amongst a large number of replicate samples from one culture there is a Poisson distribution of the numbers of mutant colonies, so if there were an average of M mutant colonies per sample the variance would be M. Similarly, the variance of the number of mutants per culture in a large number of parallel cultures would be equal to the average number m of mutants per culture. Finally, the mutant frequency is proportional to the number n of colonies (or cells) counted in arriving at the viable (or total) count of a sample, so the variance amongst replicate counts is also n. It follows that the purely statistical part of the standard error in mutant frequency, expressed as a fraction, is $\left(\frac{1}{M}+\frac{1}{m}+\frac{1}{n}\right)^{\frac{1}{2}}$.

In a typical experiment one would have numbers such as $M = 50$, $m = 100$, $n = 200$ corresponding to a fractional standard error of 0·187.

Numbers of nuclei and lengths of organisms. The lengths of a representative number of bacteria from each sample were routinely measured microscopically and numbers of nuclear bodies were also determined in some experiments using the methods described earlier (Munson & Maclean, 1961).

RESULTS

Continuous cultures were first grown in the absence of radiation for at least fifteen to twenty generation times under the desired constant environmental conditions. During this period each culture closely approached a steady state, the growth rate, population density and average bacterial length tending to apparently constant values. Having established this steady state gamma irradiation commenced with the lowering of the sources to positions giving the required dose rate.

Decrease of growth rate and viability during irradiation. During an irradiation lasting a few generation times the growth rate of a culture remained unchanged for the first one or two generation times then fell gradually and finally reached a constant value. The fall in growth rate was approximately proportional to the dose rate at each temperature, being 0·02/hr at 200 rad/hr at 37° and 22° and approximately half this value at 16°. After the exposure it slowly recovered to its original value. An example of these changes can be seen in the upper half of Fig. 2. The viable fraction of the population also decreased during irradiation, although, with the low dose rates used in mutation studies, the fall was less than 10 % so that reliable measurements could not be made and after the first few irradiations viable counts were abandoned. Additional observations covering a much larger range of dose rates were made with strain WP2 and also with its *try*$^+$ revertants and by interpolation it was possible to estimate more accurately the viabilities at the low dose rates used in the present experiments. These measurements, which form part of another experimental study to be described elsewhere, confirmed that the viabilities of *try*$^-$ and *try*$^+$ organisms during the current experiments were indeed close to 100 %.

Frequency of try$^+$ *revertants on M plates.* The time course of the frequency of *try*$^+$ revertants amongst the total population during a typical experiment is shown in the lowest graph of Fig. 2. Before irradiation the *try*$^+$ frequency remained almost constant at approximately 10^{-8} over a period of 20 hr, the spontaneous mutation rate being $\sim 10^{-10}$/bacterium/generation time. With the onset of irradiation at 425 rad/hr it rose steadily after an initial delay of less than one generation time. After irradiation the frequency remained almost constant for at least several generation times. A linear regression was fitted to the observations during irradiation, each observation being weighted inversely as the estimated variance $\frac{M^2}{n^2}\left(\frac{1}{M}+\frac{1}{m}+\frac{1}{n}\right)$. The slope of this line corresponds to an apparent mutation rate of $1{\cdot}6 \times 10^{-9}$/mutable unit/hr, which in accordance with the definition of mutation rate used above can be expressed as $3{\cdot}7 \times 10^{-11}$/mutable unit/rad. The latter should be slightly smaller than the true value of ρ since the number of *try*$^+$ colonies is here compared with the sum of viable and non-viable *try*$^-$ organisms instead of the number of viable ones alone. The correction factor is therefore the reciprocal of the viable fraction of the whole population, which for this experiment is probably less than 1·05. An independent estimate of induced mutation rate was derived from the overall change in *try*$^+$ frequency using similar weighting factors for the observations before and after irradiation. This gave $\rho = 3{\cdot}4 \times 10^{-11}$/mutable unit/ rad., no correction being necessary since the viabilities at these times were very close to 100 %.

Experiments of this type have been carried out at culture temperatures of 16, 22 and 37° using different supplements to the growth medium, a variety of exposure times and dose rates covering the whole available range. The results were subjected to a detailed statistical analysis from which values of mutation rate were found as in the example above. Those within each temperature group were compared by analyses of variances. No significant differences of mutation rate within any group were found. The combined values of mutation rate with standard errors for each temperature are given in Table 1, those based on observations during irradiation (column 3) being uncorrected for loss of viability. Evidence of a dependence of mutation rate ρ upon dose rate d was sought by fitting the observations during irradiation to a multiple regression of mutation rate on dose rate per generation time τd and temperature T, assuming a linear relation of the form

$$\rho = G_0 + G_1 \tau d + G_2 T, \tag{1}$$

where G_0, G_1 and G_2 are constants. There was no evidence for a dependence of ρ upon τd although the evidence was highly significant for a dependence of ρ upon T.

The residual variance about each regression was compared with the theoretical value of unity and in only two cases in eighteen was there a significant indication that the true variances differed from the estimated ones. The weighting factors used appeared to be generally appropriate and there was no evidence of large random errors other than those considered.

There was no indication that ρ was dependent upon the population density of the culture within the range 10^7/ml. to $2{\cdot}5 \times 10^8$/ml. or upon the concentration of tryptophan between 2 μg./ml. and 20 μg./ml. The presence of a supplementary pool of amino acids (Kada, Doudney & Haas, 1961), each at 6 μg./ml., also had no effect. A 50 % nutrient broth supplement gave a barely significant reduction of 10 % at 37° and 22° whilst a single experiment at 16° showed a 30 % reduction. This lack of response to supplements was not shared by the growth rate, which increased with the amino acids pool by 20 % and with broth by 85 %.

Frequency of try$^+$ revertants on enriched M plates. After allowing for 'plate' mutants it was found that the frequency of induced mutants on enriched plates was always greater than that on M plates. Figure 3 shows the rise in frequency as scored on M plates (method (1) above) and M plates supplemented with tryptophan (methods 2(*a*) and 2(*b*) above) during an irradiation at 425 rad/hr at 25° in M medium supplemented with 6 μg./ml. of tryptophan. Both graphs appear to be of the same shape with a relatively steep initial rise indicating more than one mutable unit per bacterium (Appendix). This is consistent with observations on the average number of nuclei per bacterium.

Distribution of population with respect to length and number of nuclear bodies. The average length of bacteria of strain WP2 in unirradiated continuous cultures at 37 and 22°, namely 3·0 μ, was almost independent of population density up to 3×10^8/ml. in M medium with added tryptophan (2–20 μg./ml.) and was not measurably different when an amino acids pool was included. In these growth media the distribution with respect to length was closely similar to that of its parent strain *Escherichia coli* B/r when the latter was grown at 37° in minimal medium at a population density of 3×10^8/ml. (Maclean & Munson, 1961). The average number of nuclear bodies per organism in unirradiated continuous cultures at 37° was approxi-

mately 2 so that the average length per nucleus was rather larger than that previously reported for strain B/r (Munson & Maclean, 1961). At 16° the average length was 3·6 μ.

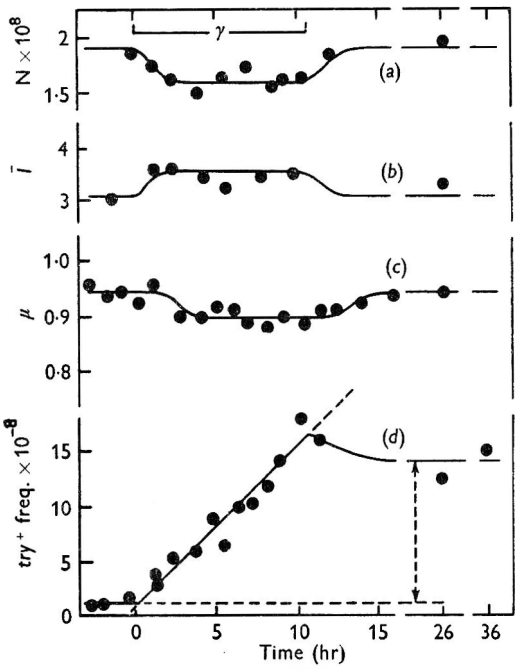

Fig. 2

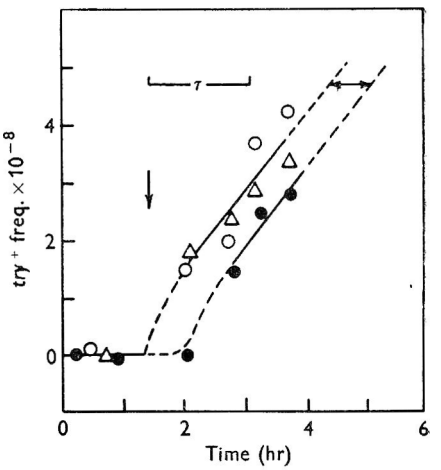

Fig. 3

Fig. 2. Changes in population density (*a*), average length (in microns) (*b*), growth rate (*c*) and revertant frequency ($try^+/try^- + try^+$) (*d*), of Escherichia coli WP2 during an exposure to gamma radiation at 425 rad/hr. The culture was grown at 37° in M medium + 6 μg. DL-tryptophan/ml. + amino acids 'pool'. The fall and subsequent rise in population density occurred at the beginning and end of the irradiation and coincided with the rise and fall in average length as expected for a constant opacity of the culture. Changes in growth rate showed a delay of one to two generation times. Mutation rates were estimated from the slope of the dashed line and from the overall change of frequency.

Fig. 3. The frequency of induced revertants scored during gamma irradiation on M and on M + tryptophan plates. The outflows from five continuous cultures of Escherichia coli WP2 growing in M medium + 6 μg. tryptophan/ml. at 25° were pooled and samples on Millipore filters were put on M agar (method 1, ●) and on M agar enriched with 0·2 (method 2*a*, ○) or 20 μg. tryptophan/ml. (method 2*b*, △). The graphs have been drawn on the assumption that the initial rises are steeper than the final slope, i.e. that there is more than one mutable unit per bacterium. The start of irradiation and the length of one generation time are shown.

Dependence of growth rate upon population density and tryptophan concentration. Growth rates of cultures of *Escherichia coli* WP2 at 37° in M medium supplemented with 2, 6 or 20 μg. tryptophan/ml. were measured at different population densities between 10^7 and 10^9/ml. Growth rates were the same at all three tryptophan concentrations and all population densities up to $2{\cdot}5 \times 10^8$/ml. but, as the population density was further increased, the growth rate with 2 μg. tryptophan/ml. diminished rapidly and became almost zero at $2{\cdot}7 \times 10^8$/ml. This sudden fall could only be interpreted in terms of tryptophan exhaustion at 2·7 ×

10^8/ml. For the higher concentrations of tryptophan curves very similar to those found previously for *E. coli* B/r (Maclean & Munson, 1961) were obtained, the growth rate falling gradually at densities greater than 3×10^8/ml. One may conclude from these observations that there was no limitation of growth due to deficiency of tryptophan or other constituents of the growth medium at the population densities usually employed in the present experiments.

DISCUSSION

The broad conclusion from the present experiments is that environmental factors other than temperature have an insignificant or marginal effect on the induced mutation rate for the reversion *try*$^-$ to *try*$^+$ in *Escherichia coli* strain WP2. Since the temperature factor is important in any theory of the nature of gene mutations, possible outstanding sources of error in the data of Table 1 may be considered. The first of these is the selective advantage or disadvantage of *try*$^+$ organisms particularly those induced in the early stages of an irradiation. In most experiments the number of revertants induced during say the first quarter of the irradiation period was between 10 and 100, and, if by chance an appreciable fraction of these had a marked selective advantage or disadvantage, the gradient of the graph of *try*$^+$ frequency, as in Fig. 2, would have altered with time. No statistical evidence of curvature of these graphs was found, the dispersal of the observed mutant frequencies about the regression lines being within the expected limits in 16 out of 18 cases. After irradiation, significant increases or decreases in *try*$^+$ frequency sometimes took place over a period of 30 or 40 generation times but these changes corresponded to weak selection pressures which would have been too small to produce significant changes in apparent mutation rate during the irradiation period.

Although viable counts have shown that the lethal effect of continuous gamma irradiation was small, it is satisfactory to find that the discrepancies between estimates of mutation rate by the two methods, only one of which was affected by loss of viability, are also small. One may therefore conclude that neither selection nor the lethal action of gamma radiation influenced the observed mutation rates substantially.

Lastly, when cultures of this strain are held in a state of arrested growth and exposed to rather large flash doses of ultraviolet light or X-rays, the expression of the mutation *try*$^-$ to *try*$^+$ is dependent upon a number of environmental factors during the first generation time of renewed growth (Witkin, 1956; Doudney & Haas, 1959; Kada, Doudney & Haas, 1961; Kada, Brun & Marcovich, 1961). The present experiments have shown that two such factors, namely, the presence of tryptophan and the temperature, are also effective with actively growing cultures exposed continuously to low-level gamma irradiation. In Fig. 3 the initial rise of *try*$^+$ frequency as estimated on enriched plates precedes the rise on minimal plates by about half a generation time. This result is to be expected if the genetic function of newly induced mutants is expressed during residual growth on enriched agar after plating and by contrast colonies on minimal plates could arise only from mutants in which genetic function was fully expressed before plating. The time interval separating the two parallel graphs would then represent the time required for expression of newly induced mutants in the growth medium apart from possible complications, e.g. the continuance of processes leading to expression despite lack

of exogenous tryptophan. This phenotypic delay will have no effect however upon the gradient of the graphs provided the delay remains unchanged.

As a result of changes in the routine of sample collection it became evident that there was a progressive fall in the score of *try*$^+$ colonies from samples taken during an irradiation and held at 0° for some hours. Errors in *try*$^+$ frequency arising from this were minimized by collecting for a constant time and keeping this as short as possible. Samples taken before irradiation and a few generation times after irradiation did not show the effect. One may therefore assume that only newly induced mutants were susceptible and if so no error was incurred in the estimates of mutation rates.

Thus it appears that the lethal effect of gamma radiation is the only likely source of systematic errors and this only affects the results for mutation rate derived from the slopes of the graphs. By allowing an arbitrary but not unrealistic average figure of 5% for this error one obtains the 'best' values for ρ given in the fifth column of Table 1.

Table 1. *Generation times and induced mutation rates from try$^-$ to try$^+$ for cultures grown in M medium + tryptophan at different temperatures*

Temperature	Generation time τ (hr)	Mutation rate/mutable unit/rad $\times 10^{-11}$			
		From slope	From overall change	ρ	$\rho\tau$
37°	0·72	3·60 ± 0·12	4·06 ± 0·16	3·89 ± 0·18	2·8
22°	2·0	2·28 ± 0·12	2·53 ± 0·38	2·42 ± 0·20	4·8
16°	5·0	1·13 ± 0·12	1·04 ± 0·16	1·13 ± 0·17	5·5

The uncorrected estimates of gamma ray-induced mutation rates in the third column have been combined (after allowing for a 5% error, see Discussion) with those of the fourth column to give the 'best' values in the fifth column. The mean values and their standard errors were derived from the following numbers of experiments: 11 at 37°, 7 at 22° and 7 at 16°.

Few investigations have been reported on the dependence of mutation rate upon temperature for bacteria. Anderson & Billen (1955) using large flash doses of X-rays found that the absolute numbers of induced revertants amongst four auxotrophic strains of *Escherichia coli* were greatest between 18 and 24°. In retrospect their results seem of dubious significance mainly because revertant colonies were scored on minimal agar plates which did not permit protein synthesis and thus probably inhibited expression. Spontaneous reversion rates of *Escherichia coli* 15 *his*$^-$ were measured with some precision by Ryan & Kiritani (1959), who concluded that the product of mutation rate and generation time was constant between 15 and 37°, in accordance with prediction from the copy-error hypothesis. This constancy was not found with induced reversions of *E. coli* WP2 *try*$^-$, as may be seen from the figures in the last column of Table 1.

Although the 'loss' of newly induced *try*$^+$ revertants has only been observed in samples kept at 0° for some hours, it is likely that a small proportion will also fail to express themselves at higher temperatures. Thus the sharp fall in mutation rate between 22 and 16° may be related to failure of expression during the average period of $\tau/2$ between the initial damaging event by radiation and the collection of the bacterium in the sample of outflow. Unless a similar explanation is invoked for the whole temperature range up to 37°, one would be forced to conclude either

that the initial damage, presumably to DNA, is also subject to repair or restitution at a rate which is temperature dependent or that this initial damage is less severe at lower temperatures.

APPENDIX

The induction of mutants in bacterial populations exposed to ionizing radiation for very short times (flash doses) and also continuously over many generation times will now be examined.

For simplicity it will be assumed that all bacteria of a synchronously dividing population are identical and that each undergoes binary fission to give two equal daughters at regular intervals τ. If each bacterium has p mutable units at the time of a flash dose D (i.e. at zero time) and the mutation rate per mutable unit per rad (unit dose) is ρ when averaged over one generation time, the probable number of gene mutations in the viable population of N cells is $D\rho pN$. Since the mutations occur randomly the probability of two occurring in the same cell is $D\rho^2p^2$, which in practice is negligibly small.

When bacteria are plated before cell divisions occur and each mutated gene gives rise to a mutant colony the apparent frequency of mutant bacteria amongst the population, namely (number of mutant colonies)/(number of viable bacteria plated) is $D\rho p$. If, however, the population continues to grow and divide for a time τ before plating it would then number $2N$ whilst the number of mutant cells would still be $D\rho pN$, assuming that the processes leading to mutation are irreversible and $p \geqslant 2$. The mutant frequency at time τ would then be $D\rho p/2$. If $p = 2^r$ where r is an integer then segregation of mutant genes would only be complete at time $r\tau$, the mutant frequencies at times 2τ, 3τ, ..., $r\tau$ being $D\rho p/2^2$, $D\rho p/2^3$, ... $D\rho p/2^r$. At times later than $r\tau$, the mutant frequency would be unchanged at $D\rho p/2^r$, i.e. at frequency $D\rho$, since $p = 2^r$. This is identical with the frequency of mutated genes immediately after their irradiation, namely $D\rho pN/pN = D\rho$.

Let us suppose that a continuous culture consists of bacteria each of which has either p or $2p$ mutable units per cell depending upon the relative phases of the replication and division cycles. We assume that all bacteria both mutant and non-mutant divide and replicate their genes at regular intervals τ, but since the population is asynchronous with a completely random distribution of division times the numbers of bacteria with p and with $2p$ mutable units per cell will always be constant apart from statistical fluctuations. Let N_1 and N_2 be the numbers of viable cells with $p(\ = 2^r)$ and $2p(\ = 2^{r+1})$ units respectively and let ρ, the mutation rate per mutable unit per rad, be assumed to be independent of radiation dose rate. If irradiation at dose rate d starts at zero time then at time t (where $t > \overline{r+1}\tau$) the numbers of mutants arising from the two classes of bacteria, namely m_1 and m_2, will be increasing at the rates

$$\frac{dm_1}{dt} = N_1\rho pd - \tfrac{1}{2}N_1\rho pd - \frac{1}{2^2}N_1\rho pd \ldots - \frac{1}{2^r}N_1\rho pd, \tag{2}$$

and

$$\frac{dm_2}{dt} = 2N_2\rho pd - N_2\rho pd - \tfrac{1}{2}N_2\rho pd \ldots - \frac{1}{2^r}N_2\rho pd, \tag{3}$$

where in each equation the first term represents the rate of induction of new mutants at time t, the second term the rate of loss in the outflow of mutants which

were induced at time $t-\tau$, the third term the rate of loss of mutants induced at time $t-2\tau$, ..., and the last term the rate of loss of those induced at time $t-r\tau$ in the case of equation (2) and at time $t-\overline{r+1}\tau$ in the case of equation (3). Mutants induced at times before $t-\overline{r+1}\tau$ will have completely segregated at time t and will not contribute additional mutants to the steady population since they divide at the same rate as non-mutants.

It can be readily shown that the right-hand side of equation (2) is $N_1\rho pd/2^r$ and of equation (3) is $N_2\rho pd/2^r$, but since $p = 2^r$ the rate of production of mutants from both classes of cell is

$$\frac{dm_1}{dt}+\frac{dm_2}{dt} = (N_1+N_2)\rho d = N\rho d. \tag{4}$$

Thus at times greater than $\overline{r+1}\tau$ the mutant frequency increases at a rate equal to the mutation rate per mutable unit, irrespective of the generation time or of the interval between cell division and replication of the gene concerned.

The above argument with slight modifications would apply if r were non-integral and unequal daughters arose at division and so the conclusion is valid under most conditions encountered in experimental continuous cultures for both induced and spontaneous mutations.

The mutation rate per mutable unit is also simply related to the change in mutant frequency resulting from the irradiation. If the time of irradiation were Δ and the total dose to the culture Δd, the mutant frequency measured at times later than $\Delta+\overline{r+1}\tau$ would be greater than the initial frequency by $\Delta\rho d$ (as for a flash dose Δd), since mutational damage is assumed to be irreversible and independent of the dose rate.

At the beginning of an irradiation the mutant frequency increases at a rate greater than $d\rho$ by a factor equal to the average number of mutable units per bacterium since the segregation of mutable units during the first small fraction of a generation time is negligible. The rate of increase of mutant frequency thereafter falls steadily until it reaches the value $d\rho$.

An important factor neglected in the above analysis is selection. If the mass growth rate (Powell, 1956) of the whole culture were μ and the selective advantage of the mutants were equivalent to a difference, real or apparent, of $\mu_m-\mu$ in growth rate, then the rate of increase in the number of mutants m at times greater than $\overline{r+1}\tau$ would be given by

$$\frac{dm}{dt} = N\rho d+(\mu_m-\mu)m, \tag{5}$$

which has the solution

$$m = N\rho d(e^{\overline{\mu_m-\mu}t}-A)/(\mu_m-\mu), \tag{6}$$

where A is a numerical constant. The number of mutants would therefore change with time in a perceptibly non-linear manner unless $\mu_m-\mu$ were small.

Advice on a suitable design for the amplifiers of the control equipment was kindly given by Mr F. S. Williamson. Much of the apparatus was designed and made by Mr R. J. C. Hudson. To them both we wish to express our gratitude. We also acknowledge the help of Mr D. G. Papworth, who was responsible for much of the statistical analysis.

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The Composition of *Staphylococcus aureus* in Relation to the Water Activity of the Growth Medium

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SUMMARY

Staphylococcus aureus organisms, grown in basal medium of water activity (a_w) 0·993 and in basal medium adjusted to several different a_w values by addition of NaCl, were analysed for the following components: sodium, potassium, calcium, magnesium, total amino acids, inorganic phosphate, chloride, water, DNA, RNA, protein. Cell water decreased from 1·66 to 0·83 g./g. dry weight when a_w was decreased from 0·993 to 0·90. Internal concentrations of solutes generally increased with decrease in a_w, potassium and phosphate concentrations being greatest at 0·92 a_w and sodium, chloride, magnesium, and amino acids at 0·90 a_w, the lowest a_w value studied. Increases in potassium and amino acid concentrations resulted largely from the decreased water contents of cocci grown at low a_w values. Intracellular sodium and chloride concentrations were much lower than and proportional to the NaCl concentration in the medium. The predominant cell solute was potassium at 0·92 a_w and above, and sodium below 0·92 a_w. The data are discussed in relation to the inhibition of bacterial growth at low a_w values.

INTRODUCTION

Staphylococcus aureus organisms possess a high osmotic pressure, or low water activity (a_w), when grown in normal media. Mitchell & Moyle (1956) suggested from vapour pressure equilibration of washed cocci that their water activity was equivalent to that of 1·0 molal sucrose solution, namely about 0·98 a_w. Christian & Waltho (1962*b*) obtained a corrected freezing point of unwashed, heated staphylococci of $-3·27°$, corresponding to about 0·97 a_w. Both values are appreciably lower than the a_w values of the growth media, which were 0·999 and 0·993, respectively. Clearly, these organisms maintain a large turgor pressure when grown in dilute media, and recent data (Christian & Waltho, 1962*a*) suggest that potassium ion and amino acids may be osmotically important components of such cocci, while sodium, phosphate, and chloride are less prominent.

Staphylococci are extremely salt tolerant, growing at a_w values down to 0·88 to 0·86 (Scott, 1953). As bacteria appear to be hypertonic to their growth medium, irrespective of its a_w value (Christian & Ingram, 1959), the concentrations of low molecular weight solutes in staphylococci grown at low a_w values are of some interest. The present paper reports partial analyses of *Staphylococcus aureus* grown

at a_w values of 0·993 to 0·90. Sodium, potassium, calcium, magnesium, total amino acids, inorganic phosphate, chloride, water, DNA, RNA, and protein were determined.

METHODS

Organism and growth conditions. The test organism was *Staphylococcus aureus* strain 49/1974 (Scott, 1953). The basal medium was brain + heart infusion broth of 0·993 a_w, adjusted where required to a_w values of 0·97, 0·94, 0·92, and 0·90 by addition of NaCl, unless otherwise stated in the text. Scott (1953) discussed in detail the control of the a_w value in culture media. Volumes (600 ml.) of medium were inoculated from a 16 hr broth culture and aerated by shaking at 30° until the population reached the stationary phase. The cocci were harvested by centrifugation, washed in a NaCl solution isotonic with the growth medium, and suspended in 40 ml. of the NaCl solution. When harvested from media adjusted to 0·97 a_w with sucrose or to 0·92 a_w with KCl, the washing was omitted and the cocci were resuspended in 40 ml. of the original supernatant fluid. Eight equal coccal pellets were obtained by centrifuging 5 ml. portions of the concentrated suspension.

Analytical methods. Interstitial space was determined by phosphate dilution and was used to correct water content, dry weight, and chemical analyses for contributions of the contaminating interstitial solution. These techniques were described in detail by Christian & Waltho (1962*a*), as were methods for extraction and analysis of sodium, potassium, total amino acids (as ninhydrin-positive substances), inorganic phosphate, and chloride. In studies of dextran-impermeable water, dextran was determined by the anthrone method (Scott & Melvin, 1953). Additional analyses performed were for magnesium and calcium by atomic absorption spectroscopy and protein by the method of Lowry, Rosebrough, Farr & Randall (1951). Deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) were extracted by the method of Burton (1956) and determined with the diphenylamine and orcinol reagents, respectively. The nitrogen content of trichloroacetic acid-extracted cocci was determined by the Kjeldahl procedure. All analyses were performed in duplicate.

The water sorption isotherm at 25° of heated cocci was obtained by the isopeistic technique of Robinson & Sinclair (1934). The coccal preparations were equilibrated with saturated solutions of potassium dichromate, potassium dihydrogen phosphate, potassium nitrate, and barium chloride. The a_w value of saturated potassium dihydrogen phosphate was found in this laboratory to be 0·952 at 25°; those of the other solutions were given by Stokes & Robinson (1949).

RESULTS

Cell water content

When phosphate dilution is used to determine the extent of interstitial space in a centrifuged pellet, it is assumed that the volume found represents that space which is rapidly and passively permeated by other solutes of low molecular weight. In preliminary experiments with 0·02 M-Na,K phosphate buffer and water-washed cocci, the volumes accessible to sodium and potassium were much greater than that accessible to phosphate. It appeared that surface charges or differences in perme-

ability were affecting the distribution of these ions. However, the values for the three ions were not appreciably different with 0·993 a_w buffer (about 0·2M). This concentration of phosphate (with the addition, where necessary, of NaCl to give the solution the same a_w value as the growth medium), was used routinely in the determination of interstitial volume.

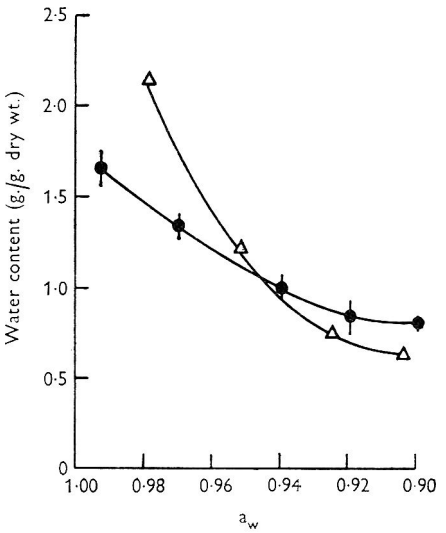

Fig. 1

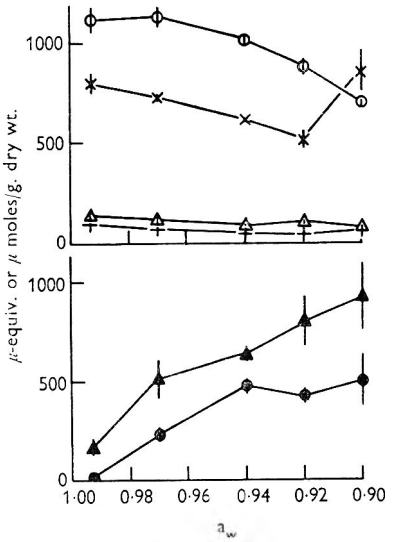

Fig. 2

Fig. 1. Water content of Staphylococcus aureus in relation to the external a_w level. ●, Cells grown in broth adjusted with NaCl to a_w levels from 0·993 to 0·900. Standard errors of mean values are shown by vertical lines. △, Cells grown at 0·993 a_w, heated and equilibrated to four a_w levels.

Fig. 2. Amounts of six solutes in cells of Staphylococcus aureus grown in broth adjusted with NaCl to a_w levels from 0·993 to 0·90. ▲, Sodium; ○, potassium; +, magnesium; ×, total amino acids; △, inorganic phosphate; ●, chloride. Standard errors of mean values are shown by vertical lines. Sodium, potassium and chloride are expressed as μ-equivalents, the remainder as μmoles.

The water content of staphylococci as a function of the a_w value in the medium is shown in Fig. 1. Reduction of a_w from 0·993 to 0·90 by addition of NaCl decreased cell water by 50 % from 1·66 to 0·83 g./g. dry weight. This change in cell water was related to a_w value, not to NaCl concentration, since similar values were obtained with sucrose (0·97 a_w) and KCl (0·92 a_w) additions (Table 1). Increasing the a_w value to 0·998 by dilution of the medium with water increased the cell water content to 2·44 g./g. dry weight (Table 3).

For comparison, Fig. 1 includes the water sorption isotherm for staphylococci grown in basal medium (0·993 a_w), washed in water and heated to destroy the osmotic barrier. At high a_w values, whole cocci held much less water than heated cocci, suggesting that the a_w within the whole coccus was substantially below that of the growth medium. The situation was reversed at low a_w values, and could be explained if cocci grown at 0·92 and 0·90 a_w had higher solute contents than the heated preparation, which was grown at 0·993 a_w.

Mitchell & Moyle (1956) found the difference between phosphate-impermeable and dextran-impermeable water of staphylococci to be 0·18 ml./g. dry wt. This was about 12 % greater than the phosphate-impermeable or intracellular water, and was interpreted as the water content of the cell wall. In the present studies, similar experiments indicated that the water in the cell wall was 11 % greater than the intracellular water after growth at 0·993 a_w, and 14·5 % greater after growth at 0·90 a_w.

Internal solutes of Staphylococcus aureus

The sodium, potassium, magnesium, amino acid, chloride, and inorganic phosphate contents of cocci grown at various a_w values in NaCl-adjusted media are shown in Fig. 2. Values are expressed on the basis of cell dry wt. Sodium and chloride contents increased with decrease of a_w value to 0·90, probably reflecting the increased NaCl concentration in the medium. The other components decreased between 0·993 and 0·90 a_w, with the exception of the amino acid pool, which increased steeply below 0·92 a_w. Assuming an average molecular wt. of 120 for the amino acids, the solutes of Fig. 2 represent about 16 % of the dry wt. of cocci grown at 0·993 a_w and 18 % when grown at 0·90 a_w.

Figure 3 presents the apparent intracellular concentrations of these solutes, obtained by expressing the values of Fig. 2 in terms of the cell water contents of Fig. 1, and assuming that all of these ions and molecules were in free solution within the cell. The concentrations are *molal*, i.e. moles of solute/kg. cell water. On this basis, intracellular sodium and chloride concentrations increased approximately linearly down to 0·90 a_w. The mean ratios of internal to external concentration were 0·41 for sodium and 0·23 for chloride. Increases occurred in potassium, amino acids, and phosphate concentrations as the a_w value was decreased to 0·92. The ratios of these three concentrations were 100:71:12 at 0·993 a_w and 100:60:11 at 0·92 a_w. Decreasing the a_w value to 0·90 decreased the potassium and phosphate concentrations, while the amino acids increased by over 70 %. As the amounts of these three constituents in the media were constant at all a_w values, the changes observed in intracellular concentrations were due to either the value of a_w or the concentration of NaCl in the growth media. The calcium content of cocci, although not precisely determined, was less than 6 μ-equiv./g. Cocci were much richer in magnesium, which occurred in highest concentration at 0·90 a_w and in lowest at 0·92 a_w. Thus, the trend in magnesium concentration on decreasing the value of a_w was broadly the reverse of that for potassium and phosphate.

The effect on cell composition of the solute used to control a_w level

In experiments described above, a_w was adjusted to desired values with NaCl only. Thus, resulting changes in composition could be ascribed equally to the NaCl concentration as to the a_w value. Table 1 provides comparisons of water content and sodium and potassium concentrations in staphylococci grown at a_w values controlled by the addition of various solutes. Clearly, water content was a function of a_w value, not of the solute used. Cell sodium remained approximately the same at all three a_w values when NaCl had not been added to the medium. Hence it depended primarily on the external salt concentration, not the a_w value. In contrast, cell potassium was influenced both by the a_w value and by high concentrations of KCl in the medium.

Table 1. *Water, sodium and potassium contents of Staphylococcus aureus grown in media* adjusted to several a_w with NaCl, KCl or sucrose*

a_w value	Adjusting solute	Cell water (g./g. dry wt.)	Sodium (m molal)	Potassium (m molal)
0·993	Nil*	1·66	100	671
0·97	NaCl	1·35	382	837
0·97	Sucrose	1·37	84	841
0·92	NaCl	0·86	930	1020
0·92	KCl	0·90	78	1922

* Basal medium was brain + heart infusion broth.

Influence of the basal medium on concentrations of potassium and amino acids in staphylococci

The ratio of potassium to amino acids was essentially constant in cocci grown at 0·92 a_w and above (Fig. 3). The concentrations of these solutes in the growth media were also constant. To determine the importance of external concentrations of potassium and amino acids in controlling internal concentrations, cocci were analysed after growth at 0·993 a_w in media whose potassium:amino acid ratio was varied almost 20-fold (Table 2). Very small changes in cell composition resulted from these treatments. Thus at these values, external concentrations of potassium and amino acids did not influence appreciably the amounts accumulated by the cocci. This result contrasts with the effect of very high external potassium concentrations discussed above.

Table 2. *Effect of potassium and amino acid concentrations in the growth medium on the composition of Staphylococcus aureus grown at 0·993 a_w*

	Medium*			
	A	B	C	D
In medium				
Potassium (mM)	23·8	6·0	26·8	6·5
Amino acids (mM)	27·2	6·8	6·8	31·3
Potassium: amino acids	0·87	0·87	3·94	0·21
In cells				
Potassium (μ-equiv./g. dry wt.)	1120	1150	1240	1020
Amino acids (μmole/g. dry wt.)	588	582	564	503
Potassium:amino acids	1·91	1·91	2·19	2·04

* Media: A, Brain + heart infusion broth (BH); B, 1/4 strength BH + NaCl; C, 1/4 strength BH + NaCl + KCl; D, 1/4 strength BH + NaCl + vitamin-free Casamino acids (Difco). The additions of NaCl were used to bring the a_w value to 0·993 in each case.

Composition of staphylococci grown in dilute media

As most common laboratory media have a_w values well above that of brain + heart infusion broth (0·993 a_w), water, sodium, and potassium contents were determined for comparison in cocci harvested from media of 0·998 and 0·9965 a_w. These were obtained by diluting the basal medium to one-quarter and one-half strength, respectively. The results are compared in Table 3 with those obtained with 0·993 a_w medium. Increase in a_w value resulted in an increase in cell water and a decrease

in cell solute concentrations. However, in spite of the marked fall in sodium and potassium content of the medium as it was diluted to one-quarter strength, both sodium and potassium/g. dry wt. cocci remained relatively constant.

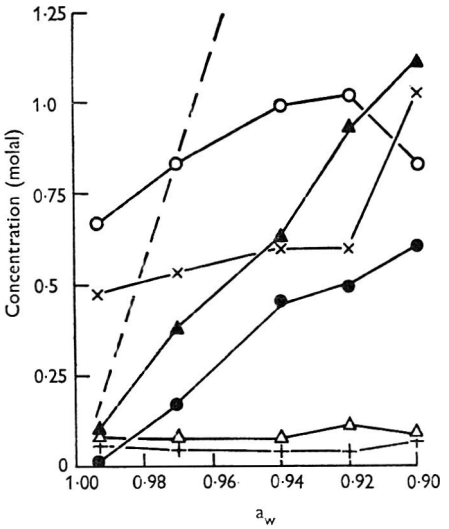

Fig. 3

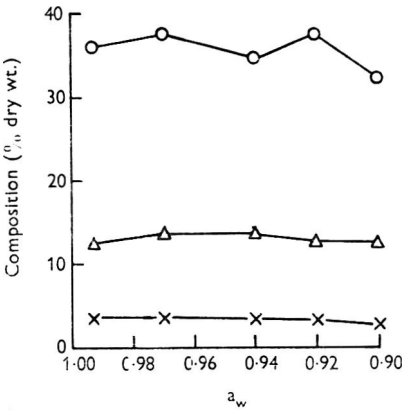

Fig. 4

Fig. 3. Concentrations of six solutes in cells of Staphlococcus aureus grown in broth adjusted with NaCl to a_w levels from 0·993 to 0·90. ▲, Sodium; ○, potassium; +, magnesium; ×, total amino acids; △, inorganic phosphate; ● chloride. The NaCl concentration in the medium is shown by broken line.

Fig. 4. Protein, DNA, and RNA content of cells of Staphylococcus aureus grown in broth adjusted with NaCl to a_w levels from 0·993 to 0·90. ○, Protein; ×, DNA; △, RNA.

Table 3. *Water, sodium and potassium contents of Staphylococcus aureus grown in media of high a_w value*

Dilution of basal medium* with water	a_w value	Cell water (g./g. dry wt.)	Sodium		Potassium	
			(mmolal)	(μ-equiv./g. dry wt.)	(mmolal)	(μ-equiv./g. dry wt.)
Nil	0·993	1·66	100	166	671	1116
1:1	0·9965	1·92	71	137	553	1060
1:3	0·998	2·44	62	151	426	1040

* Basal medium was brain + heart infusion broth.

Some macromolecular constituents

The steep increase in the amino acid pool below 0·92 a_w (Fig. 3) suggested that incorporation of amino acids into protein might be inhibited under these conditions. Protein contents of cocci grown at the various a_w values were determined and are shown, together with the DNA and RNA values, in Fig. 4. The average protein content at the five values of a_w was 35·3 % of the dry wt. The most marked change was a 14 % decrease in protein between 0·92 and 0·90 a_w. DNA averaged only 3·13 % over the five a_w values, and, like protein, decreased by 15 % below 0·92 a_w.

RNA contents were similar throughout, averaging 12·9 % of coccal dry wt. At all five a_w values, the total nitrogen content (calculated) of protein constituents was equal to about 64 % of the trichloroacetic acid-insoluble nitrogen as determined by Kjeldahl digestion and titration.

DISCUSSION

The experiments of Mitchell & Moyle (1956) suggested that the 18 % of the dry weight of staphylococci which could be extracted with trichloroacetic acid represented the low molecular weight solutes responsible for the cell osmotic pressure; 18 % is probably a slight underestimate since some solutes (e.g. sodium ions; Christian, 1962), would be removed by the preliminary water washing. The sum of the six solutes studied here was equivalent to 16–18 % of the coccal dry weight, and presumably these form the bulk of osmotically important solutes in the organisms. In view of the relatively low intracellular chloride and phosphate contents, high concentrations of other anions must be present to effect an ionic balance. *Staphylococcus aureus* organisms grown at 0·993 a_w contain sufficient fixed anions (not removed by butanol treatment and water washing) to balance about one-quarter of their potassium (Christian, 1962). Preliminary experiments indicate that other important anions include organic acids and the dicarboxylic amino acids, glutamic and aspartic acid.

It is tempting to calculate from the analytical data the intracellular a_w of cocci grown at the various a_w values. However, this will not be fruitful until the nature of the various anions and amino acids is known. Meanwhile, an estimate of the a_w value in cocci grown in basal medium may be obtained from the data of Fig. 1. Whole cocci contained 1·66 g. water/g. dry wt. Similar cocci, after heat treatment, contained this amount of water when in equilibrium with a solution of 0·967 a_w. This is close to the cell a_w value deduced from cryoscopic experiments with similar preparations (Christian & Waltho, 1962*b*). The curves of Fig. 1 converge and intersect at low a_w values probably because of the much lower sodium content of heat-treated cocci, which were grown at 0·993 a_w and washed in water. At 0·90 a_w, the whole cocci contained about 1·1 molal sodium, which was absent from the heated preparation. Addition of this concentration of a sodium salt similar to NaCl would increase the equilibrium water content of heated cocci at 0·90 a_w by about 0·25 g./g. dry wt. This would slightly more than compensate for the difference between the curves near 0·90 a_w. This indicates that cocci grown at low a_w values maintain a small turgor pressure, as predicted by the cryoscopic determinations of Christian & Ingram (1959).

Of the solutes examined, only sodium and chloride ions were found at lower concentrations in the cocci than in the medium. The only analogous data for bacteria are those of Schultz & Solomon (1961) and Schultz, Wilson & Epstein (1962), who examined exponential-phase organisms of *Escherichia coli* grown in a medium containing NaCl at concentrations below 0·15 M. Intracellular sodium and chloride concentrations were less than and approximately proportional to the external NaCl concentrations. The ratios were about 0·5 and 0·3, respectively, which are similar to those found in the present study.

The low apparent intracellular concentrations of sodium and chloride might be explained if these ions were confined to compartments in which their concentrations

were the same as in the medium. While staphylococci have an appreciable requirement for potassium (Shooter & Wyatt, 1956), it has been reported that they do not require sodium (Shooter & Wyatt, 1957). Also, sodium is readily removed from staphylococci by treatments which leave the potassium content unaltered (Christian, 1962). Thus sodium could reside in a non-specific compartment. In cocci grown at 0·92 a_w, the sums of intracellular sodium and potassium were very similar, whether the medium was adjusted with NaCl or KCl. Presumably, in cocci grown in KCl the excess potassium ions would be in the non-specific compartment. This would divide intracellular potassium into two pools, one non-specific and the other specifically accumulated. There are insufficient data here to test the compartment hypothesis, but this system clearly offers opportunities for further study. However, it should be borne in mind that the apparent sodium and chloride contents of cocci depend upon the position of boundaries determined by studies of phosphate impermeability. Thus they might merely reflect a gradation of sodium, chloride, and phosphate penetration that is governed by ionic properties of the cell surface.

In contrast to the low chloride content of staphylococci grown in high NaCl concentrations are the very high values of chloride found in extreme halophiles (Christian & Waltho, 1962*a*). It may be relevant that, relative to cell potassium content, the latter strains accumulated very small amino acid pools.

Potassium concentrations in staphylococci appeared to be a function of the a_w value whenever the external sodium:potassium ratio was high. Neither large variations in the external potassium:amino acids ratio nor decrease of a_w value to 0·92 by addition of NaCl altered appreciably the potassium:amino acid ratio within the cocci. Hence the coccal amino acid concentration was also related to the a_w value, and the mechanisms which control the accumulation of potassium and amino acids appeared to be linked in some way. The reciprocal changes in concentrations of these solutes observed below 0·92 a_w were therefore evidence of a marked disturbance of metabolism.

Between 0·92 and 0·90 a_w the amino acid pool increased by 336 μmoles/g. dry wt., while protein decreased by 52 mg./g. These changes would be equivalent if the mean molecular weight of the amino acids concerned were 155. This value is of the correct order, suggesting that most of the sharp increase in the amino acid pool resulted from decreased protein synthesis. A low a_w value might inhibit amino acid incorporation directly. However, since decrease of a_w value from 0·993 to 0·90 inhibits respiration by more than 80 % (unpublished data), it is more likely that low respiration rates limit the energy available for protein synthesis. Decreased respiration would also decrease the accumulation of potassium and inorganic phosphate. Influx of amino acids may be decreased also, but a much lower rate of utilization could result in a net increase in pool size. It seems probable that further investigations in this region (0·92–0·90 a_w) would reveal which steps in metabolism are primarily affected by restriction of the a_w value.

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Activation of Soil Microflora by Fungus Spores in Relation to Soil Fungistasis

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SUMMARY

Bacterial numbers in natural soil supplemented with living fungus spores or cell-free aqueous washings of spores, then incubated for 8 hr or more, were several-fold higher than those in non-supplemented soil. Rates of oxygen uptake in natural soil supplemented with washed or unwashed fungus spores, cell-free aqueous washings of spores, killed spores, or diluted nutrients, were several-fold higher than those in non-supplemented soil. Increased respiration occurred rapidly on addition of these supplements to soil. A single brief water washing of urediospores of *Puccinia rubigo-vera* or of conidia of *Neurospora* sp. extracted about 10 % of the spore dry weight. Washed bacteria or *Streptomyces* spp., when incubated with fungus spores in absence of added nutrients, inhibited fungus spore germination, whereas sterile filtrates of bacteria were not inhibitory. The results indicate that fungus spores, by virtue of nutrients in their exudates, stimulate rapid activity of microbes in soil, and that the enhanced microbial activity causes inhibition of fungal spore germination.

INTRODUCTION

The cause and nature of the widespread soil fungistasis (mycostasis) are not yet clearly understood. The usual explanation proposed for inhibition of fungus spore germination by natural soils is the presence therein of unstable fungistatic materials of microbial origin (Dobbs, Hinson & Bywater, 1960). Park (1961) and Griffin (1962*a*) suggested that these materials might be analogous with staling-products of cultures. Nevertheless, the presence of such materials in soil has not been unequivocally demonstrated. Lingappa & Lockwood (1961) offered evidence, based primarily on an analysis of the mechanism of fungistasis on agar discs or cellophan in contact with soil, that inhibitory substances might not be present throughout the soil mass, but might be generated in the vicinity of fungus spores by soil microbes activated by compounds exuded from the spores. The present report provides evidence that fungus spores and spore exudates can rapidly stimulate the soil microflora, and that microbial stimulation is causally related to inhibition of fungal spore germination. A brief report of some of this work has been published (Lingappa & Lockwood, 1962).

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METHODS

Conidia of *Glomerella cingulata*, *Fusarium solani* f. *pisi*, *Helminthosporium victoriae*, and *Neurospora* sp.; and urediospores of *Puccinia graminis tritici* and of *P. rubigo-vera* were used to supplement natural soil samples. Conidia of *G. cingulata*, *F. solani*, and *H. victoriae* were collected by quickly rinsing them from the surface of slopes of potato glucose agar (PGA) with cold (2°) sterile distilled water. Conidia of *Neurospora* sp. are produced near the top of the test tube above the agar surface, and were collected by tapping the inverted tube. Rust fungi were cultured on Little Club wheat plants in a greenhouse. Spore washings were prepared by shaking spores in 10 ml. cold sterile distilled water for 15 min., centrifuging-off the spores, then filtering the solution through 0·45 μ Millipore filters or Seitz sterilizing filters. Washings were air-dried, weighed, then brought to the desired concentration by dissolving in distilled water.

The soil used was sifted Conover loam containing 25 % moisture. Supplements per g. oven-dry soil are indicated as fresh weight of rust urediospores or *Neurospora* sp. conidia, or oven-dry weight of other spores or spore-washings. Urediospores and *Neurospora* sp. conidia contained 10–15 % moisture. Experiments were repeated two to five times, with reproducible results.

Dilution plates. The desired amounts of spores or spore washings in 0·5 ml. distilled water were mixed with 1·3 g. moist soil in small beakers. After incubation for 2–24 hr at 28° in a moist atmosphere, dilutions to 1×10^{-6} or 1×10^{-7} were made in sterile distilled water. One ml. of the final dilution was mixed in a Petri dish with 15 ml. melted (43°) 2 % water agar or sodium albuminate agar containing 100 μg. cycloheximide/ml. The antibiotic was used to prevent growth of fungi from the supplement. Six plates were used for each treatment. Plates were incubated at 28° for 7–10 days, when total colony numbers were determined.

Respirometry. Manometric measurements of oxygen uptake by soil were made with a Warburg apparatus and standard procedures (Umbreit, Burris & Stauffer, 1957). Duplicate 15 ml. reaction vessels each contained 2·7 g. moist soil. Flasks were placed in ice while 0·4 ml. spore suspensions or spore washings were added and mixed with the soil. After 10 min. equilibration of flasks in the water bath at 30°, readings were made at 30 min. intervals. Data are expressed as μl. oxygen uptake/g. soil for each time interval.

Germination inhibition with washed micro-organisms. Samples of the following micro-organisms were used in inhibition tests: *Rhizobium trifolii*, *Bacillus subtilis*, two unidentified bacteria designated as isolates G-1 and G-2, four isolates of Streptomyces. A mixed culture of bacteria from soil was also used. Bacterial isolates G-1 and G-2 were obtained from dilution plates made from soil supplemented with *Glomerella cingulata* conidia, and have the following properties: non-motile straight rods, 0·3–0·4 $\mu \times$ 1·0–3·0 μ; do not form spores, Gram-negative, aerobic and non-fermentative. On nutrient agar G-2 produces a light yellow diffusible pigment, whereas G-1 does not. G-1 has a higher temperature optimum than G-2. Both are tentatively placed in the genus *Pseudomonas*.

Pure cultures of bacteria were grown for 1–2 days, and the Streptomyces isolates for 7 days, on yeast extract + maltose + glucose agar slopes. Then organisms or spores were removed with cold 0·01 M-phosphate buffer (pH 7·2). Mixed soil

bacteria were grown by incubating about 50 mg. moist soil in 5 ml. nutrient broth for 24 hr. The organisms were filtered through Whatman No. 1 paper to remove soil particles. Bacteria and Streptomyces spores were washed three times with cold buffer by vigorous shaking followed by centrifugation. The washed organisms were adjusted in concentration to scale reading 1·0 optical density at 500 mμ, using a Bausch & Lomb Spectronic 20 colorimeter standardized with a water blank. Adjusted suspensions of mixed soil bacteria contained about 2×10^8 organisms/ml. as determined with a haemocytometer; bacterial isolates G-1, G-2, and *Rhizobium trifolii* contained about 14×10^8 organisms/ml. The washed suspensions were diluted, usually 1/10, 1/100 and 1/1000 with cold buffer. Fungus conidia were removed with cold buffer from 2- to 10-day-old cultures on potato glucose agar, centrifuged once, then adjusted with buffer to 5×10^4 or 2×10^5 conidia/ml.

The inhibitory effect of washed micro-organisms on the germination of fungal spores was tested by incubating dilutions of the bacterial suspensions together with fungus spores in wells of plastic slide plates. *Helminthosporium victoriae*, *Glomerella cingulata* or *Fusarium solani* were tested by mixing 0·1 ml. each of conidia and bacterial suspensions. Spores of these three fungi, washed once, germinated well in buffer or distilled water. *Mucor ramannianus*, *Penicillium frequentans* or *Verticillium albo-atrum* were tested by mixing 0·05 ml. each of bacterial suspensions and conidial suspension in designated positions on the surface of 2 % Bacto agar (Difco) or 2 % Special agar (Noble; Difco) in Petri dishes. Spores of these fungi germinated poorly in buffer or water, but germinated well on water agar surfaces.

The effect of sterile filtrates of washed microbial suspensions on fungal spore germination was also determined. Filtrates were prepared by passing portions of bacterial suspensions adjusted to 1·0 optical density at 500 mμ through sterilized 0·45μ Millipore filters. Equal volumes (0·05 ml.) of filtrates and fungal spore suspensions were added to wells of slide plates containing conidia of *Glomerella cingulata* or *Helminthosporium victoriae* or to water agar surfaces containing conidia of *Penicillium frequentans*, *Verticillium albo-atrum*, or *Mucor ramannianus*. At intervals throughout the germination period the old filtrate in wells of slide plates was replaced with 0·1 ml. fresh filtrate from the same bacterial suspensions, incubated at 24°. On agar surfaces, 0·025 ml. of fresh filtrate was simply added at intervals since the agar absorbed the liquid. After incubation for 3–5 hr for *H. victoriae* and 16 hr for other fungi, spores and germ tubes were killed and stained with phenolic rose bengal or methylene blue in lactophenol. Germination counts of at least 200 conidia of each species per treatment were made.

RESULTS

Population counts in fungal spore-supplemented soil

In seven tests, natural soil was supplemented with 20–200 mg. ($1–4 \times 10^9$) conidia of *Glomerella cingulata* or *Neurospora* sp., or urediospores of *Puccinia graminis*. Conidia of *G. cingulata* were not washed and might have carried some nutrients from the culture, but the method of collecting urediospores and the Neurospora conidia eliminated this possibility. Spore-supplemented soil incubated for 16 hr gave three to ten times greater total colony numbers than non-supplemented soil. Greater increases were observed when spore-supplemented soil was incubated

for 24 hr before plating. Differences were usually greater when soil dilutions were plated on sodium albuminate agar than on water agar. Colony numbers from non-supplemented natural soil at 0 and 16 hr, and spore-supplemented soil at 0 hr, did not differ appreciably. Results of a typical experiment with soil supplemented with Neurospora conidia are given in Table 1. When soils were plated at 0, 2, 4, 8 and 16 hr following supplementation with Neurospora or *G. cingulata* conidia, the earliest increase in colony numbers occurred at 8 hr. For example, soil supplemented with Neurospora conidia, then incubated 8 hr gave a sixfold increase on sodium albuminate agar and a twofold increase on water agar. Analysis of variance was applied to 16 hr data for spore-supplemented and non-supplemented soil, using mean plate counts from different experiments as replicates. Values for spore-supplemented soil were significantly higher (5 % level) than those for non-supplemented soil.

Table 1. *Effect of supplementing natural soil with conidia of Neurospora sp. and incubation for* 16 *hr on the total numbers of micro-organisms which appeared on dilution plates*

Soil was supplemented with 45 mg. ($1{\cdot}8 \times 10^9$) conidia/g. soil, and was diluted 1×10^{-6} (water agar) and 1×10^{-7} (sodium albuminate agar) for plating. Values are mean of six plates.

	Plating medium			
	Water agar		Sodium albuminate agar	
	Colonies/plate			
	0 hr	16 hr	0 hr	16 hr
Treatment				
Natural soil	36	42	67	72
Natural soil + *Neurospora* conidia	32	125	64	546

Since spore exudates seemed the likely cause of the increased microbial population in spore-supplemented soil, concentrated cell-free aqueous washings of conidia of *Fusarium solani* and of urediospores of *Puccinia graminis* were placed, in filter paper discs, on the surface of water agar containing diluted soil suspensions. After 48 hr incubation, in the area surrounding the discs, urediospore washings gave a twofold increase, and conidial washings gave a fivefold increase, in total colony numbers as compared with controls. The colonies were also larger on plates containing spore washings. No inhibition zones were observed as was reported in similar tests with washings from teliospores of *Ustilago zeae* (Lingappa & Lockwood, 1961).

Single aqueous washings from urediospores of *Puccinia rubigo-vera* or Neurospora conidia contained, respectively, 9·6 and 9·1 % of the dry weight of the spores. Washings from conidia of *Glomerella cingulata* contained 14 %, and those from *Fusarium solani* contained 42 % of the spore dry weight, but these might have included solutes from the culture. Ten mg. of *P. rubigo-vera* or *Neurospora* sp. spore washings were used to supplement 1·3 g. samples of moist soil. Soil dilution plates of water agar, after 16 hr incubation with Neurospora washings, gave a fivefold increase in colony numbers; urediospore washings gave a tenfold increase. The increased numbers of colonies in all experiments were bacterial colonies; actino-

mycetes decreased. No fungus colonies appeared on plates because of the use of cycloheximide to prevent fungal growth from the supplements. To determine whether spore-sized non-nutritive particles could stimulate microbial development, 1·3 g. soil samples were supplemented with 100 mg. ($2{\cdot}5 \times 10^7$) acid-washed 30μ diam. glass beads. In three tests no increases in colony numbers were observed in the supplemented soil incubated up to 24 hr before making dilution plates.

These results show that fungus spores added to soil stimulate multiplication of the bacteria in the soil, and suggest that soluble nutrients from the fungal spores cause the stimulation.

Oxygen uptake by fungal spore-supplemented soil

In various tests, oxygen uptake by soil supplemented with 1–10 mg. (6×10^5–$1{\cdot}7 \times 10^8$) conidia of *Fusarium solani*, *Helminthosporium victoriae*, *Glomerella cingulata* or *Neurospora* sp. showed rapid and relatively great increases over that by non-supplemented soil (Figs. 1–5). Oxygen uptake by spore-supplemented soil was 7- to 33-fold greater than non-supplemented soil within 30 min., from 3- to 35-fold greater in 1 hr and 3- to 20-fold greater in 2 hr. Subsequent differences in respiration rates to the end of experiments (4–6½ hr) were of a similar order. Since alkali in the centre wells of the reaction vessels might decrease CO_2 concentration below those normally existing in soil and thereby affect the metabolic responses of the soil micro-organisms, some experiments were done with a CO_2 buffer (Umbreit *et al.* 1957) in the centre well so as to maintain the flask atmosphere at 1 % (v/v) CO_2. However, the respiratory responses of soil amended with *F. solani* spores (Fig. 2) or peptone were similar in both conditions.

Unwashed fungal spores were used in some of the experiments described above. To eliminate possible contaminating nutrients from the cultures, tests were done with unwashed Neurospora conidia which were never in contact with the agar medium, and with washed conidia of *Fusarium solani*. Soil supplemented with 7·5 mg. ($1{\cdot}6 \times 10^8$) washed *F. solani* spores gave 33- and 35-fold increases in oxygen uptake in 30 and 60 min., respectively (Fig. 1). A lesser increase was given by 1 mg. ($2{\cdot}1 \times 10^7$) washed *F. solani* spores (Fig. 2). Similarly, 3·6 mg. ($1{\cdot}5 \times 10^8$) Neurospora conidia gave respective increases of 10- and 20-fold in 30 and 60 min. (Fig. 5).

The increased oxygen uptake in spore-supplemented soil might be due to the respiration of the soil microflora or to the respiration of the fungal spores themselves. The relative contribution of each was difficult to assess, since the respiratory activity of fungal spores in a fungistatic condition is unknown. Sterilized soil supplemented with live fungal spores had high respiration rates, but these spores were physiologically active rather than inactive. Cycloheximide in water at 100 or 1000 μg./ml. was used to simulate a fungistatic medium. Conidia of *Helminthosporium victoriae* or *Neurospora* sp. did not germinate in cycloheximide solutions, but at the end of 4 hr the spores were still viable. Respiration in spore-supplemented cycloheximide solutions was very low (Figs. 3, 5). If these inhibited spores can be considered as metabolically similar to those in natural soil (this is by no means certain) inhibited spores in soil may also respire at a very low rate.

To rule out respiration of the added fungal spores, soil was supplemented with killed (boiled) conidia or with cell-free aqueous washings of conidia. Oxygen uptake in soil supplemented with 10 mg. ($1{\cdot}7 \times 10^8$) boiled *Glomerella cingulata* conidia was

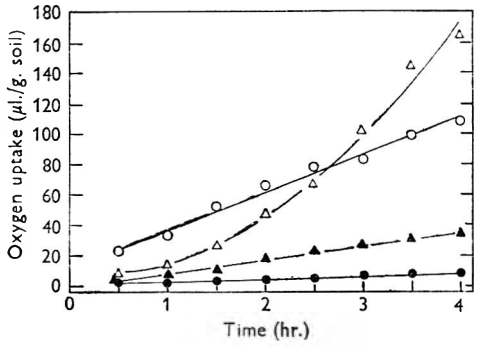

Fig. 1

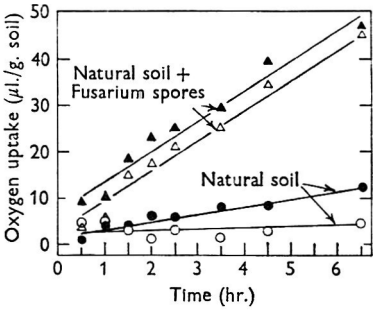

Fig. 2

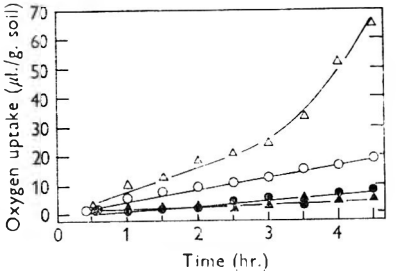

Fig. 3

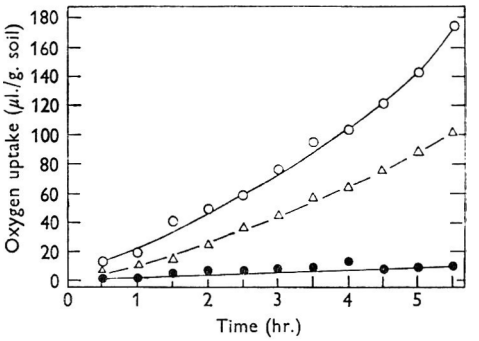

Fig. 4

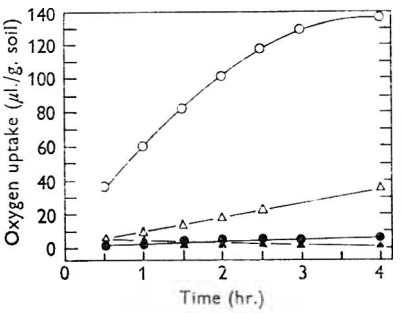

Fig. 5

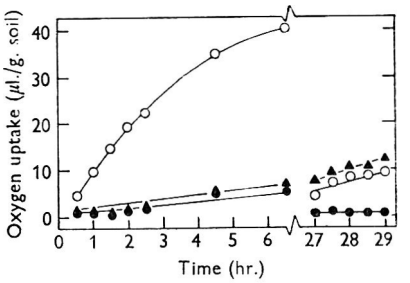

Fig. 6

Figs. 1–6. Oxygen uptake in natural Conover loam soil amended as follows (Values/g. oven-dried soil):

Fig. 1. Washed F. solani conidia (7·5 mg.; $1{\cdot}6 \times 10^8$ spores); washings from once-washed *F. solani* conidia (1 mg.); peptone (20 mg.). ●, Natural soil; ○, natural soil + Fusarium spores; ▲, natural soil + spore washings; △, natural soil + peptone.

Fig. 2. Washed F. solani conidia (1 mg.: $2{\cdot}1 \times 10^7$ spores); with KOH or CO_2 buffer in centre well. ●, ▲, KOH in centre well; ○, △, CO_2 buffer in centre well.

Fig. 3. Helminthosporium victoriae conidia (0·7 mg.; 6×10^5 spores); *H. victoriae* conidia (0·7 mg.) in cycloheximide 100 μg./ml. solution; casein hydrolysate (2 mg.). ●, Natural soil; ○, natural soil + Helminthosporium spores; ▲, Cycloheximide + Helminthosporium spores; △, natural soil + casein hydrolysate.

Fig. 4. Living or killed G. cingulata conidia (10 mg.; $1{\cdot}7 \times 10^8$ spores). ●, Natural soil; ○, natural soil + Glomerella spores; △, natural soil + boiled spores.

Fig. 5. Neurospora sp. conidia (3·6 mg.; $1{\cdot}5 \times 10^8$ spores); Neurospora sp. conidia (3·6 mg.) in 1000 μg./ml. cycloheximide solution; Neurospora conidial washings (3·5 mg.). ●, Natural soil; ○, natural soil + Neurospora spores; ▲, cycloheximide + Neurospora spores; △, natural soil + spore washings.

Fig. 6. Neurospora sp. conidial washings (3·5 mg.); 30 μ diam. glass beads (100 mg.; $2{\cdot}5 \times 10^7$ beads). Manometers were removed at 6½ hr, attached again at 27 hr. ●, Natural soil; ○, natural soil + Neurospora washings; ▲, natural soil + glass beads.

increased fivefold over that in non-supplemented soil in 1 hr (Fig. 4). Soil supplemented with 1 mg. of washings from previously once-washed *Fusarium solani* or with 3·5 mg. of washings from Neurospora conidia gave respective increases in oxygen uptake over non-supplemented soil of 7- and 15-fold in 1 hr (Figs. 1, 5, 6). These spore washings would not contain nutrients leached from the culture. These results indicate that the soil microflora was stimulated by materials present in spore exudates, and suggest that living spores might have, qualitatively at least, the same effect. Increased oxygen uptake in soil supplemented with 1–2 mg. peptone or casein hydrolysate gave further evidence that the increased respiration of spore-supplemented soil was nutritional (Figs. 1, 3).

In two tests, 100 mg. ($2{\cdot}5 \times 10^7$) glass beads of 30 μ diam. were added to soil. Respiration rates of bead-supplemented and non-supplemented soil were essentially similar during $6\frac{1}{2}$ hr incubation (Fig. 6). However, when determinations were made 20 hr later, a 10- to 18-fold increase in oxygen uptake was shown by the bead-supplemented soil. Apparently, non-nutritive surfaces provided a stimulus for microbial activity (Park, 1956), but the increases here were slight until several hours after amendment. It was concluded that enhanced respiration of microbes in soil rapidly follows supplementation of soil with fungus spores, and that this is caused by soluble materials from the spores.

Inhibition tests with soil microbes

If stimulation of soil microflora by fungus spores accounts for soil fungistasis, then a causal relationship must be shown between the stimulated soil microbes and inhibition of fungus spore germination. This was tested by incubating freshly washed soil micro-organisms with fungus conidia in the absence of added nutrients. Fresh suspensions of washed mixed bacteria from soil (Table 2), washed pure cultures of bacterial isolates G-1 or G-2, or *Rhizobium trifolii* (Table 3), strongly inhibited the germination of conidia of *Glomerella cingulata* or *Helminthosporium victoriae* in buffer solution, or conidia of *Mucor ramannianus*, *Penicillium frequentans*, or *Verticillium albo-atrum* on water agar. Results were the same whether Noble (Difco) agar (a specially purified agar) or Bacto (Difco) agar was used. The degree

Table 2. *Inhibition of fungal spore germination by washed mixed soil bacteria*

Suspensions of soil bacteria were prepared by incubating 10 mg. soil in 10 ml. nutrient broth for 24 hr, filtering off the soil, washing the organisms three times in cold 0·01 M-phosphate buffer (pH 7·2), then adjusting to scale reading 1·0 optical density at 500 mμ with a Bausch & Lomb Spectronic 20 colorimeter (about 2×10^8 organisms/ml.). Conidial suspensions were made from fungi grown on potato glucose agar slopes and were washed once with buffer and adjusted to $5–20 \times 10^4$ conidia/ml. Equal volumes (0·05 ml.) of bacterial and conidial suspensions were mixed for incubation. At least 200 spores were counted for each entry. Values are a representative result from four to six tests.

	Germination (%) in solution		Germination (%) on water agar		
Bacterial dilution	*G. cingulata*	*H. victoriae*	*P. frequentans*	*H. ramannianus*	*V. albo-atrum*
1/1	9	2	1	0	5
1/10	26	38	0	4	20
1/100	42	61	1	44	52
1/1000	60	79	57	56	93
Buffer	62	87	84	80	95

of inhibition was proportional to the concentration of bacteria. At the highest concentration of mixed or pure cultures of bacteria (optical density scale reading = 1), germination of all five test fungi in most tests was less than 20 %, whereas germination in controls was 50–65 % for *G. cingulata* and 75–95 % for other fungi. Germination of all five test fungi was inhibited to some degree by 1/100 dilutions of mixed soil bacteria. Germination of *P. frequentans* and *M. ramannianus* was inhibited by a 1/1000 dilution of mixed soil bacteria (Table 2). Germination of *G. cingulata* and *H. victoriae* was inhibited to some degree by a 1/100 dilution of bacterial isolates G-1 or G-2 and by a 1/10 dilution of *R. trifolii* (Table 3). In other tests, similar inhibition of germination of conidia of *G. cingulata* and *H. victoriae* was

Table 3. *Inhibition of fungal spore germination by washed organisms of pure cultures of bacteria*

Bacteria were grown on nutrient agar slopes, collected and washed three times in cold 0·01 M-phosphate buffer (pH 7·2), then adjusted to scale reading 1·0 optical density at 500 mμ with a Bausch & Lomb Spectronic 20 colorimeter (about 14×10^8 organisms/ml.). Conidial suspensions were from fungi grown on potato glucose agar slopes and were washed once with buffer and adjusted to $5–20 \times 10^4$ conidia/ml. Equal volumes (0·05 ml.) of bacterial and conidial suspensions were mixed in wells of slide plates for incubation. At least 200 spores were counted for each entry. Values are a representative result from three to five tests.

Bacterial dilution	Germination (%) of *G. cingulata* and *H. victoriae* conidia in presence of indicated bacterium					
	Bacterium G-1		Bacterium G-2		*Rhizobium trifolii*	
	G. cingulata	*H. victoriae*	*G. cingulata*	*H. victoriae*	*G. cingulata*	*H. victoriae*
	Germination (%)					
1/1	2	2	18	2	28	1
1/10	5	7	27	6	32	40
1/100	20	28	28	48	45	71
1/1000	30	82	51	86	42	73
Buffer	51	85	59	87	47	77

obtained with washed spores of four Streptomyces isolates or with washed *Bacillus subtilis*. Thus, inhibition resulted from some activity of the bacteria or actinomycetes initiated after washing and which required an effective time period no longer than that for germination of the fungal spores. The speed of the inhibition was especially well shown with *H. victoriae*, which will emit a visible germ tube in 30–60 min., and will give one several times the length of the spore in 3–4 hr. Even with this capacity for rapid germination, *H. victoriae* was strongly inhibited in the presence of washed bacteria. The fact that the bacteria were washed three times in cold buffer solution would seem to rule out preformed staling-products of the cultures as inhibitory factors. The possibility existed, however, that metabolic products (staling-products, antibiotics, etc.) generated by the washed bacteria during incubation with the fungal spores might account for the inhibition of fungal spore germination. This was tested by applying fresh sterile (Millipore) filtrates from suspensions of mixed or pure cultures of washed bacteria to the incubating fungal spores, once or at 30–60 min. intervals throughout the germination period (Tables 4, 5). Inhibition of germination was never observed, whether the filtrate was applied to

Table 4. *Effect of sterile filtrates of washed mixed soil bacteria on germination of fungus spores*

Suspensions of soil bacteria were prepared by incubating 10 mg. soil in 10 ml. nutrient broth for 24 hr, filtering off the soil, washing the organisms three times in cold 0·01 M-phosphate buffer (pH 7·2), then adjusting to scale reading 1·0 optical density at 500 mμ with a Bausch & Lomb Spectronic 20 colorimeter (about 2×10^8 organisms/ml.). Conidial suspensions were prepared from fungi grown on potato glucose agar slopes and were washed once with buffer and adjusted to $5–20 \times 10^4$ conidia/ml. At least 200 spores were counted for each entry. Values are from one of three tests.

	Spores of				
	G. cingulata	*H. victoriae*	*M. ramanianus*	*P. frequentans*	*V. albo-atrum*
	Germination (%)				
	In solution		In water agar		
Filtrate applied once*	67	95	—	—	—
Filtrate applied at intervals†	89	95	85	65	97
Buffer control	68	72	73	68	95

* Fresh bacterial suspension was passed through sterile 0·45μ Millipore filter. Equal volumes (0·05 ml.) of filtrate and conidial suspension were mixed for incubation.

† Immediately after preparation and at 30 min. (*H. victoriae*) or 60 min. (other fungi) intervals thereafter, a portion of the same bacterial suspension was passed through a sterilized 0·45μ Millipore filter, and used to replace or supplement the incubation medium.

Table 5. *Effect of filtrates of suspensions of washed bacteria on germination of fungus spores*

Bacteria were grown on nutrient agar slopes, collected and washed three times in cold 0·01 M-phosphate buffer (pH 7·2), then adjusted to scale reading 1·0 optical density at 500 mμ with a Bausch & Lomb Spectronic 20 colorimeter (about 14×10^8 organisms/ml.). Conidial suspensions were prepared from fungi grown on potato glucose agar slopes and were washed once with buffer and adjusted to $5–20 \times 10^4$ spores/ml. At least 200 spores were counted for each entry. Values are from one of three tests.

	Bacterial filtrate					
	Bacterium G-1		Bacterium G-2		*Rhizobium trifolii*	
	Fungal spores					
Treatment	*G. cingulata*	*H. victoriae*	*G. cingulata*	*H. victoriae*	*G. cingulata*	*H. victoriae*
	Germination (%)					
Filtrate applied once*	65	95	63	92	73	96
Filtrate applied at intervals†	85	92	83	94	66	95
Buffer control	58	83	58	82	68	86

* Fresh bacterial suspension was passed through sterile 0·45μ Millipore filter. Equal volumes (0·05 ml.) of filtrate and conidial suspensions were mixed for incubation.

† Immediately after preparation and at 30 min. (*H. victoriae*) or 60 min. (*G. cingulata*) intervals thereafter, a portion of the same bacterial suspension was passed through a sterilized 0·45μ Millipore filter, and used to replace the germination medium.

spores in wells of slide plates or to spores on water agar. In fact, germination as well as the rate and vigour of germ tube extension was usually stimulated by these filtrates. For example, in a typical experiment, the mean length of germ tubes of *H. victoriae* in buffer was 98 μ, whereas, in filtrates of mixed soil bacteria applied once, it was 161 μ; stimulation was also observed when filtrates were applied repeatedly.

The inhibition of fungus spore germination by washed bacteria and streptomyces and the absence of inhibitory substances from filtrates of incubated suspensions of washed bacteria suggest a causal relation between microbial activity and fungal spore inhibition, and that the presence of the fungal spores was required to initiate the inhibitory action.

DISCUSSION

The results presented in this paper make three main points: (1) fungus spores, by virtue of their exudates, stimulate activity and growth of micro-organisms in soil; (2) stimulation of bacterial metabolic activity occurs quickly after placing fungal spores in soil; (3) bacterial stimulation by fungus spores apparently is causally related to inhibition of fungal spore germination. These results support the authors' previous hypothesis, namely, that soil fungistasis is caused by soil microbes activated in the vicinity of fungus spores (Lingappa & Lockwood, 1961). It does not seem necessary to invoke the pre-existence in soil of fungistatic substances such as 'staling-products' in order to account for the inhibition of fungal growth. Some workers have reported inhibition of germination (Stover, 1958; Jackson, 1959; Dobbs & Griffiths, 1960; Dobbs *et al.* 1960; Griffin, 1962*b*) or growth (Jefferys & Hemming, 1953; Park, 1956; Stover, 1958) of fungi in some non-sterile aqueous extracts of soil, but soil extracts have also been reported to be non-inhibitory or even stimulatory (Dobbs *et al.* 1960; Hack & Williams, 1960; Lingappa & Lockwood, 1961). Moreover, when inhibitory soil extracts were passed through sterilizing filters or autoclaved, the inhibitory activity was usually eliminated (Park, 1956; Stover, 1958; Jackson, 1959; Dobbs & Griffiths, 1960; Griffin, 1962*b*). Thus, fungal growth inhibition in non-sterile extracts might have been due to microbial activity (Jackson, 1959) rather than to fungistatic substances extracted from soil. In one case (Dobbs & Griffiths, 1960), some inhibition of spore germination with Seitz-filtered soil extract was shown under conditions of decreased aeration; however, these authors also reported failure of unfiltered or filtered soil extracts to inhibit fungal spore germination in air.

Evidence reported in the present paper of enhancement of respiration and growth of soil microbes by water washings of fungal spores suggests that spores contain readily extractable nutrient materials. It is likely that exudation of such materials from fungal spores continues after washing. Washed *Fusarium solani* conidia enhanced oxygen uptake in soil supplemented with them, and second washings from *F. solani* conidia contained substances stimulatory of respiration of soil micro-organisms. Conidia of *Glomerella cingulata* also released more material in a second washing (Richardson & Thorn, 1962). The quantities of materials exuded by spores may be substantial. Single aqueous washings of *Puccinia graminis* urediospores or of Neurospora conidia each contained about 10 % of the dry weight of the unwashed spores. Water extracts of conidia of *Neurospora sitophila* (McCallan & Wilcoxon,

1936; Wain & Wilkinson, 1946) or *G. cingulata* (Richardson & Thorn, 1962; Thorn & Richardson, 1962) contained amino acids; those of *N. sitophila* also contained organic acids (Wain & Wilkinson, 1946). These substances are readily utilizable by a variety of micro-organisms. Loss of cellular contents, including amino acids and nucleic acids, has been reported from spores treated with fungicides (Burchfield & Storrs, 1957; Miller & McCallan, 1957; Tröger, 1958; Sisler & Cox, 1960; Richardson & Thorn, 1962; Thorn & Richardson, 1962) or antibiotics (Lowry & Sussman, 1956; Gottlieb *et al.* 1961). These losses seem to represent increased exudation over that which occurs when spores are in distilled water (Miller & McCallan, 1957; Richardson & Thorn, 1962; Thorn & Richardson, 1962). In some cases at least, loss of cell constituents by sublethal concentrations of fungicides did not impair germinability of spores (Burchfield & Storrs, 1957; Miller & McCallan, 1957). Thus, in soil, fungal spore exudates might stimulate soil microbes to produce growth-inhibitory but sublethal concentrations of antibiotics, which in turn might induce greater leakage of spore contents, thus further activating the soil microflora.

Evidence for rapid stimulation of microbes in soil by added nutrients was obtained in Warburg experiments with soil supplemented with fungus spores, spore washings or dilute nutrients. Oxygen uptake increased before the numbers of micro-organisms increased. Similar work (Rovira, 1953; Stevenson, 1956) indicated that the activity of microbes in soil can be rapidly stimulated; these workers showed that oxygen uptake in remoistened air-dried soil was several-fold higher than that of fresh soil, the increase beginning almost immediately after remoistening. The enhanced respiration was attributed to increased metabolic activity of the soil microbes, and Stevenson (1956) further showed that bacterial numbers in air-dried soil were lower than in fresh soil and did not begin to increase until at least the 3rd hour of incubation. Non-biological oxygen uptake was ruled out and a nutritional basis for the response was suggested by the presence of amino acids in water extracts of the air-dried soil, and their absence from extracts of fresh soil. Similar rapidly enhanced activity of micro-organisms has been shown in soil supplemented with glucose (Rovira, 1956), amino acids or mixtures of carbohydrates with organic acids (Katznelson & Rouatt, 1957), and ethanol or sodium acetate (Stevenson & Katznelson, 1958). For example, soil supplemented with dilute glucose solution maintained a sixfold higher oxygen utilization rate than non-supplemented soil when determinations were made from 30–210 min. (Rovira, 1956).

A causal relation between microbial activation by fungus spores and inhibition of spore germination is indicated by experiments in which washed bacteria prevented fungal spore germination in the absence of added nutrients. The inability of cell-free filtrates of inhibitory bacteria to effect inhibition would suggest that autolytic products, staling-products and the like were not involved.

It is not known which soil microbes are specifically involved in soil fungistasis, or indeed whether activity resides exclusively with any special group. All of 67 isolates of actinomycetes, bacteria or fungi, whether inhibitory or not in agar-streak tests, were able to restore a level of fungistasis to sterilized soil, though some were more effective than others (Lockwood & Lingappa, 1963). In the present work, fungal spore germination was inhibited by various mixed and pure cultures of bacteria and streptomyces. One might regard bacteria as most likely involved in mycostasis because of their short generation times and their multiplication in spore-

supplemented soil. However, increased numbers of micro-organisms were not involved in the early enhanced activity of supplemented soil in the present work, or in experiments with remoistened air-dried soil (Stevenson & Katznelson, 1958). Moreover, bacteria were less effective than actinomycetes and fungi in restoring fungistasis to sterilized soils (Lockwood & Lingappa, 1963). The means by which soil microbes inhibit the germination of fungal spores in soil is not known, though most workers attribute this inhibition to some fungistatic microbial product. It is also possible that soil microbes create nutrient deficiencies in the vicinity of the spore by rapid utilization of nutrients in spore exudates which may be required for spore germination. Either of these views would be compatible with the interpretation of soil fungistasis presented herein.

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Identification of D-Alanine as the Auto-Inhibitor of Germination of *Bacillus globigii* Spores

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SUMMARY

Thick suspensions of spores of *Bacillus globigii* did not germinate completely in nutrient media because the first spores to germinate released a substance which inhibited germination of the remainder. The inhibitory substance was identified as D-alanine by chromatography, high voltage electrophoresis, and its destruction by D-amino acid oxidase. When ^{14}C-L-alanine was used to induce germination of *B. globigii* spores, the resulting inhibitory D-alanine was ^{14}C-labelled with the same specific activity as the ^{14}C-L-alanine used. Auto-inhibition of *B. globigii* spore germination is therefore due to racemization of exogenous L-alanine, which forms sufficient D-alanine to arrest the germination process in thick suspensions of spores.

INTRODUCTION

More than 99 % of the individuals in suspensions of most types of bacterial spores will germinate in solutions of one or two amino acids or riboside (Hills, 1950); however, germination of suspensions of spores of some organisms may be incomplete in such media and also in complex media (Powell, 1950; Halvorson, 1959). Incomplete germination may have several causes (Murrell, 1961). It has often been noticed that high concentrations of spores germinate less completely than low concentrations. Stedman, Kravitz, Anmuth & Harding (1956) and Stedman, Kravitz, Harding & King (1957) found that spores of *Bacillus globigii* germinating in L-alanine + glucose exhibited pronounced auto-inhibition of germination at high spore concentrations. Their results suggested that, during germination of thick suspensions of *B. globigii* spores, a substance was released into the medium by the first spores to germinate which inhibited germination of the remainder. Stedman *et al.* (1956) showed that the inhibitor was competitive with L-alanine and suggested that it might be a high molecular weight substance, possibly with a terminal serine residue (Stedman *et al.* 1957). The strain of *B. globigii* in our possession also showed auto-inhibition of spore germination, and, since naturally occurring inhibitors of germination are rare, we re-examined the phenomenon to try and identify the auto-inhibitor. When this was proved to be D-alanine, its origin in the supernatant fluid of germinating spore suspensions was investigated.

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METHODS

Organisms and preparation of spores. Organisms used included *Bacillus subtilis* v. *niger* (syn. *B. globigii*), *B. subtilis* v. *aterrimus* NCTC 2590, *B. subtilis* strains M2 and S5, *B. cereus* NCTC 945 and *B. cereus* strains S20 and PX. The strain of *B. globigii* was obtained from W. Bale (Microbiological Research Establishment, MRE, Porton, Wiltshire) and *B. cereus* PX from R. E. Strange (MRE, Porton). The other strains were from the National Type Culture Collection (NCTC), Colindale, London, or were laboratory isolates. Spore suspensions were prepared, cleaned and stored as described by Hitchins, Gould & Hurst (1963), and the dry weights of suspensions were determined by drying samples to constant weight at 105°.

Media and measurement of germination. All spore suspensions except those of *Bacillus globigii* were heated at 70° for 30 min. before use. *B. globigii* spores germinated less rapidly after heating and were therefore used unheated at equiv. 75 μg. dry weight spores/ml. Germination medium for *B. globigii* spores consisted of L-alanine (10 mM), glucose (mM), sodium phosphate buffer (pH 7, 30 mM). Germination medium for the other spores was composed of L-alanine (500 μM), L-tyrosine (500 μM), inosine (20 μM), glucose (500 μM), sodium phosphate buffer (pH 7, 30 mM). Germination was measured by examining suspensions with the phase-contrast microscope and recording the % of phase-dark (germinated) spores. About 250 spores were counted for each estimation. Alternatively, the extinction of suspensions was measured with an absorptiometer ('Biochem', Hilger and Watts Ltd., Camden Road, London) and 560 mμ peak transmission filter. Germination was accompanied by a decrease in the extinction of suspensions.

Preparation of the auto-inhibitor. Spores of *Bacillus globigii* were suspended in germination medium at high concentrations (equiv. 1·1 mg. dry wt. spores/ml.). After 1 hr at 30° only about 20 % of the spores had germinated, indicating that auto-inhibition had occurred (Fig. 1). The spores were removed by centrifugation and the supernatant fluid concentrated tenfold in a rotary evaporator at 40°. Auto-inhibitor in the concentrate was partially purified by one-dimensional chromatography on Whatman No. 3 MM paper with butanol + acetic acid + water (4 + 1 + 5 by vol.) as solvent (Davis & Freer, 1960). Thin strips of the chromatograms were cut off and sprayed to locate ninhydrin-positive material, then the remainder of each chromatogram was cut into 1 in. squares which were soaked in water for 1 hr at 4° with stirring. Eluted material in the water was finally concentrated in the rotary evaporator to the volume of the initial concentrate. Pyridine + ethanol + water (10 + 7 + 3, by vol.) was also used as a solvent for chromatography. A Locarte instrument (Locarte Co., 24 Emperor's Gate, London, S.W. 7) was used for high voltage paper electrophoresis on Whatman 3 MM paper and with solvent consisting of M-formic acid + acetic acid buffer (pH 1·85).

Biological assay of the auto-inhibitor. Solutions containing auto-inhibitor were assayed by mixing with fresh germination medium at 30° containing equiv. about 60 μg. dry weight *Bacillus globigii* spores/ml. and the rates of germination were measured by the extinction change and phase-contrast methods. In the absence of auto-inhibitor this low concentration of spores germinated almost completely (Fig. 1).

Use of D-amino acid oxidase. Action of the D-amino acid oxidase from hog kidney (L. Light and Co. Ltd., Colnbrook) was measured by following oxygen uptake in a

Warburg apparatus at 35°. Incubation mixtures in the flasks consisted of D-amino acid oxidase (16 mg.), sodium pyrophosphate buffer (pH 8·3, 20 μmole) and either DL-alanine (150 μmole), L-alanine (150 μmole) or auto-inhibitor solution (1 ml. concentrate) in a total of 2·5 ml. (Yoshimoto, 1958). Under these conditions 80 % of the D-alanine was oxidized to pyruvic acid in 5 hr.

Estimation of pyruvic acid. Pyruvic acid formed by the action of D-amino acid oxidase on D-alanine was estimated by reaction with 2,4-dinitrophenylhydrazine (Hopkin and Williams Ltd., Chadwell Heath, Essex) after removing the enzyme by precipitation with trichloroacetic acid (Koepsell & Sharp, 1952). The derivative was extracted into ethyl acetate, and from this into sodium carbonate solution (M). After development with sodium hydroxide (final concentration, 0·4 M) for 10 min. the intensity of the colour was compared with standards and blanks at 435 mμ in a Unicam SP 500 (Unicam Instruments Ltd., Cambridge).

Isotope studies. L-Alanine uniformly labelled with ^{14}C (Radiochemical Centre, Amersham, Buckinghamshire) was diluted with unlabelled L-alanine and used as a germinant for *Bacillus globigii* spores. Radioactivity of the ^{14}C-L-alanine, the ^{14}C-D-alanine produced during germination, and the ^{14}C pyruvic acid produced by action of D-amino acid oxidase was estimated with an Ekco Type N 664 B Universal scintillation counter connected to a N 530 automatic scaler using NE 220 scintillation fluid (Nuclear Enterprises (G.B.) Ltd., Bankhead Medway, Sighthill, Edinburgh 11). Adequate controls for quenching were always included and the counts of samples were corrected accordingly. Samples were normally diluted to give about 10,000 counts/min., which was the optimal counting rate for the equipment used. The specific activity (μc/μmole) of the pyruvic acid (and hence of the D-alanine) was calculated from the colorimetric and radioactivity assays and compared with the specific activity of the L-alanine used as germinant. It was reasoned that the specific activity of the pyruvic acid (and therefore of the D-alanine) would be identical with that of the L-alanine if the D-alanine arose solely by racemization.

RESULTS

Auto-inhibition of germination

Figure 1 illustrates the effect of spore concentration on the germination of *Bacillus globigii* spores. It is clear that high concentrations of spores germinated less completely than low concentrations. The supernatant fluids of auto-inhibited cultures inhibited germination of fresh *B. globigii* spore suspensions in L-alanine + glucose medium or in yeast glucose broth, confirming the work of Stedman *et al.* (1956). Culture supernatant fluids and partially purified auto-inhibitor solution retained inhibitory activity after evaporation to dryness *in vacuo* at 40° and after storage for at least two months at 4°. The auto-inhibitor was not extracted by ether or chloroform. The auto-inhibitor migrated at the same rate as alanine on chromatograms run in two different solvents and during high voltage electrophoresis on paper. It diffused through Visking dialysis membrane and when the diffusate was autoclaved (126° for 20 min.) some inhibitory activity was lost, as found by Stedman *et al.* (1956). However, auto-inhibitor eluted from the alanine band on chromatograms was more heat stable than the auto-inhibitor in the crude supernatant fluid. This suggested that the apparent heat sensitivity of auto-inhibitor in crude super-

natant fluids was due to its reaction with a medium component which was presumably separated from the auto-inhibitor by chromatography. Eluates from the remainder of chromatograms contained no detectable inhibitor of germination. The partially purified auto-inhibitor also depressed the rate of spore germination of 4 out of 6 other *Bacillus* species studied (Table 1). All these results indicated that the auto-inhibitor was D-alanine.

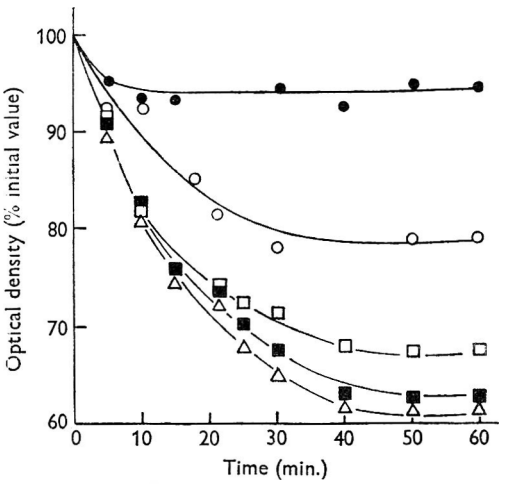


Fig. 1. Effect of spore concentration on the germination of Bacillus globigii spores. Incubation was at 30° in the defined medium and initial spore concentrations (equiv. mg. dry wt./ml.) were: ●, 1·1; ○, 0·4; □, 0·12; ■, 0·06; △, 0·05.

Table 1. *Effect of Bacillus globigii auto-inhibitor on the germination of B. subtilis and B. cereus spores*

Purified auto-inhibitor preparation was used at 1 ml./5 ml. of suspension.

	Decrease in extinction (%) after 30 min.	
	No auto-inhibitor added	Auto-inhibitor added
B. subtilis NCTC 2590	44	24
B. subtilis M 2	29	1
B. subtilis S 5	20	3
B. cereus S 20	35	8
B. cereus PX	45	42
B. cereus NCTC 945	65	60

Identification of the auto-inhibitor as D-alanine

When a concentrated spore suspension was germinated in medium containing D-amino acid oxidase (1·6 mg./ml.), auto-inhibition was decreased. When auto-inhibitor eluted from the alanine band of chromatograms was incubated with D-amino acid oxidase, oxygen was absorbed and, at the same time, loss of inhibitory activity occurred (Tables 2, 3). The keto acid formed was identified as pyruvic acid by the 2,4-dinitrophenylhydrazine method. This confirmed that the auto-inhibitor was D-alanine.

Table 2. *Effect of* D-*amino acid oxidase on auto-inhibitor and alanine*

Warburg flasks contained: D-amino acid oxidase, 16 mg.; sodium pyrophosphate buffer, pH 8·3, 20 μmole: DL-alanine, 150 μmole, or L-alanine, 150 μmole, or purified auto-inhibitor solution, 1 ml. Total volume of flask contents was 2·5 ml.: incubation at 35°.

Incubation time at 35° (min.)	Oxygen uptake (μl.) in presence of D-amino acid oxidase plus		
	Purified auto-inhibitor	DL-Alanine	L-Alanine
0	0	0	0
5	0·65	1·3	−0·05
15	2·20	5·35	−0·25
30	3·75	10·30	−0·20
70	6·05	22·75	−0·15
90	6·75	28·70	−0·25
120	7·30	36·15	−0·25

Table 3. *Effect of* D-*amino acid oxidase on the activity of the purified auto-inhibitor*

Purified auto-inhibitor was treated with D-amino acid oxidase for 2 hr as described in Methods.

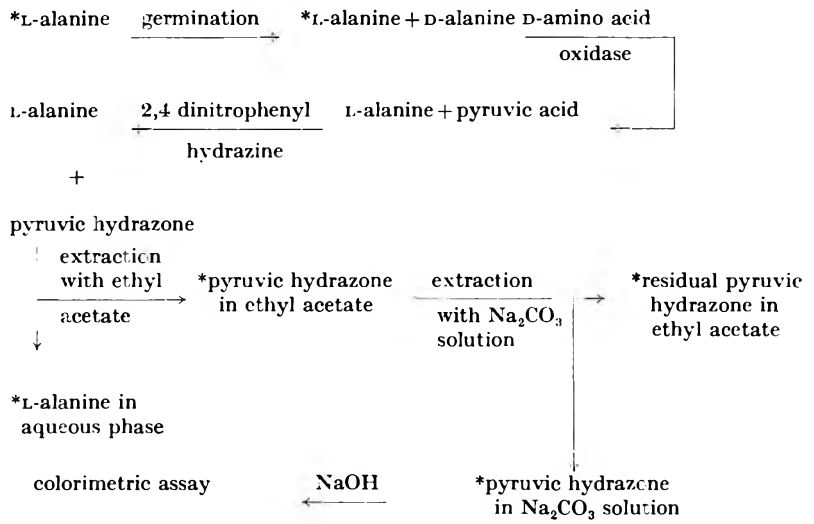
Incubation time at 37° (min.)	Germination (%) of *Bacillus globigii* spores in yeast glucose broth +		
	Untreated auto-inhibitor	D-Amino acid oxidase-treated auto-inhibitor	No auto-inhibitor
0	4	4	4
15	12	52	—
60	16	65	88

Origin of D-*alanine in supernatants of germinating Bacillus globigii spores*

Three possibilities existed for the origin of the D-alanine: (1) that the D-alanine arose by racemization of exogenous L-alanine; (2) that the D-alanine was liberated from endogenous spore material; (3) a combination of (1) and (2). To resolve these possibilities uniformly labelled ^{14}C-L-alanine was used as the germinant. If possibility (1) were correct, the resulting D-alanine should have been ^{14}C-labelled with the same specific activity as the ^{14}C-L-alanine used as germinant. If possibility (2) were correct, the D-alanine arising from unlabelled spore material should have been unlabelled. If possibility (3) were correct the D-alanine should have been ^{14}C-labelled but with a lower specific activity than the ^{14}C-L-alanine.

During purification of the auto-inhibitor strips of the chromatograms were scanned with a scintillation counter and only the alanine band was radioactive. The auto-inhibitory D-alanine and residual ^{14}C-L-alanine in supernatant fluids were eluted from the chromatograms. The D-alanine was estimated by measuring oxygen uptake in the presence of D-amino acid oxidase and also by estimating the resulting pyruvic acid by the 2,4-dinitrophenylhydrazine method. Since the hydrazine method effectively separated the pyruvic acid from any residual ^{14}C-L-alanine, the radioactivity of the pyruvic acid (and hence of the D-alanine) could be determined. Figure 2 illustrates the experimental steps and Table 4 summarizes the results.

Fig. 2. *Scheme for estimation of D-alanine by conversion to pyruvic acid*



* Samples assayed for radioactivity.

Table 4. *Conversion of ^{14}C-L-alanine into ^{14}C-D-alanine during germination of Bacillus globigii spores*

See Methods for experimental procedure.

	Amount (μmole), ^{14}C activity (μc) and specific activity (μc/μmole)	
	Before germination	1 hr after start of germination
L-Alanine		
μmole	200	140*
μc	20	13·95
μc/μmole	0·10	0·10*
D-Alanine (measured as pyruvate)		
μmole	0	39·8
μc	0	5·17
μc/μmole	0	0·13
D-Alanine (measured by O_2 uptake during enzymic oxidation)		
μmole	0	37·0
Recovery of ^{14}C label (%)	100	96
Recovery of L- + D-alanine (%)	100	90

* Specific activity of L-alanine after germination was assumed to be 0·1 and the μmole of L-alanine calculated on this assumption.

It is clear that 96 % of the ^{14}C-L-alanine supplied as germinant was accounted for after germination as unused ^{14}C-L-alanine + ^{14}C-D-alanine. Furthermore, the specific activity of the ^{14}C-D-alanine (estimated as ^{14}C-pyruvic acid) was 130 % of the specific activity of the ^{14}C-L-alanine originally present. This showed that the D-alanine produced by germinating *Bacillus globigii* spores arose predominantly from the L-alanine used as germinant and not from endogenous spore material.

A mixture of 8 mM-L-alanine + 2 mM-D-alanine, the concentrations found in auto-inhibited cultures, prevented germination (Table 5).

Table 5. *Germination of spores of Bacillus globigii in mixtures of* L-*alanine and* D-*alanine*

Fresh spores of *B. globigii* were incubated in D-alanine and L-alanine mixtures at 30°. The mixture of 8 mM-L-alanine + 2 mM-D-alanine is equivalent to that found in auto-inhibited cultures.

Concentration of mixtures (mM)		Extinction (%) after incubation for			
L-Alanine	D-Alanine	0 min.	15 min.	30 min.	60 min.
10	0	100	77	72·8	70
8	2	100	93·5	93·5	92·5
6	4	100	96·5	96·5	96·0
Purified supernatant of an auto-inhibited culture		100	93·5	90·0	89·0

DISCUSSION

The properties of the auto-inhibitor showed that it was D-alanine. This was confirmed by the labelling experiment, which further showed that the D-alanine originated from the L-alanine supplied as germinant and not from spore constituents, although D-alanine is present in spore mucopeptide (Strange & Thorne, 1957). When D-amino acid oxidase was added to *Bacillus globigii* spore suspensions at the start of germination, the auto-inhibitory effect was decreased; Stedman *et al.* (1956) were unable to show this. Such a discrepancy may be due to differences in the strains of *B. globigii* used or to different activities of the oxidase preparations or even to different mechanisms of auto-inhibition.

D-Alanine is a well-known inhibitor of spore germination (Hills, 1949) which probably acts by competing with L-alanine for the active site of L-alanine dehydrogenase (O'Connor & Halvorson, 1961). Stedman *et al.* (1956) showed low concentrations of alanine racemase in *Bacillus globigii* spores but Church, Halvorson & Halvorson (1954) did not detect it in the spores of this organism. Under the conditions of our experiments, however, the only way D-alanine could be formed from L-alanine was by the racemization reaction catalysed by alanine racemase; no attempt was made to extract the enzyme from spores.

One value of racemase in spores has been pointed out by Wolf & Mahmoud (1957): it enables spores to germinate slowly in D-alanine. In addition, however, it will prevent complete germination of dense populations of spores. Spores will often germinate in environments unsuitable for further growth; incomplete germination can therefore have a survival value because the ungerminated spores will survive and be able to germinate later. Auto-inhibition, the phenomenon in which large fractions of a spore population remain ungerminated, even when germinants are present in excess, has been observed in other *Bacillus* species (Murrell, 1961). Alanine racemase commonly occurs in spores of the genus *Bacillus* (Wolf & Mahmoud, 1957) and Powell (1957) obtained evidence that this enzyme was involved in auto-inhibition of germination of *B. subtilis* spores. In explaining auto-inhibition

of germination of spores of other species the mechanism described here for *B. globigii* must therefore be considered, particularly when L-alanine is known to be required for germination of the spores. Generally, bacterial spores respond to sublethal heating by germinating more rapidly than when unheated (Curran & Evans, 1944). *Bacillus globigii* spores, however, respond to sublethal heating by germinating less rapidly than when unheated. It is not known whether this phenomenon is related to auto-inhibition (for instance, by heat activation of the racemase), but heat-induced dormancy is not restricted to *B. globigii*, for Finley & Fields (1962) noticed a similar phenomenon with spores of *B. stearothermophilus*.

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Enzymes of the Tricarboxylic Acid Cycle in Acetic Acid Bacteria

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SUMMARY

The activities of seven enzymes which catalyse reactions of the tricarboxylic acid cycle were assayed in extracts of five lactaphilic and five glycophilic strains of acetic acid bacteria. Except for isocitrate dehydrogenase (which was not detected in glycophilic extracts) all other enzymes were found in all strains; but in general these enzymes were much more abundant in the lactaphilic extracts. In particular, extracts of glycophiles possessed only feeble citrate synthase, aconitate hydratase, fumarate hydratase and L-malate dehydrogenase activities and only their 2-oxoglutarate and succinate dehydrogenase activities were comparable to the corresponding activities in extracts of lactaphiles. Oxaloacetate decarboxylase activity was also greater in lactaphiles than in glycophiles. Two enzymes which oxidized L-malate were found: that in *Acetobacter acidum-mucosum* was an NADP-linked dehydrogenase, while the other more generally distributed enzyme required no added co-factors and may be cytochrome-linked. The evidence indicates that the tricarboxylic acid cycle may make a greater quantitative contribution to the metabolism of lactaphilic than to that of glycophilic organisms.

INTRODUCTION

There is nutritional and biochemical evidence for distinguishing two groups of acetic acid bacteria. Members of one group (the lactaphiles) have simple nutritional requirements, grow well on lactate and whole organisms oxidize intermediates of the tricarboxylic acid (TCA) cycle and effect reversible transamination between L-aspartate and 2-oxoglutarate. Members of the other group (the glycophiles) grow on glucose but not on lactate, have somewhat more complex nutritional requirements, and their ability to effect aspartate-2-oxoglutarate transamination and to oxidize TCA cycle intermediates is poorly developed or absent (Rainbow & Mitson, 1953; Brown & Rainbow, 1956; Cooksey & Rainbow, 1962). The lactaphiles and glycophiles thus distinguished correspond respectively to the genera *Acetobacter* and *Acetomonas* recognized by Leifson (1954) and by Shimwell (1958) on the criteria of flagellation and ability to oxidize acetate.

From their results, Cooksey & Rainbow (1962) suggested that the lactaphiles, but perhaps not the glycophiles, possessed an operative TCA cycle. The present work was designed to assess TCA cycle activity in acetic acid bacteria, as indicated by the individual activities of seven enzymes of the cycle in cell extracts, and so to test out this hypothesis.

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METHODS

Materials. Substrates were commercially available preparations. Before use in enzyme reaction mixtures, organic acids were adjusted with KOH solution to the pH value of the reaction mixture. Acetyl coenzyme A was synthesized by the method of Stadtman (1957).

Test organisms and cultivation. The organisms used were the strains of *Acetobacter* studied by Cooksey & Rainbow (1962) and a strain of *A. suboxydans*, which was derived from the original strain of Kluyver & van Leeuw and kindly given by Dr J. L. Shimwell (British Vinegars Ltd, Frome).

Experimental cultures were grown at 28° until good growth was obtained (2–7 days) in a medium having the following composition (g./100 ml.): glucose, 1; syrupy lactic acid (A.R.), 1; Difco yeast extract, 0·5; $(NH_4)_2HPO_4$ (A.R.), 0·2; L-glutamic acid, 0·1; $MgSO_4.7H_2O$ (A.R.), 0·05. These ingredients were dissolved in tap water and adjusted to pH 5·8 with KOH solution.

Preparation of cell extracts. After growth, organisms were harvested and washed as described by Cooksey & Rainbow (1962) and suspended in about 5 ml. of an ice-cold solution of appropriate buffer. The organisms in this suspension were disrupted in the Mullard/MSE Ultrasonic Disintegrator (Measuring and Scientific Equipment Ltd., London). During disruption (usually about 30 min.), the tube containing the material was cooled continuously in a freezing mixture. The resultant suspension was centrifuged at 15,000 *g* for 20 min. at 0–5°, yielding a pale reddish-brown supernatant fluid which was used for the enzyme assays. The protein content of this liquid was determined by the method of Lowry, Rosebrough, Farr & Randall (1951). For this determination, a solution of human plasma was used as a protein standard, the nitrogen content of this solution being determined by the micro-Kjeldahl procedure.

Enzyme assays. The enzyme nomenclature used is that recommended in the Report of the Commission on Enzymes of the International Union of Biochemistry, Pergamon Press, 1961, but trivial names are used for the dehydrogenase systems studied, since the experimental results do not permit precise identification of the enzymes concerned. In all cases, enzyme activities were calculated from initial reaction velocities determined over a period when plots of amount of chemical change against time were linear.

Spectrophotometric assays. These were done with the SP 500 quartz spectrophotometer (Unicam Ltd., Cambridge) or with the Cary recording spectrophotometer (Applied Physics Corporation, Monrovia, California). Fumarate hydratase (L-malate hydro-lyase, EC 4.2.1.2) and aconitate hydratase [citrate (isocitrate) hydro-lyase, EC 4.2.1.3] activities were assayed by a method based on that of Racker (1950), depending on measurement of increase in extinction at 240 mμ attendant on the conversion of L-malate to fumarate by fumarate hydratase and of citrate to *cis*-aconitate by aconitate hydratase. Citrate synthase (citrate oxaloacetate-lyase, EC 4.1.3.6) and malate synthase (L-malate glyoxylate-lyase, EC 4.1.3.2) activities were determined by a method based on that applied for malate synthase activity by Dixon & Kornberg (1959), in which progress of the reaction is followed by the fall in optical density at 232 mμ attendant on the cleavage of the thioester bond of acetyl coenzyme A during reaction with oxaloacetate. The

method was also applied to determine isocitrate lyase (L_s-isocitrate glyoxylate-lyase, EC 4.1.3.1) activity, the glyoxylate formed from isocitrate being converted to its phenylhydrazone, which was measured at 324 mμ. L-Malate and 2-oxoglutarate dehydrogenase systems were assayed by measuring at 600 mμ the loss of colour on reduction of 2·6-dichlorophenol-indophenol (DCPIP; Sanadi & Littlefield, 1951), and NADP-linked L-malate and isocitrate dehydrogenase systems by measurement of the increase in optical density at 340 mμ on reduction of NADP (Rao, 1955).

Manometric assays. These were done with a conventional Warburg constant-volume respirometer technique. Succinate dehydrogenase activity and L-malate oxidation were assayed by measurement of oxygen uptake, the former with succinate as substrate in the presence of phenazine methosulphate as electron carrier (Singer & Kearney, 1957) and the latter with L-malate as substrate. Oxaloacetate decarboxylase (oxaloacetate carboxy-lyase, EC 4.1.1.3) activity was measured in terms of rate of evolution of CO_2 from oxaloacetate.

Chromatography. Whatman No. 1 paper was used in conventional one-dimensional descending and ascending techniques. Oxo acids were chromatographed as their 2,4-dinitrophenylhydrazones in an ascending system as described by Smith (1958). The position of the acids was indicated by a brown area on a pale brown background.

RESULTS

Citrate synthase activities

By using the method of Ochoa, Stern & Schneider (1951), Cooksey & Rainbow (1962) readily detected citrate synthase in extracts of lactaphilic, but not in those of glycophilic, acetic acid bacteria. However, with this method, low citrate synthase activity might be overlooked in extracts possessing oxaloacetate decarboxylase activity because of decomposition of the oxaloacetate substrate. Consequently, the method of Dixon & Kornberg (1959), which is more sensitive and is not complicated by enzymic decarboxylation of oxaloacetate, was used to determine citrate synthase activities. The results (Table 1) show that activity was detected in extracts of glycophiles, but that the activity of all the lactaphiles far exceeded that of the most potent glycophile.

Aconitate hydratase and fumarate hydratase activities

Extracts of all the test strains possessed fumarate hydratase and aconitate hydratase activities, although those of the lactaphilic strains were considerably greater than those of glycophilic strains (Table 2). The fumarate hydratase and aconitate hydratase activities of *Acetobacter mobile*, while feebler than those of other lactaphiles, nevertheless exceeded those of any glycophile. Extracts from *A. rancens* showed a broad optimum of fumarate hydratase activity over the range pH 7·2–7·6. Activity was partially inhibited by 0·001 M-malonate, 0·017 M-D-malate and by 0·01 M-fluoride, but not by dialysis. In these properties, the enzyme resembled that prepared from other sources (Massey, 1953*a*, *b*). The properties of the aconitate hydratase of *A. rancens* resembled those of the mammalian enzyme (Morrison, 1954) in that activity was optimal over the range pH 7·0–7·6 and dialysis caused partial inhibition, which was annulled by addition of 0·003 M-Fe^{2+}.

Succinate dehydrogenase activities

Table 3 shows that extracts of all test organisms possessed succinate dehydrogenase activity. In general, extracts of lactaphiles were more active than those of glycophiles, but the difference between the two groups was not great.

Table 1. *Citrate synthase activities of cell extracts of acetic acid bacteria*

Reactions were carried out at 22° and pH 7·1 in 1 cm. quartz cuvettes containing, in a final volume of 1·5 ml.: cell extract; tris buffer (pH 7·1; 50 μmole); oxaloacetate (2 μmole); $MgCl_2$ (2 μmole); and acetyl coenzyme A (0·1 μmole), addition of which was used to start the reaction. Progress of the reaction was followed by fall in extinction at 232 mμ (E_{232}). Activity is given as μmole of acetyl coenzyme A hydrolysed/hr/mg. protein. The fall in E_{232} on hydrolysis of 1 μmole of acetyl coenzyme A in 1·5 ml. of water is 3·0.

Extract	Protein added (mg.)	Fall in E_{232} in 6 min.	Citrate synthase activity
Lactaphiles			
A. acidum-mucosum	0·015	0·133	29·0
A. ascendens	0·016	0·283	60·4
A. mobile	0·015	0·044	9·6
A. oxydans	0·025	0·357	47·9
A. rancens	0·028	0·249	30·7
Glycophiles			
A. capsulatum	0·090	0·008	0·3
A. gluconicum	0·060	0·020	1·1
A. suboxydans	0·062	0·018	0·9
A. turbidans	0·062	0·004	0·2
A. viscosum	0·072	0·009	0·5

Table 2. *Aconitate hydratase and fumarate hydratase activities of extracts of acetic acid bacteria*

Reactions were carried out at 22° and pH 7·0 in 1 cm. quartz cuvettes containing in a final volume of 3·0 ml.: cell extract; potassium phosphate buffer (pH 7·0; 50 μmole); and substrate (50 μmole). The reaction was started by addition of substrate (L-malate for fumarate hydratase or citrate for aconitate hydratase activity) and progress of the reaction was followed by increase in extinction at 240 mμ (E_{240}). Activity is given as μmoles of fumarate or *cis*-aconitate formed/hr/mg. protein. In 3·0 ml. of water, the extinction of 240 mμ of 1 μmole of fumarate and of 1 μmole of *cis*-aconitate are 0·703 and 1·18 respectively.

		Fumarate hydratase		Aconitate hydratase	
Extract from	Protein added (mg.)	Increase in E_{240} after 10 min.	Activity	Increase in E_{240} after 10 min.	Activity
Lactaphiles					
A. acidum-mucosum	0·103	0·105	8·7	0·332	16·4
A. ascendens	0·101	0·159	13·2	0·261	13·1
A. mobile	0·091	0·041	3·8	0·026	1·2
A. oxydans	0·112	0·312	23·7	0·356	16·1
A. rancens	0·101	0·127	10·7	0·236	11·8
Glycophiles					
A. capsulatum	0·202	0·011	0·4	0·006	0·1
A. gluconicum	0·130	0·007	0·5	0·009	0·4
A. suboxydans	0·101	0·015	1·3	0·007	0·4
A. turbidans	0·273	0·034	1·1	0·018	0·3
A. viscosum	0·274	0·028	0·9	0·017	0·3

Table 3. *Succinate dehydrogenase activities of extracts of acetic acid bacteria*

Warburg vessels contained in 3 ml. final volume: cell extract; phosphate buffer (pH 7·6; 150 μmole); potassium succinate (pH 7·6; 150 μmoles added from side arm); phenazine methosulphate (2 mg. added from side arm); KCN (3 μmole, pH 7·6). Reactions were carried out at 37° in subdued light and followed by uptake of oxygen, corrections being made in each case for the small volume of gas (1·5–10 μl.) evolved by the extract. Activity is given as μmole O_2 absorbed/hr/mg. protein.

Extract from	Protein added (mg.)	μl. O_2 absorbed/hr	Succinate dehydrogenase activity
Lactaphiles			
A. acidum-mucosum	1·62	57·4	1·58
A. ascendens	0·78	60·5	3·53
A. mobile	1·48	74·9	2·26
A. oxydans	1·63	46·2	1·27
A. rancens	1·46	85·4	2·61
Glycophiles			
A. capsulatum	1·32	49·9	1·68
A. gluconicum	0·83	24·5	1·32
A. suboxydans	1·68	29·6	0·80
A. turbidans	4·97	40·8	0·37
A. viscosum	4·98	41·8	0·38

Table 4. *Nicotinamide nucleotide-linked* L*-malate and isocitrate dehydrogenase activities of extracts of acetic acid bacteria*

Reactions were carried out at 22° in 1 cm. quartz cuvettes containing in a final volume of 0·41 ml.: tris buffer (pH 7·1; 25 μmole); L-malate or DL-isocitrate (pH 7·1; 25 μmole); and NAD or NADP (0·1 μmole) added to initiate the reaction. Reaction was followed by increase in extinction at 340 mμ (E_{340}). Activity is given as μ moles of reduced nicotinamide nucleotide produced/hr/mg. protein. 1 μmole of reduced NAD or NADP in 3·0 ml. water in the 1 ml. cuvette had an E_{340} of 2·1.

	Isocitrate dehydrogenases					NADP-linked L-malate dehydrogenase		
	NAD-linked			NADP-linked				
Extract from	Protein added (mg.)	Increase in E_{340} in 5 min.	Activity	Increase in E_{340} in 5 min.	Activity	Protein added (mg.)	Increase in E_{340} in 5 min.	Activity
Lactaphiles								
A. acidum-mucosum	0·025	0·114	3·59	0·530	16·7	0·25	0·277	0·85
A. ascendens	0·024	0·104	3·36	0·553	17·9	1·21		
A. mobile	0·097	0·274	2·21	0·232	1·9	0·97	No activity	
A. oxydans	0·028	1·75	49·8	0·241	6·7	1·37	detected over	
A. rancens	0·032	0·205	5·03	0·322	7·9	0·44	10 min.	

Glycophiles: All reaction mixtures contained at least 0·5 mg. protein added as enzyme extract, but no activity was detected over 10 min.

Isocitrate dehydrogenase activities

Both NAD- and NADP-dependent isocitrate dehydrogenases were detected in extracts of lactaphiles, but neither was detected in those of glycophiles (Table 4). However, the progress curve of the NADP-linked reaction was sigmoidal, the lag period, before maximum rate of reaction was attained, increasing with enzyme dilution. Consequently, failure to detect isocitrate dehydrogenation by extracts of glycophiles was not proof of its absence, although it did indicate that the amount

of the enzyme in glycophilic extracts was indeed low, especially large amounts of protein having been added as extract in an attempt to detect it.

Malate-oxidizing enzymes

Several attempts to detect an NAD-dependent L-malate dehydrogenase failed, but an NADP-dependent enzyme was detected only in extracts of *Acetobacter acidum-mucosum* with a high protein content (Table 4). Maximum activity of this enzyme was observed at pH 7·0–7·5. Activity was inhibited by dialysis, but restored by addition of 0·0003 M-Mn^{2+}, and inhibited by 0·001 M-*p*-chloromercuribenzoate. Like isocitrate dehydrogenase, the progress curve for enzyme activity was sigmoidal, the length of the lag phase being diminished by using greater enzyme concentrations and by incubating the enzyme with L-malate before starting the

Table 5. *Activities of the dichlorophenolindophenol-linked* L-*malate and 2-oxoglutarate dehydrogenases in extracts of acetic acid bacteria*

For L-malate dehydrogenase, reactions were carried out at 22° in 1 cm. quartz cuvettes containing in a final volume of 3·0 ml.: cell extract; tris buffer (pH 7·0; 100 μmole); L-malate (pH 7·0; 20 μmole); $MnSO_4$ (1 μmole); 2·6 dichlorophenolindophenol (DCPIP, 0·1 μmole, added to start the reaction). For 2-oxoglutarate dehydrogenase, reactions were carried out as above, except that L-malate and tris buffer were replaced by 2-oxoglutarate (pH 7·0; 60 μmole) and phosphate buffer (pH 7·0; 100 μmole), $MnSO_4$ was omitted and thiamine pyrophosphate (2 μmole) was added. The progress of the reactions was followed by measuring the difference in extinction at 600 mμ (E_{600}) between the reaction mixtures and appropriate blanks consisting of the same mixtures, but lacking substrate. Activity is given as μmole of DCPIP reduced/hr/mg. protein. A solution containing 0·1 μmole DCPIP per 3 ml. water had an E_{600} of 0·51.

	2-oxoglutarate dehydrogenase			L-malate dehydrogenase		
Extract from	Protein added (mg.)	Fall in E_{600} in 5 min.	Activity	Protein added (mg.)	Fall in E_{600} in 5 min.	Activity
Lactaphiles						
A. acidum-mucosum	0·259	0·036	0·32	0·248	0·187	1·48
A. ascendens	0·395	0·085	0·51	0·121	0·086	1·36
A. mobile	0·543	0·020	0·08	0·485	0·046	0·19
A. oxydans	0·370	0·054	0·35	0·032	0·105	6·51
A. rancens	0·184	0·030	0·38	0·159	0·041	0·51
Glycophiles						
A. capsulatum	0·516	0·038	0·17	0·261	0·040	0·30
A. gluconicum	0·260	0·012	0·10	0·272	0·002	0·01
A. suboxydans	0·252	0·029	0·27	0·368	0·021	0·11
A. turbidans	0·353	0·062	0·41	0·306	0·025	0·16
A. viscosum	0·315	0·075	0·56	0·277	0·048	0·34

reaction by adding NADP. Oxaloacetate was identified as a product of the reaction by paper chromatography and since the reaction was completely inhibited by 0·033 M-oxaloacetate, but not by 0·033 M-pyruvate, it appeared to be catalysed by an L-malate : NADP oxidoreductase different from the L-malate : NADP oxidoreductase (decarboxylating) (EC 1.1.1.40) found in Chromatium by Fuller & Kornberg (1961).

Failure to detect a nicotinamide nucleotide-dependent malate-oxidizing system in strains other than *Acetobacter acidum-mucosum* prompted a search for an alterna-

tive hydrogen-acceptor for L-malate oxidation. Methylene blue, 2,3,5-trimethyltetrazolium chloride and neotetrazolium chloride failed in this role, but DCPIP was suitable for the dehydrogenation of both L-malate and of 2-oxoglutarate (see below). In experiments with L-malate, the same enzyme extracts were used as for the tests for NADP-linked L-malate dehydrogenase. Oxaloacetate was detected by paper chromatography as a reaction product and the enzyme was found in extracts of lactaphiles and of glycophiles, the former, with the exception of *A. mobile*, being richer in the enzyme (Table 5).

Table 6. *Oxidation of L-malate by extracts of Acetobacter rancens, A. acidum-mucosum and A. capsulatum*

Warburg vessels contained a final volume of 3·0 ml., made up of cell extract, tris buffer (pH 7·1; 100 μmole) and the following additions as indicated: L-malate (pH 7·1; 10 μmole); NAD (1 μmole); DL-isocitrate (pH 7·1; 10 μmole); NADP (1 μmole); KCN (3 μmole; pH 7·1); guanidine (30 μmole). Temperature 28°. The reaction was started by adding L-malate (or DL-isocitrate) from the side arm and was followed by measurement of O_2 uptake.

Extract from	Reaction mixture	Added (mg.)	μl. O_2 absorbed/hr	μmole O_2 absorbed/mg. protein/hr
A. rancens	No additions	1·54	2	0·06
	+ L-malate	1·54	56	1·63
	+ L-malate + NAD	1·54	56	1·63
	+ L-malate + NADP	1·54	58	1·68
A. acidum-mucosum	No additions	1·80	3	0·09
	+ L-malate	1·80	200	4·96
	+ L-malate + KCN	1·80	20	0·50
	+ L-malate + guanidine	1·80	205	5·06
	+ L-malate + cytochrome *c*	1·80	190	4·71
	+ DL-isocitrate	1·80	0	0
	+ DL-isocitrate + NAD	1·80	0	0
A. capsulatum	No additions	1·09	0	0
	+ L-malate	1·09	30	1·23

Manometric experiments with extracts of *Acetobacter acidum-mucosum*, *A. rancens* and *A. capsulatum* showed that L-malate oxidation was accompanied by uptake of oxygen (Table 6), which was unaffected by addition of NAD, NADP or cytochrome *c*, but inhibited 90 % by 0·001 M-cyanide. Guanidine (0·01 M), which in mammalian systems inhibits electron transfer between reduced nicotinamide nucleotides and cytochrome *c* (Hollunger, 1955), did not inhibit the reaction. In similar experiments, isocitrate was not oxidized, even when NAD was added. The possibility that the oxidation of L-malate was mediated by cytochromes other than cytochrome *c* was indicated by the following experiment. An extract of *A. acidum-mucosum* with a high protein content (10 mg./ml.) was shaken in air to ensure that all its cytochromes were in the oxidized state. A portion of this preparation was incubated with buffered L-malate (pH 7·1) and the absorption spectrum of the reaction mixture was plotted between 450 and 600 mμ, using as reference solution the same reaction mixture from which L-malate was omitted. The difference spectrum so obtained had peaks at 525 and 555 mμ similar to those observed for the cytochromes of other acetic acid bacteria by Smith (1954), King & Cheldelin (1956), Fewster (1958) and by Stouthamer (1961). No difference spectrum was obtained

when isocitrate was substituted for L-malate in the reaction mixture. Similar results were obtained with extracts of *A. oxydans*. These results indicate that the aerobic oxidation of L-malate by extracts of acetic acid bacteria was accompanied by spectral changes suggestive of the formation of reduced cytochromes.

2-Oxoglutarate dehydrogenase activities

Oxidation of 2-oxoglutarate using DCPIP as hydrogen acceptor led to the formation of succinate, identified by paper chromatography. Extracts of all strains possessed a similar order of 2-oxoglutarate dehydrogenase activity (Table 5).

Table 7. *Oxaloacetate decarboxylase activities of extracts of acetic acid bacteria*

Warburg vessels contained in 3·0 ml. final volume: cell extract; potassium acetate buffer (pH 5·6; 100 μmole); $MnSO_4$ (1 μmole); and oxaloacetate (pH 5·6; 10 μmole added from side arm). Temperature 35°. Activity is given as μmoles CO_2 evolved/hr/mg. protein, and is corrected for spontaneous decomposition of oxaloacetate.

Extract from	Protein added (mg.)	μl. CO_2 evolved/hr	Oxaloacetate decarboxylase activity
Lactaphiles			
A. acidum-mucosum	0·162	57	15·6
A. ascendens	0·312	47	6·9
A. mobile	0·742	354	21·3
A. oxydans	0·612	334	24·4
A. rancens	0·486	175	16·1
Glycophiles			
A. capsulatum	0·662	10	0·7
A. gluconicum	0·414	38	4·1
A. suboxydans	0·435	37	3·8
A. turbidans	0·497	14	1·3
A. viscosum	0·498	12	1·1

Table 8. *Isocitrate lyase activities of extracts of acetic acid bacteria*

Reactions were carried out at 22° and pH 6·85 in 1 cm. quartz cuvettes containing, in a final volume of 3·0 ml.: cell extract; phosphate buffer (pH 6·85, 200 μmole); DL-isocitrate (pH 6·85, 5 μmole); $MgCl_2$ (15 μmole); L-cysteine (pH 6·85, 6 μmole); and phenylhydrazine (10 μmole). Reactions were initiated by addition of cell extract and their progress followed by measurement of increase in extinction at 324 mμ (E_{324}) over that of a blank containing no substrate. Activity is given as μmole of glyoxylate produced/hr/mg. protein. The molar extinction coefficient of glyoxylate phenylhydrazone is $1·7 \times 10^4$ (Dixon & Kornberg, 1959).

Extract from	Protein added (mg.)	Increase in E_{324} in 5 min.	Activity
Lactaphiles			
A. acidum-mucosum	0·86	0·069	0·17
A. ascendens	0·78	0·046	0·13
A. mobile	0·74	0·091	0·26
A. oxydans	0·61	0·020	0·70
A. rancens	0·97	0·069	0·15

Glycophiles: reaction mixtures contained 0·24–1·98 mg. protein added as enzyme extract, but no activity was detected in 10 min.

Oxaloacetate decarboxylase activities

Because of its possible influence on assays of citrate synthase by the method of Ochoa *et al.* (1951) already mentioned, assays of oxaloacetate decarboxylase activities were done with freshly prepared bacterial extracts. Table 7 shows that extracts of lactaphiles were more active in decarboxylating oxaloacetate than were those of glycophiles. Action took place optimally at pH 5·6 and was stimulated by addition of Mn^{2+}. Activity of the extracts diminished rapidly on storage even at $-15°$.

Isocitrate lyase and malate synthase activities

These enzyme activities were tested by the methods of Dixon & Kornberg (1959). The results (Table 8) show that extracts of lactaphiles, but not of glycophiles, possessed isocitrate lyase activity. Malate synthase activity was not detected in extracts of either lactaphiles or glycophiles, although an extract prepared from a strain of *Acetobacter aceti* grown on Hoyer's ethanol+inorganic salts medium (Frateur, 1950) had an activity of 67 μmoles malate synthesized/hr/mg. protein.

The organisms used in the present work grew, at best, only feebly on the culture medium when acetate replaced glucose and lactate as energy source, although Brown & Rainbow (1956) found that acetate could stimulate growth of lactaphiles in a casein hydrolysate medium. The lack of malate synthase in organisms grown in glucose+lactate may well be the reason why our lactaphiles did not grow on ethanol or acetate as sole carbon source (Brown & Rainbow, 1956). *Acetobacter aceti*, however, possesses both malate synthase (see above) and isocitrate lyase (Smith & Gunsalus, 1955) and therefore grows on acetate as sole carbon source (Frateur, 1950).

DISCUSSION

The results may be summarized conveniently by Table 9, which shows that all the lactaphilic acetic acid bacteria examined possess the enzymic equipment for carrying out the reaction sequence of the TCA cycle. In contrast, the glycophilic strains were in general much less well endowed with these enzymes, especially citrate synthase, aconitate hydratase, fumarate hydratase and L-malate dehydrogenases, while isocitrate dehydrogenase activity was not detected in any glycophilic extract. In the glycophiles, only the 2-oxoglutarate and succinate dehydrogenase activities were of a similar order to those found in lactaphiles. As a lactaphile, *Acetobacter mobile* was somewhat exceptional in that, although all the enzymes of the cycle were detected in it, their activities were considerably lower than those of the other lactaphiles and, in two cases, lower than those of glycophiles. *A. mobile* thus appears to have properties relating it to the glycophiles, as had already been noted from nutritional studies (Rainbow & Mitson, 1953).

There are previous reports dealing with the TCA cycle in acetic acid bacteria. Thus, its presence was reported by King, Kawasaki & Cheldelin (1956) in *Acetobacter pasteurianum*, by Rao (1955) in *A. aceti* and by Stouthamer (1959) in strains of Frateur's (1950) oxydans and mesoxydans groups. All these appear to be 'over-oxidizing' (i.e. they oxidize acetate to CO_2 and water) strains of Acetobacter, which fall into our lactaphilic group. On the other hand, reports on strains of Frateur's 'non-over-oxidizing' suboxydans group consistently indicate that they lack the

TCA cycle (King & Cheldelin, 1952; Stouthamer, 1959). The present results tend to bear out these contentions, although it remains possible that members of the suboxydans (glycophilic) group have severely restricted ability to carry out the reactions of the TCA cycle, rather than that they completely lack this ability. In particular, a more exhaustive search for isocitrate dehydrogenase activity in glycophylic extracts is desirable. The results are also consistent with the view (Rainbow & Mitson, 1953; Brown & Rainbow, 1956; Cooksey & Rainbow, 1962) that the

Table 9. *Comparative activities of TCA cycle enzymes in extracts of acetic acid bacteria*

For each enzyme, activities are expressed relative to that of the most active extract (100). C, citrate synthase; A, aconitate hydratase; ID/NAD and ID/NADP, isocitrate dehydrogenase (NAD- and NADP-linked); OD, 2-oxoglutarate dehydrogenase; SD, succinate dehydrogenase; F, fumarate hydratase; MD, L-malate dehydrogenase (DCPIP-linked).

	C	A	ID/NAD	ID/NADP	OD	SD	F	MD
Lactaphiles								
A. acidum-mucosum	48	100	7	93	58	45	37	23
A. ascendens	100	80	7	100	92	100	56	21
A. mobile	16	8	4	10	15	64	16	3
A. oxydans	79	98	100	38	63	36	100	100
A. rancens	51	72	11	44	68	74	45	8
Glycophiles								
A. capsulatum	<1	1	0	0	31	48	2	5
A. gluconicum	2	2	0	0	19	37	2	<1
A. suboxydans	2	2	0	0	49	23	5	2
A. turbidans	<1	2	0	0	74	10	4	3
A. viscosum	1	2	0	0	100	11	4	5

lactaphilic and glycophilic acetic acid bacteria are two nutritional and biochemical types, corresponding respectively to the genera *Acetobacter* and *Acetomonas* as defined by Leifson (1954) and by Carr & Shimwell (1961). Nevertheless, this view does not preclude the possibility of the existence of forms, like our *A. mobile*, possessing properties intermediate between those of the two groups.

It is interesting that only *Acetobacter acidum-mucosum* of our strains possessed an NADP-dependent L-malate dehydrogenase, although Rao (1955) found a similar enzyme in *A. aceti*. All other strains, and *A. acidum-mucosum* itself, oxidized L-malate possibly in a cytochrome-dependent system in which DCPIP or molecular oxygen acted as terminal acceptor. Two malate oxidizing systems have also been described in *Pseudomonas ovalis* by Kornberg & Phizackerley (1961) but, in this case, the nicotinamide nucleotide-linked enzyme was an oxidative decarboxylase.

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Action of the Polyene Antibiotics Filipin, Nystatin and N-Acetylcandidin on the Yeast Cell Membrane

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SUMMARY

The effects of the antifungal polyene antibiotics N-acetylcandidin, nystatin and filipin on the yeast cell membranes were compared with the reversibility by K^+ and NH_4^+ of the inhibition of glycolysis caused by these antibiotics. The results confirm the existence of two functional classes of polyenes which correspond to the number of carbon atoms in the molecule; intermediate types of action can be illustrated within the group of large polyenes. The inhibition of yeast glycolysis by N-acetylcandidin, a large polyene, was annulled by K^+ and NH_4^+, even at high antibiotic concentrations; inhibition by filipin, a small polyene, was not so annulled. The inhibition of glycolysis by polyenes was associated with the loss of cell K^+; filipin caused concurrent loss of inorganic phosphate and of accumulated sugar (L-sorbose) and initiated ATPase and pyruvate decarboxylase activity. Although N-acetylcandidin treatment did not produce loss of accumulated sorbose, added sorbose leaked into treated cells when uptake by way of the hexose transport system had been blocked (by glucose). Thus, definite membrane damage had occurred. Nystatin, a large polyene, was intermediate in effect: at low concentrations its action approached that of N-acetylcandidin; at high concentrations, its action was similar to that of filipin. It is suggested that the polyenes present a full spectrum of effects which relate to the degree of physical damage to the cell membrane. They range from filipin which destroys general structural integrity of the membrane even at the minimum concentration necessary to inhibit glycolysis (and growth) to N-acetylcandidin which produces only minimal damage so that relatively specific defects are observed, e.g. those related to K^+ loss and sorbose leakage.

INTRODUCTION

Considerable evidence is now available that the polyene antifungal antibiotics combine with the yeast cell membrane and decrease its ability to take up and retain critical metabolites (Lampen, 1962; Lampen & Arnow, 1963; Kinsky, 1962*a*; Stachiewicz & Quastel, 1963). Direct evidence for an interaction between polyene and membrane has been provided from studies with protoplasts and isolated membrane fractions (Kinsky, 1962*a*, *b*; Lampen, Arnow, Borowska & Laskin, 1962). This interaction appears to involve the membrane sterols. This explains the protective effect of sterols (Gottlieb, Carter, Sloneker & Ammann, 1958; Lampen, Arnow

& Safferman, 1960) and the insusceptibility of bacteria, which are generally almost devoid of sterols.

One of the earliest detectable effects of polyene action on baker's yeast is the rapid loss of K^+ from the organisms. This loss is reflected in decreased glycolysis (Marini, Arnow & Lampen, 1961; Harsch & Lampen, 1963). Polyenes have been separated into two major classes on the basis of the annulment by K^+ and NH_4^+ of their inhibition of yeast glycolysis (Lampen & Arnow, 1963). This separation can be correlated with the number of carbon atoms per molecule. Inhibition of glycolysis by the large polyenes (those which contain 46 or 47 carbon atoms per molecule) is either prevented or annulled by the presence of K^+ and NH_4^+ ions. Inhibition by the small polyenes (containing 34–37 carbon atoms per molecule) is not prevented or annulled by K^+ and NH_4^+, nor by a mixture of the known glycolytic co-factors or by boiled yeast juice. The reversibility (annulment of inhibition) or non-reversibility can be understood if binding of the large polyenes produces a relatively specific damage to the K^+ accumulation system of the cell, whereas the small polyenes cause more generalized defects in the cell membrane and an irreparable loss of some cell constituents. Evidence in support of this hypothesis has been reviewed (Lampen, 1962; Lampen & Arnow, 1963; Harsch & Lampen, 1963).

The proposed difference in the action of the large and small polyenes could be tested by determining the effect of these polyenes on the glucose-inhibited transport of non-metabolizable sugars. L-Sorbose is transported across the yeast cell membrane by a facilitated diffusion process which is inhibited competitively by glucose (Cirillo, 1961*a*). The ability of glucose to inhibit sorbose transport into or out of the cell provides a criterion of cell membrane integrity and, therefore, of the effect of the two classes of polyenes on yeast membrane structures.

The present experiments were designed to compare the actions on the cell membrane of three polyenes: (1) filipin, one of the polyenes most active in initiating lysis of protoplasts (Kinsky, 1962*a*); (2) N-acetylcandidin (NAC), whose action on glycolysis is readily annulled by K^+ and NH_4^+ ions; (3) nystatin, whose action is reversible at low concentrations but which causes leakage of many cell constituents at higher concentrations; however, even then, glycolytic inhibition can still be partially annulled by a mixture of the usual glycolytic cofactors.

METHODS

Organisms. Commercial (Anheuser-Busch, Inc., Old Bridge, New Jersey) baker's yeast obtained as pressed filter cake was washed four times in distilled water before use.

Saccharomyces cerevisiae strain LK 2G 12 (from Dr H. O. Halvorson, University of Wisconsin, Madison) and strain AB 14 (obtained as a pure culture isolated from a cake of the commercial Anheuser-Busch yeast) were grown for 16 hr on Sabouraud's glucose agar slopes (Difco Laboratories, Inc., 920 Henry St., Detroit, Michigan). The growth from one slope was transferred to 100 ml. of Wickerham medium (Wickerham, 1951) and incubated for 16 hr at 28° on a rotary shaker; 10 ml. of this culture was added to 100 ml. of Wickerham medium and incubated as above for 4 hr to produce a culture of log-phase organisms.

All weights of organisms are expressed as equivalent dry weight.

Manometry. The conventional Warburg respirometer was used at 30°. Each vessel contained equiv. 2 mg. yeast in 3·0 ml. of 0·06 M-tris + succinate + tartrate or Na^+-tris buffer (Harsch & Lampen, 1963) at pH 7·5, and 0·04–0·1 M-glucose.

Potassium and phosphate analyses. Samples of packed organisms were suspended in demineralized water and placed in a boiling water bath for 5–10 min. Upon cooling, the extracts were cleared by centrifugation, diluted appropriately and analysed. K^+ was analysed by using the Beckman Model B flame spectrophotometer with an oxygen-acetylene burner. Inorganic phosphate (P_i) was determined by the method of Fiske & SubbaRow according to Umbreit, Burris & Stauffer (1957).

ATPase activity. ATPase activity is expressed as the μg. of inorganic phosphate released from ATP in 30 min. at 30° by equiv. 1 mg. yeast. The assay mixture contained 20 mg. yeast material (either as intact organisms or a sonically-treated suspension), 2 ml. of ATP stock solution and 1 ml. of 0·03 M-$MgCl_2$ in a total volume of 10 ml. Samples were taken at 30 min. for measurement of inorganic phosphate. The ATP stock solution contained 467 mg. ATP dissolved in 5 ml. of 0·3 M-tris + succinate buffer (pH 7·0). To this was added 0·7 ml. of M-KCl and 0·08 ml. of 5 N-NaOH and the final volume brought to 25 ml. with 0·3 M-tris + succinate buffer (pH 7·0).

Sorbose uptake. To 1 ml. of a 1 % (v/v) yeast suspension in a 12 ml. centrifuge tube was added 4 ml. of 0·02 M-tris + succinate + tartrate (pH 7·5) containing sufficient L-sorbose to give a final concentration of 3–15 % (w/v). The yeast suspension was incubated in a sloped position on a shaker at 30° for 1 hr, after which the organisms were packed by centrifugation and washed with three 5 ml. portions of ice-cold 0·9 % (w/v) NaCl. The washed organisms were resuspended in distilled water and extracted in a boiling water bath for 20 min. After cooling, the heated organisms were removed by centrifugation and the extracts analysed for sorbose by the Dische & Devi (1960) procedure for ketoses. In some experiments in which glucose was included in the sorbose incubation medium, the extracts were analysed for glucose by the Glucostat method (Worthington Biochem. Co., Freehold, New Jersey). Sorbose and glucose content are expressed as mg. sugar/ml. cell water calculated on the basis that cell water represents 47 % of the packed cell volume (Cirillo, 1962).

Sorbose efflux in glucose + iodoacetic acid solution. In some experiments yeast which was washed in ice-cold distilled water after incubation for 1 hr in sorbose was resuspended in a sorbose-free medium containing 15 % (w/v) glucose and 10^{-3} M-iodoacetic acid (IAA) at 30°. At the end of this second incubation, the organisms were washed in ice-cold saline and extracted as described above.

Polyene and cholesterol additions. N-Acetylcandidin was dissolved in 0·2 M-tris + succinate + tartrate buffer (pH 7·5) according to the procedure of Harsch & Lampen (1963). Filipin and nystatin were dissolved in either dimethyl sulphoxide or absolute methanol. Cholesterol was dissolved in absolute ethanol. Control organisms received the same amount of solvent or buffer as added with the antibiotic or cholesterol. The concentrations of polyenes or cholesterol are expressed as μg./mg. dry weight yeast.

Polyene sources. N-Acetylcandidin was provided by Dr E. Borowski and Dr C. P. Schaffner (Institute of Microbiology, Rutgers, The State University, New Bruns-

wick, N.J.); nystatin by the Squibb Institute for Medical Research (New Brunswick, N.J.) and filipin by the Upjohn Co. (Kalamazoo, Michigan).

RESULTS

Changes in K^+ and inorganic phosphate concentrations and ATPase availability

The polyenes NAC, nystatin, and filipin all initiated a rapid loss of cellular K^+ from log-phase yeast of a strain LK 2G 12, but their effects on inorganic phosphate (P_i) concentrations and on ATPase differed (Table 1). Thus, 20 μg. NAC/mg. dry wt. yeast produced complete K^+ loss in 90 min., but 100 μg./mg. did not cause a significant decrease of P_i content. Cellular P_i amounts decreased sharply with 20 μg. nystatin or filipin (Table 1), and in other experiments, with as little as 5 μg. either polyene/mg. dry wt. yeast. These findings were confirmed by measurement of the actual loss of P_i to the suspending medium.

Table 1. *The effect of antifungal polyene antibiotics on cellular K^+ and inorganic phosphate (P_i) concentration and ATPase availability*

Washed log-phase yeast of strain LK 2G12 (equiv. 50 mg. dry wt.) was shaken at 30° in 25 ml. of 0·06 M-tris + succinate buffer (pH 7·5) containing 0·12 M-glucose and the polyenes as listed. Nystatin and filipin were dissolved in 0·2 ml. dimethyl sulphoxide, NAC in the tris-succinate buffer. Samples were removed at indicated times for K^+ analysis. After 60 or 90 min. incubation, the organisms were washed and resuspended at equiv. 10 mg. dry wt./ml. in demineralized water. ATPase activity (see Methods for unit) was determined on these intact organisms after 90 min. contact with the antibiotics and on the suspension after sonic treatment for 5 min. at 0° in the Mullard disintegrator. Inorganic phosphate (P_i) content of the treated organisms after 90 min. contact with the antibiotics was determined on the sonically-treated extract.

Treatment (quantities μg./mg. dry wt. organism)	Time (min.) 0	60	90	90	90	
	μg. K^+/mg. dry wt. cells			μg. P_i/mg. dry wt. cells	ATPase activity intact cells	sonic extract
I. None	24·1	.	21·0	9·9	1·3	33·1
+ NAC (20)	.	.	2·8	7·3	0·2	19·7
+ NAC (100)	.	.	2·3	9·5	0·9	34·1
II. None	21·1	20·6	.	7·8	0·25	45·3
+ Dimethyl sulphoxide (0·2 ml.)	.	19·4	.	7·7	2·4	35·5
+ Nystatin (20)	.	< 1	.	2·4	18·7	22·9
+ Filipin (20)	.	< 1	.	0·4	24·9	16·5

The specific ATPase of yeast is associated with the membrane fraction (Bolton & Eddy, 1962); in log-phase organisms of our strain of yeast it is not accessible to external substrate unless the organisms are damaged, e.g. by toluene or sonic treatment. NAC did not cause this membrane ATPase to become accessible (Table 1); nystatin-treated and filipin-treated organisms, in contrast, showed substantial ATPase activity. The total activity of these organisms (as measured in sonic extracts) actually decreased during treatment. Although this decrease may represent a destruction of enzyme, the situation is probably more complex since Dr E. Racker (personal communication) has found in yeast extracts an inhibitor of ATPase

similar to that present in beef heart mitochondria (Pullman & Monroy, 1963). ATPase activity was not released into the suspending medium except with 20 μg. filipin/mg. dry wt. yeast; even then the loss only amounted to about 5 % of the newly detectable ATPase of the treated organisms.

The action of various concentrations of nystatin and filipin was compared in the experiment presented in Table 2. Nystatin produced almost complete K^+ loss at 1 μg./mg. dry wt. yeast. Only a low ATPase activity was seen at this concentration or at 5 μg.; 20 μg. nystatin/mg. dry wt. yeast was required for a major effect. Although the cellular K^+ concentration may respond to a slightly lower filipin concentration than does ATPase (see the results with 0·2 μg. filipin/mg.), the corresponding membrane alterations must occur almost in parallel since 1 μg. filipin, which caused only partial K^+ loss, also made a large part of the membrane ATPase accessible.

Table 2. *Differential effect of nystatin and filipin on K^+ content and ATPase activity of yeast*

Experimental conditions as in Table 1. At 5 μg. nystatin or filipin, 0·2 ml. dimethyl sulphoxide was present, at lower concentrations correspondingly less. ATPase was measured on intact organisms at 60 min.

Treatment (quantity μg./mg. dry wt. cells)	μg. K^+/mg. cells		ATPase of treated cells.
	0 min.	60 min.	60 min.
Dimethyl sulphoxide (0·2 ml.)	25·3	24·6	0·3
Nystatin (0·2)	.	21·5	0·4
Nystatin (1·0)	.	2·7	2·9
Nystatin (5·0)	.	2·3	3·5
Filipin (0·2)	.	20·3	0·6
Filipin (1·0)	.	7·5	10·4
Filipin (5·0)	.	2·1	17·7

Annulment of NAC inhibition of glycolysis

The action of nystatin, filipin and NAC on glycolysis has previously been described. With *Saccharomyces cerevisiae*, log-phase organisms, rapid inhibition in neutral Na^+ + buffer was obtained with 3–5 μg. nystatin, 6–8 μg. NAC, or 5–8 μg. filipin/mg. dry wt. yeast (Lampen & Arnow, 1963). Inhibition by nystatin and NAC was annulled by K^+ and NH_4^+, but the inhibitory action of filipin was not annulled or prevented by a mixture of glycolytic cofactors.

Of special interest for the present comparison is the observation that the inhibition of glycolysis by a high concentration of NAC (100 μg./mg. dry wt. yeast) was completely annulled by 0·01 M-NH_4Cl (Fig. 1) even when addition was delayed until 60 min. after CO_2 production had ceased. KCl was effective at 0·01 M when added with the polyene or shortly after the inhibition had been established, but by 60 min. after polyene addition 0·1 M-KCl was necessary to produce even partial annulment. There was no response to the glycolitic co-factors which are needed for the annulment of the inhibition by high concentrations of nystatin (Sutton, Arnow & Lampen, 1961).

Inhibition by filipin of sorbose retention

The effect of filipin on sorbose retention was measured with commercial yeast incubated in 15% (w/v) sorbose for 1 hr. With untreated organisms, sorbose enters the cell and equilibrates with the external medium; washing with saline at 4° results in only negligible loss of sorbose (Cirillo, 1962). Filipin prevented the retention of

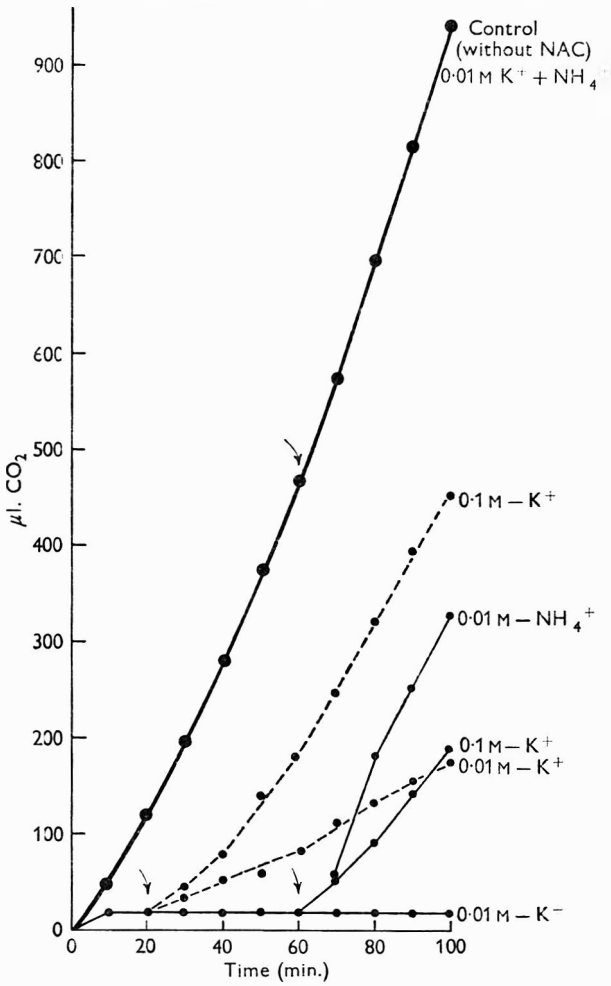

Fig. 1. Annulment by K^+ or NH_4^+ of N-acetylcandidin (NAC) inhibition of glycolysis by a yeast. Each vessel received 2·0 mg. of log-phase organisms of strain LK2G12 and 120 μmoles glucose in 3·0 ml. of 0·06 M-Na^+ + tris buffer (pH 7·5), and 200 μg. NAC at zero time. KCl or NH_4Cl (0·01 or 0·1 M) was added either just after inhibition was maximal (20 min.) or after 60 min. (indicated by the arrows).

sorbose during cold washing (Table 3; no cholesterol added). The decrease in sorbose content in filipin-treated organisms paralleled the inhibitory effect of filipin on glycolysis in commercial yeasts.

Since sterols protect against polyene inhibition of yeast glycolysis (Lampen *et al.* 1960; Gottlieb *et al.* 1961), cholesterol protection from filipin action on sorbose

retention was tested. When cholesterol 40 μg./ml. was added to the yeast suspension together with filipin, cholesterol protected the membrane against all concentrations of filipin tried. However, when the cholesterol was added 30 min. after filipin there was no protection (Table 3; cholesterol addition: zero time and 30 min., respectively).

Table 3. *Inhibition by filipin of sorbose retention by yeast*

Washed commercial yeast (0·01 ml. packed volume) was incubated at 30° for 1 hr in 5 ml. of 0·03 M-tris + succinate buffer (pH 7·5) in 0·06 M-NaCl containing 150 mg. sorbose/ml. and filipin at the concentrations indicated. Cholesterol, when indicated, was added at 40 μg./mg. dry wt. yeast. After 1 hr the organisms were packed by centrifugation, washed at 4° with three 5 ml. portions of 0·9 % (w/v) NaCl and extracted with distilled water in a boiling water bath. Sorbose was analysed in the extract.

	Filipin (μg./mg. dry wt. yeast)	Cholesterol addition: No addition	At zero time	At 30 min.
		Sorbose content (mg./ml. cell water)		
Exp. 1	0	36	—	—
	10	29	35	—
	20	20	37	—
Exp. 2	0	61·8	—	—
	20	13·8	56·4	21·0
	30	4·8	55·8	11·4
Exp. 3	0	49·8	—	—
	40	1·2	42·6	1·8
Exp. 4	0	78·6	—	—
	50	1·2	75·0	—

Table 4. *Sensitivity to filipin of sorbose retention by commercial yeast and log-phase organisms of Saccharomyces cerevisiae strain AB 14*

Washed commercial yeast or log-phase organisms of *S. cerevisiae* strain AB 14 were treated as described in Table 3. Cholesterol additions were made at 30 min.

Filipin concentration (μg./mg. dry wt. organism)	Commercial yeast		Log-phase strain AB 14	
	No addition	+ Cholesterol	No addition	+ Cholesterol
	Sorbose content (mg./ml. cell water)			
0	36	—	18·6	—
10	29	35	1·2	1·2
20	20	37	1·2	1·0

Log-phase organisms of strain AB 14 were much more sensitive to filipin action on sorbose retention than was the parent commercial baker's yeast (Table 4). With 10 μg. filipin/mg. dry wt. organism, retention by log-phase organisms was eliminated, whereas 20 μg. filipin/mg. decreased retention by commercial yeast only about 50 %. It can also be seen that 40 μg. cholesterol/mg. dry wt. organism protected the commercial yeast but not the log-phase organisms of strain AB 14.

Effect of N-acetylcandidin on sorbose retention

The retention of sorbose by log-phase organisms of strain AB 14 and commercial yeast during washing in ice-cold saline was unaffected by treatment with NAC. At 25 μg. NAC/mg. dry wt. organism in the presence of 0·06 M-Na^+, glycolysis was inhibited completely in both kinds of yeast cell. Sorbose retention at 4° was not altered by NAC concentrations as high as 100 μg./mg. dry wt. organism (Table 5, column 3).

Table 5. *Glucose inhibition of sorbose uptake by N-acetylcandidin (NAC)-treated yeast*

Washed commercial yeast (0·01 ml./tube) was incubated for 1 hr at 30° in 2 ml. 0·02 M-tris + succinate + tartrate buffer (pH 7·5), in 0·06 M-NaCl and the indicated concentrations of NAC. At 1 hr, 2 ml. of sorbose solution (15 %, w/v) or 2 ml. of a mixture of sorbose (15 % w/v) + glucose (15 % w/v) were added and the incubation continued at 30° for 30 min. The organisms were then packed by centrifugation, washed by three 5 ml. portions of 0·9 % (w/v) NaCl at 4° and extracted with distilled water in a boiling water bath. The extracts were analysed for sorbose or glucose as described under Methods.

	N-acetylcandidin (μg./mg. dry wt. yeast)	Incubation medium: Sorbose	Incubation medium: Sorbose + glucose	% Inhibition	Incubation medium: Sorbose + glucose
		Sorbose content (mg./ml. cell water)			Glucose content (mg./ml. cell water)
Exp. 1	0	32·4	3·6	88·9	0
	25	36·0	7·8	78·4	4·2
	100	34·8	10·8	69·0	4·2
Exp. 2	0	36·6	3·6	90·2	0
	25	42·0	8·4	80·0	5·4
	100	36·0	15·6	57·0	5·4

That NAC did not affect sorbose retention by the yeast cell membrane during washing at 4° does not exclude the possibility that membrane damage has occurred but is not evident at 4°. This is suggested by the observation that K^+ loss induced by NAC is markedly temperature sensitive, and is negligible at 0° (Harsch & Lampen, 1963).

Damage to the cell membrane can be assessed at 30° by taking advantage of the fact that sorbose uptake is strongly inhibited by glucose. In the experiment of Table 5, sorbose uptake by intact organisms decreased about 90 % in the presence of an equal concentration of glucose. (This inhibition is the result of competition between glucose and sorbose for the hexose carrier system involved in sugar transport (Cirillo, 1961*b*, 1962).) The glucose block was, however, only partially effective in the presence of NAC. The sorbose content of glucose-blocked organisms treated with 25 μg. NAC mg. dry wt. organism was twice that of untreated controls; at 100 μg. NAC the increase was three- to fourfold (Table 5). Thus NAC had caused sufficient damage to the cell membrane to permit leakage of sorbose into the yeast cell. This damage to the yeast membrane by NAC does not appear to be extensive. This is indicated by the absence of sorbose loss during cold washing and by the low concentration of free glucose in the treated organism. These organisms contained about 5 mg. free glucose/ml. cell water, i.e. about one-tenth the value to be expected

if equilibrium had been reached by free diffusion through tears in the membrane. The limited extent of membrane damage in NAC-treated organisms at 30° is also indicated by the continued ability of intracellular glucose to inhibit sorbose efflux from sorbose-loaded cells into sorbose-free medium (unpublished results).

Effects of nystatin on sorbose retention at 4° and 30°

The sorbose content of nystatin-treated yeast incubated with sorbose alone or with sorbose + glucose is shown in Table 6. At nystatin 100 μg./mg. dry wt. organism there was complete sorbose loss during washing at 4°; the loss from organisms treated with 5 μg. nystatin/mg. was less extensive. At nystatin 2·5 μg./mg., the lowest value at which nystatin inhibits glycolysis in commercial yeast within 1 hr, there was no sorbose loss from the organisms during cold washing. On the other hand, the elevated sorbose content of nystatin-treated organisms incubated at 30° in sorbose + glucose shows that even 2·5 μg. nystatin caused extensive entry into the organism. This entry was substantially greater than that seen with 100 μg. NAC/mg. dry wt. organism.

Table 6. *Production of sorbose leakage by nystatin at 4° and 30°*

Washed commercial yeast (0·01 ml. packed volume) was incubated with constant shaking at 30° in 2 ml. 0·02 M-tris + succinate + tartrate buffer (pH 7·5) in 0·06 M-NaCl containing the indicated concentrations of nystatin. At 1 hr 2 ml. of a sorbose or a sorbose + glucose solution were added and the incubation continued for either 10 or 30 min. (In experiment 1 the final concentration of each sugar was 2·5 % (w/v); in experiments 2 and 3 it was 5 % (w/v).) After the incubation the organisms were packed by centrifugation, washed with three 5 ml. portions of 0·9 % (w/v) NaCl at 4° and extracted in distilled water in a boiling water bath. The extracts were analysed for ketose.

	Nystatin (μg./mg. dry wt. yeast)	Time (min.)	Incubation medium: Sorbose content (mg./ml. cell water)		% Inhibition by glucose
			Sorbose	Sorbose + glucose	
Exp. 1	0	30	9·3	0·6	93
	100	—	0·3	0·3	—
Exp. 2	0	30	19·2	1·3	93
	2·5	—	24·1	13·3	45
	5·0	—	12·8	9·1	29
Exp. 3	0	10	18·4	2·2	88
	2·5	—	18·4	12·2	34

DISCUSSION

The differences in the action of filipin, nystatin and N-acetylcandidin on inorganic phosphate and sorbose leakage and on the development of ATPase activity by whole yeast organisms support the contention that there are two major groupings of polyenes. A comparison of the various effects of these polyenes is presented in Table 7. Some hitherto unpublished observations (Lampen & Arnow) on the initiation of pyruvate decarboxylation (at pH 5–7) by yeasts are included. The data for protoplast lysis are those reported by Kinsky (1962*a*) for *Neurospora crassa* and unpublished observations by Sih, Arnow & Lampen for yeast. There is clearly a

correlation between non-reversibility of the inhibition of glycolysis, the loss of inorganic phosphate, inability to retain L-sorbose, the activation of ATPase and pyruvate decarboxylase and the lysis of protoplasts. Filipin (a small polyene) exhibits all of these effects; N-acetylcandidin (NAC; large polyene) none of them. Nystatin, though a large polyene, is intermediate in its action, being similar to NAC at low concentrations and similar to filipin at high concentrations. From the studies on lysis of protoplasts and of erythrocytes by amphotericin B (Kinsky, 1962*a*; Kinsky *et al.* 1962), this large polyene can be placed between nystatin and filipin. Candicidin (46 carbon atoms) resembles NAC in that high concentrations do not initiate pyruvate decarboxylation (Larsen & Demis, 1963). Thus, although two major groupings can be described, the polyenes should primarily be considered to exhibit a graded series of actions with filipin and NAC as the present examples of the extreme types. Filipin causes the destruction of the cell membrane; to this event all other effects of its action can probably be attributed. NAC produces only slight damage to membrane integrity, and this results in relatively specific permeability defects (Table 7). Since both small and large polyenes react with membrane sterols (Gottlieb *et al.* 1958; Lampen *et al.* 1960, 1962), the molecular basis of the differences in mode of action remains undefined.

Table 7. *Comparison of effects of various polyenes on yeast*

	Polyene*				
	NAC		Nystatin		Filipin
Effect	Low	High	Low	High	
K^+ loss	+	+	+	+	+
Inhibition of glycolysis	+	+	+	+	+
Annulment by K^+ and NH_4^+ of glycolysis inhibition	+	+	+	−	−
Annulment by cofactors of glycolysis inhibition	+	+	+	+	−
Inorganic phosphate loss	−	−	−	+	+
Sorbose leakage at 4°	−	−	−	+ + +	+ + +
Sorbose leakage at 30°	±	+	+ +	+ + +	+ + +
ATPase activity	−	−	−	+	+
Pyruvate decarboxylation	−	−	±	+	+
Protoplast lysis†	±	Slow	Slow	Moderate	Fast

* Sorbose leakage is assessed as − to + + +; other criteria generally as −, ±, or + only.

† Protoplasts of yeast (Sih, Arnow & Lampen, unpublished observations); protoplasts of *Neurospora crassa* (Kinsky, 1962*a*).

The preferential reactivation of NAC-inhibited glycolysis by NH_4^+ rather than by K^+ (Fig. 1) probably explains the observation of Harsch & Lampen (1963) that K^+ accumulation did not occur when 0·01 M-KCl was added to yeast pretreated for 60 min. with 100 μg. NAC/mg. dry wt. organism. Since glycolysis would not have resumed, there was no energy source available to drive the accumulation process.

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The Germination of Sporangiospores of *Rhizopus arrhizus*; Spore Swelling and Germ-Tube Emergence

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SUMMARY

The germination of sporangiospores of *Rhizopus arrhizus* was investigated, and differing requirements found for the initiation of germination, spore swelling and germ-tube emergence. The initiation of germination, as indicated by the commencement of swelling and by the spores becoming permeable to methylene blue, requires the presence of glucose or fructose. Maximal spore swelling requires in addition the presence of a nitrogen source, PO_4^{3-} and K^+ or Na^+. If these requirements are satisfied, the increase in spore diameter with time is approximately linear for 8 hr, implying the maintenance of a constant rate of water uptake per unit area of spore surface for this period. If germinating spores are transferred to a medium lacking glucose, swelling soon ceases. The rate of swelling is identical at widely differing osmotic pressures. It is suggested that water uptake by the germinating spore is an active process, requiring energy. Germ-tube emergence from some spores can be obtained with glucose alone, but for germ-tube production from all spores other nutrients must also be supplied. If glucose is present, spores can take up sufficient nutrients in 1–2 hr to permit complete germination in the absence of exogenous nutrients several hours later. Depending on conditions, germ-tube production can occur after either slight or massive spore swelling. The effect of anaerobic conditions on germination was also examined and found to permit only partial spore swelling and greatly diminished germ-tube production.

INTRODUCTION

Spore germination in fungi has been the subject of an extensive literature; see reviews by Cochrane (1958, 1960), Gottlieb (1950), Hawker (1950) and Lilly & Barnett (1951). The present authors, proposing to extend the studies of Carlile & Sellin (1963) on an endogenous inhibition of spore germination in the fungi, found it essential to obtain more information on some aspects of normal germination. In most fungi, germ-tube emergence is preceded by swelling of the spore. This process has received very little attention, and although there was an interesting quantitative study of spore swelling in *Myrothecium verrucaria* by Mandels & Darby (1953), further work on the topic is needed. Spore swelling is particularly striking in the Mucorales, and *Rhizopus arrhizus*, the member of the Mucorales used in the present study, was a suitable organism for investigating the process. In addition to observing changes in spore diameter, the occurrence of germ-tube emergence was also recorded, as the significance of the whole process lies in this event. In the present

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study the emergence of the germ-tube will be referred to as such, and 'germination' will be used as a general term for the events which culminate in the production of a germ-tube.

METHODS

Organism. The fungus used was an isolate of *Rhizopus arrhizus* Fischer, obtained from soil at Ibadan. *Rhizopus arrhizus* appears to resemble closely the better known *R. nigricans* and references to literature on this species will be made at several points in the text. Stock cultures of *R. arrhizus* were maintained on agar slopes of the basal medium indicated below in screw-capped MacCartney bottles.

Preparation of inoculum. Sporangia were gently scraped with an inoculating loop from stock cultures and dispersed in sterile distilled water. The spore concentration was determined by means of a haemocytometer or by turbidity measurements and adjusted to give 3×10^6 spores/ml.

Media. The standard medium used had the following composition (%, w/v): D-glucose, 1·0; NH_4Cl, 0·1; KH_2PO_4, 0·15; $MgSO_4.7H_2O$, 0·05; distilled water. This is a modification of the medium used by Stadler (1952) in studies with *Rhizopus nigricans*, differing in that asparagine was replaced by its molar equivalent of NH_4Cl. Except in experiments on anaerobic conditions, where a liquid medium was preferred, media were solidified with 1·2 % Oxoid Agar No. 3 (referred to as standard medium agar). No deliberate addition of micronutrients to media was made, but confirmatory experiments in which the inorganic micronutrients used by Carlile (1962) were added, showed their omission to have been without effect on the results of experiments.

The assessment of germination. Strips of sterile moist permeable cellophan were placed on agar medium in Petri dishes and two drops of spore suspension were spread on each strip. The Petri dishes were then placed in an incubator at 30°, and the strips removed for microscopic examination either hourly or at 8 hr. Strips were then mounted in dilute methylene blue, or when immediate examination was not possible, in cotton blue in lactophenol. Twenty, 50 or 100 spores were examined at random, the number depending on the variability of the material and the degree of accuracy required. They were recorded as having produced germ-tubes when an emerging germ-tube was perceptible. Spore diameter was measured with a micrometer eyepiece and oil-immersion objective. Spores in optical section were usually nearly but not quite circular, and the maximum diameter was measured.

All experiments were repeated and most of the numerical results reported are averaged from two or more experiments. Spore diameters are given to the nearest 0·5 μ and germ-tube emergence to the nearest 10 %. The data on spore-diameter is of high reliability; the standard deviation was calculated for many of the results and found to be between 0·4 and 1·1 μ. Records of no germ-tube emergence or of complete germ-tube emergence are also of high reliability, but records of intermediate percentages, such as those obtained on glucose agar, are less reliable.

Anaerobic conditions. Experiments on the effect of anaerobic conditions were made in 150 ml. Erlenmeyer flasks containing 18 ml. liquid medium to which 2 ml. of spore suspension had been added. To maintain anaerobic conditions, industrial nitrogen was passed through three wash bottles each containing 100 ml. sodium dithionite solution (14 g. $Na_2S_2O_4$ and 1·4 g. indigocarmine in 100 ml. 10 % KOH)

to remove traces of oxygen from the gas, and which was then bubbled through the medium for at least 30 min. before inoculation and for the duration of the experiment.

RESULTS

Germination on the standard medium

The ungerminated spore of the isolate of *Rhizopus arrhizus* used was a prolate spheroid with major axis about 5·5 μ and minor axes about 4·5 μ. When placed in dilute methylene blue it did not stain.

One hour after inoculation on to standard medium agar, the spores stained with methylene blue and had begun to swell. Initially the rate of swelling was slightly

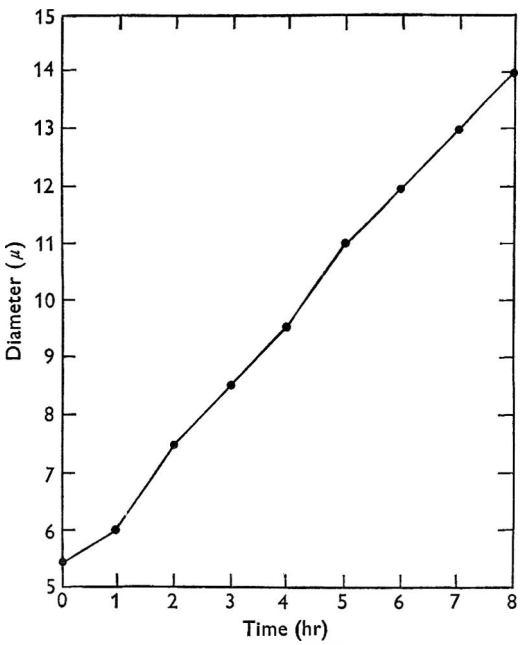

Fig. 1. *Rhizopus arrhizus:* increase in spore diameter with time, on standard medium at 30°.

greater along the minor axes than along the major axis, so that 3 hr after inoculation the spores were spheres of diameter about 8·5 μ. The emergence of germ-tubes was first observed at 4 or 5 hr, and all spores produced germ-tubes by 8 hr. Swelling of spores ceased at about 8 hr, when they had a diameter of about 14 μ. By this time spores were no longer strictly spherical, some distortion having occurred at the time of germ-tube emergence, and most of the germ-tubes were so long that accurate measurement was difficult.

The increase in diameter with time is indicated in Fig. 1, and as will be noted is approximately linear. This was confirmed by successive measurements on individual spores made with the highest magnification dry-objective available. It is therefore clear that the emergence of the germ-tube did not perceptibly influence the linear increase in spore diameter. Hence the spore and the germ-tube may be considered separate systems as regards the uptake of water involved in their increase in size.

The linear increase in the diameter of the spore was accompanied by a change in shape that was relatively slight. When this change in shape is ignored, which seems justifiable as a first approximation, then the linear diameter increase implies that the rate of increase in volume of the spore at any time was proportional to the surface area at that time. Hence water uptake proceeded at a rate that was constant/unit surface area throughout a period in which the volume of the spore increased almost 20-fold, and presumably considerable dilution of the spore contents occurred. It seems likely therefore that the water uptake was not osmotic, but was an active process, requiring the expenditure of energy. The rate indicated by the data reported above is about 1·1 μ^3/μ^2/hr, or about 10 water molecules/mμ^2/sec.

Table 1. *The effect of components of the standard medium, singly and in combination, in bringing about germination of spores of Rhizopus arrhizus*

Nutrients	Spores staining with methylene blue (%)	Average diam. of spores (μ)	Spore with germ-tubes (%)
None	0	5·5	0
$MgSO_4$	0	5·5	0
NH_4Cl	0	5·5	0
$NH_4Cl + MgSO_4$	0	5·5	0
$KH_2PO_4 + MgSO_4$	0	5·5	0
$NH_4Cl + KH_2PO_4 + MgSO_4$	0	5·5	0
KH_2PO_4	30	7·5*	30
$KH_2PO_4 + NH_4Cl$	50	7·5*	50
Glucose	100	7·5	20
Glucose + $MgSO_4$	100	7·5	20
Glucose + KH_2PO_4	100	7·5	20
Glucose + $KH_2PO_4 + MgSO_4$	100	8·5	100
Glucose + NH_4Cl	100	9·5	100
Glucose + $NH_4Cl + MgSO_4$	100	9·0	100
Glucose + $NH_4Cl + KH_2PO_4$	100	13·5	100
All	100	13·5	100

* The figure given is the average for those spores stainable with methylene blue, and not for all spores. Only the fraction that stained with methylene blue were swollen, and all of these had produced germ-tubes.

Nutrient requirements for germination

On the standard medium agar all spores swelled to about 14 μ diameter and produced germ-tubes within 8 hr. On water agar neither swelling nor germ-tube emergence occurred, nor did the spores become stainable with methylene blue. The role of the components of the standard medium in bringing about germination was therefore investigated. Agar media containing the four nutrients, singly and in all combinations, were prepared, and their effects on the spores recorded 8 hr after inoculation. The results obtained are indicated in Table 1. It will be noted that consistent germination did not occur in absence of glucose. In the presence of KH_2PO_4 alone, however, some spores swelled and produced germ-tubes, a response that was prevented by the presence of $MgSO_4$, but not of NH_4Cl. This response by a fraction of the spores was not always obtained, and attempts to ascertain the reason for this inconsistent and anomalous germination in the absence of glucose were not successful. Possibly a considerable carry-over of nutrients from the

sporangium, on or within some spores, sometimes occurred. It is, however, reasonable to conclude that initiation of germination normally requires glucose.

Glucose alone brought about the swelling of spores to about 7·5 μ, and some of them produced germ-tubes. The provision of $MgSO_4$ or of KH_2PO_4 in addition to glucose did not lead to enhanced germination; but on glucose + NH_4Cl swelling to about 9·5 μ occurred and almost all spores produced germ-tubes. Production of germ-tubes by all spores was also observed when $KH_2PO_4 + MgSO_4$ was provided additional to glucose, but in this instance enhanced swelling relative to glucose alone was not observed. The addition of KH_2PO_4 to a medium containing glucose + NH_4Cl resulted in spore swelling and germ-tube emergence equal to that which occurred on standard medium.

Experiments were then carried out on the replacement of constituents of the standard medium with other compounds. The ability of sugars other than glucose to initiate germination was tested. On fructose agar spores swelled to about 7·5 μ and 30 % of them produced germ-tubes, but the initiation of germination did not occur on sucrose or lactose agar. This is in conformity with the report by Margolin (quoted by Lilly & Barnett, 1951) that *Rhizopus nigricans* will grow with glucose or fructose, but not sucrose or lactose, as carbon source, and of the finding by McCallan, Miller & Weed (1954) that sucrose was without effect on the rate of oxygen uptake by *R. nigricans* spores. Clearly glucose exercises its effect on germination through being a utilizable source of carbon/energy.

In preliminary experiments made on the medium used by Stadler (1952) swelling to 14 μ and germ-tube emergence from all spores was observed. Hence asparagine is a fully adequate substitute for NH_4Cl as a constituent of the standard medium and the significance of these compounds lies in their being adequate nitrogen sources.

When KH_2PO_4 was replaced by KCl or by $NH_4H_2PO_4$ as a constituent of the standard medium, spores swelled to only 11·5 μ, showing that both the K^+ and the PO_4^{3-} radicals were needed for full swelling. Replacement of KH_2PO_4 by NaH_2PO_4 as a constituent of the basal medium resulted in swelling to 13·5 μ, however, showing that Na^+ could replace K^+.

The results of these nutritional observations may be summarized: the germination process can be initiated by glucose or fructose, either of which will suffice for swelling to 7·5 μ, and also germ-tube emergence by some spores; the additional provision of a nitrogen source allows swelling to 9·5 μ to occur and all the spores to produce germ-tubes; the further addition of PO_4^{3-} and K^+ or Na^+ allows full swelling to occur.

The effect of transferring spores from one medium to another during germination

A simple technique that yielded information of interest was to transfer cellophan strips bearing spores from one medium to another at different times during germination. The results of making one such transfer, from standard medium agar to water agar, are given in Table 2. Exposure for 1 hr to standard medium was sufficient to bring about subsequent germ-tube emergence from about 80 % of the spores, and exposure for 2 hr resulted in germ-tube production from all spores. Clearly, enough nutrients were taken up in about an hour to allow subsequent germ-tube emergence. However, when spores were transferred from glucose agar to standard medium agar lacking glucose the percentage of spores which produced germ-tubes

was no greater than when the spores had been left on glucose agar, or when transferred from glucose agar to water agar. Hence, for utilization of nutrients other than glucose to occur, glucose had to be simultaneously present in the medium.

It will also be noted from Table 2 that the average increase in spore diameter after transfer from standard medium agar to water agar did not exceed 1 μ. The same result was obtained by transferring spores from standard medium agar to standard medium but lacking glucose. Glucose was therefore necessary for continued swelling and hence for continued water uptake.

Table 2. *The effect of transferring spores of Rhizopus arrhizus from standard medium to water agar*

Time of retention on media (hr)		Condition at time of transfer		Condition at 8 hr	
Standard medium	Water agar	Average spore diameter (μ)	Spores with germ-tube (%)	Average spore diameter (μ)	Spores with germ-tubes (%)
0	8	5·0	0	5·0	0
1	7	6·0	0	7·0	80
2	6	8·0	0	8·0	100
3	5	8·5	0	9·0	100
4	4	9·0	20	9·5	100
5	3	10·0	40	10·5	100
6	2	12·0	50	12·5	100
8	0	14·0	100	14·0	100

The effect of glucose concentration and osmotic pressure on germination

The effect on germination of various glucose concentrations is indicated in Table 3. Features of interest are the production of germ-tubes at glucose concentrations inadequate for normal swelling, and the marked decrease in frequency of germ-tube emergence at the very high glucose concentrations which only slightly diminished swelling. It has already been suggested that active water uptake, requiring energy, occurs, but the possibility that there is also an osmotic component of water uptake was not excluded. An experiment was therefore made on the effect of different osmotic pressures on germination at glucose concentrations which were limiting for germ-tube emergence (0·01 %, w/v) and for spore swelling (0·2 %, w/v). High osmotic pressures were obtained by adding sucrose, which had already been shown to be inactive in bringing about germination, in the amounts necessary to give the osmotic pressure that would be obtained with a standard medium containing 10 % glucose; the results are given in Table 4. The fact that the rate of water uptake was identical at widely different osmotic pressures is strong evidence that the process was entirely an active one. The alternative possibility, that sucrose, a non-utilizable disaccharide, penetrated the organism so rapidly that equilibration occurred, seems improbable.

The effect of anaerobic conditions on germination

Wood-Baker (1955) reported that under anaerobic conditions the spores of *Rhizopus nigricans* swelled but did not produce germ-tubes. Since, however, she gave no indication of the extent of swelling, a re-examination of the effect of anaerobic conditions on germination of *R. arrhizus* spores was made. Liquid standard

medium was used; aerobic conditions were obtained by passing air, and anaerobic conditions by passing pure nitrogen, through the liquid. At 8 hr the *R. arrhizus* spores under aerobic conditions had swelled to about 13 μ and about 90 % of them had produced germ-tubes. Under anaerobic conditions spore swelling was to about 8 μ and only about 10 % of the spores had produced germ-tubes. Hence although swelling of *R. arrhizus* spores did occur under anaerobic conditions, as reported by Wood-Baker for *R. nigricans*, it was incomplete. The discrepancy between the results of the present authors and those of Wood-Baker as regards germ-tube emergence may be due to the different temperatures used; Wood-Baker's experiments were made at 20° and 25° and the authors' at 30°. Experiments made by the present authors at room temperature (27–28°) resulted in *R. arrhizus* spores swelling to 7·5 μ, a value similar to that obtained at 30°; but, as in Wood-Baker's experiments, no germ-tube emergence was observed.

Table 3. *The effect of glucose concentration on germination of spores of Rhizopus arrhizus*

Glucose (%, w/v)	Average diameter of spores (μ)	Spores with germ-tubes (%)
0·0	5·5	0
0·0005	5·5	0
0·001	7·0*	20
0·002	7·0*	30
0·005	7·0*	70
0·0075	7·5*	80
0·01	9·0*	90
0·05	9·0	100
0·1	9·0	100
0·2	11·0	100
0·5	13·5	100
0·75	13·5	100
1·0	13·5	100
10·0	13·0	100
20·0	12·5	40
30·0	12·0	20

* The figure given is for those spores stainable with methylene blue, and not for all spores. Only the fraction that stained with methylene blue were swollen, and all of these had produced germ-tubes.

Table 4. *The effect of sucrose on germination of spores of Rhizopus arrhizus*

Sugars (%, w/v) present in medium		Average diameter of spores (μ)	Spores with germ-tubes (%)
Glucose	Sucrose		
0·01	0·0	9·0	90
0·01	18·98	9·0	90
0·2	0·0	11·5	100
0·2	18·98	12·0	100

DISCUSSION

Initiation of germination. Within 1 hr of being placed on a medium containing glucose, the spore of *Rhizopus arrhizus* becomes stainable with methylene blue, and begins to swell by uptake of water. These developments suggest that an early step in the germination process is a change in the permeability of the spore. Sussman (1954) showed that soon after germination had been initiated in the spores of *Neurospora tetrasperma* by heat treatment an increased permeability to various ions could be shown. The work of Rothstein (1954) on yeast indicated that glucose could be metabolized at the surface of the organisms to provide energy for the active uptake of ions. It is possible, therefore, that the initiation of the germination of the spores of *R. arrhizus* results from permeability changes brought about by the metabolism of exogenous glucose, perhaps at the spore surface.

Water uptake. The uptake of water by the spore of *Rhizopus arrhizus* requires the presence of glucose, and ceases soon after the spore is transferred to a medium lacking glucose. The rate of water uptake/unit area spore surface remains unchanged as the volume of the spore increases (and, presumably the cell contents become diluted) and does not differ at widely differing osmotic pressures. These facts suggest that water uptake by the spore is an active process and not osmotic; this deserves further investigation in other fungi. The active uptake of water might be the basis of some unexplained phenomena such as the finding that the spores of powdery mildews can take up water rapidly (Delp, 1954) but lose water very slowly, even over desiccants (Yarwood, 1950).

The emergence of germ-tubes from the spores of *Rhizopus arrhizus* can take place in the absence of exogenous glucose, some hours after transfer to a glucose-free medium has resulted in the cessation of swelling. Since the growth of these germ-tubes will involve water uptake, it is clear that the mode of water uptake by germ-tubes, and hence, presumably, by the fungus mycelium, may differ from that which occurs in the spore. This view is supported by the low percentage of germ-tube emergence at high glucose concentrations, which did not prevent almost complete spore swelling.

The relationship between spore swelling and germ-tube emergence. A tentative hypothesis was advanced by Yarwood (1950) to account for the widespread occurrence in fungi of spore swelling before germ-tube emergence. He pointed out that the spores of powdery mildews which have a high water content produce germ-tubes without prior swelling and suggested that in other fungi water has to be absorbed to bring the spore to a degree of hydration similar to that of the powdery mildews before germ-tube emergence can occur. This hypothesis does not appear tenable in view of the present finding that the spores of *Rhizopus arrhizus*, which normally increase to many times their original volume before producing germ-tubes, can do so after a relatively slight increase in size. Hypotheses to account for the swelling of spores before germ-tube emergence will have to be consistent with the fact that germ-tube production can occur after either slight or massive swelling.

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Some Properties of a New Bacteriocin Formed by *Bacillus megaterium*

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SUMMARY

A new bacteriocin, megacin C, was recognized in certain strains of *Bacillus megaterium*; it was initially observed as the agent responsible for killing sensitive organisms in mixed culture. The bacteriocin was liberated during growth of cultures; but later, after reaching a maximum value, it disappeared from the culture media. Megacin C was produced by strains already known to produce another bacteriocin, megacin A. The activity spectra of the two megacins differed markedly and non-megacin A (MA^-) mutants still produced megacin C. A preliminary grouping of megacin types is proposed. Megacin A is comparable to the original megacin first studied by Ivánovics & Alföldi (1954). Megacin A produced inhibition zones surrounding colonies on solid medium but its formation in liquid medium required induction with ultraviolet (u.v.) radiation. Megacin B, whose activity spectra differed from those of megacins A and C, can be detected only on a solid medium; it is not formed in liquid culture even after u.v.-irradiation. Megacin C can be detected only on a particular solid medium and its formation in liquid media is not induced by u.v.-irradiation.

INTRODUCTION

Bacteriocins are substances, produced by many bacteria, which are highly specific antibacterial proteins active against strains of the homologous species (Jacob, Lwoff, Siminovitch & Wollman, 1953). Sensitive bacteria may be killed by the adsorption of a few molecules of bacteriocin to specific receptor sites, but the actual mode of action is not yet understood. The wide distribution of bacteriocins has been reviewed by Hamon & Peron (1963) and their general properties by Ivánovics (1962). The bacteriocins most extensively studied are the colicins produced by members of the family Enterobacteriaceae (Fredericq, 1957). Several different colicins have been isolated or partially purified and in each case the active protein has been found to be associated with a lipo-polysaccharide protein complex identifiable with a cell wall component (Nüske, Hösel, Venner & Zinner, 1957; Goebel & Barry, 1958; Hutton & Goebel, 1961; Barry, Everhart & Graham, 1963). This suggests that colicins may be 'normal' components of the cell envelope of colicinogenic strains. In some cases the production of colicin may be induced by ultraviolet (u.v.) irradiation (Fredericq, 1954, 1955; Kellenberger & Kellenberger, 1956). Since it has been shown that both spontaneous and induced formation, i.e. leading to release of soluble colicin, can be lethal to the producing organisms (Ozeki, Stocker & de Margerie, 1959) it might be suggested that this is the result of a de-repressed and uncontrolled formation of a normally minor cell wall component, rather than a

unique synthesis of protein. Ability to produce colicin is determined by a genetic factor (colicinogenic factor) which behaves as a cytoplasmic element and which can be transferred from colicinogenic (*col*$^+$) to non-colicinogenic (*col*$^-$) bacteria by cell to cell contact (Fredericq & Betz-Bareau, 1953*a*, *b*, 1956; Alföldi, Jacob & Wollman, 1957). Some colicinogenic factors also facilitate transmission of chromosomal genes from *col*$^+$ to *col*$^-$ organisms (Ozeki, Howarth & Clowes, 1961). Thus the distinction between *col*$^+$ factors and fertility (*F*) factors is, in fact, only marginal (see Sneath, 1962).

Bacteriocinogenic strains of *Bacillus megaterium* were first reported by Ivánovics & Alföldi (1954) but only one type of megacin has so far been described. This megacin is inducible by u.v.-radiation (Ivánovics & Alföldi, 1955) and is a simple protein free from lipopolysaccharide (Holland, 1961). The bacteriocin acts by destroying the cytoplasmic membrane of sensitive bacteria (Ivánovics, Alföldi & Nagy, 1959*a*; Holland, 1962).

This paper describes evidence of some new types of megacin and suggests a classification. The demonstration of the new megacin arose out of studies of the possible function of megacinogenic factors as fertility agents in *Bacillus megaterium*. In a preliminary communication (Holland & Roberts, 1963) we reported a killing effect by certain megacin-producing strains which was apparently manifest through the actual contact of megacinogenic and non-megacinogenic bacteria. We have now been able to show that the killing activity was due to a hitherto undetected megacin. The production and mode of action of this new bacteriocin, megacin C, are quite different from those of previously reported megacins.

METHODS

Organisms. The following organisms used in this work were obtained from other laboratories as follows. *Escherichia coli* K 12 and *Salmonella typhimurium* 903 from Dr R. C. Clowes; *Bacillus megaterium* Mut. and the lysogenic *B. megaterium* strain 10 from Professor A. Lwoff; *B. megaterium* De Bary and *B. cereus* strains 8012 and 8122 from the National Collection of Industrial Bacteria (NCIB); *B. subtilis* 1379 from Dr R. G. Tucker; *B. cereus* 7587 and *B. subtilis* 8236 from the National Collection of Type Cultures (NCTC); *B. megaterium* strain 207-M and strain 216 were described previously (Holland, 1961); two non-megacin A producing mutants, strains 216-MA$^-$ (17) and 216 MA$^-$ (28) were from Professor G. Ivánovics.

The remaining strains of *Bacillus megaterium* were isolated by the present authors. Soil samples in distilled water were heated at 80° for 15 min. and after standing for 60 min. the large debris was removed by decantation and a loopful of each remaining suspension spread on plates of nutrient broth agar. After incubation for 20 hr at 37° one colony was taken from each plating and purified by streaking out twice on nutrient broth agar. Criteria for the identification of *B. megaterium* included acetylmethylcarbinol production, colonial form, cellular morphology and motility. Megacinogenic strains were tested and all found to grow on a defined medium (Alföldi, 1958).

Recognition of megacinogenic organisms. Large colonies (5–6 mm. diam.) of different strains of *Bacillus megaterium* were obtained by stabbing cultures into nutrient broth agar plates and incubating for 36 hr at 37°. The organisms were

killed with a heavy dose of ultraviolet (u.v.) radiation which would also inactivate most bacteriophages produced by lysogenic strains. The irradiated colonies were then overlayered with a phage-resistant megacin-sensitive strain (*B. megaterium* 207-M) from an overnight culture diluted 20-fold in fresh broth before use. After 14 hr at 37° the irradiated colonies which produced inhibition zones in the overlay, i.e. M^+ strains, were noted.

U.v.-irradiation. The source of u.v.-radiation was a Hanovia Chromatolite (Holland, 1961). For killing large colonies and the irradiation of spore suspensions the lamp was set at 25 and 50 cm., respectively, and the irradiation was continued for 10 min.

Isolation of mutants. Non-megacinogenic mutants (MA^-) of strains producing megacin A were isolated from irradiated spore suspensions as described by Ivánovics & Nagy (1958). Streptomycin-resistant mutants (*SR*) were selected by progressive exposure to increasing concentrations of streptomycin; nutrient broth (50 ml.) was inoculated with an overnight culture and streptomycin (final concentration 1 μg./ml.) added after 4 hr incubation at 37°. The culture was allowed to reach to the stationary phase and this was used as inoculum for the successive treatment with streptomycin at 2, 5 and 10 μg./ml. The resistant strain was plated on nutrient broth agar + streptomycin to 10 μg./ml., purified by re-streaking twice and its properties compared with those of the parent organism.

Determination of antibacterial spectra of different megacins. Stab cultures of M^+ strains which produced A and B megacins were set up as already described and overlayered with an overnight culture (diluted 20-fold in fresh broth) of different strains. After incubation for 14 hr at 37° the activity spectrum for each producing strain was determined, from the inhibition zones produced, after examination under standard lighting conditions. For determination of megacin C activity, stab cultures of MA^- organisms were set up on Oxoid nutrient agar plates and incubated for 24 hr at 37°. The macro-colonies were u.v.-irradiated, overlayered and the activity spectra obtained as before.

Measurement of concentration of cultures. The optical density of bacterial suspensions and cultures was determined in an EEL colorimeter, with a neutral density filter.

Media. For the determination of megacin C activity spectra, Oxoid Nutrient Agar (CM3; Oxo Ltd., London) was used. The nutrient broth agar, other media and all other general cultural methods were as described previously (Holland, 1961, 1962).

RESULTS

Grouping of megacinogenic strains of Bacillus megaterium

Killing in mixed culture by megacinogenic strains. Megacinogenic (M^+) strains were initially recognized by their ability to inhibit the growth on solid medium of a standard indicator strain, *Bacillus megaterium* 207-M. Although free megacin formation in liquid cultures was extremely low or not detectable, it was found that in mixed culture with M^+ strains of *B. megaterium*, the M^- bacteria were often killed (Holland & Roberts, 1963). This effect was studied in short-term experiments in which young growing cultures of M^-SR (streptomycin-resistant) organisms were mixed with equal numbers of washed M^+SS (streptomycin sensitive) organisms. The mixed cultures were incubated and the survival of M^-SR organisms determined by plating diluted samples on nutrient broth agar + streptomycin. The results

shown in Fig. 1 were obtained with *B. megaterium* De Bary *SR* as the M^- organism; similar results were obtained with two other M^- strains. The M^+ strains were clearly divisible into three groups: group 1 (non-killers) did not kill the M^- bacteria; group 2 (delayed killers) killed the M^- bacteria only after the latter had multiplied for 30–45 min.; group 3 (rapid killers) killed the M^- organisms immediately the two strains were mixed.

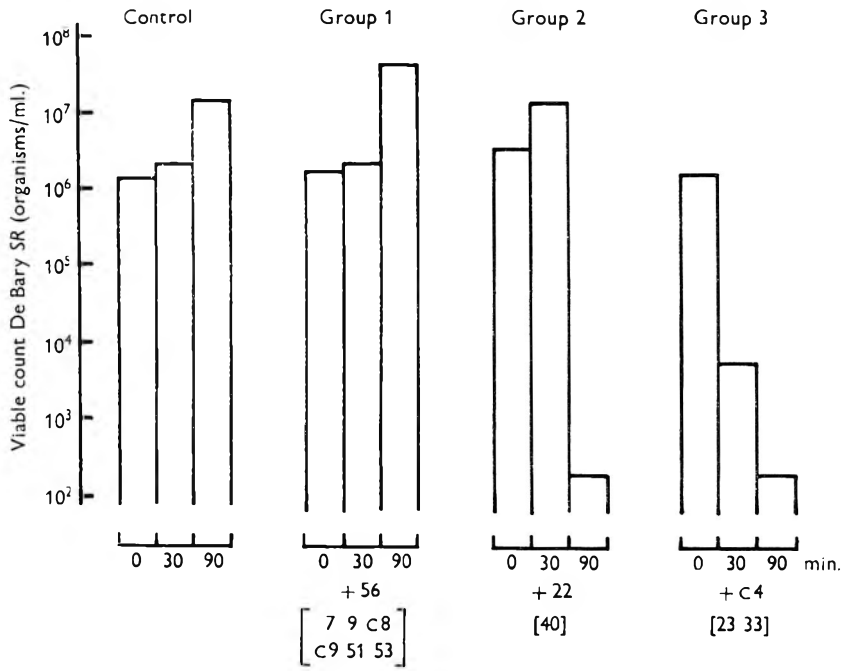

Fig. 1. Preliminary grouping of megacinogenic (M^-) strains of Bacillus megaterium after mixed culture with B. megaterium De Bary *SR*. Growing cultures of B. megaterium De Barry *SR* in nutrient broth were mixed with an equal volume (5 ml.) of different B. megaterium M^+ strains. The M^+ organisms were washed once, resuspended to a final concentration of about 10^6 organisms/ml. in warm (37°) broth before mixing. Samples were taken at intervals, diluted and plated on nutrient broth agar+streptomycin 10 μg./ml. The viable count of strain De Bary *SR* was determined after 14 hr at 37°; 1 strain from each M^+ group is illustrated, strains giving similar results are in brackets; control, untreated.

Inducibility of M^+ strains of Bacillus megaterium. As already stated megacinogenic strains usually form no detectable megacin when grown in liquid media, but with some strains megacin formation can be induced under such conditions by u.v.-irradiation (Ivánovics & Alföldi, 1955). All the M^+ strains were therefore also tested for u.v. inducibility. Young growing cultures (EEL reading, 0·5) were irradiated in a warm Petri dish for 1 min. under conditions described previously (Holland, 1961). Irradiated and non-irradiated control cultures were re-incubated and their growth followed with an EEL absorptiometer. Finally, the organisms were removed by centrifugation and the supernatant fluids assayed with the megacin-sensitive indicator strain 207-M. Representative results are shown in Fig. 2; in every case the M^+ strains behaved in a characteristic way according to the

group to which they had already been assigned. Group 1 strains (non-killers) were not inducible; group 3 strains (rapid killers) were all inducible and lysed about 90 min. after u.v.-irradiation, releasing large amounts of megacin; group 2 strains (delayed killers) also lysed about 90 min. after irradiation but there was no increase in megacin titre. It was found subsequently that non-megacinogenic mutants of delayed killers no longer lysed after u.v.-induction (Fig. 6). It appears therefore that the *M* factor is defective in some way in the parent strain. Thus although the expression of the M^+ factor on induction of group 2 strains led to lysis of the bacteria, no active megacin was released.

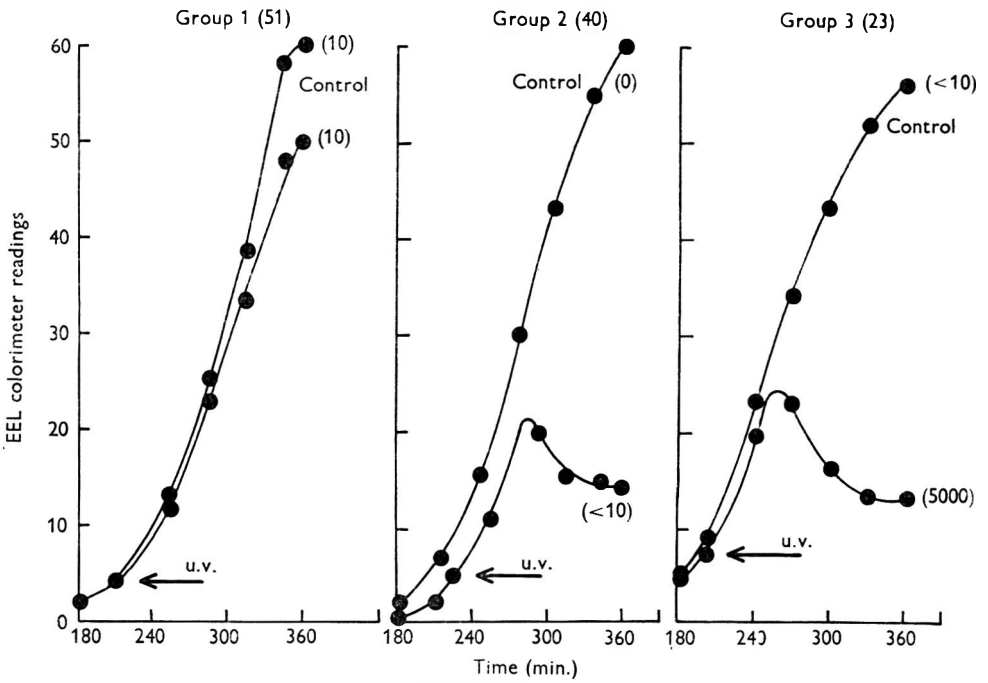

Fig. 2. Response to u.v. irradiation of different groups of megacinogenic strains of Bacillus megaterium. Young cultures of B. megaterium M^+ strains growing in nutrient broth were u.v.-irradiated for 60 sec., re-incubated at 37° and growth followed in an EEL absorptiometer. Results with one strain from each group are shown; controls, unirradiated. The figures by the curves are megacin titres, in units/ml. of the final supernatant fluids, with B. megaterium 207-M as indicator organism.

The strains of group 3 (rapid killers) and group 2 (delayed killers) behaved essentially like the u.v.-inducible strains described by Iványovics and his co-workers (cf. Ivanovics, 1962); the megacin they produce will be described as megacin A. The group 1 strains (non-killers) will be described as megacin B producers.

The nature of killing in mixed culture by strains of Bacillus megaterium which produced megacin A

Although killing was associated with certain megacinogenic strains, several pieces of evidence indicated that megacin A itself was not responsible. No antibacterial agent was detected in the supernatant fluids from the mixed cultures or in cultures

of the killer strain alone when these were tested against two megacin-sensitive indicator strains, *Bacillus megatarium* 207-M and Mut. Moreover, strains either resistant or sensitive to megacin A in plate tests were killed with equal rapidity in mixed culture. Finally, no killing took place when organisms were grown in a common medium but separated by bacterial filters; this indicated that close contact of M^+ and M^- bacteria was necessary for killing (Holland & Roberts, 1963).

The effect of 'concentration' of killer strain on the survival of sensitive organisms. The effect of the number of organisms present of the rapid killer strain *Bacillus megaterium* c 4 (*SS*) on the survival of the M^- strain *B. megaterium* Mut. SR was

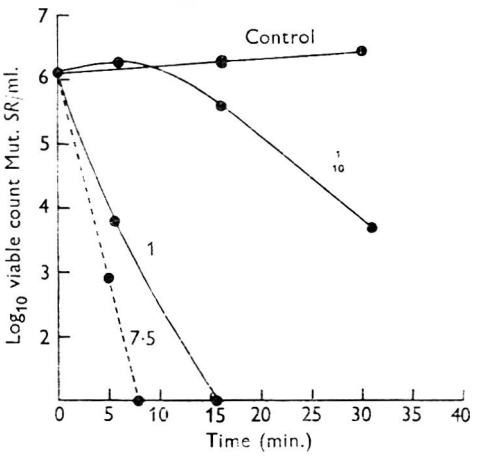

Fig. 3

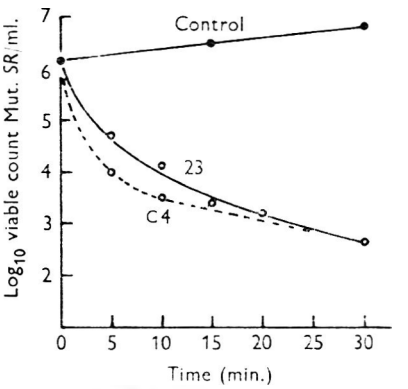

Fig. 4

Fig. 3. Effect of number of organisms of M+, Bacillus megaterium c 4 (Group 3) on the survival in mixed culture of B. megaterium Mut. SR. A growing culture of B. megaterium c 4 was harvested, washed and resuspended to different concentrations in warm (37°) broth; each suspension mixed with an equal volume (5 ml.) of a growing culture of B. megaterium Mut. SR. Survival of B. megaterium Mut. SR was determined as in Fig. 1. Control was strain Mut. SR alone; figures by the curves indicate the ratio, M+: M− organisms present at time 0.

Fig. 4. Effect on the viability of Bacillus megaterium Mut. SR mixed with non-growing cultures of M+ (Group 3) strains c 4 and 23 of B. megaterium. Growth of strains 23 and c 4 was first halted by cooling to 20°, harvested, washed and resuspended in broth at 20°. The non-growing suspensions were added to growing cultures of B. megaterium Mut. SR and the decrease in viability of this organism determined as in Fig. 1. Control, mixed with 5 ml. cold (20°) broth at time 0; $M^+ : M^- = 1$.

examined. A growing culture of *B. megaterium* c 4 was harvested by centrifugation, washed with fresh warm broth and finally resuspended in warm broth. The bacteria were mixed with a young growing culture of the sensitive organism and the mixture shaken at 37°. Samples were taken at intervals, diluted and plated on nutrient agar + streptomycin to determine the survival of *B. megaterium* Mut. SR (Fig. 3). The onset and rate of killing was markedly affected by the relative proportion of M^+ organisms added. At a multiplicity of 7·5 (i.e. $M^+ : M^-$), killing was very rapid and no M^- bacteria could be recovered after 15 min. At lower multiplicity some increase in viable count of the M^- organism was observed before killing began and the rate of killing was decreased.

Similar results were observed whether washed or unwashed M^+ bacteria were

used and whether the experiments were completed in static or in aerated cultures at 37°. It was noted that young cultures of *Bacillus megaterium* c 4 were sensitive to temperature shock, and harvesting and washing were consequently done with broth, as described above, warmed to 37°. The viable count of cultures washed in broth at 20° decreased considerably and the culture did not resume growth for several hours after re-incubation. Nevertheless, such 'shocked' cultures still killed

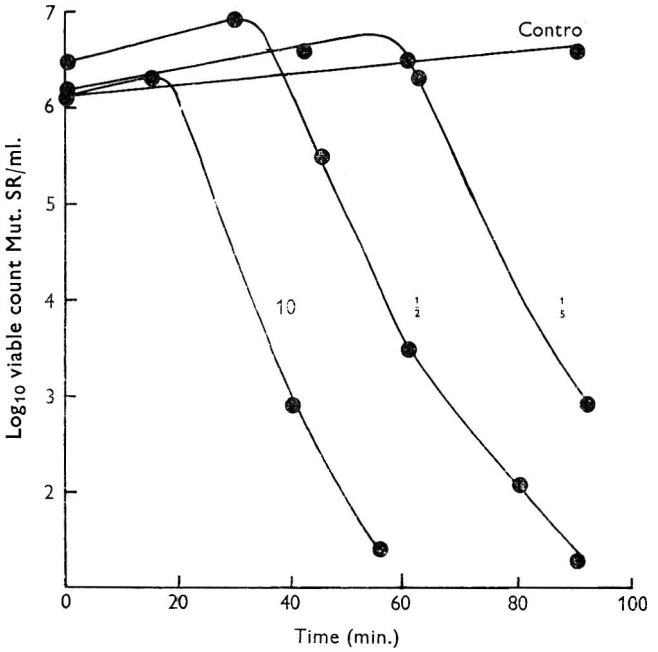

Fig. 5. Effect of the number of organisms of M^+ strain, Bacillus megaterium 22 (group 2) on the survival of B. megaterium Mut. SR in mixed culture. Experiment was carried out as in Fig. 3. Control, B. megaterium Mut. SR growing alone, figures by the curves are the ratio M^+ : M^- organisms present at time 0.

at an initial rate comparable to that of growing cultures, but killing ceased after 5–10 min. whilst a considerable number of M^- bacteria survived (Fig. 4). This suggests that a growing culture of *B. megaterium* c 4 was not necessary for killing but that the concentration of the killing agent was limited in non-growing populations. These results, together with the experiments with cultures separated by bacterial filters (see above) and continued failure, at this time, to demonstrate any soluble lethal agent, indicated that the killing factor was something closely associated with the whole organism.

The effect of multiplicity on the killing of *Bacillus megaterium* Mut. SR in mixed culture with the delayed killer *B. megaterium* 22 was then tested (Fig. 5). As found previously, with roughly equal amounts of M^+ and M^- bacteria there was a lag of 30–45 min., whilst the sensitive organism multiplied, before killing began. By adjusting the multiplicity of the killer strain the lag period could be increased or decreased and thus the difference between the delayed and rapid killers made greater or smaller. The distinction between delayed and rapid killing was therefore quantitative rather than qualitative.

Isolation and properties of non-megacinogenic mutants

Although megacin A had been excluded as the killing agent it was possible that the megacinogenic factor itself was responsible, as a result of some form of zygotic induction or lethal zygosis (Jacob & Wollman, 1961). A series of non-megacinogenic mutants (MA^-) of megacin A-producing strains were therefore isolated from heavily irradiated spore suspensions (Table 1). Non-megacinogenic mutants occurred at a

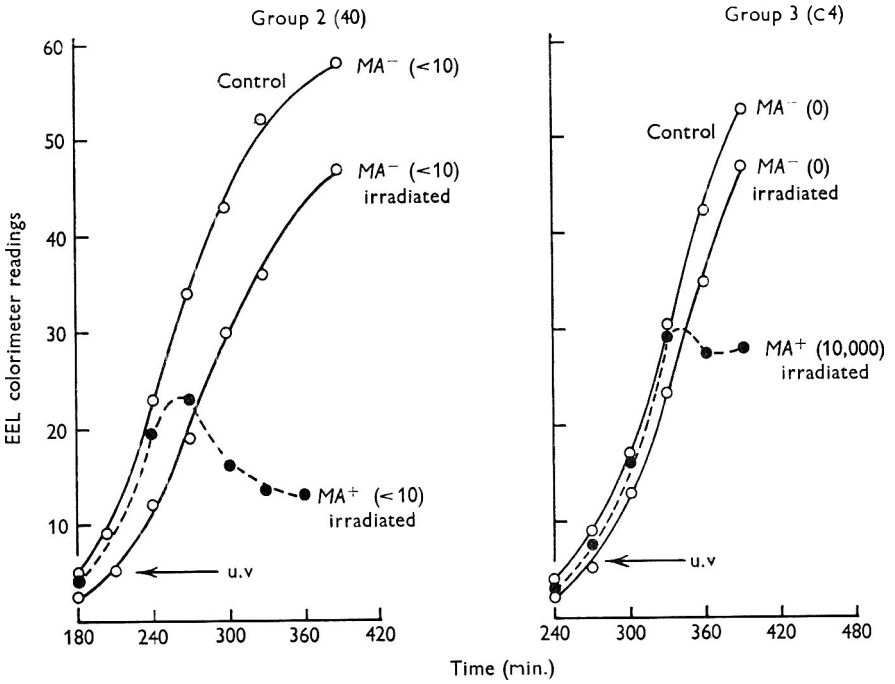

Fig. 6. Response to u.v.-irradiation of MA⁻ mutants of megacinogenic strains of Bacillus megaterium. Induction was carried out as in Fig. 2. Controls, un-irradiated; figures by the curves are the megacin titres, in units/ml. of the final supernatant fluids when B. megaterium 207-M was the indicator bacterium.

Table 1. *Isolation of non-megacinogenic mutants of Bacillus megaterium*

Spore suspensions (10 ml.) in distilled water were u.v.-irradiated for 10 min. in open Petri dishes placed 50 cm. from the u.v. source. The proportion of surviving colony formers was about $1/10^4$; these were plated on nutrient broth agar; after overnight incubation at 37° the colonies were replicated on to plates previously seeded with the megacin-sensitive organism, Bacillus megaterium 207-M. Mutants which produced no inhibition zones were selected.

Strain	No. of spores irradiated (per ml.)	Total no. of surviving organisms examined	M⁻ mutants isolated
c4	$3{\cdot}2 \times 10^6$	710	2
22	$2{\cdot}0 \times 10^5$	950	3
40	$3{\cdot}0 \times 10^6$	634	1
23	$2{\cdot}0 \times 10^6$	960	1
33	$8{\cdot}0 \times 10^5$	410	1

high frequency among the surviving populations, a result noted previously by Ivánovics & Nagy (1958) who used irradiated and non-irradiated spore suspensions. Spontaneous loss of megacinogeny during serial subculture on Oxoid broth agar slopes was also noted. In both cases, in addition to loss of ability to produce megacin A on solid media, the MA^- mutants, including the group 2 producers (delayed killers), no longer lysed after u.v.-irradiation (Fig. 6). The MA^- strains thus appeared to have lost completely the megacin A factor and in the majority of cases the mutants were found to have become very sensitive to the megacin A produced by the parent organism. In these respects therefore megacin A factors behave in the same way as prophage and other episomes (Jacob, Schaeffer & Wollman, 1960).

Table 2. *Killing of Bacillus megaterium Mut. SR by non-megacinogenic mutants in mixed culture*

Growing cultures of Bacillus megaterium Mut. SR (about 4×10^6 organisms/ml.) in nutrient broth were mixed with equal numbers of washed M^+ SS or M^- SS organisms. The mixed cultures were shaken at 37°, the viable count of Mut. SR determined at intervals by plating diluted samples on nutrient broth agar + streptomycin (10 μg./ml.).

	Time (min.)			
Killer strain	0	60	90	120
	Viable count Mut. SR (bacteria/ml.)			
None	$3{\cdot}3 \times 10^6$	10^7	$2{\cdot}3 \times 10^7$	$3{\cdot}6 \times 10^7$
c4 M^+	$2{\cdot}5 \times 10^6$	< 10	—	—
c4 MA^-	$6{\cdot}5 \times 10^6$	< 10	—	—
23 M^+	$2{\cdot}8 \times 10^6$	$1{\cdot}7 \times 10^2$	—	—
23 MA^-	$2{\cdot}9 \times 10^6$	10^3	$3{\cdot}0 \times 10^1$	—
33 M^+	$2{\cdot}0 \times 10^6$	10^1	—	—
33 MA^-	$2{\cdot}7 \times 10^6$	10^3	< 10	—
40 M^+	$2{\cdot}5 \times 10^6$	$7{\cdot}5 \times 10^6$	$2{\cdot}0 \times 10^6$	$5{\cdot}2 \times 10^4$
40 MA^-	$3{\cdot}1 \times 10^6$	$1{\cdot}6 \times 10^7$	$1{\cdot}6 \times 10^7$	$4{\cdot}5 \times 10^6$
22 M^+	$4{\cdot}7 \times 10^5$	$2{\cdot}1 \times 10^6$	4×10^2	—
22 MA^-	$4{\cdot}8 \times 10^5$	$2{\cdot}6 \times 10^6$	$4{\cdot}7 \times 10^6$	—

Killing by non-megacinogenic mutants (MA^-) in mixed culture. The short-term mixed culture experiments were repeated with different MA^- strains and the non-producer *Bacillus megaterium* Mut. SR (Table 2). In one case, *B. megaterium* 22 (delayed killer), loss of the *MA* factor resulted in loss of the ability to kill in mixed culture. However in all other cases *B. megaterium* Mut. SR was killed at rates comparable with those observed with the original strains; in most cases therefore we were able to confirm that some factor other than megacin A or its genetic determinant was concerned in killing.

Demonstration of a new bacteriocin megacin C

The non-megacinogenic mutants (MA^-) were routinely tested for reversion to wild type and in the course of these tests, after several subcultures, strains *Bacillus megaterium* c 4 MA^- and 33 MA^- were observed to produce a previously undetected antibacterial agent. It was first noted on broth agar plates by the formation of very narrow, turbid, inhibition zones surrounding the colonies of these strains in the otherwise complete lawn of indicator *B. megaterium* Mut. SR. It was subsequently

found that all organisms in a population of MA^- bacteria gave rise to colonies which produced these rather indistinct inhibition zones and which were quite unlike those formed by the parent M^+ bacteria. This new antibiotic was active only against strains of *B. megaterium*, although its activity spectrum was quite different from that of megacin A (see below). This new bacteriocin of *B. megaterium* will be described as megacin C.

Table 3. *The formation of megacin C in growing cultures of Bacillus megaterium*

M$^+$ and M$^-$ strains of Bacillus megaterium were grown in nutrient broth at 37°. Optical density measurements made at intervals and samples centrifuged and the supernatant fluid assayed. Megacin C activity was determined by the method described previously for megacin A (Holland, 1961) but using the strain, highly sensitive to megacin C, B. megaterium Mut., as indicator.

	B. megaterium strain 33 M$^+$		B. megaterium strain 33 MA$^-$	
Time (hr)	EEL (optical density) reading	Megacin C titration	EEL (optical density) reading	Megacin C titration
0	0	0	0	0
2	0	0	1·5	0
3	0·5	20	1·5	20
4	9·5	400	13	400
5	36	200	39	200
6	58	5	63	20
7	63	0	67	0
8	65	0	70	0
	Strain 40 M$^+$		Strain 40 MA$^-$	
0	0	0	0	0
2	0	0	0	0
3	0	0	0	0
4·5	14	0	1·5	0
5	25	0	5	0
6	52	1	29	0
7	61	5	59	20
8	71	1	74	1

Production of megacin C in liquid medium. By using *Bacillus megaterium* Mut. SR as indicator organism megacin C was detected in the supernatant fluids of young cultures of the MA^- strains, and could also now be detected in cultures of all the corresponding MA^+ original strains. It should be stressed that these results were quite contrary to our earlier findings when, under apparently similar conditions and with the same indicator strain, no soluble antibiotic activity was formed (Holland & Roberts, 1963). Formation of megacin C by both MA^+ and MA^- strains of *B. megaterium* is shown in Table 3. In the original rapid killers (group 3, megacin A-producers) and their MA^- derivatives, megacin C formation began in the late lag period, reached a maximum in the early exponential phase, but thereafter disappeared rapidly. In only one of the delayed killers (group 2 megacin A-producers) was megacin C activity found consistently. In this strain, *B. megaterium* 40, and its MA^- derivative, megacin C formation began late in the exponential phase, possibly reaching a maximum in the early stationary phase. Thus there was a clear

correlation between megacin C formation and the killing in mixed cultures observed previously with both delayed and rapid killers. None of the non-killers (megacin B producers) formed any megacin C during growth in liquid media.

Activity spectra of different megacins

The activity spectra of strains producing megacin A and B were determined against 53 strains of *Bacillus megaterium* as described previously (Methods). The six megacin-A producers tested inhibited the growth of all but two of the 53 *B. megaterium* strains tested. Iványovics and co-workers obtained similar results and also found that, with few exceptions, megacin A was not active against other species of bacteria (Nagy, Alföldi & Iványovics, 1959; Nagy, 1959). The range of activity of the nine strains which produced megacin B was markedly less than that of megacin A, and only certain strains appeared to be sensitive. Eighteen strains, including all the A-producing strains, were found to be resistant to megacin B. The species specificity of this bacteriocin has not yet been determined.

Although megacin C formation was difficult or impossible to demonstrate on nutrient broth agar, large clear inhibition zones were obtained when Oxoid Nutrient Agar was used. The activity spectra of six megacin C-forming strains, obtained as described above, was determined against 28 strains of *Bacillus megaterium*. Most non-megacin producing strains were sensitive to megacin C. Some strains especially *B. megaterium* strain Mut., were highly sensitive to megacin C; others, including all the megacin-producing strains, were resistant. The most remarkable feature about the activity spectra of megacin C was the uniform pattern of activity displayed by different producers. There was also a noticeable absence of reciprocal activity between different producing strains. This suggested that the megacin C produced by different strains is very similar. In contrast, both A-forming and B-forming strains showed considerable variation in the size and morphology of the inhibition zones produced, and both groups showed strong reciprocal antagonisms when tested against strains which also produced the homologous megacin. This suggests that these groups contain different forms of the particular megacin.

In limited specificity tests megacin C was found to be inactive against the following organisms overlayered on colonies which produced megacin C: *Escherichia coli* K 12; *Salmonella typhimurium* 903; *Bacillus cereus*, 8012, 7587, 8122; *B. subtilis*, 1379, 8236.

Megacin C formation by Bacillus megaterium 216

The properties of *Bacillus megaterium* 216, the prototype megacin A-producing strain, have been extensively studied (review by Ivanovics, 1962). The formation of megacin C by this strain is somewhat anomalous and production of the bacteriocin in liquid culture has not been conclusively demonstrated, when either the parent or the *MA*$^-$ mutant strains have been tested. A bacteriocin may in fact be formed during growth of non-induced cultures of this organism, but this appears to be predominantly megacin A and is not liberated by the *MA*$^-$ mutant strains. Nevertheless, the two *MA*$^-$ mutants, strain 216-MA$^-$ (17), 216-MA$^-$ (28) did produce a second bacteriocin on solid medium under the same conditions and with an activity

spectrum identical with those of other megacin C-producing organisms. It is conceivable that in strain 216 megacin C is tightly bound to the bacteria during liquid culture and is not released into the medium.

DISCUSSION

The system for colicin classification devised by Fredericq (1953) is based upon cross-resistance tests with bacterial mutants resistant to specific colicins. It has proved impossible to isolate stable megacin (A, B, C) resistant mutants of *Bacillus megaterium*; similar difficulties in isolation of bacteriocin-resistant strains have been observed with *Pasteurella pestis* (Burrows & Smith, 1962 and personal communication), *Pseudomonas aeruginosa* (Miss A. Patterson, personal communication) and *B. megaterium* (Professor G. Ivánovics, personal communication). Fredericq (1957) pointed out that colicins classified by use of resistant mutants may differ considerably in chemical and biological properties although having in common the ability to fix to a particular receptor site. Furthermore, grouping by this method breaks down with strains which produce more than one type of bacteriocin. From our studies of bacteriocinogenesis in *B. megaterium* several different types of megacin have been recognized, and, together with the abundant information available from the work of Ivánovics and his colleagues, we feel justified in proposing a preliminary grouping of megacin types. The main criteria for this grouping were: the conditions required for the formation of megacin, the activity spectra, and where possible the mode of action of the megacin. The three types so far recognized have proved to have remarkably consistent group properties and characteristic activity spectra when tested against a range of indicator bacteria. The strains which produce megacin A were all u.v.-inducible, and in all cases studied in the present work and by Nagy *et al.* (1959), this megacin destroys the cytoplasmic membrane of sensitive bacteria. The group B megacins are not inducible by u.v. radiation, and have so far only been demonstrated on solid media. Megacins A and B show considerable reciprocal activity within each group and it is apparent that they comprise a number of different types of the same compound. In contrast the C megacins form a very homogeneous group, different strains apparently producing a similar antibacterial agent. Megacin C was characteristically produced during logarithmic growth in liquid culture although later it disappeared, perhaps as the result of readsorption to the bacteria. Megacin C, which appears to be a protein whose activity is destroyed by proteolytic enzymes (unpublished) has a mode of action quite different from megacin A and is more reminiscent of colicin (Holland, 1963). The association of megacin C with u.v.-inducible megacin A is interesting, but the significance of the association is not yet clear. The two megacin types are, however, separable and there seems no reason why bacteria which produce only megacin C should not be isolated from nature if the appropriate indicator strain can be found.

It was reported previously that actual cell to cell contact of certain 'killer' (M^+) and sensitive bacteria (M^-) of *Bacillus megaterium* was required for killing to occur (Holland & Roberts, 1963). This conclusion resulted primarily from experiments in which killing was prevented by separating the M^+ and M^- bacteria with bacterial filters. At the same time no soluble activity was detectable in either single or mixed cultures containing 'killer' organisms. However, when the experiments were

repeated several months later killing did occur across bacterial filters and easily detectable amounts of soluble megacin C were found in the culture medium. This was also the case when cultures of killer strains were grown from dried spore stocks set aside at the beginning of the investigation. It has now been clearly established that the killing factor was a new bacteriocin which we designate megacin C. The killer strains were clearly releasing greater quantities of megacin C in the later experiments although as far as is possible to ascertain there was no change in cultural conditions. If it be postulated that megacin C, like some colicins, may be a component of the cell surface of the producing bacteria, it is possible that an equilibrium may exist between bound and soluble states. In the current experiments the equilibrium has apparently shifted, in early logarithmic growth, to favour the soluble state. In the latter stages of growth the equilibrium favours the bound form and the megacin is consequently re-adsorbed. There are two, more probable, explanations for the results of the early experiments where an apparent cell contact killing took place. It is possible that very small amounts of the soluble form of megacin C were present in the cultures and this was prevented from acting across the bacterial filters by adsorption to the filter. Alternatively, the bound form of megacin C may have been able to kill sensitive organisms as the result of cell to cell contact in mixed culture.

Marjai & Ivánovics (1962) reported the formation of a bacteriocin by one strain of *Bacillus megaterium* which has some of the properties of megacin C; it was not u.v.-inducible, but did appear in the early logarithmic cultures and was readily adsorbed by sensitive bacteria. However, this compound was easily sedimented in the ultracentrifuge and it was suggested that it may be a defective phage particle. Megacin C is not, however, sedimented in the ultracentrifuge (unpublished) and there is no other evidence to suggest that megacin C is a defective phage particle.

Ivánovics, Alföldi & Nagy (1959*b*) suggested that megacin (megacin A) formation is controlled by a highly degraded prophage; nevertheless, this megacin (or any other bacteriocin) does not contain nucleic acid and is therefore quite distinct from bacteriophage. The distinction is not so clear at the genetic level with bacteriocinogenic factors having many of the properties of prophages (cf. Fredericq, 1957; Ivánovics & Alföldi, 1957; Ivánovics, 1962). It is also difficult to distinguish between bacteriocins and products of highly defective phage particles which lack DNA. Within the scope of our present studies, however, it should be pointed out that the question of nomenclature and whether bacteriocinogenic factors and prophages have evolved in common or independently is not of great significance.

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Bacteriophage Sensitivity and Biochemical Group in *Xanthomonas malvacearum*

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SUMMARY

Isolates of *Xanthomonas malvacearum*, of which 18 representative cultures were examined in detail, were classified into two groups differing in: capacity to oxidize lactose, colony form on first isolation, proteolytic activity, and lysis by bacteriophages. The seven group 1 isolates examined in detail gave confluent lysis at routine test dilution by 7 of 13 phages isolated from diseased cotton plant material, and were weakly proteolytic and did not oxidize lactose in common with other group 1 isolates. All but one of the 11 group 2 isolates gave confluent lysis at RTD by the 6 phages to which the group 1 isolates were resistant but not by the other 7 phages. All isolates of group 2 oxidized lactose and were relatively strongly proteolytic. Phages active against *X. malvacearum* were detected in 87 out of 127 collections of infected leaves examined, and there was some correlation between the presence of groups 1 or 2 xanthomonads and the group-specificity of the phage isolated from the same material. An isolate of group 2 *X. malvacearum* which was resistant to some of the group 2-specific phages was apparently lysogenic.

INTRODUCTION

Xanthomonas malvacearum (E. F. Smith) Dowson, the cause of bacterial blight of cotton (*Gossypium* spp.), has been much studied from the pathological aspect but little as a subject for comparative bacteriological examination. Strains of the pathogen are known to occur which differ in pathogenicity for different varieties of cotton (Brinkerhoff, 1963), but it is not known whether such differences are correlated with characteristics of the pathogen determinable in the laboratory. As a first step isolates of *X. malvacearum* of diverse geographical origin were compared by using a variety of techniques including sensitivity to phages isolated from diseased cotton material, with a view to determining subspecific taxa. In this paper two groups of *X. malvacearum* are defined and their present known distribution is given.

METHODS

Bacteria and phages. Since October 1962 212 collections of diseased cotton material or cultures of *Xanthomonas malvacearum* have been received for examination. Eighteen representative isolates (Table 1) were selected and examined in detail. For isolation and subcultivation sucrose (2 %) peptone agar was used (Hayward, 1960). On this medium cultures of *X. malvacearum* produced copious yellow mucoid growth after 2–3 days at 25° or 30°. All the phages used were isolated

in this laboratory with the exception of phages B 648 P, B 649 P and B 650 P, provided by C. Logan (formerly of the Cotton Research Station, Namulonge, Uganda). Leaf suspensions were enriched with a young liquid culture of the isolate of *X. malvacearum* previously obtained from the source material, and treated with chloroform following the technique of Crosse & Hingorani (1958). It was later found to be unnecessary to enrich the suspension with a young culture of *X. malvacearum* or to make the suspension in a nutrient medium; the following technique was

Table 1. *Sources of isolates of Xanthomonas malvacearum and phages*

Isolate no. and group designation		Territory of origin	Date of isolation	Cotton material as source of phage
B 648	Group 1	Uganda (Namulonge)	1959	Soil and leaf debris
B 649	Group 2	Uganda (Kigumba)	1959	Soil and leaf debris
B 650	Group 2	Tanganyika (Ukiriguru)	1959	Soil and leaf debris
B 1329	Group 1	Northern Nigeria (Katsina Province)	1962	Leaves
B 1330	Group 1	Northern Nigeria (Bauchi Province)	1962	Leaves
B 1331	Group 1	Northern Nigeria (Sardauna Province)	1962	Leaves
B 1334	Group 2	Sudan (Shambat)	1962	—
B 1451 B	Group 1	Tanganyika (Nguduma)	1963	Leaves
B 1451 D	Group 2	Tanganyika (Nguduma)	1963	Leaves
B 1457	Group 1	Tanganyika (Ilonga)	1963	Cotton seed, leaves and stem
B 1528	Group 2	United States, ATCC 9924	1945	—
B 1529	Group 2	United States, ATCC 12132	1955	—
B 1542 A	Group 2	Sudan (Kadugli)	1963	—
B 1543	Group 1	Sudan (El Effein)	1963	Leaves
B 1656	Group 2	Uganda (Namulonge)	1963	—
B 1657	Group 2	Uganda (Namulonge)	1963	Leaves
B 1738	Group 2	Australia (N.S.W.)	1963	Leaves
B 1821	Group 2	Kenya (Kibos)	1963	Leaves

ATCC = American Type Culture Collection, Washington 7, D.C., U.S.A.

adequate. Portions of heavily infected leaf material about 2 cm.^{2} were crushed with a glass rod in 10 ml. quantities of sterile distilled water, allowed to stand at room temperature for 24 or 48 hr, centrifuged, and 5·0 ml. of the supernatant fluid shaken vigorously with 0·5 ml. chloroform in a sterile $\frac{1}{4}$ oz. bottle. When the chloroform had settled about 0·02 ml. of suspension was pipetted on to the surface of pour plates of *X. maivacearum*. Phages were purified by streaking from single plaques on pour plates at least three times in succession. Titres of purified phage suspensions obtained by lysis of 24 hr liquid cultures followed by centrifugation at 2500 *g* for 15–20 min. were in the range 10^9 to 5×10^{10} phage particles/ml. suspension. For phage assays the agar medium of Crosse & Hingorani (1958) was used, adjusted to pH 7·2 with 40 % NaOH. The same medium without agar was used for propagation of phage in liquid culture. Oxoid agar No. 3 (Oxo Ltd., London) at 1 % (w/v) was used in the solid medium, 15 ml./9 cm. diam. Petri dish for pour plates. Phages stored over chloroform (0·1–5 ml. suspension) were diluted to 10^{-7} in phage liquid medium and Pasteur pipettes delivering about 50 drops/ml. were used to add phage suspensions to the surface of pour plates dried open at 37° for 2 hr. All phage assay tests were carried out at 25°.

Physiological tests. The methods used were those described previously (Hayward & Hodgkiss, 1961; Hayward, 1962) with modifications described below. Nitrate

and nitrite reduction tests were made at 6 days, the concentration of sodium nitrite being lowered to 0·01 g./l. For detection of urease activity the method of Christensen (1946) was used, cultures being incubated for 14 days; for detection of lipolytic activity, the method of Sierra (1957) with Tween 80 (Honeywill Atlas Ltd.). For determination of the egg yolk reaction, Oxoid nutrient agar No. 2 (CM 2) was supplemented with glucose (10·0 g./l.) and sodium chloride (5·0 g./l.). Oxoid sterile egg yolk (5 ml.) was added to 50 ml. molten cooled basal medium, and three plates poured. After incubation for 4 days the surface growth was wiped away and the agar beneath the confluent growth examined for changes due to lipase or lecithinase activity. Growth at 37° was determined by inoculation from a pipette or with a loop from agar growth on to slopes incubated in a water bath; the latter technique gave more consistent results. For assay of starch hydrolysis a densely opaque suspension was inoculated with a 3·0 mm. diam. wire loop to the centre of cavities cut in soluble starch agar plates with a No. 2 cork borer. After 42 hr at 28° plates, each with three cavities and inoculated with three isolates, were flooded with Gram's iodine solution and zones of hydrolysis measured (Hayward, 1962). Casein and gelatin plates inoculated from a light suspension in sterile water were examined at 3 and 6 days. The surface growth was wiped away with cotton wool and gelatine plates were flooded with acid mercuric chloride solution (Frazier, 1926) to determine areas of hydrolysis. On casein agar plates areas of clearing were observed. The method for determination of carbohydrate oxidation reactions in a 0·1 % peptone bromothymol blue medium was described by Hayward (1962); tubes were discarded after incubation for 21 days. A dense pellicle of yellow surface growth and acid reaction 0·5 cm. beneath the surface growth was recorded as a positive reaction. Carbohydrate media and liquid media were inoculated with 2–3 drops of a light distilled-water suspension from a Pasteur pipette. Oxidase activity was examined by the method of Kovacs (1956) with 48 hr growth on Oxoid nutrient agar No. 3 (CM 3). Gelatin stab cultures were made in the following medium: Oxoid bacteriological peptone, 5·0 g.; Difco yeast extract, 3·0 g.; gelatine (British Drug Houses Ltd.), 120·0 g.; distilled water, 1 l.; to pH 7·0–7·4 with 40 % NaOH. Sterilization was by autoclaving 10 ml. volumes in 1 oz. bottles at 121° for 15 min.

RESULTS

The characteristics of the 18 isolates of *Xanthomonas malvacearum* are summarized in Table 2. Eleven of the isolates were consistently positive for lactose oxidation, the other seven consistently negative in repeated examinations. The lactose-positive isolates differed from the lactose-negative cultures in rate and extent of hydrolysis of casein and gelatine. In the latter group, at 3 days on casein agar, no zone of clearing was observed when the confluent growth was wiped away. At 6 days there was a zone of clearing confined to the boundary of the confluent growth, whereas lactose-positive cultures produced a zone of clearing extending for several mm. beyond the confluent growth at 2–4 days of incubation. Similarly, on gelatin agar, lactose-positive cultures hydrolysed gelatin more rapidly than lactose-negative cultures, the same difference being apparent in gelatin stab cultures. At 10 days there was no liquefaction or a drop of liquid only at the surface of the lactose-negative cultures; by contrast the lactose-positive cultures produced a zone of

liquefaction 5·0–10·0 mm. in depth. In general these differences in proteolytic activity were reproducible and were invariably according to group in freshly isolated cultures, provided that the casein and gelatin agar media were inoculated from light suspensions in distilled water. The difference in proteolytic activity was less clear-cut, or not apparent, when agar media were heavily inoculated with a wire loop from agar growth. In addition some lactose-negative cultures maintained on artificial media apparently acquired increased proteolytic activity, and one lactose-negative strain maintained on artificial medium for 11 years was comparable in proteolytic activity to lactose-positive cultures, although otherwise unchanged in characters typical of this group. There was no correlated difference in starch hydrolysis; the 18 isolates produced clearly defined zones of hydrolysis of diameter 20·0–25·0 mm. (mean value 22·5 mm.) at 42 hr.

Table 2. *Characteristics of* 18 *isolates of Xanthomonas malvacearum*

Positive	Variable	Negative
Motility	Lactose (11 strains consistently positive, 7 consistently negative)	Urease
Mucoid, yellow growth on sucrose peptone agar	Casein and gelatin hydrolysis (11 strains strongly positive, 7 weakly positive)	Nitrite from nitrate
Aesculin hydrolysis	Mannitol† (11 strains consistently negative, 7 strains variable reaction at different times of testing)	Nitrite destruction
H_2S production	Egg-yolk reaction‡	Indole
Starch hydrolysis		Acetoin
Growth at 37° *		Oxidation of inulin, dulcitol, sorbitol, α-methyl-D-glucoside, *meso*inositol, salicin, rhamnose, erythritol, sorbose
Tween 80 hydrolysis		Kovacs oxidase test (at 5–10 sec.)
Oxidation of glucose, mannose, fructose, sucrose, arabinose, galactose, maltose, cellobiose, dextrin, raffinose†, glycerol, melibiose†, xylose		

* Growth at 37° erratic except from heavy inoculum.

† B 1451 B and B 1451 D negative in raffinose medium; some other strains give a late partial reaction. B 1451 B negative in melibiose medium. The seven cultures variable in mannitol oxidation were all group 1.

‡ Reaction varied according to composition of the medium (see text).

All 18 isolates of *Xanthomonas malvacearum* were negative in the Kovacs oxidase test at 5 sec. In a minority of isolates there was a trace reaction in 5–10 sec., but all were positive in 10–60 sec. Aesculin was hydrolysed by all strains in 4–8 days, and lipolytic activity in the Tween 80 medium was apparent in 2–4 days of incubation. The test for gluconate oxidation was negative or weakly positive (trace of orange precipitate, with blue suspension) at 7–10 days, in agreement with the results of Dye (1962) for the genus *Xanthomonas*. In Oxoid nutrient agar supplemented with glucose and sodium chloride none of the cultures produced any change in the egg-yolk medium, which was initially clear, after incubation for 4 days. However, when the test was repeated with the basal medium for detection of hydrolysis of Tween 80 (Sierra, 1957), which became opaque on addition of sterile egg-yolk emulsion, all isolates of group 1 produced a zone of clearing and then a densely opaque zone of precipitation extending from the margin of the confluent bacterial growth during incubation for 2–4 days. Group 2 isolates produced no change in the medium at 4 days. The difference in reaction between group 1 and 2 isolates on an opaque egg-yolk medium is potentially of diagnostic

value but has yet to be confirmed on a larger collection of isolates. Slight differences in colony form between lactose-negative and lactose-positive cultures were apparent in 2- to 3-day cultures at 25 or 30° on sucrose peptone agar on first isolation. Lactose-negative cultures (group 1) when viewed by transmitted light with the naked eye showed a finely striate texture. This character was best seen in 40–48 hr cultures at 30° or in 60 hr cultures at 25°; the appearance was less distinct in older cultures. Lactose-positive cultures were homogeneous when examined in the same way and were less convex than group 1 colonies, which were in general of a more mucoid consistency and sometimes of a deeper yellow than group 2 colonies. These differences were of great value in the screening of isolation plates for the presence of group 1 or 2 colonies, but they were lost on serial transfer on sucrose peptone agar due to the enhancement of mucoid consistency in group 2 isolates on this medium, and to the occurrence of colony variants.

The plaques formed by phage isolates on the propagating strains of *Xanthomonas malvacearum* were generally clear circular with entire margins, and were 3·0–5·0 mm. diam. after 24 hr at 25°. Growth of resistant colonies in areas of lysis varied with the phage and propagating strain but they appeared only on prolonged incubation in some cases. Rosberg & Parrack (1955) did not detect the growth of resistant colonies of *X. malvacearum* until 12 days had elapsed, when lysed liquid cultures were poured on to the surface of potato glucose agar plates. Other workers have commented on the size and regularity of the plaques formed by phages active on Xanthomonas species. For example, the phage for *X. phaseoli* var. *fuscans* described by Klement & Lovas (1960) produced plaques of 5·0–6·0 mm. diam. at 18–20 hr in a semi-solid agar medium. Mandell & Eisenstark (1953), however, found that the size and form of plaques produced by *X. pruni* phages were markedly affected by such factors as temperature, age of inoculum, concentration of plating bacteria and salt concentration.

The activity of 14 bacteriophages at routine test dilution (10^{-5} dilution of a suspension containing 10^9 to 5×10^{10} phage particles/ml.) on the 18 cultures of *Xanthomonas malvacearum* is shown in Table 3. There is a correlation between phage sensitivity and biochemical group. Phages lysing isolates of group 1 lysed all other group 1 strains and none of group 2, whereas phages lysing group 2 cultures were inactive on group 1 isolates with the exception of isolate B 1543. With this isolate, small discrete plaques were obtained at the routine test dilution with three of the six group 2-specific phages. The reactions obtained with undiluted suspensions were less clear cut. Some of the group 2 cultures gave discrete plaques when tested against the undiluted group 1-specific phages. Another effect, distinct from confluent lysis due to multiplication of phage, was observed with many high-titre suspensions on cultures which were unaffected at the RTD. Clear zones of lysis were obtained, but, unlike true phage lysis, the zone did not extend in diameter on further incubation and the area of lysis was restricted to the zone of adsorption of the drop of phage suspension. At 10^{-1}, 10^{-2} and 10^{-3} dilutions the effect became progressively less clear cut and ultimately faded out without the intermediate formation of discrete plaques. This effect was interpreted to be either lethal adsorption or bacteriocin activity; the former explanation was indicated by the following experiment. All 18 cultures of *X. malvacearum* were grown for 3 days in 10 ml. phage liquid medium, centrifuged, and 5 ml. quantities of supernatant fluid shaken

with 0·1 ml. chloroform. About 0·02 ml. of each suspension was then added to pour plates of the cultures of *X. malvacearum*. No zones of inhibition of growth were observed, showing that inhibition by high-titre phage suspensions was due to lethal absorption. Isolate B 1334, a group 2 isolate which was resistant to two of the group 2-specific phages and partially susceptible to the other four was shown in this experiment to be apparently lysogenic. Numerous small discrete plaques of 0·5–1·0 mm. diam. were formed by isolate B 1334 on six of the group 2 cultures (B 649, B 650, B 1821, B 1657, B 1738, B 1656) but none of the cultures of group 1 was lysed by the suspension.

Table 3. *Lytic activity of* 14 *phages at routine test dilution on* 7 *isolates of Xanthomonas malvacearum group 1 and* 11 *isolates of X. malvacearum group 2*

	Phage													
	B 648 P	B 1329 P	B 1330 P	B 1331 P	B 1451 B/P	B 1457 P	B 1543 P	B 649 P	B 650 P	B 1821 P	B 1451 D/P	B 1657 P	B 1738 P	B 930 P
X. malvacearum isolates	Territory of origin(*)													
	U	N	N	N	T	T	S	U	T	K	T	U	A	NY
Group 1														
B 648	CL	CL	CL	CL	CL	CL	CL	–	–	–	–	–	–	–
B 1329	CL	CL	CL	CL	CL	CL	CL	–	–	–	–	–	–	–
B 1330	CL	CL	CL	+++	CL	CL	CL	–	–	–	–	–	–	–
B 1331	CL	CL	CL	CL	CL	CL	CL	–	–	–	–	–	–	–
B 1451 B	CL	CL	CL	CL	CL	CL	CL	–	–	–	–	–	–	–
B 1457	CL	CL	CL	CL	CL	CL	CL	–	–	–	–	–	–	–
B 1543	CL	CL	CL	CL	CL	CL	CL	(++)	(+++)	–	(++)	–	–	–
Group 2														
B 649	–	–	–	–	–	–	–	CL	CL	CL	CL	CL	CL	–
B 650	–	–	–	–	–	–	–	CL	CL	CL	CL	CL	CL	+++
B 1821	–	–	–	–	–	–	–	CL	CL	CL	CL	CL	CL	+++
B 1451 D	–	–	–	–	–	–	–	CL	CL	CL	CL	CL	CL	+++
B 1657	–	–	–	–	–	–	–	CL	CL	CL	CL	CL	CL	+++
B 1738	–	–	–	–	–	–	–	CL	CL	CL	CL	CL	CL	+++
B 1542 A	–	–	–	–	–	–	–	CL	CL	CL	CL	CL	CL	–
B 1528	–	–	–	–	–	–	–	CL	CL	CL	CL	CL	CL	–
B 1529	–	–	–	–	–	–	–	CL	CL	CL	CL	CL	CL	–
B 1656	–	–	–	–	–	–	–	CL	CL	CL	CL	CL	CL	+++
B 1334	–	–	–	–	–	–	–	+++	SCL	(+++)	(+++)	–	–	–

* U, Uganda; N, Nigeria; T, Tanganyika; S, Sudan; K, Kenya; A, Australia; NY, Nyasaland.
CL, Confluent lysis; SCL, Semi-confluent lysis; –, no confluent lysis or discrete plaques; ++, +++, numerous large discrete plaques (> 1·0 mm. diam.); (++), (+++), numerous small discrete plaques (< 0·5 mm. diam.).

A total of 260 isolates from the 212 collections received was examined for the correlation between biochemical group and lysis by phages; 104 isolates were classified as group 1 and 156 as group 2. All but three of the group 1 isolates gave confluent lysis at the RTD with the group 1-specific phages with which they were tested, and not with the group 2-specific phages. Two of these three aberrant isolates had been maintained on artificial medium for more than 10 years. All three were

resistant to one or other of the group 1-specific phages. Some other cultures which were typical of group 1 on first isolation showed a tendency to become resistant to group 1 phages as evidenced by an increase in the opacity of areas of confluent lysis when they were retested after several months on artificial medium. The isolates classified as group 2 included many which on first isolation and in repeated tests were resistant to one or other or all of the group 2-specific phages (e.g. isolate B 1334; see Table 3). Such differences may provide the basis for subdivision of group 2 isolates.

Experiments were made to determine the sensitivity of other Xanthomonas species to six representative *X. malvacearum* phages and the sensitivity of 18 *X. malvacearum* isolates to phages isolated from plant material infected with other plant pathogens. Four group 1-specific phages (B 648 P, B 1329 P, B 1330 P, B 1331 P) and two group 2-specific phages (B 649 P, B 650 P) were tested at high titre (0·02 ml. of a suspension containing 10^9 to 5×10^{10} phage particles/ml.) on pour plates of 50 xanthomonads representing 20 known species and 3 undescribed species. The bacteria tested were the following: *X. phaseoli* f.sp. *sojense* (9 strains), *X. hederae* (1), *X. citri* (1), *X. papavericola* (1), *X. campestris* (3), *X. phaseoli* (4), *X. ricini* (4), *X. cyamophagus* (1), *X. vignicola* (2), *X. cassiae* (1), *X. punicae* (1), *X. khayae* (1), *X. patelii* (1), *X. cyamopsidis* (1), *X. vasculorum* (2), *X. phaseoli* var. *fuscans* (4), *X. sesami* (2), *X. desmodii-rotundifolii* (1), *X. nigromaculans* f.sp. *zinniae* (3), *X. pruni* (2), five isolates of unidentified *Xanthomonas* spp. The six *X. malvacearum* phages were without macroscopic effect on 38 of the isolates. With the remaining twelve isolates zones of inhibition of growth were observed which appeared to be due to lethal adsorption and not to phage replication. When cultures representative of these twelve isolates were tested against dilutions of the six phages the zones of inhibition of growth became progressively less clear cut and ultimately faded out without the appearance of discrete plaques. Undiluted high titre suspensions of two *X. pruni* bacteriophages and one each for *X. vasculorum*, *X. cassiae*, *X. campestris*, *X. citri*, *X. phaseoli* and *X. phaseoli* var. *fuscans* did not produce confluent lysis or discrete plaques on the 18 cultures of *X. malvacearum* listed in Table 1. When some of these high-titre suspensions were diluted to 10^{-1} and 10^{-2}, however, scattered discrete plaques of 1·0–1·5 mm. diam. were formed on some isolates of group 2. This suggests that undiluted lysates contain an inhibitor of the development of host-range mutants or that such mutants are inhibited by the majority of phage particles not capable of growth on the bacterium.

Four isolates of phages active against *Xanthomonas phaseoli* f.sp. *sojense*, isolated from soya bean leaves showing symptoms of bacterial pustule infection, were tested. Two were without effect but the other phages (B 998 P and B 930 P) gave confluent lysis of some group 2 isolates of *X. malvacearum*. Phage B 930 P was diluted and tested at the RTD: with six of the eleven group 2 isolates this phage gave numerous discrete plaques but the suspension was without activity on the seven group 1 cultures (Table 3). These experiments suggest that bacteriophages for *X. malvacearum* are highly specific and that isolates of this Xanthomonas species are in general not susceptible to lysis by phages isolated from hosts other than cotton infected with various Xanthomonas species.

Seed, stems, or leaves of cotton infected with *Xanthomonas malvacearum* frequently contain phages active against the bacterial pathogen. Material infected

with group 1 *X. malvacearum* may contain a phage active against this group only, and similarly cotton material infected with group 2 *X. malvacearum* may contain a group 2-specific phage. Material infected with xanthomonads of both groups has been shown to contain two phages, which show group 1 and 2 specificity, respectively. This consistency of phage presence in bacterial blight-infected cotton material is illustrated in the experiments described below. In the first experiment 20·0 g. quantities of eight seed samples from Tanganyika were added to 100 ml. quantities of phage liquid medium in 350 ml. conical flasks. To each suspension 10 ml. cultures of an isolate of *X. malvacearum* group 1 (B 1457) and an isolate of

Table 4. *Lytic activity of chloroform-treated suspensions derived from cotton seed washings*

Seed sample	Activity on group 1 isolate B 1457	Activity on group 2 isolate B 1451 D
UKA G2.1/17/904	CL	–
UKA 59/209	CL	+ + +
UKA 59/206	CL	–
UKA 59/488	CL	–
UKA 59/494	CL	+
UKA 59/513	CL	–
UKA 59/515	CL	–
IL 47/10	CL	–

CL, Confluent lysis; + + +, +, discrete plaque formation.

group 2 (B 1451 D) were added. After incubation overnight at 25° the seed liquor was centrifuged and 5 ml. quantities of supernatant fluid added to 0·2 ml. chloroform in sterile $\frac{1}{4}$ oz. bottles. After vigorous shaking drops of chloroform-treated supernatant fluid were added to pour plates of the two xanthomonad isolates used for enrichment. The results given in Table 4 show that all the seed samples contained a phage active against group 1 and two of them also contained phages active against group 2 though in smaller numbers. The phages from UKA 59/209 and UKA 59/494 which lysed the group 2 isolate (B 1451 D) were isolated and purified by propagation on the same culture. At high titre neither phage exhibited activity against group 1 isolates. However, the presence of the bacterial pathogen in the seed samples could not be confirmed by isolation because of the rapid overgrowth of isolation plates with yellow, swarming, fast-growing saprophytic bacteria. In other experiments portions of cotton leaves from 127 heavily infected collections were suspended in 10–20 ml. sterile distilled water in 1 oz. bottles. After 1–6 days at room temperature about 5 ml. of the leaf liquor was added to 0·3–0·5 ml. chloroform, shaken vigorously, allowed to settle and drops added to the surface of separate pour plates of a group 1 isolate and of a group 2 isolate. After 24–48 hr at 25° the plates were examined for the presence of one or more discrete plaques. Forty-one of the 52 collections from which group 1 only had been isolated contained phages active against the group 1 isolate with which they were tested, but phages active on group 2 were not detected. Twenty-nine of the 63 collections containing group 2 *X. malvacearum* contained phages active against the group 2 isolate with which they were tested; 4 were active on group 1 only and three were active on both groups. Twelve collections were examined which contained both group 1 and group 2 *X.*

malvacearum, and of these nine were active on group 1 only, none were active on group 2 only, and one was active on both groups. The majority of the collections were examined for the presence of phages 1–6 months after the date of collection, having been stored at room temperature in the period after collection.

The lack of correlation in some cases between the specificity of the phage and the xanthomonad isolated may partly be explained by the fact that some of the leaves were infected with organisms of both groups or that different leaves of the same collection were infected with group 1 or group 2 organisms; this has been shown to be so in some cases. The method described above assumes that any group 1 or group 2 isolate that has been shown to be susceptible to all the isolated phages of corresponding specificity will be adequate for the detection of phages in cotton leaves. However, this is not always the case; for example, chloroform-treated extracts prepared from eleven collections of leaves from Thailand, from each of which group 1 xanthomonads had been previously isolated, did not lyse the group 1 isolate B 1451 B, which in other experiments had proved adequate for the detection of group 1-specific phages. When the same extracts were tested on pour plates of one of the group 1 isolates obtained from this group of eleven collections, nine of them gave confluent lysis.

DISCUSSION

There are other reports of the occurrence of virulent phages in cotton plant material infected with *Xanthomonas malvacearum* (Lagière, 1960, pp. 37–40), but these reports cannot be related to the present work because the phages described and their propagating strains were not available for comparison. Rosberg & Parrack (1955) isolated a phage from 2-year-old dried diseased cotton leaves, but were unable to isolate similar phages from fresh collections from the field or the greenhouse. The factors leading to the appearance and distribution of phages on cotton material are not understood. Such phages are presumably virulent mutants of temperate phages. In view of the consistent occurrence of phages on cotton it is possible to speculate that they have a role in the reduction of disease incidence in the field, in modification of the intensity of epidemics or in the reduction of numbers of the bacterial pathogen in moribund leaves or trash remaining after harvest, but the fact that the bacterial pathogen can be isolated readily from leaves heavily infected with phage indicates that this effect is localized.

Experience in this laboratory has shown that bacterial blight of cotton is not an isolated example of a disease in which predation of the pathogen by a virulent phage is a common occurrence. Recently phages active against *Xanthomonas citri*, *X. phaseoli*, *X. cassiae*, *X. campestris*, *X. ricini*, *X. phaseoli* var. *fuscans*, and *X. vasculorum* have been isolated from host plant material for each of these pathogens. If it can be demonstrated as shown above for *X. malvacearum*, that such phages are specific for their bacterial hosts, then detection of phage in moribund plant material may be used as an alternative to isolation of the bacterial pathogen in diagnosis. The survival period (dry life) of phages, in common with that of other viruses, would be expected to be greater than that of bacteria. Although an aged specimen may be devoid of the bacterial pathogen, detection of the specific phage would enable a retrospective diagnosis to be made. In the case of bacterial blight of cotton the detection of phages active against *X. malvacearum* groups 1 and 2 in

cotton material is strong presumptive evidence of active infection by either or both groups, or of a previous infection in material in which the bacterium is no longer viable or could not be detected by conventional plating methods. Such a method would be an alternative to the method described by Katznelson & Sutton (1951) for the demonstration of internally borne bacterial infections of seed, but is likely to be of less wide application.

Diseased plant material is known to be a source of phages active against plant pathogenic bacteria. Phages for *Xanthomonas translucens* have been isolated from cereal seed (Sutton & Katznelson, 1953), for *X. phaseoli* var. *fuscans* from bean seed (Klement, 1957), for *X. carotae* from leaves and inflorescences of carrot, and for *X. vesicatoria* from diseased fruit or leaves of pepper and tomato (Klement, 1959). A bacteriophage active against numerous species of Xanthomonas has been described (Sutton, Katznelson & Quadling, 1958), but other phages for species of this genus on which a detailed study of host range has been carried out have proved to be species or strain-specific (Eisenstark & Bernstein, 1955; Klement & Lovas, 1960). By contrast, phages for plant pathogenic species of Pseudomonas may show cross-reaction with other species and with green-fluorescent soil pseudomonads (Stolp, 1961). However, most of Stolp's phages were isolated from soil rather than from plant material. Billing (1963) found that, whereas the majority of plant pathogens that she examined were lysed by one or more of the phages isolated from different sources using plant pathogens as propagating strains, *Pseudomonas aeruginosa* and many of the pseudomonads from sources other than plant material were not. One of the phages isolated by Billing from plant material lysed many plant pathogenic species of Pseudomonas but none of the isolates from soil and water. However, it is apparent from the work of Klement & Lovrekovich (1960) that phages for plant pathogenic species of Pseudomonas cross-react with other plant pathogens of this genus more frequently than do phages for species within the genus *Xanthomonas*.

The use of phages obtained from lysogenic strains of *Xanthomonas malvacearum* or of phages from sources other than bacterial blight-infected cotton material may serve to differentiate strains within groups 1 and 2, and such work should be carried out because of possible correlation with differences in the reaction of cotton varieties. Inoculation experiments done in Tanganyika (Cross, 1963, and personal communication) showed that there are differences in varietal reaction between group 1 and group 2 isolates, but group 2 isolates show pathogenic variability. All the United States isolates examined so far have been classified into group 2; they include ATCC no. 9924, 12131, 12132 and eleven collections of plant material infected with physiologic races of *X. malvacearum* (including the races 1, 2, 4, 6, 10 of Brinkerhoff, 1963). The pathogenic variability of group 1 has yet to be determined. Preliminary work has indicated the utility of phage-sensitivity tests in an epidemiological investigation (Hayward, 1963). Knowledge of the distribution of groups 1 and 2 is at present incomplete. Collections from three widely separated areas in Australia have proved to be group 2. In the Republics of Mali and the Sudan both groups have been found; similarly in Tanganyika, Uganda and Northern Nigeria. Group 1, but not 2, has been obtained from Northern Rhodesia, Nyasaland, Ceylon and Thailand, but the number of collections examined from these areas is too small for any firm conclusion to be drawn at present.

The characteristics of *Xanthomonas malvacearum* described above are in general similar to those of most Xanthomonas species. According to Dye (1962), who has made a survey of the genus, laboratory methods are of little value for the identification of the innumerable species in this genus. However, the present work suggests that examination of isolates of one species from many parts of the world can give information of use in epidemiological investigations and even lead, as in this case, to the delineation of subspecific taxa differing consistently in phage sensitivity and in biochemical characters readily determinable in the laboratory.

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Temperature Sensitivity of Mutants of *Bacillus anthracis* Caused by a Block in Thymine-Nucleotide Synthesis

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SUMMARY

Mutants were isolated from the non-capsulated strain Vollum of *Bacillus anthracis* which were unable to grow above 34° in the absence of certain pyrimidines. At elevated temperatures, one of the mutants, VC⁻TdR⁻, was found to be dependent on thymidine while the other, VC⁻T⁻, required thymine. Both mutants, however, grew normally in the absence of pyrimidines at near to room temperature. A relatively high concentration of thymine was needed in order to overcome the thymidine requirement of mutant VC⁻TdR⁻ at 37°, whereas a combination of a low concentration of thymine with different deoxyribosides (deoxyadenosine, deoxyguanosine, deoxycytidine) gave good growth of the mutant. This observation is suggestive of the presence of a particular enzyme, trans-*N*-deoxyribosylase, in the mutant VC⁻TdR⁻, an enzyme which appears to be of limited distribution in nature. The second mutant, VC⁻T⁻, utilized added thymine readily at 37° and the base could not be substituted by its nucleoside, thymidine. In fact, thymidine and deoxyribonucleosides inhibited the growth of mutant VC⁻T⁻ in the presence of thymine.

Both mutants also grew well at 37° in the presence of thymidine-5-phosphate, which indicated that the *de novo* pathway of pyrimidine synthesis is blocked above 34° somewhere in the pathway between deoxycytidine-phosphate and thymidine-5-phosphate. This block in the pyrimidine synthesis occurring at elevated temperatures caused an unbalanced synthesis of macromolecules accompanied by an abnormal cell-wall formation. At 37°, germinated spores showed an abnormal elongation of the initial cell concomitant with a gradual loss of viability. At this temperature cell-wall formation was also abnormal at limiting concentrations of pyrimidines and minor deficiencies in cell-wall structure of the mutants were still apparent even in the presence of a large excess of pyrimidine. This, however, did not involve any change in virulence of mutant VC⁺TdR⁻ in the homoiothermic mouse.

It is assumed that the mutants produce an altered enzyme protein corresponding to a block in *de novo* synthesis of pyrimidine, or that an inhibitor is produced at high temperatures which diminishes and finally prevents the action of the normal enzyme.

INTRODUCTION

A number of amino acids, salts, glucose and glutamine were defined by Gladstone (1939) as the minimal nutritional requirement of *Bacillus anthracis*. A certain proportion of each amino acid in the basal medium was found to be essential since inhibition of growth is caused by some of the amino acids in excess (Gladstone, 1939). The pure amino acid mixture was replaced later by casein hydrolysate,

which was not only practicable, and by coincidence resembled the mixture giving optimal growth (Brewer *et al.* 1946). The growth of the bacteria in media consisting of amino acids, salts and glucose alone is poor but growth is very much stimulated by the addition of several growth factors. Thiamine was found to be the most potent stimulator but optimal growth was achieved only in the presence of further metabolites, primarily purine and pyrimidine bases (Brewer *et al.* 1946; Belton & Strange, 1954; Puziss & Wright, 1954; Thorne & Belton, 1957).

Growth factors having a simple stimulatory effect on the growth of *Bacillus anthracis* cannot be considered as essential nutrients for this pathogen. In view of this fact, an attempt was made to isolate mutants with definite essential requirements, e.g. requirements which are not stimulatory but indispensable. In the course of such an investigation Iványovics, Varga & Marjai (1963) succeeded in isolating a few mutants, each with a clear essential requirement, e.g. for lactoflavin, uracil or purine bases. These mutants were found capable of growing only in the presence of the essential metabolite; therefore they could be considered as auxotrophs of *B. anthracis*.

In one experiment, after replication from yeast extract peptone medium to chemically defined casein hydrolysate agar medium, three individual colonies grew at room temperature but not at 37°. A detailed study of these isolates initially revealed a thymidine dependency of this mutant at 37°. As has been described recently (Iványovics, 1963), unbalanced growth could be induced in this mutant by a temperature shift. During the course of these studies a secondary temperature-sensitive derivative appeared which was dependent on thymine instead of thymidine. Both mutants grew well in the presence of thymidine-5-phosphate. A detailed description of these mutants will be given in the present paper.

METHODS

Organisms. A non-capsulated mutant of *Bacillus anthracis*, strain Vollum, which was described earlier (Iványovics, 1962) served as a parent for obtaining mutants. This strain will be referred to as VC^- in this paper.

Chemicals. Commercial grades of analytical purity were employed for preparing defined media. Purine and pyrimidine bases were purchased from Fluka A.G. Chemische Fabrik (Basel); thymidine and thymidine-5-phosphate were products of Biochemical Corporation (Cleveland). The other deoxyribosides were kindly provided by Dr H. Amos (Harvard Medical School, Boston).

Basal casein hydrolysate medium (BCM). The double strength medium consists of: Casamino acids, vitamin-free (Difco), 10·0 g.; L-glutamic acid, 1·0 g.; DL-tryptophan and L-cystine, 0·05 g.; sodium citrate, 0·8 g.; K_2HPO_4, 4·0 g.; Na_2SO_4, 0·02 g.; $MgSO_4.7H_2O$, 0·02 g.; ferric ammonium citrate, 10·0 mg.; $MnSO_4$, 4·0 mg.; distilled water 1 l. Each ingredient was dissolved separately and mixed in the given order. The pH of the medium was adjusted to 7·2 with N-NaOH, distributed in 200 ml. volumes, and sterilized at 110° for 30 min. Double strength medium was diluted with the same volume of either sterile distilled water or 3 % agar solution; 10 ml. of 20 % glucose solution was added aseptically per litre of medium. Commercial agar was washed (several times) before use.

Complete casein hydrolysate medium (CCM). Double strength BCM was completed

with thiamine, *p*-aminobenzoic acid, nicotinic acid amide, each at 1 μg./ml.; folic acid, pyridoxin, pyridoxal, pantothenic acid, each at 0·5 μg./ml.; lactoflavin, 0·25 μg./ml.; biotin, 0·01 μg./ml.; cyanocobalamine, 0·005 μg./ml.; guanine, adenine, hypoxanthine, thymine and uracil, each at 5 μg./ml.

When partially completed BCM medium was needed, individual metabolites were added to the basal medium. Pyrimidine base-free medium was made by omitting uracil and thymine from CCM.

Yeast extract peptone medium. YP broth or agar was prepared as described previously (Ivánovics & Alföldi, 1955).

Method for determining nutritional requirement of mutants. The essential requirements of mutants at 37° were examined either in liquid or on solid media. Assay of the essential metabolites in liquid medium was carried out as follows: various amounts of single or of mixtures of metabolites were measured into a series of 160 × 16 mm. test tubes. Thymine and thymidine were added to the tubes before sterilization while solutions of other nucleosides and thymidine-5-phosphate were sterilized separately by filtration through membrane filters (Membran filter, Göttingen, Gruppe 8). These metabolites were added to the sterile media aseptically before inoculation. The volume in each tube was made up to 2·5 ml. with distilled water, and then 3 ml. of double strength CCM (without pyrimidine bases) was added. Tubes were covered with aluminium foil and sterilized at 110° for 30 min. Inoculation of tubes was made with a dilute spore suspension containing about 50 colony-formers (0·5 ml. of a spore suspension prepared in 2·3 % glucose was added to each tube).

The incubation of the cultures was carried out in a maximally sloped position at 37°, and the rate of growth estimated by the optical density at 600 mμ of the cultures.

When the nutritional requirements of mutants were investigated on agar plates, metabolites to be tested were measured into Petri dishes, mixed with melted casein hydrolysate agar and the plates inoculated with about 50 viable spores.

Testing for capsule formation of bacteria. Bacterial streaks were made on yeast extract casein hydrolysate agar and incubated in an atmosphere containing 25 % CO_2 (Ivánovics, 1962).

Spore materials. Unless otherwise stated spores were always used for inoculation. Well-dispersed spore suspensions of each isolate investigated were prepared according to Ivánovics (1962). Spore suspensions in distilled water were stored at 4°, and diluted appropriately with distilled water before use.

RESULTS

Isolation of a temperature-sensitive mutant from strain VC^{-}. The procedure described by Wachsman & Mangolo (1962) for isolating auxotrophs from *Bacillus megaterium*, using 8-azaguanine as a mutagen and used by us with *Bacillus anthracis* (Ivánovics *et al.* 1963), was effective in isolating the thermosensitive mutants. Bacteria were irradiated with ultraviolet light, treated with 8-azaguanine, and the surviving organisms finally allowed to spore. These spores were plated on yeast extract peptone agar (YP agar), and after incubation at 37° the colonies were replicated on to BCM agar. In one experiment involving 250 colonies, three failed

to appear on the replica plates after 24 hr incubation at 37°. However, the missing colonies did develop on keeping the plates at room temperature for 48 hr. These mutants were apparently not capable of forming colonies on minimal agar at 37° but were able to do so at a lower temperature.

All three temperature-sensitive isolates appeared to be identical on first examination, so only one was subjected to a detailed study. All the subsequent experiments described in this paper were carried out with a stock spore preparation. This material was obtained by plating mutant spores on BCM agar, and incubating at 26° for 6 days. The spore suspension was prepared from a pool of 30 colonies.

Analysis of spore material of the temperature-sensitive mutant. When samples of spore material were plated on YP agar and incubated either at 37° or 26° for 48 hr, an identical number of colony-formers was observed. The same number of colony-formers was also seen on BCM plates at 26°. In contrast, no colonies were formed when an inoculum giving rise to 10^7 colonies at 26° was distributed on BCM plates and incubated at 37°. Thus, among 10^7 viable spores of temperature-sensitive mutant no back mutants were detected. On the other hand, about $1/10^5$ spores gave rise to colonies at 37° when plated on CCM agar instead of BCM agar. The colonies which developed on CCM agar at 37° reached a diameter of 4–6 mm. by 24 hr. These colonies did not grow when they were transferred to BCM agar and incubated at 37°. This showed that the spores of temperature-sensitive mutants consist of a population in which two distinct types of colony-formers are present. The growth requirement at 37° of the majority type could be met by thymidine. This type will be referred to as VC⁻TdR⁻. The minority type, designated VC⁻T⁻, is characterized by its dependency on a low concentration of thymine at 37°. When a number of BCM plates were inoculated from individual colonies of VC⁻TdR⁻ and incubated at 26° until sporulation was complete, the proportion of VC⁻T⁻ spores varied from 10^{-5} to 10^{-7} in the cultures. It seems that VC⁻T⁻ bacteria appeared in the cultures of VC⁻TdR⁻ as secondary mutants of strain VC⁻. Table 1 illustrates the growth requirements at 37° of the parent, strain VC⁻, and those of its derivatives.

Table 1. *The growth of the parent strain, VC⁻, and of the mutants, VC⁻TdR⁻ and VC⁻T⁻, on basal casein (BCM) agar supplemented with different metabolites, incubated at 37° for 24 hr*

Medium	The rate of growth		
	VC⁻	VC⁻TdR⁻	VC⁻T⁻
BCM	+	−	−
BCM + B_1	+ +	−	−
BCM + B_1 + adenine	+ + +	−	−
BCM + B_1 + guanine	+ + +	−	−
BCM + B_1 + uracil	+ +	−	−
BCM + B_1 + thymine	+ +	−	+ + +
BCM + B_1 + cytosine	+ +	−	−
BCM + B_1 + thymidine	+ +	+ + +	−
CCM	+ + +	−	+ + +
CCM without B_1	+ +	−	+
YP	+ + + +	+ + + +	+ + + +

Rate of growth: +, colonies 1 mm. in diam. or smaller; + +, 1–3 mm.; + + + 3–7 mm.; + + + + > 7 mm. B_1 = 1 μg./ml. thiamine; the concentration of purine or pyrimidine bases was 5 μg./ml. each.

It can be seen that the requirements of the mutants are highly specific. In addition, Table 1 demonstrates that thiamine appears to be an effective stimulatory nutrient for the parent, and for its mutant strains.

Standard CCM medium which contains as little as 2·5 μg. thymine/ml. and about $1/10^5$ of stock spore material gave rise to colonies (4–6 mm. in diameter) after 24 hr at 37°, even at this low concentration of thymine. In fact, as a result of plating stock spore material at increasing concentration of thymine, the number of colony formers was found to be approximately proportional to the concentration of thymine. In the presence of 20 μg. thymine/ml. about 1 % of viable spores gave rise to colonies; at 100 μg./ml. 30–40 %; and at 200 μg./ml. nearly 100 % of spores formed colonies.

The growth of spores of VC$^-$TdR$^-$ at a high concentration of thymine was slow and they were only 0·5–2 mm. diam. after 24 hr at 37°. Bacteria of strain VC$^-$TdR$^-$ grown at a high concentration of thymine preserved their original characters. When their colonies were transposed to plates containing a low concentration of thymine, they did not grow. On the other hand, a good growth appeared on BCM agar containing as little as 2·5 μg. thymidine/ml.

Some cultural characteristics of the mutants. The effect of temperature on the growth of mutants and parent strains was studied in liquid BCM and CCM (Table 2).

Table 2. *The growth of the parent strain VC$^-$ and its derivatives at different temperatures after incubation for 48 hr*

Medium	Temperature (°C.)	Optical density values		
		VC$^-$	VC$^-$TdR$^-$	VC$^-$T$^-$
BCM	26·0	0·40	0·25	0·30
	29·0	0·34	0·26	0·30
	32·2	0·31	0·10	0·09
	34·2	0·12	0·00	0·00
	37·0	0·07	0·00	0·00
CCM	26·0	0·78	0·62	0·65
	29·0	> 0·76	0·64	0·58
	32·4	> 0·65	0·32	0·50
	34·2	> 0·66	0·00	0·48
	37·0	> 0·78	0·00	0·32

Values marked with > were obtained in cultures where marked aggregation of the bacteria occurred despite energetic shaking before estimating their O.D.

The cultures were made as described under Methods and incubated in the sloped position.

With increased temperature the growth response of bacteria was considerably affected. The mutants did not grow at 34° or above on BCM. In CCM the growth response of the parent strain was almost the same at all temperatures investigated. The growth of VC$^-$T$^-$ at elevated temperatures was apparently due to the presence of thymine in CCM.

The spores of temperature-sensitive mutants gradually died when they were exposed to 37° in BCM. The loss of viability of VC$^-$TdR$^-$ spores followed first order kinetics. Between 97·5 and 98·5 % of spores lost their viability after 24 hr incubation at 37° (Fig. 1). A similar result was observed under identical conditions with spores of VC$^-$T$^-$.

When suspensions of spores in distilled water were exposed to 40° for 24 hr no appreciable decrease in number was seen.

It could be concluded from these observations that the inoculum died during germination and not because the spores were extremely heat sensitive. This was confirmed by microscopical examination of an inoculum made at intervals during incubation at 37°. Samples were removed at intervals from heavily seeded BCM plates by touching the surface with coverslips and the preparations stained by Gutstein's (1926) method. A gradually increasing number of germinated spores was observed and after about 2 hr all the spores appeared to have germinated. Germination was followed by an abnormal elongation of vegetative bacteria, six to eight times that seen in normal cultures of this pathogen. Very few intersepta were visible and the cell wall stained poorly (Pl. 1, fig. 1). After 4–6 hr, the organisms ceased to grow and a gradual disintegration of the bacteria ensued; by the next day most of the cells were broken and disintegrated. The same sequence of events could be observed when 3–4 % (w/v) sucrose was incorporated into the medium.

In contrast, normal growth of temperature-sensitive mutants occurred even on minimal medium at 26°, this being demonstrable both by the colony form and by the morphological appearance of the bacteria. Cultures of mutants VC$^-$TdR$^-$ and VC$^-$T$^-$ on BCM agar after 48 hr incubation at 26° appeared to be identical with those of the parent strain VC$^-$. These opaque and convex colonies reached about 3–4 mm. in diameter and contained characteristic anthrax organisms; long evenly segmented 'bamboo-like' threads with distinct parallel intersepta (Pl. 1, fig. 2).

The amounts of pyrimidines required by the temperature-sensitive mutants at 37°. Half-maximum growth was reached by mutant VC$^-$TdR$^-$ in liquid defined media in the presence of approx. 0·1 μg. thymidine/ml. (Fig. 2*b*). Strain VC$^-$T$^-$ exhibited a similar growth response to thymine but at a ten times higher concentration than that of VC$^-$TdR$^-$ to thymidine (Fig. 2*a*). It was found that addition of pyrimidine bases had no significant effect on the growth response of strain VC$^-$TdR$^-$ to thymidine.

Individual organisms of the mutant strains were found to be abnormal when they were cultivated at 37° on either BCM or on CCM agar containing various concentrations of pyrimidines. At limiting concentrations of the pyrimidines, cell-wall formation was markedly affected. Mutant VC$^-$TdR$^-$, grown at 37° in the presence of 0·5–1·0 μg. thymidine/ml., formed stunted colonies consisting of many bizarrely shaped bacteria. Thus, long filaments without intersepta, heavily distorted and disintegrated cells were seen in preparations stained for cell walls (Pl. 1, fig. 3). Gram staining also revealed extreme abnormalities in the cell structure (Pl. 1, fig. 4). By increasing the concentration of thymidine in CCM agar, malformation of the cell wall was diminished. Nevertheless, even at concentrations as high as 10–50 μg thymidine/ml., cell-wall formation was not completely normal. A few bacteria showed a poor cell-wall stain, but, in addition, the majority of filaments were not uniformly septated, in marked contrast to bacteria grown at 26° (Pl. 1, figs. 5, 6).

Similar observations were made when the thymine-dependent mutant, VC$^-$T$^-$, was grown at 37° in the presence of different concentrations of this base. Severe deficiencies in the cell wall were apparent at concentrations of 1–3 μg. thymine/ml. and minor abnormalities in the cell-wall formation were still to be seen at 200 μg. thymine/ml.

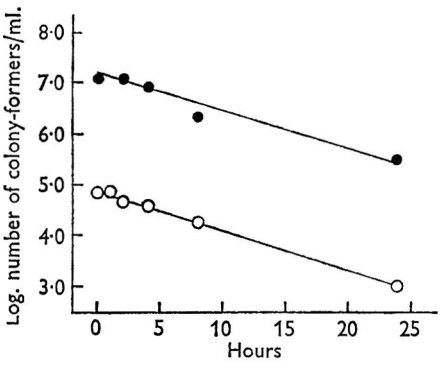


Fig. 1

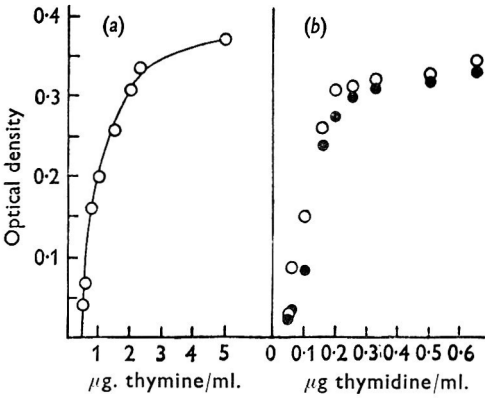


Fig. 2

Fig. 1. The decrease in the number of colony-formers of VC-TdR- spores after incubation at 37° in basal medium. ●, Samples of spore material were plated on BCM agar, and incubated at 37°. Plates were taken at intervals and reincubated for 48 hr at 26°. The number of colony-formers given in the figure is directly related to the total number of colony-formers in the original spore material. ○ 100 ml. of liquid BCM was inoculated with 0·05 ml. of spore material and incubated at 37°. Samples were taken at intervals, and plated on YP agar. The plates were incubated at 37° for 24 hr, and scored for colony-formers.

Fig. 2. The growth response of strains VC-TdR- and VC-T- to thymidine and thymine respectively. CCM without pyrimidine base was used as medium. Incubated at 37° for 24 hr.

(*a*) Strain VC-T-; (*b*) strain VC-TdR-. ○, In the presence of 10 μg./ml. uracil and thymine; ●, without these bases.

The microscopic examination of cells of the temperature-sensitive mutants revealed that cell-wall formation at 37° was slightly abnormal even when an excess of the pyrimidine was added. The minor abnormalities in cell-wall formation therefore cannot be considered as a direct consequence of pyrimidine depletion alone.

Analysis of the block in the pyrimidine synthesis of the mutants. The results of observations with both mutants, VC-TdR- and VC-T-, can be dealt with briefly as follows:

(1) Thymidine-5-phosphate (TdRP) supported the growth of both mutants at 37°. The minimal concentration of TdRP supporting full growth of VC-TdR- and VC-T- bacteria was about 5 μg./ml. at 37°. This requirement of strain VC-TdR- appeared to be about ten times greater than that for thymidine. It should be stressed again that thymidine in contrast to TdRP did not support the growth of VC-T-.

When spores of both mutants were plated on pyrimidine-free CCM agar containing 5 μg. TdRP/ml., all viable spores formed colonies, indicating an identical response of individual spores to this nucleotide.

(2) The thymidine requirement of strain VC-TdR- at 37° could not be replaced by other deoxyribosides. In the absence of thymine in medium CCM, no growth was observed in the presence of 20 μg./ml. of deoxyadenosine (AdR), deoxycytidine (CdR), or deoxyguanosine (GdR). On the other hand, good growth occurred in media containing a low concentration of thymine when the deoxyribosides were added (Table 3).

Table 3. *The growth of mutant VC⁻TdR⁻ as measured by final optical density in the presence of thymine and of individual deoxyribosides*

	Additional substances (μg./ml.)								
		TdR	AdR		CdR		GdR		d
Thymine (μg./ml.)	None	5	20	40	20	40	20	40	40
0	0·00	0·28	0·00	0·00	0·00	0·00	0·00	0·00	0·00
5	0·00	—	0·23	0·26	0·06	0·06	0·06	0·23	0·00
10	0·00	—	0·33	0·28	0·23	0·25	0·18	0·23	0·00

Different amounts of thymine and various deoxyribosides were added to CCM without pyrimidines and the tubes were inoculated with 30 spores of the mutant, VC⁻TdR⁻. Incubated at 37° for 24 hr. Abbreviations (see text): TdR, thymidine; d, deoxyribose.

Thymine at a low concentration, while not supporting growth alone, did so in combination with any of the deoxyribosides. The rate of growth was found to be dependent on the concentration of both thymine and the deoxyribosides. According to the individual deoxyriboside used there was some variation in the rate of growth, the most effective being AdR. The pentose, deoxyribose itself, was found to be ineffective in supplementing thymine.

Similar observations were found when combinations of thymine and deoxyribosides in solid medium were investigated. On CCM agar plates containing 10 μg. thymine/ml. and 20 μg. AdR/ml. all viable spores inoculated gave rise to well-developed colonies (3 mm. diam.). Only a proportion of spores developed when AdR was substituted either with CdR or with GdR. Thus, only half the viable spores formed colonies in the presence of CdR plus thymine after 24 hr at 37°, and only 20–30 % in the presence of GdR plus 10 μg. thymine/ml.

These observations indicated some heterogeneity in the spore population as to their ability to utilize thymine in the presence of deoxyribosides. The variation appears, however, to be more quantitative than qualitative. By increasing the concentration of either thymine or deoxyriboside, or by extending the incubation period, the percentage of colony-formers can reach 100.

When thymine was replaced by 2 μg. thymidine/ml. in CCM, the growth of VC⁻TdR⁻ was neither inhibited nor enhanced by addition of the other deoxyribosides.

(3) The thymine-requiring mutant, VC⁻T⁻, did not grow in the absence of this base at 37°, but the nucleotide could replace thymine while the nucleoside, thymidine, was ineffective. Moreover, thymidine actually prevented growth at 37° in the presence of thymine. This antagonism appeared to be competitive. When increasing amounts of thymidine were added to a series of tubes containing CCM plus 400, 200, 100 and 50 μg. thymine/ml. and inoculated with VC⁻T⁻ spores, the growth of bacteria was proportional to the ratio of base and nucleoside (Fig. 3).

According to the concentration of thymine used, 1 mole of thymidine successfully prevented the 50 % utilization of 391, 399, 305 and 260 mole of thymine.

This inhibition of thymine utilization by strain VC⁻T⁻ was not restricted to thymidine, since all deoxyribonucleosides showed a similar effect. Nucleosides AdR, CdR and GdR at a concentration of 1·5 μg./ml. gave a 50 % inhibition in the

presence of 50 μg. thymine/ml., and 2 μg. nucleosides/ml. completely suppressed the growth of bacteria. No inhibition of growth was, however, observed when deoxyribose instead of deoxyribosides was added to the system.

The pathogenicity of mutant VC⁻TdR⁻. Neither the non-capsulated parent strain VC⁻ nor its mutant VC⁻TdR⁻ killed mice in a dose of a million organisms but two out of five mice were killed by about 10^8 organisms of mutant VC⁻TdR⁻ injected intraperitoneally; the bacteria recovered formed mucoid colonies in the presence of 25 % CO_2. This mucoid growth was allowed to sporulate, and samples of the spore material were plated on BCM, CCM and YP agar and incubated at 37°.

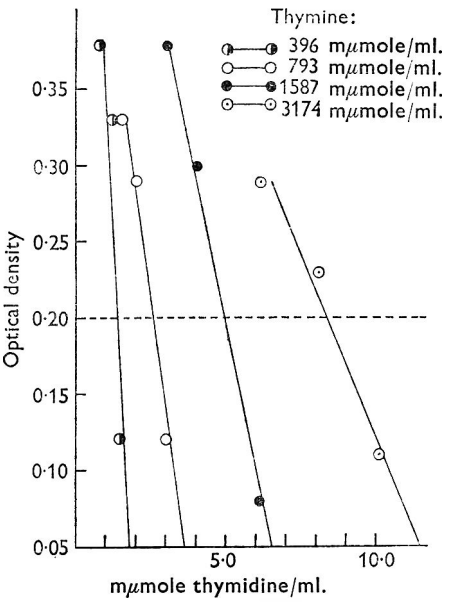


Fig. 3. About 50 % inhibition of growth (0·2 optical density values) of strain VC⁻T⁻ by thymidine at different concentrations of thymine.

Table 4. *The virulence of mutant VC⁺TdR⁻ for mice*

No. of spores inoculated subcutaneously	Mice dying (dead/total)
5×10	2/3
5×10^2	2/3
5×10^3	3/3
5×10^4	3/3

The number of colonies formed differed considerably; about $1/10^5$ grew on BCM agar; these were revertants to the prototrophic type; about 1 % of the spores grew on CCM agar; these were found to be thymine-dependent at 37°. These observations show that there was a mutation to thymine dependency and also a reversion to prototrophy during growth in the animal body. It appears that a series of independent mutations took place in the animal infected with spores of VC⁻TdR⁻. The sequence of mutations appeared to be: VC⁻TdR⁻ → VC⁺TdR⁻ → VC⁺T⁻ → VC⁺; the latter being the wild-type strain Vollum.

Groups of mice were then infected subcutaneously with increasing doses of mutant VC⁻TdR⁻. The result is shown in Table 4. It can be seen that a small number of spores was enough to kill mice. The virulence of the mutant VC⁺TdR⁻ does not differ significantly from that of wild-type strain Vollum (Ivánovics, 1962). Blood from these mice was also plated on YP agar and the bacteria which grew out were checked for capsule production and for additional requirements at 37°. A proportion (about 10^{-3}) of colony-formers was found to be the thymine-dependent, VC⁺T⁻ mutant.

This experiment showed that the strain VC⁺TdR⁻ while dependent on thymidine at 37° was capable of multiplying in the body of the homoiothermic mouse.

DISCUSSION

Although this study may not reflect the fine details of the minimal nutritional requirements of the prototrophic strain VC⁻ at 26° and 37°, it nevertheless appears that this strain of *Bacillus anthracis* has a somewhat more complex requirement at 37° than at a lower temperature. In this respect *B. anthracis* appears to be similar to *Pasteurella pestis*, a pathogen having a definitely higher nutritional requirement at 37° than below 32° (Hills & Spurr, 1952). It has been found (Ivánovics, Varga & Marjai, to be published) that the adverse effect of incubation at 37° on *B. anthracis*, strain VC⁻, was very much diminished by the presence of thiamine. On addition of adenine and guanine as well as thiamine, the difference in the rate of growth at 26° and 37° was only small. These facts suggest that the 'stimulatory' effect of some metabolites on the growth of *B. anthracis* may be explained by assuming that a 'salvage' pathway is put into operation in the presence of exogenous metabolites. Thus, the inhibition of the *de novo* pathway caused by elevated temperature is circumvented by addition of the appropriate metabolite.

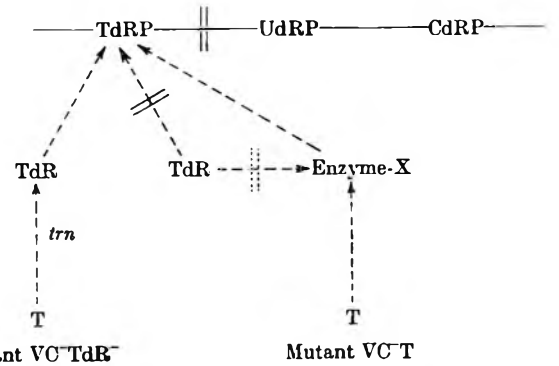
The striking feature of the mutants described in this paper was their dependency on thymidine or thymine at a temperature above 34°. By decreasing the temperature of incubation, their dependency on pyrimidines could be diminished proportionally.

The primary mutant, VC⁻TdR⁻, had a requirement for thymidine at 37°; thymine can replace thymidine only at an unusually high concentration, as this base was utilized poorly and slowly. The secondary mutant, VC⁻T⁻, derived from the spore material of VC⁻TdR⁻ by selection at a low concentration of thymine exhibits a marked specificity to this base. Thymine could not be replaced by its nucleoside, thymidine. Nevertheless, both mutants grew similarly and well at 37° on addition of thymidine-5-phosphate. This observation is rather striking, for the direct utilization of nucleotides by Enterobacteriaceae has long been open to question. On the other hand, experimental evidence indicates that *Lactobacillus bulgaricus* directly incorporates uracil-nucleotides (Rose & Carter, 1954). There seems to be, therefore, a difference in the incorporation of nucleotides by different bacteria.

It appears that the *de novo* pathway of pyrimidine synthesis is blocked at nucleotide level somewhere in the pathway between thymidine-5-phosphate and deoxycytidine-phosphate. In trying to guess the position of this block in the mutants, other *de novo* pathways beside that represented by Scheme 1 should be considered. It is known (Cohen & Barner, 1957) that *Escherichia coli* is capable of converting

5-methyl-deoxycytidine to thymidine. Therefore, the position of the block represented by Scheme 1 has been arbitrarily chosen.

Scheme 1.



Key: CdRP, Deoxycytidine-phosphate; UdRP, deoxyuridine-phosphate; TdRP, thymidine-5-phosphate; T, thymine; TdR, thymidine; *trn*, trans-*N*-ribosylase; ═, block in the conversion; ⋯, enzyme inhibition but block in the TdR conversion; ——, *de novo*; - - -, salvage pathway.

Mutant VC⁻TdR⁻ utilized thymidine readily and the main salvage pathway in this mutant at elevated temperature therefore appeared to be the conversion of thymidine to its corresponding nucleotide.

It is safe to assume that in the utilization of thymine in mutant VC⁻TdR⁻ the enzyme, trans-*N*-deoxyribosylase, is involved. This enzyme has been described in *Lactobacillus helveticus* and *L. delbrückii* by MacNutt (1952), and renamed by Roush & Betz (1958). The enzyme is able to catalyse the transfer of the deoxyribosyl group in deoxynucleosides to purine or pyrimidine bases. The role of this enzyme is therefore concerned in the utilization of deoxyribonucleosides (McNutt, 1952). It was found that the spores of mutant VC⁻TdR⁻ were capable of forming colonies at a high concentration of thymine, although the clones derived from individual spores varied in their ability to utilize this base. In fact, the utilization of thymine was poor and slow. Nevertheless, when a deoxyriboside (deoxyadenosine, deoxyguanosine, deoxycytidine) was added to the culture of mutant VC⁻TdR⁻, even a low concentration of thymine permitted growth. A combination of deoxyriboside and thymine was effective in supporting growth while deoxyriboside or thymine alone was ineffective.

It should be stressed, however, that mutant VC⁻TdR⁻ was able to grow, albeit poorly, in the absence of added deoxyribonucleoside, when a high concentration of thymine was present in the medium. The utilization of thymine in this case can probably be explained by a combination of purine-deoxyribosides supplied from the pool.

Mutant VC⁻T⁻ was not capable of utilizing thymidine, although both the base and the nucleotide permitted growth at elevated temperature. Moreover, thymidine not only failed to support growth, but its presence competitively inhibited the utilization of thymine. This effect was not limited to thymidine but was brought about also by the other deoxyribosides tested. It could be inferred from these

observations that an enzyme (designated as enzyme-X in the Scheme) which incorporates thymine in the salvage pathway in this mutant has binding sites for both base and deoxyribosides. The failure to utilize thymidine may be due to absence of an adequate kinase in this mutant. Since thymine could not be utilized via nucleosides, it should be supposed that thymine reaches the main pathway in the mutant VC$^-$T$^-$ bypassing the nucleoside level and the base is thus converted directly to nucleotide. If this be the case, the existence of an enzyme (similar to the known phosphoribosyl pyrophosphorylases) should be considered which is capable of attaching to the base a deoxyribose-5-phosphate moiety.

The pyrimidine dependency of mutants at 37° was associated with a malformation of cell wall. This was very pronounced at low concentrations of the pyrimidine. Restoration of cell-wall synthesis appeared to require a higher concentration of pyrimidine than that which was necessary to support the growth of the temperature-sensitive mutants. The morphological integrity of the cell wall was not totally restored even at a high excess of pyrimidines, suggesting that mutant growth at 37° was not perfectly balanced during the incorporation of pyrimidines on the salvage pathway. Nevertheless, the capsulated mutant of thymidine-dependent bacteria was found to be fully virulent in mice.

It has been shown recently (Iványovics, 1963) that an unbalanced synthesis of the essential macromolecules can be elicited by a temperature shift to 37° in a culture of mutant VC$^-$TdR$^-$ growing exponentially without thymidine at room temperature. This unbalanced growth of *Bacillus anthracis* mutants is similar to several systems already known in *Escherichia coli*, where the lack of thymine (Barner & Cohen, 1954) or the prevention of thymine synthesis (Cohen & Barner, 1956; Tomasz & Borek, 1962) causes unbalanced growth, followed by death, of bacteria. Thymine deprivation in the mutants of *B. anthracis* did not cause rapid death of the bacteria but rather a prolonged, gradual loss of viability.

Since normal growth of the temperature-sensitive mutants occurred at a low temperature, even in the absence of pyrimidines, it is obvious that the genes controlling the enzymes involved in the biosynthesis of thymine were functioning normally under these conditions. The block in the pyrimidine pathway at elevated temperature could be due to a mutation affecting a structural gene. Several mutants of micro-organisms have already been described where the mutation resulted in a synthesis of an altered but functional enzyme. Maas & Davis (1952) described a mutant of *Escherichia coli* which required pantothenate for growth above 30°. The enzyme that catalysed pantothenate synthesis was found to be much more heat-labile than that in the corresponding preparation of the wild type. Fincham (1959) reviewed the data concerning qualitative alteration of enzymes caused by mutation in micro-organisms. In a recent study, Yanofsky, Helinski & Maling (1961) described mutational changes of the A gene controlling tryptophan synthetase in *E. coli* which lead to the formation of altered proteins differing in their solubility and heat stability. Nevertheless, quantitative enzymatic activity measurements failed to reveal differences between the mutants. It might therefore be assumed that the block in pyrimidine synthesis which manifests itself only at an elevated temperature in *Bacillus anthracis* mutants derives from a mutation in a structural gene. If this is the case, a production of an excessively heat-sensitive enzyme should be considered in the pyrimidine pathway.

An alternative explanation of the thermosensitivity of mutants could be related to the production of an inhibitory substance at elevated temperature. It seems likely that in this case a gene of regulation is involved in the mutation. Unfortunately, no tool is yet available for the genetic analysis of *Bacillus anthracis*, so that this question cannot be answered at present.

I should like to thank Professor E. Borek and Dr A. Thomasz (Department of Biochemistry, Columbia University, New York) for reading the manuscript and for their valuable suggestions. I am also indebted to Dr B. Holland (Department of Biochemistry, University of Oxford) for his editorial help, and to Dr H. Amos (Department of Bacteriology, Harvard Medical School, Boston) for the deoxyriboside samples kindly sent to me.

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EXPLANATION OF PLATE

Photomicrographs of strain VC⁻TdR⁻ grown under different conditions. Cell-wall staining unless otherwise stated. Magnification ×1230.

Fig. 1. Bacteria germinated from VC⁻TdR⁻ spores on BCM agar after 4 hr incubation at 37°.

Fig. 2. 'Bamboo-like' threads of VC⁻TdR⁻ bacteria grown (in the absence of thymidine) on BCM agar. Incubated at 26° for 48 hr.

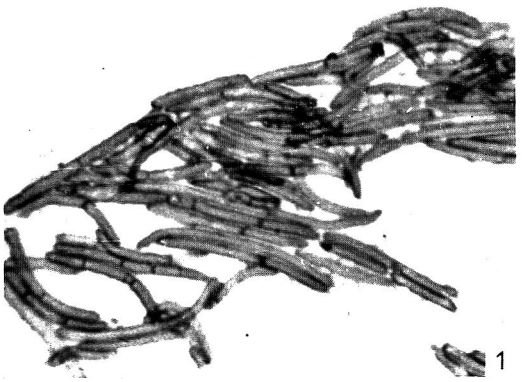
Figs. 3–6. VC⁻TdR⁻ bacteria grown on pyrimidine base-free CCM agar on addition of thymidine at various concentrations. Incubated at 37° for 24 hr.

Fig. 3. In the presence of 0·5 μg. thymidine/ml.

Fig. 4. In the presence of 0·5 μg. thymidine/ml. With Gram staining.

Fig. 5. In the presence of 10 μg. thymidine/ml.

Fig. 6. In the presence of 50 μg. thymidine/ml.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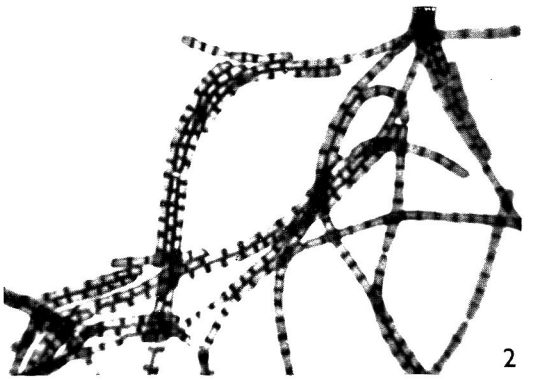
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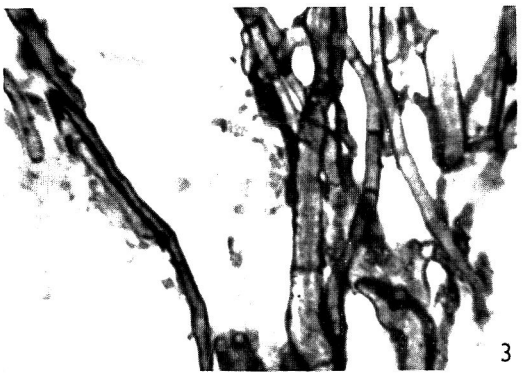
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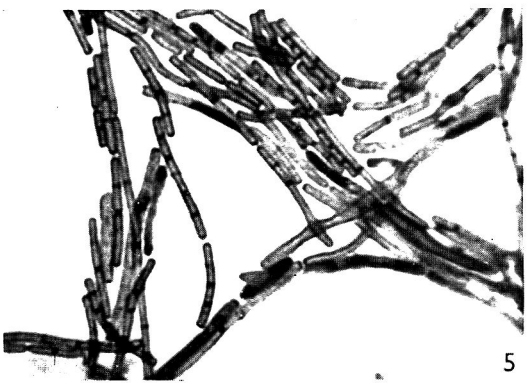
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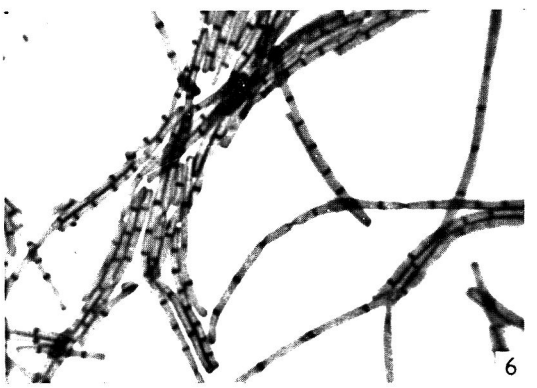
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Lysozyme Lysis of Gram-Negative Bacteria without Production of Spheroplasts

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SUMMARY

Cells of Gram-negative bacteria undergo lysis when treated with lysozyme in the presence of ethylenediaminetetraacetic acid (EDTA) and tris buffer, as shown by Repaske. However, contrary to the prevalent assumption, lysis is not necessarily preceded by formation of a spheroplast as the cell wall is damaged. Treatment of *Escherichia coli* and *Pseudomonas aeruginosa* with the lytic system was shown to cause the formation of osmotically fragile rods, rather than spheres. The extent of destruction of the cell walls of Gram-negative bacteria by lysozyme in this system is, at least in some cases, less than has been generally supposed.

INTRODUCTION

Spheroplasts have been described by Tulasne, Minck, Kirn & Krembel (1960) and McQuillen (1960) as spherical bodies formed after partial loss of the bacterial cell wall; they are osmotically fragile, and undergo lysis in media of low osmotic pressure. Protoplasts, on the other hand, are characterized by complete loss of the cell wall (Brenner *et al.* 1958; McQuillen 1960).

Repaske (1956, 1958) demonstrated that the cells of a number of unrelated species of Gram-negative bacteria could be lysed by lysozyme, in the presence of ethylenediaminetetraacetic acid (EDTA) with tris buffer (tris-(hydroxymethyl) aminomethane) at pH 8. This system was used (Mahler & Fraser, 1956; Fraser & Mahler, 1957) to prepare 'protoplasts' or spheroplasts of *Escherichia coli* in the presence of 0·5 M-sucrose. Although none of these papers reported any morphological observations on the transformation of cells to spheroplasts, McQuillen (1958) stated that '... Repaske [has] used lysozyme, to prepare spherical, osmotically shockable forms of Gram-negative bacteria such as *Escherichia coli*'. Later, McQuillen (1960) said, 'Mahler and Fraser went on to show that in the case of *E. coli* lysis could be prevented and the action arrested at a stage of spherical, osmotically shockable "protoplasts" if sucrose (0·5 M) were present'.

It is the purpose of this communication to demonstrate that treatment of Gram-negative bacteria with the lysozyme-EDTA-tris buffer system may yield osmotically fragile cells, without so complete a destruction of the rigid cell wall as to cause the production of true spheroplasts.

METHODS

The organisms used were *Escherichia coli* (ATCC 10536) and a strain of *Pseudomonas aeruginosa* isolated in our laboratories. Twenty-four hour cultures at 37° in brain heart infusion broth (Difco) were centrifuged, washed twice with water, and resuspended in half the original volume of water. Optical densities at 660 mμ were about 0·65 and 0·25, respectively, as measured with a Coleman Junior Spectrophotometer and 16 mm. test tubes.

EDTA was used as the disodium salt. A 1 % solution was adjusted to pH 8·0 before use. Tris buffer was prepared as a 1 M solution, adjusted to pH 8·0 with HCl. Crystalline egg white lysozyme (Difco) was dissolved in distilled water to give a concentration of 1 mg./ml.

For studies of lysis, a 2 ml. portion of the suspension of washed cells of *Escherichia coli* was exposed to 500 μg. EDTA and 20 μg. lysozyme/ml., in the presence of 0·033 M-tris buffer and in a total volume of 6 ml. For *Pseudomonas aeruginosa*, the concentration of EDTA was reduced to 200 μg./ml.; other conditions remained the same. Lysis at room temperature was followed by measurement of optical density at 660 mμ, as above.

For studies of morphological changes occurring during the action of lysozyme, parallel studies were carried out in the presence of 0·5 M-sucrose to prevent lysis. As controls, cell suspensions were prepared with sucrose alone, or with the EDTA-tris buffer-sucrose systems without lysozyme. After standing for 20 min., 3 ml. samples of each suspension were treated with 6 drops of 10 % formaldehyde or 1·5 ml. of 1 % buffered osmic acid fixative (Chapman, 1959) and allowed to stand overnight. The fixed cells were then washed three times in distilled water, diluted, and a specimen deposited on a carbon substrate and shadowed with platinum before examination in a Siemens Elmiskop I electron microscope.

RESULTS

The curves in Fig. 1 show the lysis of *Escherichia coli* and *Pseudomonas aeruginosa* upon exposure to lysozyme in the presence of EDTA and tris buffer, under the conditions outlined above. In each case, turbidity of the suspension decreased 70 to 75 % during 10 min. at room temperature. Microscopic examination after this period showed only amorphous debris.

However, when the presumed 'spheroplasts' derived from treatment with lysozyme-EDTA-tris buffer were supported with sucrose to prevent lysis, and fixed with osmic acid or formaldehyde, examination with the electron microscope showed that spheres had not, in fact, been formed. In Pl. 1, the first three figures show the appearance of *Escherichia coli* after exposure to sucrose alone (Pl. 1, fig. 1), EDTA-tris buffer-sucrose (Pl. 1, fig. 2), and lysozyme-EDTA-tris buffer-sucrose (Pl. 1, fig. 3). In comparison with the normal cell (Pl. 1, fig. 1), that exposed to the lytic system (Pl. 1, fig. 3) shows evidence of extensive loss of internal substance and damage to the cell wall, but still retains its identity as a rod. Organisms treated with EDTA and tris buffer alone, in the presence of sucrose (Pl. 1, fig. 2), also show some evidence of damage, as compared with the normal.

With *Pseudomonas aeruginosa*, similar findings were obtained. Plate 1, figs. 4, 5

and 6, show the results of corresponding treatments of this organism, and reveal that here, too, conversion of the bacteria to an osmotically fragile form by treatment with lysozyme-EDTA-tris buffer is not accompanied by transformation of the rods to spheres. Both the mixture of EDTA with tris buffer and the system with lysozyme caused less obvious morphological damage to *P. aeruginosa* than to *Escherichia coli*.

The osmotic fragility of both species after exposure to the lytic system was confirmed by dilution; lysis occurred rapidly on the addition of three or four volumes of water.

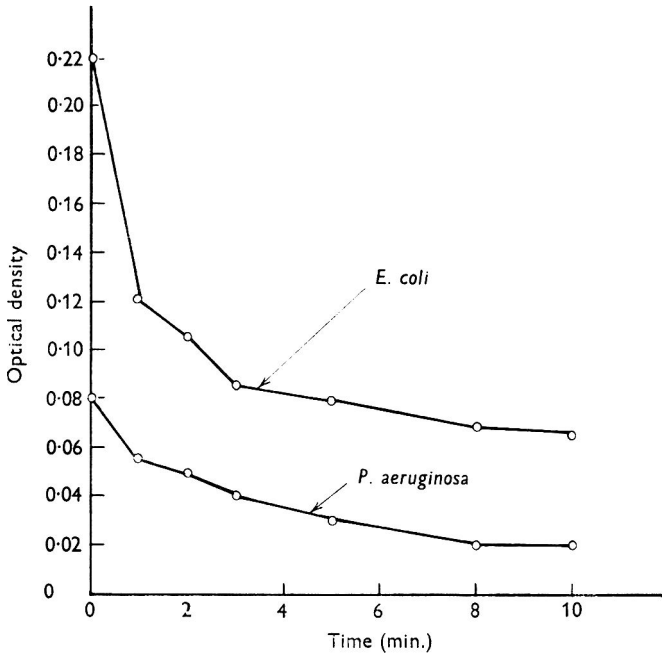

Fig. 1. Decrease in optical density at 660 mμ of suspensions of Escherichia coli and Pseudomonas aeruginosa in the presence of 500 μg. EDTA/ml. (*E. coli*) or 200 μg. EDTA/ml. (*P. aeruginosa*), 0·033 M-tris buffer, and 20 μg. lysozyme/ml.

DISCUSSION

That lysozyme can cause the conversion of Gram-negative bacteria to spheroplasts has been demonstrated by Zinder & Arndt (1956), who illustrated the formation of spheroplasts of *Escherichia coli* by lysozyme at pH 9. It has been generally assumed that the production of lysis of Gram-negative bacteria by similar means must be preceded or accompanied by the destruction of so much of the rigid cell wall that the rods are converted to spheres. McQuillen (1958, 1960) assumed that Repaske and Mahler & Fraser had induced the formation of spheroplasts in causing lysis of *E. coli* and other Gram-negative species by treatment with lysozyme and EDTA. Actually, Repaske (1956, 1958), Mahler & Fraser (1956), and Fraser & Mahler (1957) never reported the production of spheres from rods; their observations were confined to the occurrence of lysis. It may be that, under their conditions

and with their strains, spheroplasts were produced; however, this does not appear to have been established.

Murti (1960) has, indeed, shown the production of spheres from *Escherichia coli* and *Vibrio cholerae* under the influence of lysozyme and EDTA in buffered sucrose solution; however, incubation for 2–3 hr at 38° was required for this transformation. The observations of Repaske and of Mahler & Fraser, as well as those reported above, concern changes occurring within the first few minutes at room temperature. Continuation of these changes might well be expected to lead to the eventual loss of rigidity of the cell wall and the formation of spheroplasts.

The results reported here show that a stage intermediate between the intact cell and the spheroplast exists. In these cases, damage to the cell wall was sufficient to cause it to lose its effectiveness as a rigid support for the cytoplasmic membrane, but was still so incomplete that the characteristic shape of the organism was not lost. This may well be the case in other work in which osmotic fragility has been assumed to demonstrate conversion of cells into spheroplasts.

The evidence of some degree of damage to the cell wall by exposure to the system containing only EDTA and tris buffer is in agreement with the data reported by Repaske (1956, 1958), who showed significant lysis of *Pseudomonas aeruginosa* by EDTA in the absence of lysozyme. It is assumed that this action of buffered EDTA on the cell wall, causing some morphological evidence of damage and some lysis, is that which causes the cell wall to become susceptible to lysozyme.

Thanks are due to Mr A. P. Murphy for electron microscopy and photography.

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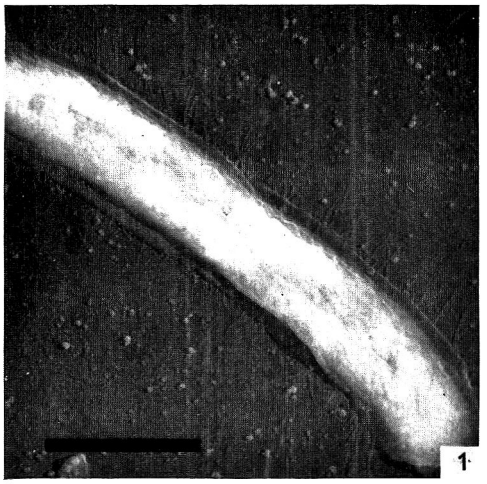

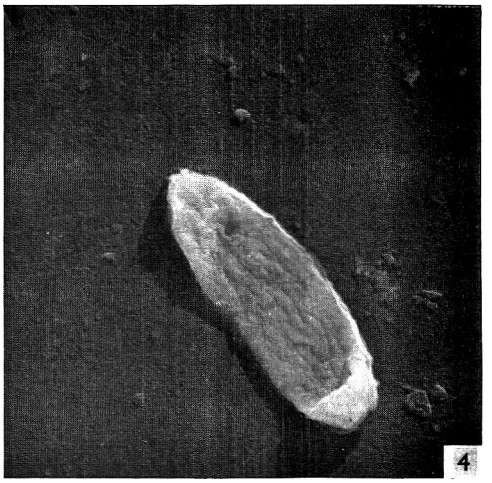

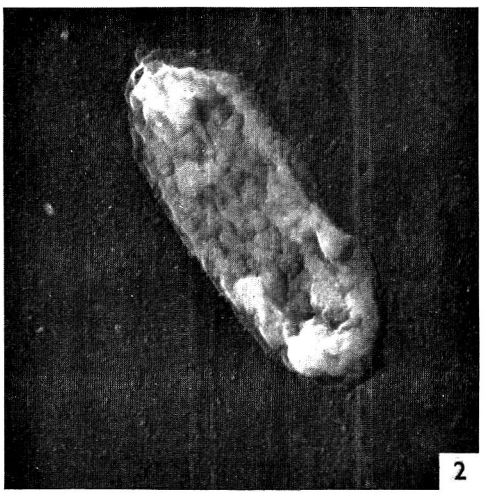

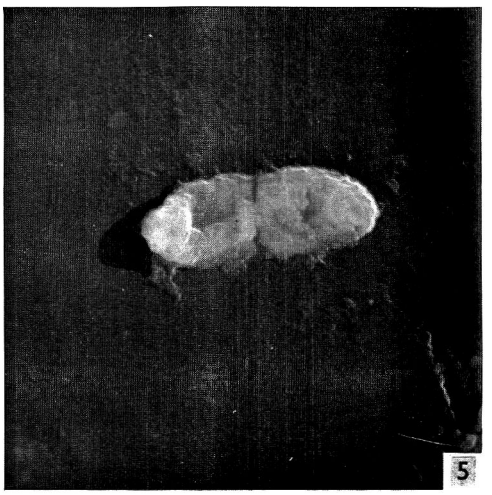

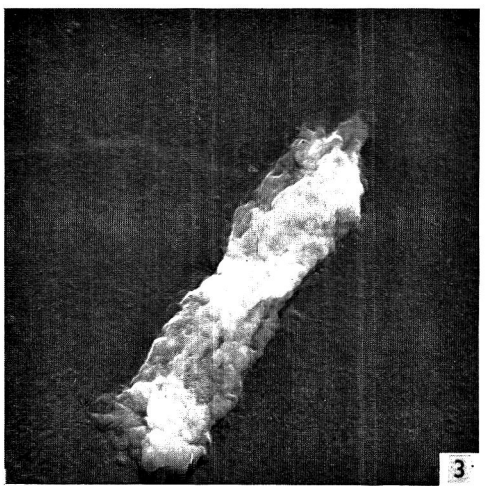

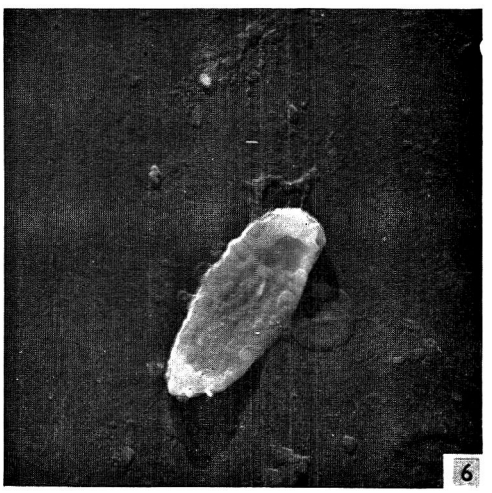

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EXPLANATION OF PLATE

Electron micrographs of platinum-shadowed *Escherichia coli* and *Pseudomonas aeruginosa* are all at the same magnification; the mark on Fig. 1 represents 1 micron. Cells fixed with osmic acid and washed.

Fig. 1. *Escherichia coli* after exposure to 0·5 M-sucrose.

Fig. 2. *Escherichia coli* treated with EDTA and tris buffer in the presence of 0·5 M-sucrose.

Fig. 3. *Escherichia coli* treated with lysozyme, EDTA, and tris buffer in the presence of 0·5 M-sucrose.

Fig. 4. *Pseudomonas aeruginosa* after exposure to 0·5 M-sucrose.

Fig. 5. *Pseudomonas aeruginosa* treated with EDTA and tris buffer in the presence of 0·5 M-sucrose.

Fig. 6. *Pseudomonas aeruginosa* treated with lysozyme, EDTA, and tris buffer in the presence of 0·5 M-sucrose.

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Regulatory Mechanisms Governing Synthesis of the Enzymes for Tryptophan Oxidation by *Pseudomonas fluorescens*

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SUMMARY

Both co-ordinate and sequential inductions govern the synthesis of the enzymes required for the oxidative dissimilation of L-tryptophan by a strain of *Pseudomonas fluorescens*. The first two enzymes of the sequence, tryptophan pyrrolase and formylkynurenine formamidase, are induced co-ordinately by L-kynurenine, the product of their successive action on L-tryptophan; L-tryptophan itself is not an inducer. Both these enzymes are present at low concentrations in uninduced organisms, so that inducer is generated endogenously when such organisms are exposed to an exogenous supply of L-tryptophan. L-Kynurenine also induces formation of the third enzyme of the sequence, kynureninase, which is not detectable in uninduced bacteria. Although it is elicited by the same inducer, synthesis of kynureninase is not co-ordinate with the syntheses of pyrrolase and formamidase; the induction of kynureninase can accordingly be considered as the first sequential inductive step in the pathway. It is immediately followed by another sequential inductive step: the synthesis of anthranilic acid oxidase, elicited by anthranilic acid, which is formed from kynurenine through the action of kynureninase.

INTRODUCTION

Tryptophan can serve as sole source of carbon and energy for the aerobic growth of many bacteria belonging to the Pseudomonas group. The oxidative dissimilation of tryptophan is catalysed by special sequences of enzymes, the physiological role of which is to convert this heterocyclic substrate to aliphatic fragments that can enter the central pathways of cellular intermediary metabolism. The initial attack is mediated by tryptophan pyrrolase and formylkynurenine formamidase, and results in conversion of L-tryptophan to L-kynurenine (Suda, Hayaishi & Oda, 1949; Stanier & Tsuchida, 1949; Stanier & Hayaishi, 1951). The further metabolism of L-kynurenine proceeds through one of two alternative pathways, the so-called 'aromatic' and 'quinoline' pathways (Fig. 1). On the aromatic pathway, a sequence of about seven enzymes operates to convert L-kynurenine into L-alanine, succinyl-CoA and acetyl-CoA (Hayaishi & Stanier, 1951; Katagiri & Hayaishi, 1956). On the quinoline pathway, a different but equally extensive sequence of enzymes catalyses the conversion of L-kynurenine, through a series of quinoline and pyridine derivatives, to glutamic and oxaloacetic acids (Behrman, 1962). Any given Pseudomonas strain capable of extensive oxidation of tryptophan uses either the aromatic or the quinoline pathway (Stanier, Hayaishi & Tsuchida, 1951).

Early manometric studies established that tryptophan oxidation is an inducible process, formation of the specific enzymes being elicited by exposure of the cells to tryptophan (Suda *et al.* 1949; Stanier & Tsuchida, 1949). It was also observed that induction is grossly sequential: thus, anthranilic acid and kynurenic acid, the first intermediates on the aromatic and quinoline pathways, respectively, do not

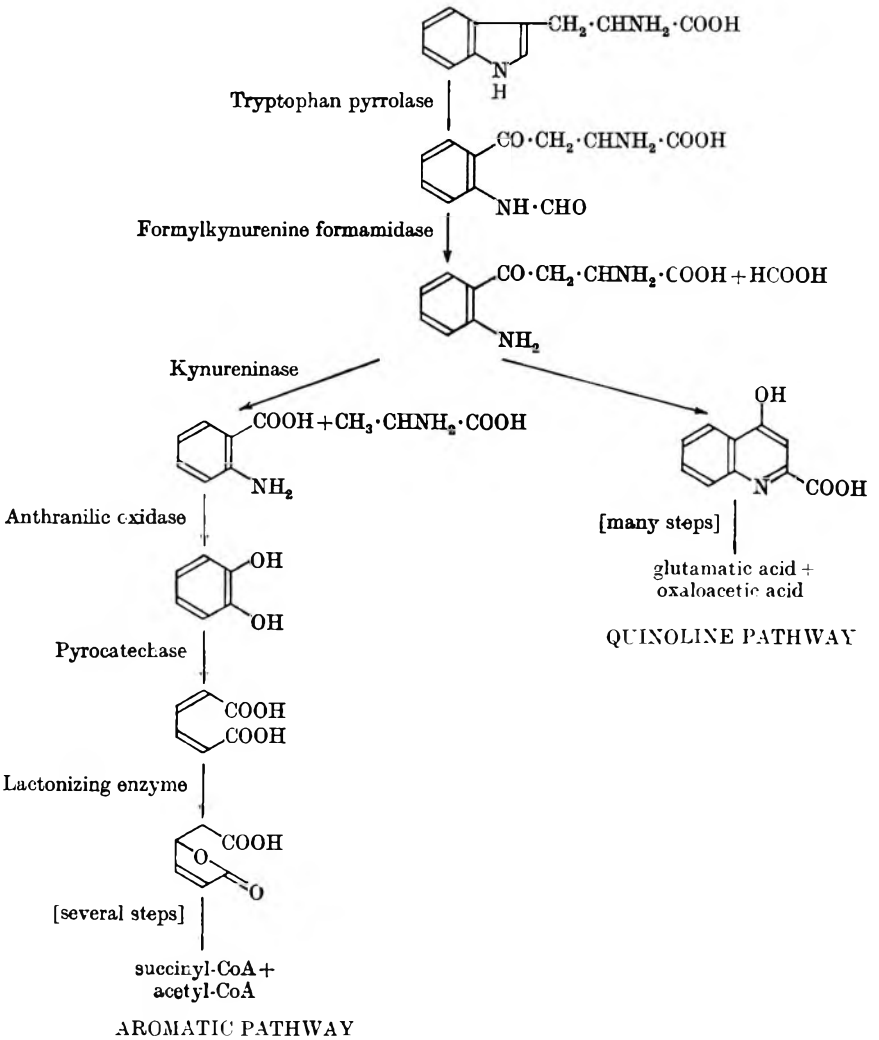

Fig. 1. A condensed metabolic map of the divergent pathways for the oxidative dissimilation of L-tryptophan by pseudomonads, showing the points of action of the enzymes discussed in this paper.

serve as inducers for the enzymes responsible for the initial attack on tryptophan, although they elicit synthesis of the whole series of enzymes operative in the subsequent steps of their respective pathways. The discovery of inductive sequentiality in these and other complex enzymatic sequences among bacteria led to the hypothesis, independently proposed by several workers (Stanier, 1947; Karlsson &

Barker, 1948; Suda *et al.* 1949), that induction proceeds in single sequential steps. According to this hypothesis, each substrate in such a metabolic sequence is at the same time a specific inducer of the enzyme which converts it to the next compound of the sequence; exposure of the cell to a primary inducer-substrate thus triggers an interlocked chain of specific inductions and specific enzymatic step-reactions. The evidence in support of this hypothesis with respect to the inducible systems for the oxidation of tryptophan was extremely fragmentary. Furthermore, one of the first studies of inductive patterns, in a Pseudomonas strain using the quinoline pathway, revealed a striking example of non-sequential induction: kynurenine proved as effective as tryptophan in inducing attack on tryptophan (Stanier & Tsuchida, 1949). During the past decade, the nature of the inductive and repressive control mechanisms that operate in other types of bacterial metabolic sequences has been extensively explored (Jacob & Monod, 1961). This has prompted us to re-investigate the mechanism of induction of the enzymes which initiate the dissimilation of L-tryptophan through the aromatic pathway; the results are reported here.

METHODS

Biological materials. The bacterium employed was *Pseudomonas fluorescens* Tr-23, the strain with which the biochemistry of the aromatic pathway was originally elucidated (Hayaishi & Stanier, 1951). Cultures were grown in defined media containing Na K phosphate buffer (0·02 M, pH 6·8), 1000 ml.; $(NH_4)_2SO_4$, 1·0 g.; Hutner's base (Cohen-Bazire, Sistrom & Stanier, 1957) 20 ml.; and an appropriate oxidizable substrate. Asparagine (0·3 %) was commonly used for the growth of 'uninduced' bacteria (i.e. bacteria not specifically induced for the enzymes of the aromatic pathway). Fully induced bacteria of the wild type were usually grown in the mineral asparagine medium supplemented with 0·1 % L-tryptophan, since preliminary experiments showed that asparagine did not repress synthesis of the enzymes of tryptophan oxidation. Tryptophan and tryptophan derivatives used in media as inducers or substrates were sterilized by filtration and added separately to the autoclaved basal media. Liquid cultures were grown on a mechanical shaker. The temperature of incubation was 30°.

Mutants of *Pseudomonas fluorescens* strain Tr-23 with specific enzymatic defects in the aromatic pathway were isolated from the parent strain after treatment with the chemical mutagen ethylmethanesulphonate (EMS). A suspension containing approximately 10^9 bacteria of the wild type in 0·033 M-phosphate buffer (pH 7·0) was mixed with an equal volume of freshly diluted EMS (approx. 0·25 M) in phosphate buffer and incubated on the shaker at 30° for 90 min. About 99·5 % of the bacteria were killed by this treatment. The mixture was then diluted in buffer, and samples containing 50–100 viable bacteria were spread on a series of plates. Two methods were used to detect tryptophan-negative mutants. In the first, primary plates were prepared on mineral asparagine agar. When colonies had developed, replicas were prepared on mineral tryptophan agar. After growth had taken place on the replica plates, tryptophan-negative mutants were detected by visual comparison of the two sets of plates. Subsequently, an even simpler method for detecting such mutants was devised. After treatment with EMS, the survivors were spread on plates of a medium containing 0·1 % L-tryptophan and 0·01 % asparagine.

The low concentration of asparagine was not in itself sufficient to allow the growth of colonies to full size, so that mutant clones unable to utilize tryptophan were immediately detected on such plates by the smallness of their colonies. All putative mutants were purified by restreaking on mineral asparagine agar, after which their inability to grow with tryptophan and their genetic stability were controlled by patching on mineral tryptophan agar plates.

Table 1. *Spectrophotometric assays for enzymes of the aromatic pathway as employed with crude bacterial extracts*

Enzyme	Reaction	Principle of assay method	Wavelength of measurement (mμ)	Reference
Pyrrolase	L-Tryptophan → L-formylkynurenine	Accumulation of kynurenine in presence of excess of formamidase and semicarbazide	365	Modified from Tanaka & Knox (1958)
Formamidase	L-Formylkynurenine → L-kynurenine + formic acid	Accumulation of kynurenine in presence of semicarbazide	365	Modified from Knox (1955)
Kynureninase	L-Kynurenine → anthranilic acid + L-alanine	Disappearance of kynurenine	365	Hayaishi & Stanier (1952)
Pyrocatechase	Catechol → *cis*, *cis*-muconic acid	Accumulation of *cis*,*cis*-muconic acid in presence of EDTA	260	G. Hegeman (unpublished observations)
Lactonizing enzyme	*Cis*,*cis*-muconic acid → muconolactone	Disappearance of *cis*,*cis*-muconic acid	260	Sistrom & Stanier (1954)

Mutants were initially screened to determine the site of the block in tryptophan metabolism as follows. Cultures were grown in liquid asparagine medium supplemented with 0·1 % L-tryptophan, the cultures were centrifuged, and the ultraviolet absorption spectrum of the supernatant medium was determined. All the early intermediates on the aromatic pathway have distinctive absorption peaks between 230 and 380 mμ, so that the nature of the intermediate accumulated by many mutants (and, by inference, the blocked enzymatic reaction) could be readily determined. In most cases, the nature of the enzymatic lesion was subsequently confirmed by the enzymatic analysis of cell-free extracts. The initial screening method did not differentiate between pyrrolase-less and formamidase-less mutants, neither of which could attack tryptophan appreciably under the conditions of culture used. These two phenotypes could, accordingly, be distinguished only by enzymatic analysis.

The experiments with mutants that are reported in this paper were performed with three phenotypes: pyrrolase-less (Py^-); formamidase-less (Fo^-); and kynureninase-less (Ky^-). The selection procedure also yielded many mutants blocked at later steps in the aromatic pathway, and specifically lacking either anthranilic acid oxidase, pyrocatechase, or lactonizing enzyme.

Enzymatic methods. Bacteria were harvested by centrifugation, washed once with 0·033 M-phosphate buffer (pH 6·8), and stored as frozen pellets until the time of extraction. The frozen bacteria were resuspended with about three times their volume of 0·02 M-phospate buffer (pH 7·0), and broken by treatment for 90–180 sec. in the MSE ultrasonic disintegrator. The resulting extracts were centrifuged at

20,000 *g* for 30 min., and the sediment was discarded. The supernatant extract, with a protein content between 12 and 20 mg./ml. as measured by the biuret method, was used for the performance of assays.

Five specific enzymes of the aromatic pathway were assayed in such crude extracts by spectrophotometric methods, as outlined in Table 1. The assay methods used for tryptophan pyrrolase and formylkynurenine formamidase depend on the conversion of these substrates to kynurenine, which has a major absorption peak at 365 mμ. The published procedures (Knox, 1955; Tanaka & Knox, 1958) are satisfactory only with preparations that are essentially free of kynureninase. This enzyme is normally very active in extracts prepared from tryptophan-induced organisms of *Pseudomonas fluorescens* Tr-23, but its activity can be virtually abolished by pre-incubation of the extract with semicarbazide. The effects of semicarbazide on pyrrolase and formamidase function could be directly evaluated using crude extracts from induced Ky$^-$ mutants, which contain high activities of these two enzymes, but lack kynureninase. Semicarbazide does not appreciably affect pyrrolase function. The rate of formamidase action is, however, rapidly decreased in the presence of semicarbazide, no doubt as a consequence of the combination of semicarbazide with the substrate. Nevertheless, the assay of formamidase can be performed in the presence of semicarbazide by measuring the rate of the reaction during the 1st min. after substrate addition. Accordingly, semicarbazide at a concentration of 5×10^{-3} M was routinely used in the assays of pyrrolase and formamidase, being added to the extract 5 min. before substrate addition. The published assay method for tryptophan pyrrolase (Tanaka & Knox, 1958) calls for supplementation of the assay mixture with formamidase, to ensure the immediate conversion of the reaction-product, formylkynurenine, to kynurenine. This usually proved unnecessary here with the bacterial extracts, since formamidase was present in excess of pyrrolase. Extracts of the Fo$^-$ phenotype were, however, completely devoid of formamidase. In this case, pyrrolase was assayed by measuring appearance of the formylkynurenine peak at 315 mμ, control experiments having shown that rates so measured were indistinguishable from rates measured in the presence of an excess of added formamidase.

Chemicals. L-α-Amino-γ-hydroxy-γ-(2-aminophenyl)-butyric acid, which will be referred to hereafter as γ-hydroxy-L-kynurenine, was prepared by treatment of 1·54 m-mole L-kynurenine sulphate in 25 ml. of 0·006 M-phosphate buffer (pH 7·0) with a fourfold molar excess of sodium borohydride at room temperature. The reduction was followed spectrophotometrically by the disappearance of the kynurenine peak at 365 mμ, and was complete in about 60 min. The reaction mixture was passed through Amberlite IR 120 in the H^+ form, and, after the column had been washed with water, the γ-hydroxy-L-kynurenine was eluted with 5 N-ammonia. The eluate was concentrated under reduced pressure, and the product crystallized from water before use.

Pure D-kynurenine was prepared from the racemic mixture through selective oxidation of the L-isomer by tryptophan-induced organisms of the wild type. The course of the reaction was followed spectrophotometrically, by measuring the decrement of absorbancy at 365 mμ, which proceeded to the theoretical value. The bacteria were removed by centrifugation, and the resultant solution of D-kynurenine was filter-sterilized and used directly for experiments. The completeness of the

resolution was confirmed by treating a sample with a large excess of bacterial kynureninase, which is specific for the L-isomer and is not inhibited by the D-isomer. Such treatment produced no change in the absorbancy at 365 mμ.

Commercial D-tryptophan, found to be contaminated by traces of the L-isomer, was completely resolved by treatment in similar fashion with tryptophan-induced organisms of the wild type, and was subsequently recrystallized several times from water.

Commercial L-formylkynurenine, found to be contaminated with L-kynurenine, was repurified by treatment with a suspension of organisms of the Fo⁻ mutant pre-induced with kynurenine, and therefore able to oxidize L-kynurenine but not L-formylkynurenine. The course of the oxidation was followed by measurement of absorbancies at 320 mμ (formylkynurenine peak) and 365 mμ (kynurenine peak).

RESULTS

Inductive patterns of the wild type

As a preliminary to enzyme studies, the rates of oxygen uptake at the expense of L-tryptophan, L-kynurenine and anthranilic acid by resting organisms of the wild-type *Pseudomonas fluorescens* Tr-23 after exposure to various inducers were determined. Exposure to either L-tryptophan or L-kynurenine established the same respiratory pattern; both these compounds, as well as anthranilic acid, were immediately oxidized at a high and steady rate. Organisms exposed to anthranilic acid immediately and rapidly oxidized this substrate, but could not initially oxidize either tryptophan or kynurenine. 'Uninduced' bacteria, grown on asparagine alone, did not immediately respire with tryptophan, kynurenine or anthranilic acid.

The specific activities of five enzymes operative in the pathway of tryptophan oxidation were then determined on extracts prepared from uninduced bacteria (grown with asparagine alone), and from bacteria grown in the same medium supplemented singly with L-tryptophan, L-kynurenine, anthranilic acid, and D-tryptophan (Table 2). All five enzymes are present at relatively high specific activities (ranging from 20 to 150 mμmoles of substrate utilized/min./mg. protein) in extracts of bacteria grown with either L-tryptophan or L-kynurenine. The enzymatic patterns induced by these two compounds were indistinguishable, both quantitatively and qualitatively. It should be specifically noted that the first two enzymes of the sequence, pyrrolase and formamidase, were fully induced by L-kynurenine, the product of their action on L-tryptophan.

Extracts from uninduced (asparagine-grown) bacteria did not contain measurable activities of kynureninase, pyrocatechase, or lactonizing enzyme. The values given in Table 2 accordingly represent the maximal possible activities, calculated from the sensitivity of the specific assay methods; they range from less than 0·01 to 0·2 % of the activities present in tryptophan-induced bacteria. The first two enzymes in the pathway (pyrrolase, formamidase) were, however, readily detectable in extracts of asparagine-grown bacteria. In nine separate analyses of extracts prepared from asparagine-grown bacteria, the specific activity of pyrrolase ranged from 0·26 to 0·7 and, of formamidase, from 2·1 to 4·2.

Extracts of bacteria induced with anthranilic acid did not contain detectable kynureninase activity, and the activities of pyrrolase and formamidase were within

the range characteristic for asparagine-grown bacteria. Both pyrocatechase and lactonizing enzyme were present at concentrations comparable with those in tryptophan-induced bacteria. Also presented in Table 2 are data obtained for an extract from bacteria grown in the presence of D-tryptophan, which is not attacked by *Pseudomonas fluorescens* strain Tr-23. The enzymatic constitution of such bacteria is indistinguishable from that of asparagine-grown bacteria.

Table 2. *Induction of enzymes of the aromatic pathway in Pseudomonas fluorescens strain Tr-23 by tryptophan and some of its derivatives*

P. fluorescens Tr-23 was grown for 12 hr in liquid mineral asparagine medium, either unsupplemented or specifically supplemented with the indicated compounds, at an initial concentration of 0·05 M. The activities were determined on cell-free extracts, and are expressed as mμmoles substrate decomposed/min./mg. protein.

	Additions to basal medium				
	None	L-tryptophan	D-tryptophan	L-kynurenine	Anthranilic acid
Enzyme	mμmoles substrate decomposed/min./mg. protein				
Pyrrolase	0·4	21	0·4	20	0·3
Formamidase	3	51	4	53	3
Kynureninase	⩽0·1*	74	⩽0·1*	60	⩽0·1*
Pyrocatechase	⩽0·05*	71	⩽0·05*	70	52
Lactonizing enzyme	⩽0·01*	144	⩽0·01*	130	118

* Activity not detectable. The value given represents the possible maximum, in view of the estimated threshold sensitivity of the assay method employed.

The results of the enzymatic analyses are in good general accord with the gross inductive patterns revealed by the respiratory activities of intact bacteria. The one unexpected finding was the presence of low concentrations of pyrrolase and formamidase in asparagine-grown bacteria. The action of pyrrolase, but not of formamidase, results in oxygen consumption. It can be calculated that the rate of oxygen uptake at the expense of tryptophan caused by the amount of pyrrolase in uninduced bacteria is negligible relative to the endogenous rate of respiration. Hence manometric analysis is too coarse a tool to detect the presence of this enzyme in such bacteria.

Either the primary substrate, L-tryptophan, or the second intermediate in the pathway, L-kynurenine, can induce all the enzymes of the aromatic pathway. However, since uninduced bacteria already possess the first two enzymes of the pathway at low concentrations, the provision of an exogenous supply of L-tryptophan immediately permits an endogenous synthesis of L-kynurenine. Hence these experiments do not permit the conclusion that L-tryptophan has an intrinsic inductive function, distinct from the indirect inductive function that it undoubtedly possesses by virtue of serving as a metabolic precursor of L-kynurenine. The non-metabolizable D-isomer of tryptophan is completely devoid of inductive function. Anthranilic acid cannot induce synthesis of any of the first three enzymes of the pathway, all of which are induced by its immediate metabolic precursor, L-kynurenine.

The gross kinetics of induction in the wild type

A culture of the wild-type *Pseudomonas fluorescens* Tr-23 in the course of exponential growth at the expense of asparagine was transferred to a flask of the same medium, supplemented with 0·05 M-L-tryptophan. Samples of the growing culture were removed after 30, 90, and 180 min. The bacteria were harvested and extracted, and the activities of pyrrolase, formamidase, kynureninase, pyrocatechase, and lactonizing enzyme determined in the extracts (Fig. 2). With respect to their initial rates of synthesis, the five enzymes clearly fell into two distinct groups. The first three enzymes were rapidly synthesized; their specific activities increased markedly 30 min. after exposing the bacteria to tryptophan. The last two enzymes, which operate below the level of anthranilic acid in the pathway, were synthesized only

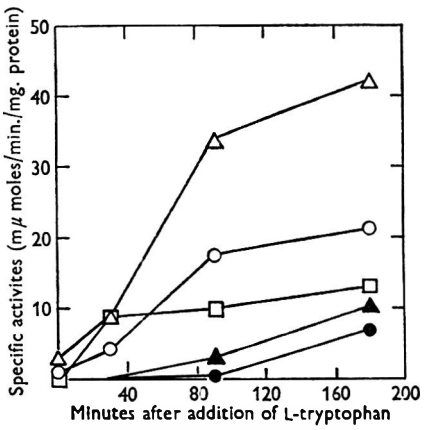

Fig. 2

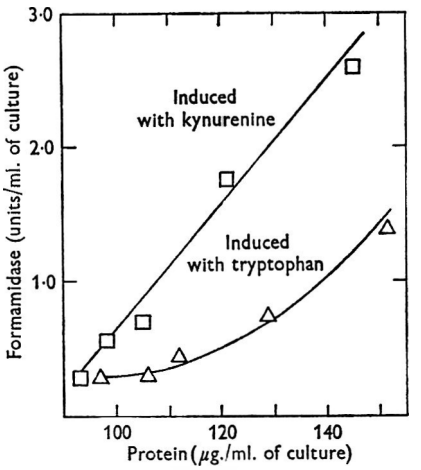

Fig. 3

Fig. 2. The gross kinetics of synthesis of five enzymes of the aromatic pathway, following induction of cells by L-tryptophan during growth in a mineral asparagine medium. ○——○, Pyrrolase; △——△, formamidase; □——□, kynureninase; ●——●, pyrocatechase; ▲——▲, lactonizing enzyme.

Fig. 3. Differential rates of synthesis of formamidase by the wild type of Pseudomonas fluorescens, following induction with L-kynurenine and with L-tryptophan.

after a considerable lag, being undetectable after 30 min. of exposure to tryptophan, and present at very low values (relative to the first three enzymes) even after 90 min. A parallel experiment, in which L-kynurenine was used as inducer in place of L-tryptophan, gave comparable results.

The general interpretation of these experiments seems evident. Effective inducer concentrations for the synthesis of pyrrolase, formamidase, and kynureninase were established in the bacteria very shortly after exposure to tryptophan or kynurenine; but effective inducer concentrations for the synthesis of pyrocatechase and lactonizing enzyme were produced only as a result of metabolic transformations of kynurenine which required a considerable time. This suggests that one or more sequential inductive steps intervened between the synthesis of kynureninase and the syntheses of pyrocatechase and lactonizing enzyme.

The severity of the transient imbalance between the enzymes of the aromatic pathway that follows the onset of induction implies that, if the enzymatic constitution of the bacteria could be stabilized after brief induction, such organisms might metabolize tryptophan or kynurenine with a massive accumulation of the first sequential inducer-substrate in the pathway after kynurenine. To test this hypothesis, bacteria which had been permitted to grow for 60 min. in the presence of 0·05 M-L-tryptophan were subjected to ultraviolet irradiation sufficient to block further protein synthesis. The irradiated bacteria were washed and resuspended in buffer, and their ability to metabolize tryptophan was determined. They oxidized tryptophan with a very low total oxygen uptake, accompanied by a virtually quantitative conversion of tryptophan to anthranilic acid, as determined by spectrophotometric examination of the supernatant medium. The dose of irradiation used did not decrease the rate or extent of tryptophan oxidation by fully induced organisms. An unirradiated suspension of the partly induced organisms oxidized tryptophan to completion under the conditions of a manometric experiment, presumably because synthesis of the later enzymes in the pathway occurred during the course of this oxidation. This experiment indicates that anthranilic acid oxidase is sequentially induced by its substrate, anthranilic acid. Additional sequential inductive steps may intervene before the synthesis of pyrocatechase and lactonizing enzyme, but we have not further examined this aspect of the problem.

Inductive responses of mutants with early blocks in the aromatic pathway

Five mutants with early and absolute blocks in the aromatic pathway were isolated. Two were pyrrolase-less (Py^-), one formamidase-less (Fo^-), and two kynureninase-less (Ky^-). The specific activities of the first three enzymes of the pathway in extracts of uninduced (asparagine-grown) and specifically induced organisms of these five mutants are shown in Table 3. The specifically induced cells were prepared by adding either L-tryptophan or L-kynurenine to 0·005 M in a culture growing at the expense of asparagine, and harvesting the organisms 3 hr later.

With respect to pyrrolase and formamidase synthesis, the two Ky^- mutants showed the same qualitative responses as did the wild type to induction by tryptophan and kynurenine: both these compounds served as inducers, and possessed more or less equal effectiveness. The Py^- mutants and the Fo^- mutant showed entirely different qualitative responses; in these mutants, tryptophan did not induce measurable synthesis of any of the first three enzymes of the pathway, whereas kynurenine was an effective inducer for all of them. Py^- and Fo^- mutants share a common metabolic property which differentiates them from both wild-type and Ky^- organisms; namely, inability to generate kynurenine from tryptophan within the organism, as a result of the genetic elimination of one of the two enzymes which catalyse this conversion.

Since the Py^- mutants are unable to synthesize the first enzyme of the pathway, they cannot initiate oxidative attack on L-tryptophan. The failure of L-tryptophan to act as an inducer for these mutants therefore shows that tryptophan itself does not possess inductive function. In the Fo^- mutant, L-tryptophan can presumably undergo a slow endogenous conversion to L-formylkynurenine, as a result of the retention of basal pyrrolase activity in this phenotype. The absence of induction by tryptophan therefore provides indirect evidence that L-formylkynurenine is not

an effective inducer for either pyrrolase or kynureninase. Taken in conjunction, the data obtained with the Py⁻ and Fo⁻ phenotypes accordingly demonstrate that L-kynurenine is the only early member of the metabolic pathway which possesses inductive function, and that it can serve simultaneously as the inducer of pyrrolase, formamidase, and kynureninase.

Table 3. *Levels of early enzymes of the aromatic pathway in Py⁻, Fo⁻, and Ky⁻ mutants of Pseudomonas fluorescens*

Organisms were grown for 3 hr in liquid mineral asparagine medium, unsupplemented and supplemented with L-tryptophan or L-kynurenine at an initial concentration of 0·005 M. The activities, determined on cell-free extracts, are expressed as mμmoles of substrate decomposed/min./mg. protein.

Strain	Addition to basal medium	Enzyme assayed*		
		Pyrrolase	Formamidase	Kynureninase
Py⁻34	None	—	4	⩽0·1
	Tryptophan	—	4	⩽0·1
	Kynurenine	—	375	47
Py⁻54	None	—	3	⩽0·1
	Tryptophan	—	4	⩽0·1
	Kynurenine	—	173	30
Fo⁻16	None	1·0	—	⩽0·1
	Tryptophan	0·5	—	⩽0·1
	Kynurenine	9	—	49
Ky⁻12	None	0·3	3	—
	Tryptophan	19	49	—
	Kynurenine	18	54	—
Ky⁻21	None	0·7	6	—
	Tryptophan	39	131	—
	Kynurenine	37	160	—
Wild type	None	0·4	3	⩽0·1
	Tryptophan	21	51	74
	Kynurenine	20	53	60

* The symbol — has been used in the column that refers to the genetically deleted enzyme of each mutant. In no case was activity of the genetically deleted enzyme detectable, either with or without induction.

It is noteworthy that, in the Py⁻ mutants, genetic elimination of the ability to synthesize pyrrolase did not prevent the synthesis of formamidase by asparagine-grown organisms: the basal amount of this enzyme in the Py⁻ mutants was in the normal wild-type range. This fact makes improbable the simplest explanation for the existence of basal pyrrolase and formamidase activities; namely, endogenous synthesis of the inducer (kynurenine) at a very low rate. So far as is known, kynurenine is biosynthesized uniquely from tryptophan through the action of pyrrolase and formamidase; consequently, the genetic elimination of either enzyme should completely prevent the intracellular formation of kynurenine. The relatively high basal values of pyrrolase and formamidase found here are thus best interpreted as the result of an imperfect mechanism of repression, rather than of a continuous endogenous production of inducer.

The inductive responses of the mutants showed some curious quantitative anomalies. One is the induced hyperproduction of formamidase characteristic of mutant Py⁻ 34 and, in lesser measure, of mutant Py⁻ 54. Both mutants showed specific

formamidase activities following induction with kynurenine which are far above the range for the wild type, whereas the basal values of this enzyme in asparagine-grown mutant organisms was normal. In mutant Ky⁻ 21, the basal and the induced values of pyrrolase and formamidase were approximately double those characteristic of the wild type.

Kinetic evidence for the role of kynurenine as primary inducer

If the interpretation offered above for the inductive responses of Py⁻ and Fo⁻ mutants is correct, it follows that in wild-type organisms exposure to kynurenine should result in an immediate triggering of induction, whereas exposure to tryptophan should lead to a slight delay in the initiation of induction, corresponding to the time required for the endogenous generation of an effective inducing concentration of kynurenine. This led us to make a more refined analysis of the early kinetics of induction in the wild type by tryptophan and kynurenine. The results of several such experiments showed that the early rate of synthesis of kynureninase and formamidase by the wild type was always markedly lower in response to induction by tryptophan than in response to induction by kynurenine. There was, in fact, an absolute lag of variable duration (10–20 min.) before induction by tryptophan became detectable, whereas induction by kynurenine was always detectable within 5–10 min. The critical measurements must be made on extracts of low enzyme content, where the errors of assay are maximal, so that the values for specific activities are relatively unreliable. However, experiments of this type always gave the same general result. Typical data are shown in Fig. 3.

The inductive affinity of L-kynurenine and of non-metabolizable analogues

Since L-kynurenine was not metabolized by Ky⁻ mutants, the rate of synthesis of pyrrolase and formamidase as a function of inducer concentration could be measured with this phenotype. Organisms were induced by growth for 6 hr in mineral asparagine containing a series of concentrations of L-kynurenine. The results (Fig. 4) show that maximal amounts of both pyrrolase and formamidase were induced by 3×10^{-5} M-kynurenine, and detectable induction occurred even with 3×10^{-6} M-kynurenine. This extraordinarily high affinity no doubt explains why induction of the wild type following exposure to L-tryptophan is initiated with so brief a lag.

The discovery that L-kynurenine, but not L-tryptophan, acted as the inducer for the synthesis of pyrrolase, formamidase, and kynureninase, led us to examine analogues of L-kynurenine as possible non-metabolizable inducers. Two chemically related compounds which cannot be detectably metabolized by the wild type are the D-isomer and the product of borohydride reduction, γ-DL-hydroxy-L-kynurenine. These compounds were accordingly tested for their inductive effect on the wild type. As shown in Fig. 5, synthesis of pyrrolase, formamidase, and kynureninase was induced by γ-hydroxy-L-kynurenine, although its affinity was far lower than that of L-kynurenine: a concentration of 3×10^{-3} M was required to induce maximal amounts of pyrrolase and formamidase. D-Kynurenine likewise induced the synthesis of all three enzymes, the saturating inducer concentration being approximately 10^{-3} M (Table 4).

Neither D-kynurenine nor γ-hydroxy-L-kynurenine induced measurable forma-

tion of pyrocatechase and lactonizing enzyme by wild-type organisms. This finding is in accordance with the evidence that at least one sequential inductive step occurs below the level of kynurenine. The absence of these two enzymes shows that the inductive effects of D-kynurenine and γ-hydroxy-L-kynurenine are not attributable to contamination by L-kynurenine.

These experiments reveal, with much greater clarity than any other type of analysis, the strict co-ordination between the differential rates of synthesis of pyrrolase and formamidase: at all inducer concentrations, the ratio between the specific activities of the two enzymes remains constant, within the limits of error of the

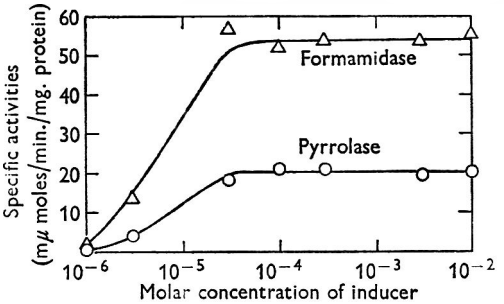

Fig. 4

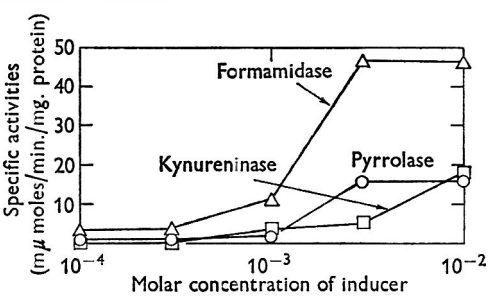

Fig. 5

Fig. 4. The specific activities of pyrrolase and formamidase induced in a kynureninase-less mutant (Ky⁻12) of Pseudomonas fluorescens by growth in the presence of different concentrations of L-kynurenine.

Fig. 5. The specific activities of pyrrolase, formamidase, and kynureninase induced in the wild type of Pseudomonas fluorescens by growth in the presence of different concentrations of γ-hydroxy-L-kynurenine, a non-metabolizable inducer.

Table 4. *Induction of Pseudomonas fluorescens wild type by D-kynurenine*

Organisms were harvested after growth for 6 hr in an asparagine mineral medium, supplemented with the indicated concentrations of D-kynurenine.

	Enzyme		
	Pyrrolase	Formamidase	Kynureninase
Concentration of D-kynurenine (M)	Specific activities of extracts mμmoles substrate utilized/min./mg. protein		
None	0·4	3	⩽0·1
10^{-5}	0·3	4	⩽0·1
10^{-4}	2	17	2
5×10^{-4}	24	72	34
10^{-3}	30	89	52

assays. As shown by the data on induction of the wild type with γ-hydroxy-L-kynurenine and D-kynurenine, this co-ordination does not extend to include kynureninase. The inductive affinity of the kynureninase-forming system for both non-metabolizable inducers was far lower than that of the system responsible for the formation of pyrrolase and formamidase. This fact implies that kynurenine and its analogues play two distinct roles in induction, acting separately to induce the syntheses of pyrrolase and formamidase on the one hand, and of kynureninase on the other.

Patterns of induction in other strains

The peculiar mechanism of induction of pyrrolase, formamidase, and kynureninase demonstrated for *Pseudomonas fluorescens* strain Tr-23 might be a strain-specific property, not shared by other pseudomonads which oxidize tryptophan through the aromatic pathway. To obtain information on this point, the inductive responses of two other Pseudomonas strains, RYS-2 and RYS-3, were examined. Like strain Tr-23, these strains were originally isolated from soil by enrichment with tryptophan as carbon source; but the three strains were obtained on different occasions from different samples of soil, so that a close genetic relationship between them is improbable. Strain RYS-3 is a fluorescent pseudomonad; RYS-2 does not produce pigment. The patterns of synthesis of pyrrolase, formamidase, and kynureninase by organisms grown at the expense of asparagine, L-tryptophan and L-kynurenine as sole carbon sources are shown in Table 5.

Table 5. *Patterns of synthesis of early enzymes on the aromatic pathway by Pseudomonas strains* RYS-2 *and* RYS-3

Strain	Substrate for growth	Enzyme: Pyrrolase	Formamidase	Kynureninase
		Specific activities, mμmoles of substrate decomposed/min./mg. protein		
RYS-2	Asparagine	0·1	0·23	⩽0·1
	L-Tryptophan	29	46	84
	L-Kynurenine	21	36	102
RYS-3	Asparagine	⩽0·05	0·15	⩽0·1
	L-Tryptophan	10	102	77
	L-Kynurenine	9	58	57

The patterns of enzyme synthesis by strain RYS-2 were qualitatively indistinguishable from those of strain Tr-23: L-tryptophan and L-kynurenine induced all three enzymes to approximately equal degrees; asparagine-grown pseudomonads contained measurable amounts of pyrrolase and formamidase, but not of kynureninase. The basal specific activities found in strain RYS-2 were significantly lower than those found in strain Tr-23, whereas the specific activities of induced organisms were approximately the same. For strain RYS-3, kynurenine also served as an inducer of all three enzymes, although the amounts were slightly lower than those induced by tryptophan. In strain RYS-3, no pyrrolase activity was detected in asparagine-grown organisms, although there was a very low amount of formamidase. If pyrrolase were really absent, the inductive mechanism which operated in strain Tr-23 obviously did not operate in strain RYS-3. However, the basal pyrrolase amount may have been too low to be detected by present assay methods. It is perhaps significant that strain RYS-3 has a very long growth lag (about 10 hr) on transfer from a medium containing asparagine to one containing tryptophan as sole carbon source; under the same conditions, strain Tr-23 has a growth lag of only 2 hr.

DISCUSSION

The mechanisms which regulate synthesis of the inducible enzymes that catalyse the early steps in the bacterial oxidation of tryptophan have proved to be unexpectedly varied and complex. The original hypothesis (Suda *et al.* 1949; Stanier & Tsuchida, 1949) of strict sequential induction was evidently an oversimplified one; sequential inductions do occur, but by no means all the enzymes of the pathway are sequentially induced. Pyrrolase, formamidase, and kynureninase, the first three enzymes of the pathway, are all synthesized in response to a single inducer, L-kynurenine. At first sight, this might suggest that the synthesis of these three enzymes is under the control of a single operon, which can be de-repressed by kynurenine. The syntheses of pyrrolase and formamidase show close co-ordination under all conditions of induction, so that the assumption of control through a single operon may be correct for these two enzymes. Co-ordination does not extend to kynureninase, however, as shown particularly clearly by the lesser affinity of its induction by the non-metabolizable kynurenine analogue, γ-hydroxy-L-kynurenine. Accordingly, it appears probable that kynurenine has two different sites of action as an inducer.

L-Tryptophan, the primary substrate in this metabolic pathway, is not able to induce the synthesis of any of the early enzymes, as shown by experiments with mutants that lack either pyrrolase or formamidase. Nevertheless, exposure of uninduced organisms of the wild type to L-tryptophan causes a rapid initiation of enzyme synthesis. This inductive effect is an indirect one, made possible because the uninduced organisms have relatively high basal amounts of pyrrolase and formamidase. Exposure to an exogenous supply of tryptophan thus permits an immediate endogenous synthesis, although at a low rate, of the actual primary inducer, L-kynurenine. Since kynureninase, the enzyme responsible for conversion of kynurenine to anthranilic acid and alanine, is undetectable in uninduced organisms, the kynurenine so formed can accumulate. Its affinity for induction of pyrrolase and formamidase is exceptionally high, and an accelerated rate of synthesis of these two enzymes consequently begins 10–15 min. after exposure of organisms to tryptophan. The non-co-ordinate sequential induction of kynureninase is also rapidly initiated, with the result that, 30–60 min. after exposure to the primary substrate, the cells possess the enzymatic equipment to convert tryptophan to anthranilic acid at a high rate. Anthranilic acid oxidase, the next enzyme of the sequence, appears to be sequentially induced by its substrate. Unfortunately, there is no easy and sensitive assay for this enzyme, so that we have been unable to study its specific synthesis. However, cells that have acquired an effective complement of the first three enzymes of the pathway as a result of brief exposure to tryptophan cannot oxidize anthranilic acid at an appreciable rate, which indicates that there is a substantial time-lag, following exposure to the primary substrate, before the sequential induction of anthranilic acid oxidase is initiated. The existence of a very marked time-lag in synthesis has been shown by direct enzymatic analysis for the next two enzymes of the pathway, pyrocatechase and lactonizing enzyme.

Recent studies (Stanier, Hegeman & Ornston, in the press) have clarified the mechanism of induction of the enzymes responsible for the oxidation of mandelic acid by *Pseudomonas fluorescens*. This biochemical pathway converges at the level

of catechol with the aromatic pathway for tryptophan oxidation. None of the enzymes of the mandelate sequence can be detected in uninduced cells. The initial induction is co-ordinate, and results in the synthesis of the first five enzymes of the pathway, responsible for the conversion of D-mandelic acid to benzoic acid. This multiple induction is elicited by D-mandelic acid, the primary substrate, as shown by studies with the appropriately blocked mutant. Hence the initial inductive events in the metabolically convergent mandelate and tryptophan pathways are mechanistically different, even though their gross physiological consequences are analogous.

Induction by the primary substrate has such obvious adaptive value in the regulation of a dissimilatory pathway that it is tempting to look for a special explanation of the 'metabolite induction' which controls synthesis of the first enzymes in the pathway for the dissimilation of tryptophan. Unlike many of the oxidizable substrates used by *Pseudomonas fluorescens*, L-tryptophan also has a general role in the metabolism of the cell. It is synthesized endogenously to serve as a precursor for protein synthesis. In the absence of an external supply of tryptophan, the organism must accordingly prevent loss of its limited internal biosynthetic supply of this amino acid as a result of oxidation through the dissimilatory pathway. Since the later enzymes of the dissimilatory pathway cannot be detected in asparagine-grown organisms, it is evident that the control mechanisms do in fact operate to prevent such loss. The first two enzymes of the pathway are present in low amounts in uninduced organisms, which might suggest that some of the endogenous tryptophan supply is diverted to the formation of kynurenine, the specific inducer of these enzymes. This interpretation is excluded, however, by the finding that the basal amount of formamidase remains unchanged in mutants which have lost the ability to form pyrrolase, and are thus incapable of converting tryptophan to kynurenine. The basal amounts of pyrrolase and formamidase are thus best interpreted as resulting from a slightly defective repressor, rather than from endogenous production of inducer. Although there are no data on the size of the tryptophan pool in *P. fluorescens*, analyses of the amino acid pools in other micro-organisms indicate that the tryptophan is one of the least abundant constituents, frequently being undetectable chromatographically. The primary physiological factors which channel endogenously synthesized tryptophan to protein synthesis in *P. fluorescens* are therefore probably: (1) maintenance of a low pool concentration; (2) extremely high affinity of the specific activating enzyme. Given these two conditions, no appreciable flow to kynurenine should occur, even though the organism contains pyrrolase and formamidase. However, if the concentration of the endogenous tryptophan pool occasionally rises transiently to a value at which pyrrolase can compete with the activating enzyme for their common substrate, the brief lag in inductive response that is introduced by metabolite induction would serve as a damper on synthesis of the enzymes of the aromatic pathway. Only a high and steadily maintained pool concentration, resulting from the presence of a high exogenous concentration of tryptophan, would trigger effectively their induction.

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Persistence of *Staphylococcus aureus* in Penicillin *in vitro*

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SUMMARY

After exposure of *Staphylococcus aureus* strain Oxford (H) to penicillin *in vitro* under conditions leading to the death of at least 99·9% of the exposed population, the offspring of surviving cocci ('persisters') showed the same sensitivity as the original population. Successive exposure of the offspring of persisters did not increase the proportion of survivors nor their resistance. Persisters were not the most resistant cocci in the original population; their offspring showed the same distribution of cocci resistance as the parent culture. The number of survivors, about 1 in 2500 cocci, was proportional to the original number when exposed to penicillin in a small volume (10–20 ml.), but not in a larger volume (40–200 ml.). Inocula of less than 10 staphylococci allowed to multiply to 10^3/ml. before addition of penicillin yielded persisters. Populations greater than about 2×10^8/ml. were not acted upon by penicillin, even when the majority of the cocci were only 4 hr old, unless the mixtures were shaken. The proportion of survivors at 37° from equal volumes of penicillin broth containing 10^5–10^8 organisms/ml. was not affected by the age and growth phase of the exposed bacteria, conditions of previous storage of the inoculum, initial exposure at low temperature, type of container, aeration by shaking, clumping of cocci, a 1000-fold increase in the dose of penicillin, or addition of streptomycin. When removed from penicillin, persisters and their offspring multiplied at the same rate as the parent culture without requiring a recovery period. The persisters were neither spheroplasts nor L forms. Indirect evidence indicates that persisters survived because at the time of first contact with the penicillin they were in a state unfavourable to initiation of division or cell wall synthesis.

INTRODUCTION

In populations of staphylococci exposed to penicillin a few cocci usually survive after 99% or more have been killed (Hobby, Meyer & Chaffee, 1942; Rammelkamp & Keefer, 1943; Rantz & Kirby, 1944; Chain & Duthie, 1945). Bigger (1944*a*, *b*) found that the offspring of such surviving cocci were as sensitive to penicillin as those of the parent culture and designated these penicillin-sensitive survivors as 'persisters'. Persisting cocci behave as if they are genotypically drug-sensitive but phenotypically drug-resistant (Davis, 1954).

In infection, persisters may be responsible in part for occasional failure of chemotherapeutic agents to eradicate bacteria in spite of apparent *in vitro* sensitivity and initial satisfactory response of the patient. In such situations, the illness may recur and bacteria isolated at the time of recurrence may appear fully susceptible to the drug used. This ability of drug-susceptible micro-organisms to survive the action of

a drug *in vivo* was designated as 'microbial persistence' by McDermott (1958). Survival often involved a state of 'drug-indifference' wherein all or many of the organisms in a given population were neither permanently damaged by a drug nor able to multiply in its presence. The 'persisters' of Bigger, unlike the 'drug-indifferent' micro-organisms of McDermott, survive under conditions leading to the death of most of the exposed population.

In the present paper, 'persisters' are defined as those members of a drug-sensitive population of bacteria in pure culture which survive the action of a drug under conditions resulting in the death of at least 99·9% of the exposed organisms and which produce offspring that are as sensitive as the original culture. Modifications of the *in vitro* methods of Bigger were selected rather than those involving complex interactions of micro-organisms and infected animals. In a preliminary report, Bigger's findings were partly confirmed (Gunnison & Jawetz, 1958). It was hoped that a re-investigation of Bigger's observations by more precise quantitative methods interpreted in the light of current knowledge of penicillin action might lead to better understanding of the nature of persisters.

METHODS

Bacteria. Staphylococcus aureus, strain Oxford (H), ♯3 R 9647, obtained from the Merck Institute for Therapeutic Research, where it had been brought in 1942 by Dr N. G. Heatley, was used. This strain was chosen because it had been tested by Bigger, it does not produce penicillinase, and its sensitivity to penicillin has remained stable (Cowan, 1945; Oeding & Ostervold, 1959). The stock culture was pretested for sensitivity to penicillin, grown in broth, and stored at −10°. The term 'parent culture' refers to subcultures of this strain which have not been exposed to penicillin.

Penicillin. Crystalline potassium penicillin G was dissolved in sterile distilled water to 1000 units/ml. and stored at −10° for 1–2 weeks. Dilutions in broth were prepared just before use. Unless otherwise stated, the test dose was 0·25K unit penicillin G/ml. (0·15 μg./ml.), about 2·5 times the minimal bactericidal dose. The amount of penicillin carried over in subcultures of 0·1 ml. samples was not bacteriostatic for the test culture.

Media. Tests were made in broth composed of proteose peptone No. 3 (Difco), 20 g.; glucose, 0·5 g.; sodium chloride, 5 g.; disodium phosphate ($Na_2HPO_4.12H_2O$), 5 g.; water, 1000 ml.; pH 7·2. Proteose No. 3 agar (Difco) was used for plate counts.

Procedure. For 'standard' tests, 9 ml. portions of broth containing 0·25 unit penicillin/ml. in 18 × 150 mm. test tubes with loose-fitting metal caps (Morton) were pre-warmed to 37° and inoculated with 1 ml. of an 18-hr broth culture (about 3×10^8 cocci/ml.) of *Staphylococcus aureus* (H) or with serial tenfold dilutions thereof. After thorough mixing, the tubes were incubated at 37° without further shaking except when samples were removed. Samples (0·5 ml.) were withdrawn at intervals and tenfold dilutions were prepared in sterile distilled water. Plate counts were made by spreading 0·1 ml. of sample or dilution with bent glass rods on the surfaces of agar plates, with or without different amounts of penicillin.

Such counts determine the number of clumps or colony-forming 'units' of

staphylococci rather than of individual cocci, and the number of viable cocci per colony-forming unit might increase or decrease without altering the plate count. Moreover, cocci which survived the action of penicillin might fail to produce colonies (Eagle, 1954). The 'number of cocci/ml.' refers to the number of colony-forming units; '99% of the population were killed' means that 99% of the original units no longer formed colonies. Differences of less than 1 log raise were disregarded because of the inherent errors of the method.

In other tests, 18–180 ml. portions of penicillin broth in flasks or bottles with various kinds of closures were pre-warmed to 37° and inoculated with 2–20 ml. of diluted or undiluted culture. Whenever the effects of variations in the conditions of exposure were tested, 10 or 20 replicates of each model system were compared. Penicillinase concentrate (Difco) was added to media when prompt inactivation of penicillin was desired.

Surviving bacteria or their offspring were tested for sensitivity in comparison with the parent culture by inoculating into series of tubes of broth containing penicillin in twofold increments, by direct plating on agar containing graded concentrations of penicillin in increments of 0·005 unit/ml., or by replica plating of colonies from penicillin-free agar on to penicillin agar. Since survivors might include both resistant organisms and 'persisters', the latter term is used only when the surviving organisms have been shown to be as sensitive as the parent culture.

Assays of residual penicillin were made after incubation for 18–24 hr. Staphylococci were removed by filtration through Seitz filters and the filtrate was assayed by the tube dilution method, with either the parent culture or a highly sensitive strain of *Streptococcus pyogenes* as the test organism. Uninoculated controls otherwise treated in identical fashion were tested simultaneously.

RESULTS

In tests by the 'standard' procedure described above, Bigger's finding (1944*a*, *b*) that a small proportion of the exposed population of *Staphylococcus aureus* (H) survived in the presence of penicillin was confirmed. After 4 hr the viable count had dropped to about 10% of the original number, after 8 hr to about 1%, and by 18 hr to above 0·1% or less (Fig. 1). Survivors remained viable for 2–30 days in the presence of penicillin with wide variation in their survival time beyond the first 24 hr. The colonies were of the same size and appearance as those of the parent culture. In spite of difficulties inherent in the technique, repeated tests under the same conditions gave fairly uniform results. In a series of 123 tests a mean number of $3{\cdot}4 \times 10^7 \pm 0{\cdot}3 \times 10^7$/ml. exposed for 18 hr yielded a mean of $0{\cdot}039 \pm 0{\cdot}02$% survivors at 95% confidence limits.

These survivors were regarded as 'persisters' because their offspring consistently showed about the same degree of sensitivity as the original population when tested in broth. For both the parent culture and the offspring of persisters the minimal concentration of penicillin which prevented growth in broth for 24 hr of an inoculum of 10^7 cocci/ml. was 0·04 unit/ml.; the minimal concentration killing 99·9% within 24 hr was 0·1 unit/ml.

Subcultures of persisters when again treated with penicillin 0·25 unit/ml. yielded approximately the same number of survivors as before. In four successive exposures

there were no significant differences in the proportion of persisters nor in the sensitivity of their offspring as determined by dilution tests in broth.

To define the sensitivities more accurately the numbers of organisms able to produce colonies on closely graded concentrations of penicillin in agar were determined. Enumeration of coccal units able to multiply on given concentrations of penicillin agar may be misleading and can only be used as a guide to the behaviour of a bacterial population (Barber, 1953). A resistant colony might arise from a single coccus in a clump of staphylococci (Eagle, Fleischman & Levy, 1952). Fine differences in resistance might be obscured by the use of a log scale at the sensitive part of the scale and of too large increments of penicillin (Hughes, 1952), as well as by a probable 10% error in plate counts. In spite of these difficulties, the method demonstrated gradations in sensitivity which were not detectable in broth tests.

In the parent culture, the distribution of cocci of different penicillin susceptibilities was similar to that reported by Demerec (1945) and by Mayr-Harting (1955). Slight differences in resistance among cocci or clumps of cocci gave a continuous spectrum, with the number able to form colonies decreasing as the concentration of penicillin increased over a narrow range as observed by Eagle *et al.* (1952). There was no inhibition on plates containing 0·02 unit penicillin/ml., 90% inhibition with 0·03 unit/ml., and complete inhibition with 0·08 unit/ml. From an inoculum of 10^6 organisms, about 0·1% formed minute colonies in 48–96 hr on plates with 0·045 unit penicillin/ml.; about 0·01% with 0·05 unit/ml.; and about 0·001% with 0·055 unit/ml. Suspensions of randomly selected colonies of the parent culture plated directly on penicillin agar showed slight variation in resistance among cocci or clumps of cocci within a given colony as Hughes (1952) had demonstrated with single cocci technique.

Since the proportion of staphylococci growing on low concentrations of penicillin agar was similar to the proportion surviving in higher concentrations of penicillin broth, persisters might represent the most resistant cocci in a given population. However, in randomly chosen colonies of survivors recovered on penicillin-free agar, suspended in broth, and plated on penicillin agar either immediately or after incubation for 18 hr the distribution of resistant cocci was similar to that of the parent culture. Hence, if persisters were the most resistant cocci present at the time of exposure, this resistance was not transmitted uniformly to their offspring.

Staphylococci closely related genetically may have similar resistance to penicillin for several cell divisions (Hughes, 1952). To test whether the more immediate offspring of persisters were any more resistant than those in 18 hr colonies, replica plating was used. After overnight exposure in penicillin broth, samples were plated on penicillin-free agar and incubated; at 2, 7 and 8 hr replica plates from the penicillin-free agar were made on to graded penicillin concentrations in nutrient agar. Replica plating from the parent culture was done at the same intervals. There were no marked differences in resistance between the earlier and the later offspring of survivors, nor between these and the parent.

To determine whether the most resistant cocci in the parent culture survived in bacterial concentrations of penicillin direct plate counts were made on penicillin (0·035 – 0·06 unit/ml.) agar at intervals during incubation in penicillin broth (Fig. 1). Penicillinase could not be added to samples, but immediate control plates from broth with and without penicillin on to penicillin-free and to penicillin agar plates

showed that diluted portions equivalent to 0·01 ml. or less of the original mixture did not contain enough penicillin to influence the initial counts. After 4 hr in penicillin broth, cocci able to form colonies on penicillin agar were rarely detected. Meanwhile, in penicillin-free broth such organisms had increased tenfold. Most of the cocci able to multiply on 0·035–0·06 unit penicillin/ml. in agar were apparently killed early in exposure to 0·25 unit/ml. in broth. Possibly, these slightly resistant cocci remained viable but were unable to form colonies on penicillin agar because of damage during exposure or of inhibition by bound penicillin + that in the agar. However, as far as could be determined, cocci in the parent population able to multiply on low concentrations of penicillin in agar were no more resistant to a bactericidal concentration in broth than the rest of the population (Fig. 1).

Colonies of survivors with maximal resistance were selected from penicillin-free plates by replica plating on to agar containing 0·05–0·06 unit penicillin/ml. Subcultures of these rare colonies were simultaneously plated directly on to graded concentrations of penicillin in agar and re-exposed to penicillin in broth. On penicillin agar, some of these subcultures behaved like the parent culture; others showed a higher proportion of cocci able to form colonies and may have contained mutants. All yielded about the same number of persisters in broth as did the parent culture. Thus, it is unlikely that persisters merely represent the most resistant of the cocci exposed.

Effect of environmental variables on the frequency of persisters

Number of staphylococci exposed. In over a hundred tests by the 'standard' procedure in 10 ml. volumes, the number of survivors from populations of 10^5–10^7 staphylococci/ml. was roughly proportional to the number present when penicillin was added (Fig. 1; Tables 1, 2). Sometimes the decrease in the number of survivors was less than tenfold when the initial number was decreased from 10^7 to 10^6/ml. With inocula below 10^5 cocci/ml. the number of persisters was too small to be detected regularly by plate counts, but they were demonstrable by subculture in broth from inocula as small as 10^3 cocci/ml. In view of the many variables, the series was analysed by a regression curve. The data fitted a straight line and indicated that the number of persisters was directly related to the number exposed in the range of 10^5–10^8 cocci/ml. in 10–20 ml. volumes. When exposed in larger volumes, however, the number of survivors was not related to the original number (see below).

To determine whether potential persisters might emerge from small initial inocula in the absence of penicillin, replicate series in 10 ml. volumes of penicillin-free broth were inoculated with numbers of cocci varying from less than 10 to 10^4/ml. and incubated at 37°. Penicillin was added to different sets of tubes at hourly intervals during multiplication. Provided that the population had reached at least 10^5 cocci/ml. before the addition of penicillin, the number of persisters at a given population density was approximately the same regardless of the size of the initial inoculum. For example, either 7 cocci/ml. or 7×10^4 cocci/ml. if allowed to multiply in 10 ml. of penicillin-free broth to about 5×10^5 cocci/ml. before adding penicillin yielded about 50 persisting cocci/ml.; if allowed to multiply to about 5×10^7/ml. they yielded about 5×10^3 persisters/ml. By addition of penicillinase to the original tubes, persisters were detected in cultures arising from less than 10 cocci even when penicillin was added when the population had reached only 10^3 cocci/ml.

From populations of about 2×10^8 cocci/ml. or higher, 15–100% of the cocci survived (Fig. 1, Table 1). Such large populations are 'indifferent' to penicillin (Chain & Duthie, 1945; McDermott, 1958), but survivors under such conditions are excluded from the category of persisters as defined here. It was only necessary to

Table 1. *The number of persisters of Staphylococcus aureus Oxford strain (H) exposed to penicillin as influenced by the size but not by the age of the population*

Organisms exposed to 0·25 unit penicillin/ml. in broth; total volume 10 ml., at 37° without shaking. 0 hr, Initial count immediately after mixing with penicillin broth; 24 hr, count after exposure to penicillin for 24 hr.

Time at 37° before exposure to penicillin (hr)	Penicillin-free agar		Penicillin agar (0·05 unit/ml.)	
	0 hr	24 hr	0 hr	24 hr
	Plate counts (cocci/ml.)			
8	$9{\cdot}0 \times 10^6$	$1{\cdot}0 \times 10^3$	$2{\cdot}8 \times 10^3$	< 10
8	c. $9{\cdot}0 \times 10^5$	$1{\cdot}9 \times 10^2$	$3{\cdot}0 \times 10^2$	< 10
8	c. $9{\cdot}0 \times 10^4$	$1{\cdot}3 \times 10^1$	$3{\cdot}0 \times 10^1$	< 10
18	$3{\cdot}0 \times 10^7$	$2{\cdot}6 \times 10^3$	$2{\cdot}8 \times 10^3$	< 10
18	$5{\cdot}6 \times 10^6$	$1{\cdot}7 \times 10^2$	$9{\cdot}6 \times 10^2$	< 10
31	c. $3{\cdot}0 \times 10^7$	$1{\cdot}2 \times 10^3$	$2{\cdot}1 \times 10^3$	< 10
31	c. $3{\cdot}0 \times 10^6$	$1{\cdot}7 \times 10^2$	$1{\cdot}0 \times 10^2$	< 10
31	c. $3{\cdot}0 \times 10^5$	$6{\cdot}0 \times 10^1$	$5{\cdot}0 \times 10^1$	< 10
No penicillin				
18	$3{\cdot}0 \times 10^7$	$4{\cdot}9 \times 10^8$	$1{\cdot}2 \times 10^3$	$2{\cdot}2 \times 10^4$
18	c. $3{\cdot}0 \times 10^6$	$3{\cdot}8 \times 10^8$	$8{\cdot}0 \times 10^2$	$7{\cdot}5 \times 10^3$

Table 2. *Effect of volume of broth and of size of population of Staphylococcus aureus Oxford strain (H) exposed to penicillin upon the number of survivors*

Culture, broth, and 0·25 unit penicillin/ml. mixed in 250 ml. flasks in volume of 110 ml.; 10 ml. transferred from each flask to a test tube immediately, incubated at 37° without shaking. Figures are mean counts from 10 replicate containers.

Number organisms/ml. exposed	Plate counts cocci/ml. on penicillin-free agar after 24 hr exposure to penicillin in	
	100 ml. volume	10 ml. volume
$4{\cdot}3 \times 10^7$	$1{\cdot}7 \times 10^4$	$3{\cdot}1 \times 10^3$
$6{\cdot}2 \times 10^6$	$2{\cdot}9 \times 10^4$	$6{\cdot}6 \times 10^2$
$9{\cdot}9 \times 10^5$	$1{\cdot}4 \times 10^4$	$1{\cdot}2 \times 10^2$
$5{\cdot}3 \times 10^4$	$9{\cdot}4 \times 10^3$	$6{\cdot}0 \times 10^1$
$7{\cdot}8 \times 10^3$	$1{\cdot}6 \times 10^3$	< 10

dilute these large populations tenfold or less, either in fresh broth or in supernatant broth obtained by centrifuging part of the culture, or to remove 10% of the cocci by centrifugation, to obtain the usual low number of persisters (Table 3). In an attempt to 'divorce' the number of bacteria from their age, young cultures were centrifuged and resuspended in broth to give high concentrations of cocci as had been done by Eagle & Musselman (1949) and Berntsen (unpublished, quoted by McDermott, 1958) with conflicting results. Cultures 2–7 hr old concentrated to

$2–5 \times 10^8$ cocci/ml. behaved like equal numbers of 18–31 hr culture cocci with survival rates of 30–50 %; those concentrated only to 5×10^7–1×10^8/ml. gave survival rates of 0·01–0·02 % (Table 3). These results, in accord with those of Eagle & Musselman (1949), were similar whether the cocci were re-suspended in the broth in which they had grown, in a filtrate of a 24 hr culture, or in fresh broth; hence, metabolites in the medium seemed to have no effect. A state of 'indifference' to penicillin seemed to depend upon the concentration of the cocci rather than upon their age.

Table 3. *Relative insusceptibility to penicillin of large numbers of organisms of Staphylococcus aureus Oxford strain* (H) *regardless of age*

Organisms were exposed to 0·25 unit penicillin/ml. in broth; *total volume* 37° without shaking. 0 hr, Initial count immediately after mixing with penicillin broth; 24 hr, count after exposure to penicillin for 24 hr.

Age of organisms (hr)	Plate counts on drug-free agar cocci/ml. 0 hr	24 hr	% of population surviving
4*	6×10^8	3×10^8	50·0
4	4×10^7	2×10^3	0·005
7	7×10^8	2×10^8	28·5
7	2×10^7	4×10^3	0·02
18	4×10^8	6×10^7	15·00
18	2×10^7	2×10^3	0·01
32	8×10^8	4×10^8	50·00
32	4×10^7	8×10^8	0·02
No penicillin			
18	4×10^8	3×10^8	—
18	3×10^7	4×10^8	—

* Organisms were concentrated by centrifugation in the broth in which they had grown; results were similar when resuspended in fresh broth.

Assay of residual penicillin after contact with populations greater than 10^8 cocci/ml. showed little decrease in its potency. Even after 24 hr at 37°, the concentration of penicillin showed at most a twofold decrease, no greater than that with uninoculated controls and well above the minimal inhibitory concentration. Hence, there was no evidence that the high survival rate was related to more rapid decay of penicillin when heavily inoculated such as Sabath & Finland (1963) observed with methicillin and other semi-synthetic penicillins.

Rogers (1959) showed that a broth culture of staphylococci at a stable population of 10^8 cocci/ml. increased to 10^9 cocci/ml. when shaken vigorously and that penicillin susceptibility reappeared with this new upsurge of growth. We confirmed his findings with both 6 hr and 18 hr cultures containing $3–4 \times 10^8$ cocci/ml. When these large populations were shaken for 10–30 min. before addition of penicillin and then incubated with continuous shaking for 18 hr only 0·005–0·1 % survived as compared with 15–50 % in unshaken cultures. Penicillin-free controls showed no increase in the number of cocci when not shaken, but a tenfold increase when shaken. These results are consistent with the frequent observation that bacteria are maximally susceptible to penicillin only when able to multiply in the absence of the drug (Hobby *et al.* 1942).

Volume of broth. The percentage of survivors was higher when the staphylococci were exposed to penicillin in 40–200 ml. of broth preheated to 37° than in 10–20 ml. (Table 2), as had been found by Bigger (1944*a*, *b*). In 30 ml. volumes the number of survivors varied from test to test. The number of survivors in 40–200 ml. volumes was not proportional to the number exposed, as it was in 10–20 ml. volumes, but fell in a range of about 5×10^3–5×10^4 cocci/ml., regardless of the initial number, provided that this was at least 5×10^4/ml. and not more than 10^8/ml. With an inoculum of about 5×10^7 cocci/ml. the survival rate was slightly higher in the larger volumes than in the smaller, although the differences sometimes fell within the standard error of the plate counts. With inocula of 10^5 cocci/ml. or less the number of survivors/ml. was usually at least 100 times greater in 40–100 ml. than in 10–20 ml. volumes, so that the organisms behaved as if they were 'indifferent' in the larger volumes.

To obtain comparable initial conditions, several series of mixtures of broth, penicillin, and bacteria were made in 20 replicate containers with a final volume of 110 ml.; from each of these, 10 ml. portions were transferred immediately to separate containers for incubation along with the remaining 100 ml. portions. The number of persisters/ml. was consistently higher from the larger portions (Table 2). The effect of the length of time of exposure to penicillin in large volumes was tested by periodically removing 10 ml. portions and continuing their incubation in tubes. All portions removed within 1–12 hr showed similar low numbers of persisters/ml.; portions removed at 14–18 hr yielded higher proportions of persisters, comparable to the count obtained from the remainder in the large original container. Hence, whatever factors may have determined the higher rate of survival in the larger volume, they did not exert their influence upon the bacterial population until after exposure for 12 hr.

Dispersion of the inoculum did not account for the higher yield of survivors in larger volumes because it was noted both with equal initial numbers/ml. and with equal total numbers/container. The volume of medium had no demonstrable effect upon the length of the lag period or the growth rate in penicillin-free controls, nor upon the early death rate during the first 8 hr in presence of penicillin. The rate of deterioration of penicillin was no greater in 100 ml. than in 10 ml. of broth, whether inoculated or not. The sensitivity of survivors from large and small volumes, as determined by both direct and replica plating on penicillin agar, was about the same; the difference could not be attributed entirely to the presence of a greater number of slightly resistant cocci in the larger volume.

Factors without demonstrable effect. The number of persisters was not significantly altered by the size or shape of the containers, by whether or not the closures were airtight, nor by continuous shaking of populations less than 2×10^8 cocci/ml. during incubation. Hence the higher number of survivors in 40–200 ml. volumes than in 10–20 ml. volumes was not related to the degree of aeration. Holding in penicillin broth at 4° or about 20° for 2–24 hr before placing at 37° had no effect upon the outcome. Bigger (1942) stated that even brief chilling before penicillin began to act resulted in a higher number of persisters; this could not be confirmed by us. With continuous exposure at about 20°, the death rate was slower, but the number of persisters was similar to that at 37°. Previous storage of the bacteria in the absence of penicillin for 85–242 days at 37°, at room temperature or at −10°, had no

influence; nor did 8–12 serial transfers at 2–6 hr intervals in penicillin-free broth at 37°. The age of the organisms and whether they were in the lag, the logarithmic, or the early stationary phase at the time penicillin was added made no demonstrable difference. No correlation was observed between the size of clumps of cocci in a given culture and the proportion of survivors. The concentration of penicillin over a range 0·1–100 units/ml. and the addition of various concentrations of streptomycin to the mixture were without effect upon the number of persisters.

Multiplication of persisters after removal from penicillin

Bacteria treated with penicillin under conditions permitting their multiplication in the absence of the drug require a recovery period after inactivation of penicillin before they resume multiplication at their normal rate; when treated under conditions not permitting multiplication, this lag is not shown (Parker & Marsh, 1946; Eagle & Musselman, 1949). Hence, determination of whether persisters require a recovery period might indicate whether or not they had been capable of multiplication during exposure to penicillin.

Staphylococcus aureus (H) organisms from 8 and 18 hr cultures were inoculated to a concentration of 10^7 cocci/ml. into broth containing penicillin 0·25 unit/ml. and incubated for 18 hr at 37°. Then penicillinase was added and plate counts were made at 30–60 min. intervals during further incubation at 37°. The rate of multiplication was compared with that of approximately equal numbers of staphylococci never exposed to penicillin. Although preliminary tests had suggested that persisters showed a longer lag than the parent culture (Gunnison & Jawetz, 1958), more rigorous controls showed this to be untrue. As one control, cocci never exposed to penicillin were inoculated in small numbers approximately equal to the number of persisters into a sterile filtrate of a mixture treated for 18 hr exactly like the test itself so that it contained metabolic products of bacterial growth, substances liberated by lysis of 99·9% of the original culture, penicillinase and inactivated penicillin. The parent staphylococci multiplied in this filtrate at the same rate as an equal number in fresh penicillin-free broth. As a second control, sterile broth containing penicillin was incubated along with each test for 18 hr, then penicillinase added, and small numbers of previously unexposed cocci inoculated into the broth. Comparison of the growth rate with that of equal numbers in penicillin-free broth showed that penicillin was completely inactivated within 10 min. or less.

Persisters which had survived for 18 hr in penicillin broth multiplied therein after the penicillin had been inactivated, with no increase in lag and at the same rate as controls inoculated with equal numbers of parent culture (Fig. 2). However, cocci removed from penicillin action by addition of penicillinase after only 30–120 min. before any significant decrease in the viable count showed a lag of 1–2 hr before resumption of their normal growth rate (Fig. 2). Hence, the population exposed for 2 hr or less behaved as if penicillin had exerted an effect, whereas persisters exposed for 18 hr behaved as if they had not been affected by the penicillin.

In other tests, 10–20 replicate tubes of penicillin broth were set up by the standard method along with an equal number of uninoculated control tubes of penicillin broth. After 24 hr at 37°, penicillinase was added to all tubes, The controls were then inoculated with previously untreated staphylococci in numbers similar to the expected number of persisters. Plate counts were made and the time of appearance

of turbidity at 37° was recorded. In three trials, persisters and controls became turbid at about the same time. Bigger (1944*a*, *b*) had noted that staphylococci removed from prolonged penicillin action often multiplied rapidly. If it be accepted that bacteria which do not require a recovery period after penicillin treatment have not been affected by the penicillin (Eagle & Musselman, 1949), these results suggest that persisters were not in a state of potential cell division during exposure to penicillin.

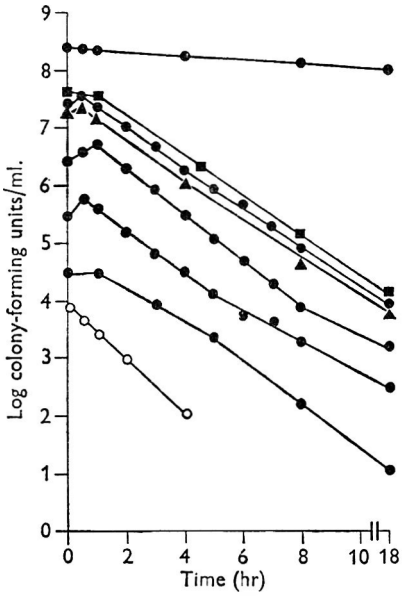

Fig. 1

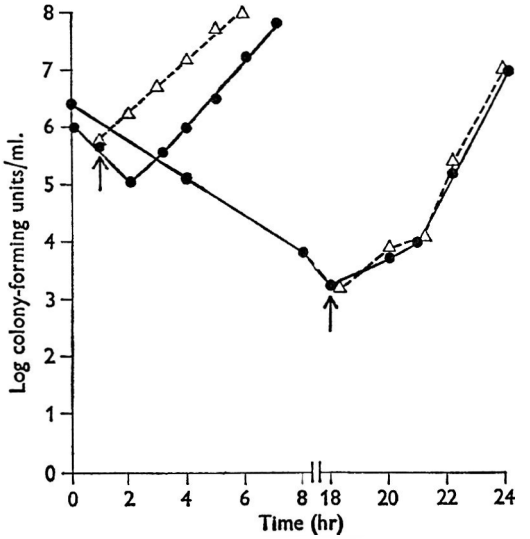

Fig. 2

Fig. 1. Effect of concentration of penicillin and size of exposed population of *Staphylococcus aureus* Oxford strain (H) upon the number of persisters. Counts made on penicillin-free agar during exposure to 0·1 unit penicillin/ml., ▲ - ▲; 0·25 unit/ml., ● - - ●; 100 units/ml., ■ - - ■. Counts made on penicillin agar (0·05 unit/ml.) during exposure of a total initial population of 5×10^7 cocci/ml. to 0·25 unit penicillin/ml., ○ - - ○. Cocci able to form colonies on penicillin agar were killed within 4 hr.

Fig. 2. Rate of growth of *Staphylococcus aureus* Oxford strain (H) after exposure to 0·25 unit penicillin/ml. in broth at 37° for 1 hr and for 18 hr upon inactivation of the antibiotic. Penicillinase was added to the mixture at the points indicated by arrows and incubation continued. △ - - - △, Control organisms not exposed to penicillin; ● - - - ●, organisms exposed to penicillin. Organisms treated for 1 hr showed a 2-hr lag, but those surviving for 18 hr showed no lag.

DISCUSSION

The persistence of some cocci of genotypically penicillin-sensitive *Staphylococcus aureus* strain Oxford (H) is readily demonstrated after at least 99·9 % of the population of which they were members have been killed by penicillin, but the reasons for this survival are not clear. Is persistence due to the non-dividing state of the survivors? Penicillin is effective only against bacteria exposed to it under conditions that would permit their multiplication in the absence of penicillin (Hobby *et al.* 1942; Lee, Foley & Epstein, 1944; Chain & Duthie, 1945; Wood & Smith, 1956).

Persistence of sensitive bacteria under such conditions has been attributed to the temporary inability of a small proportion of the organisms to divide during the exposure (Bigger, 1944*a*, *b*; Eriksen, 1946; Gunnison & Jawetz, 1958; McDermott, 1958). Penicillin inhibits synthesis of bacterial cell walls, especially at the division septum, so that bacteria which initiate the division process become osmotically fragile and are killed by lysis unless protected by hypertonic solution (Lederberg, 1957; Park & Strominger, 1957). With *S. aureus* (H), accumulation of cell wall precursor material coincides with the onset of lysis and a decrease in the number of intact cocci (Ciak & Hahn, 1962). Dormant or resting bacteria are not susceptible to this action of penicillin on cell wall synthesis and hence survive (Lederberg & St Clair, 1958).

Are persisters cells which are metabolically 'dormant' at the moment of exposure to penicillin? In experiments reported here there was no difference in generation time nor in rate of multiplication between persister offspring and parent culture after removal from penicillin. This suggests that at the time of initial effective exposure to penicillin the persisters were not susceptible to the biochemical lesion brought about by the penicillin. Cell wall production, and particularly the synthesis and assembly of muramic acid-containing mucopeptide, the presumed 'target' of penicillin action, may be an intermittent process which might lead to temporary insusceptibility of some bacteria in any population.

Does the growth phase of the exposed culture affect the number of survivors? Although the bactericidal rate is a function of the rate at which the majority of the organisms would multiply if no penicillin were present (Hobby & Dawson, 1944; Lee *et al.* 1944) the phase of growth at the time of adding penicillin had little effect upon the number of persisters. It is not surprising that organisms removed directly from storage at $-10°$ showed no more persisters than did 18 hr broth cultures, inasmuch as bacteria stored at subfreezing temperatures may initiate multiplication more rapidly when returned to 37° than unfrozen cells (Harnsell, 1951). Fewer persisters might be expected from cultures in the early log phase than in the stationary phase; but Hamburger & Carleton (1960) found that staphylococci from 4 or 18 hr cultures might survive in penicillin broth for as long as 9 days.

Do rapidly bactericidal synergistic combinations of penicillin with other antibiotics prevent the emergence of persisters as suggested by Gunnison, Kunishige, Coleman & Jawetz (1955)? Re-inspection of data and further tests show that small numbers of various bacteria persist even in the presence of synergistic pairs of drugs. With *Streptococcus faecalis*, for example, although the number of persisters was lower with penicillin + streptomycin than with either compound alone, an appreciable number survived (Gunnison, Jawetz & Coleman, 1950). Pairs of antibiotics do not act synergistically under conditions which prevent multiplication in drug-free media (Gunnison *et al.* 1955). Therefore, persistence of organisms when exposed to synergistic pairs under conditions which permit multiplication of most of the organisms in drug-free media favours the assumption that the surviving organisms were unable to initiate division.

Are potential persisters predetermined when penicillin is added to a culture or do they emerge in the presence of the compound? If persisters were present before contact with penicillin, their number under defined conditions might comprise a fixed proportion of the initial number, as it indeed did with *Staphylococcus aureus* (H)

in 10 ml. volumes of penicillin broth. In modified fluctuation tests, entirely independent cultures showed a greater variance in the number of persisters than did those inoculated from a single culture (Fraher, unpublished 1960). Although such tests are of doubtful validity (Eriksen, 1953), they suggest that persisters do not arise as a result of the presence of penicillin. On the other hand, in volumes of 40 ml. or greater the number of survivors was not proportional to the initial number. The results suggest that some factor related to higher survival rates reached a critical value in larger volumes after 12 hr of exposure to penicillin. It is possible that a rarely encountered unknown factor responsible for temporary insusceptibility, perhaps by inducing a pause in cell wall synthesis, may be supplied under these conditions.

Do persisters represent the most resistant cocci in the normal distribution curve shown by a given population? The parent culture of *Staphylococcus aureus* (H) contains a few cocci able to form colonies on concentrations of penicillin agar which are inhibitory to the majority of the organism in the population, but subcultures of these colonies may have enhanced, decreased, or unaltered resistance as compared with the original culture (Demerec, 1945; Hughes, 1952). Eagle (1954) found that after 10 serial passages of another strain of Staphylococcid in a concentration of penicillin bacteridal for most of the cocci, the sensitivity of offspring of persisters had not changed. With the *S. aureus* strain Oxford (H) slightly resistant, cocci were among the earliest to be killed. Possibly the somewhat resistant bacteria potentially or actually able to divide may be killed by doses of penicillin in which less resistant cocci survive.

Do persisters differ in any demonstrable way from other bacteria in the same population? Currently there is much interest in the possible survival of spheroplasts or of L forms in penicillin. The staphylococcal persisters under consideration are definitely not spheroplasts since they are not osmotically fragile but survive suspension in distilled water; nor are they L forms because they produce colonies of normal size within 24 hr on ordinary media when removed from penicillin. They show none of the characteristics of resistant mutants (Rake, McKee, Hamre & Houck, 1944; Barber, 1953; Mayr-Harting, 1955). Persisters do not differ from the parent culture in size and morphology of colonies, pigment and coagulase production, uniform turbidity in broth, tendency to clump, cellular morphology, or ability to utilize certain carbohydrates. Of course, they may vary in other less obvious and untested properties.

Persisters have been found among various bacteria exposed to many different antibiotics (McDermott, 1958; Hobby & Lenert, 1957). *Streptococcus faecalis*, for example, behaved much like *Staphylococcus aureus* in the presence of penicillin (Mr J. H. Tucker, personal communication). Persisters have been demonstrated in methicillin (Stewart, 1961). They occur upon exposure not only to penicillin and other antibiotics which interfere with cell wall synthesis, but also to those which act by other mechanisms.

In general, bacteria which survive the action of various harmful substances may not transfer this ability to their progeny (Eddy & Hinshelwood, 1953), After survival in acid, for example, closely related cells share similar resistance for several divisions, but this is not propagated through an overnight subculture (Powell, 1958). In populations treated with various chemicals, heat, or radiation the 10^3 organisms/ml.

which usually survive are not the maximally resistant organisms at the extreme of the normal distribution curve. It is difficult to detect resistant organisms among survivors even when they comprise 0·1 % of the original population (Wyss, 1950). Only rarely has heritable resistance of survivors of heat been tested, and this with conflicting results; but available evidence suggests that they are phenotypically deviant rather than genetically resistant (Jordan, Jacobs & Davies, 1947; Lederberg, 1949). Persisters probably are the result of random phenotypic accidents due to a multiplicity of independent causes. A given bacteriam may be more or less resistant to a lethal agent at different moments in its life, with or without relation to cell division. Bryson & Szybalski (1955) suggested that bacteria may survive in an unfavourable microenvironment due to temporary changes at the cell level such as clumping, capsule formation, or unusual accumulation of chemical substances; but that their adaptive advantage is not genetic and so transitory as to defy demonstration after subculture.

Bacterial persistence *in vivo* was reviewed by McDermott (1958) who inferred from clinical observations and experimental evidence that it occurs broadly throughout the microbial world; he speculated that it might be a reflection of a high degree of adaptive plasticity of individual microbes. It seems probable that persisters similar to those studied *in vitro* do occur in the body and that they may be in part responsible for failures in antibiotic therapy.

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Cellular Factors Affecting Nitrogen Fixation in the Blue-Green Alga *Chlorogloea fritschii*

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SUMMARY

Synchronous cultures of a nitrogen-fixing blue-green alga, hitherto known as *Chlorogloea fritschii* but more probably an anomalous species of the genus *Nostoc*, were obtained by a combination of light and temperature treatments. Variation in dimensions, dry weight, pigment content and total nitrogen content of cells was followed during the development of synchronous cultures. The nitrogen-fixing activity was greatest in the small-celled filaments which develop from the endospores and predominate during exponential growth of the alga in cultures of limited volume. Strains of the alga produced by repeated exposure to X-rays, ultraviolet radiation, or sublethal concentrations of colchicine or urethane, were found to have lower rates of nitrogen fixation per unit dry weight than the original strain but liberated relatively more extracellular nitrogenous products.

INTRODUCTION

In the absence of an exogenous source of combined nitrogen the fixation of elementary nitrogen by blue-green algae normally parallels increase in cell material, any factor affecting growth having at least an approximately corresponding effect on nitrogen fixation. It must be envisaged, however, that the relation of nitrogen fixation to increase in total cell material is under genetic control; and it is attractive, although perhaps naïve, to suppose that strains with especially pronounced capacities for nitrogen fixation may be obtained and used on the economic scale to increase the fertility of habitats such as rice-fields. Superimposed on the genetically determined relation there must be changes depending on the physiological condition of the cells and this will depend on endogenous factors as well as on environmental conditions. Work with synchronous cultures of Chlorella (Nihei *et al.*, 1954) has shown that the metabolic characteristics of cells change during the division cycle and leads to the expectation that the capacity of a cell of a blue-green alga to fix nitrogen may vary considerably during its individual growth. This paper describes investigations into these possibilities carried out with the alga *Chlorogloea fritschii*, which was the subject of the previous communication in this series (Fay & Fogg, 1962).

METHODS

Strain of alga, culture and general analytical methods. These were the same as in previous work (Fay & Fogg, 1962) except that the medium used for the mutagenesis studies was that described by Fogg (1949) diluted to half the usual concentration.

Ultraviolet irradiation. A Hanovia 30 cm. 30 watt quartz-jacketed mercury lamp

emitting mainly at 2537 Å. was used. Algal suspensions were exposed at a distance of 25 cm. from the tube in open sterile Petri dishes with occasional agitation.

X-irradiation. The characteristics of the source and radiation were: 33 kV_p, 25 mA., 0·275 mm.Al filtration, 3·1 mm. wax half value thickness, dose rate 4250 r./min. Algal suspensions were exposed in Petri dishes covered with thin sterile 'Styrafoil-S', which transmits most of the radiation (Cosslett & Nixon, 1960).

Pigment estimations. Chlorophyll and carotenoids were extracted by absolute methanol from fresh material that had been washed once in distilled water. After extraction for 10 min. the suspension was centrifuged and the supernatant fluid decanted; a second extraction resulted in complete removal of chlorophyll and carotenoids. The two extracts were combined and made to known volume with methanol. Relative measures of chlorophyll concentration in the extracts were obtained by the determination of absorption at the 665 mμ peak. For estimation of phycocyanin the material, having been washed in distilled water, was resuspended in distilled water and frozen at −20°. A drop of chloroform was added and the material was allowed to autolyse at 5° for a few days. A clear solution of phycocyanin was obtained by repeated extraction and centrifugation, making the combined extracts up to known volume. Absorption at 620 mμ was used as a measure of the relative concentration of phycocyanin.

Tracer experiments. Uptake of ^{15}N was determined according to Burris & Wilson (1957). The algal suspensions were exposed to an atmosphere containing N_2, enriched with ^{15}N, in a closed culture apparatus. The samples were subjected to Kjeldahl digestion and distillation and the ammonium-N so produced was converted to elementary nitrogen in a Rittenberg tube. Mass analysis was carried out with a MS 3 type Associated Electrical Industries Ltd. mass-spectrometer.

RESULTS

Variations in nitrogen-fixing capacity during the life-cycle

The life-cycle. The starting point may be taken as the liberation of endospores which occurs soon after the transference to fresh medium of cells from a mature culture. The endospores remain adhering to each other after disruption of the parent cell-membrane, forming a short motile filament, usually 4–6 cells long, resembling the hormogonium as found in Nostocales and Stigonematales. The cells at stage I are pale blue-green in colour and from 2·5 to 3·5μ in diameter (Pl. 1, fig. 1). After their liberation, the cells grow in size, up to about 4·5μ in diameter, and become more deeply blue-green, dividing transversely so that the filamentous character is preserved (stage II; Pl. 1, fig. 2). Later, cell diameter increases to about 6·0μ and longitudinal divisions occur. The filamentous character then gradually disappears and the shape of the cells changes from round to polygonal as the size of the groups increases (stage III; Pl. 1, fig. 3). Finally (stage IV), the cell diameter increases to about 7·2μ, the contents become yellowish green and markedly granular, and endospores are formed by repeated divisions (Pl. 1, fig. 4).

The course of growth and nitrogen fixation in cultures of limited volume. Growth and nitrogen fixation under optimum conditions (35°, 5000 metre-candles and continuous agitation) followed a normal course (Fig. 1). A lag phase of about 3 days was followed by exponential growth for about 7 days then by a period of declining

relative growth leading into a stationary phase after about 30 days. During the course of growth the proportions of the various size-classes of cells changed so that the mean cell diameter decreased during exponential growth and increased in the post-exponential phases (Fig. 2). Cell nitrogen content on a dry weight basis changed in the opposite manner (Fig. 2), suggesting that the nitrogen-fixing capacity of the cells varied, those in the earlier stages of the life-cycle evidently being more active in this respect than those at a later stage. Further study of this possibility required more homogeneous populations of cells than can be obtained in cultures of this type.

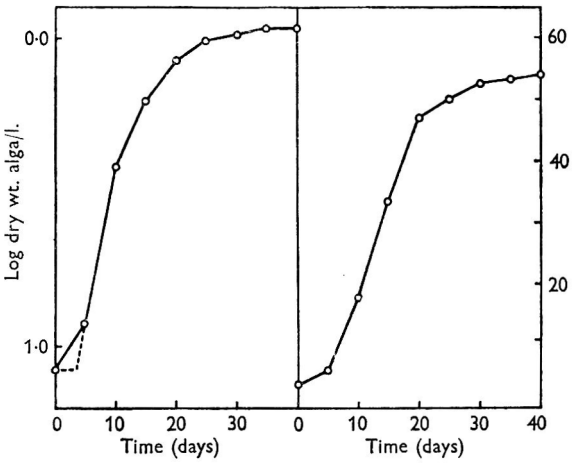

Fig. 1

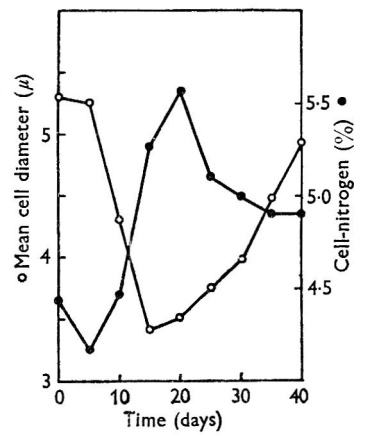

Fig. 2

Fig. 1. The course of growth of *Chlorogloea fritschii* in cultures of limited volume at 35° and 5000 metre-candles with continuous agitation. Left, dry wt.; right, total cell-nitrogen.

Fig. 2. Changes in cell size (○) and total cell-nitrogen as % of dry wt. of alga (●) during growth of *Chlorogloea fritschii* in cultures of limited volume (see Fig. 1).

Synchronous cultures

The life-cycle of *Chlorogloea fritschii* is more complex than that of Chlorella and to obtain cultures in which the majority of cells are synchronized as regards stage in the life-cycle is correspondingly more difficult. Experiments already reported (Fay & Fogg, 1962) showed that high light-intensity and high temperature favoured a high proportion of large mature cells, whereas at low light-intensity and low temperature the proportion of small cells was high. Synchronized cultures may thus be obtained by a combination of light and temperature treatments, a method similar to that used with *Chlorella ellipsoidea* by Tamiya (1961).

Cultivation of *Chlorogloea fritschii* at 35° and about 5000 metre-candles for about 20 days resulted in 75–85% of the population being stage IV cells (Pl. 1, fig. 6). Transfer of a population rich in stage IV cells to fresh medium at a lower temperature (25°) and a lower light intensity (about 300 metre-candles) resulted, after about 20 days, in cultures in which 75–85% of the cells were in stage I (Pl. 1, fig. 7). When populations rich in stage I cells were transferred to fresh medium and

the higher temperature and higher light intensity, they changed gradually and uniformly into stage IV-rich populations.

Changes in cell characteristics in synchronized cultures

Samples were withdrawn aseptically at 2- or 3-day intervals from synchronized cultures for determination of cell number, cell size, dry weight, cell nitrogen, chlorophyll and phycocyanin. The results are given in Figs. 3 and 4.

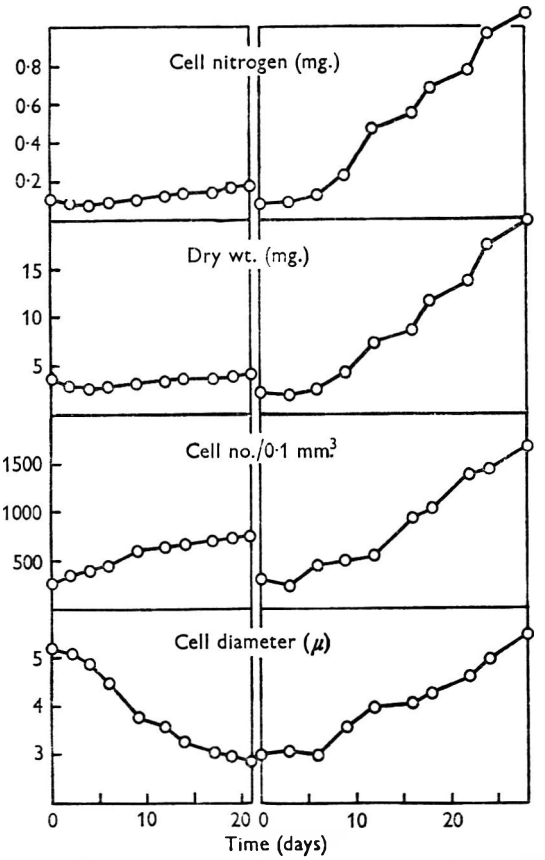

Fig. 3. Changes in cell size, cell number per 0·1 mm.³, dry wt. in mg./100 ml., and total cell-nitrogen in mg./100 ml. in a synchronized culture of *Chlorogloea fritschii*. Left, transformation of stage IV to stage I; right, transformation from stage I to stage IV.

During the transformation from stage IV to I (Figs. 3 and 4, left-hand diagrams) there was an increase in cell numbers and a decrease in mean cell diameter, corresponding to the liberation of endospores. These changes were more rapid during the first 8 days than afterwards. Dry weight and cell nitrogen showed similar slight decreases following transference to fresh medium, an occurrence which has also been observed with *Anabaena cylindrica* (Fogg & Than-Tun, 1960). After this, there was a slow rise in total dry weight and in cell nitrogen, the latter increasing relatively more as shown by an increase in cell nitrogen/unit dry weight of organism (Fig. 4). Both the chlorophyll and the phycocyanin content of the suspension increased about

threefold, i.e. the amount of pigment per cell remained at about the same value throughout the experimental period. There was, however, a distinct lag in synthesis of phycocyanin as compared with that of chlorophyll.

The stage I material obtained in the above experiment was transferred to fresh medium at high temperature and high light-intensity for observation of the transformation to stage IV cells (Figs. 3 and 4; right-hand diagrams). The curve for increase in cell numbers shows fairly distinct steps, indicating four periods of more

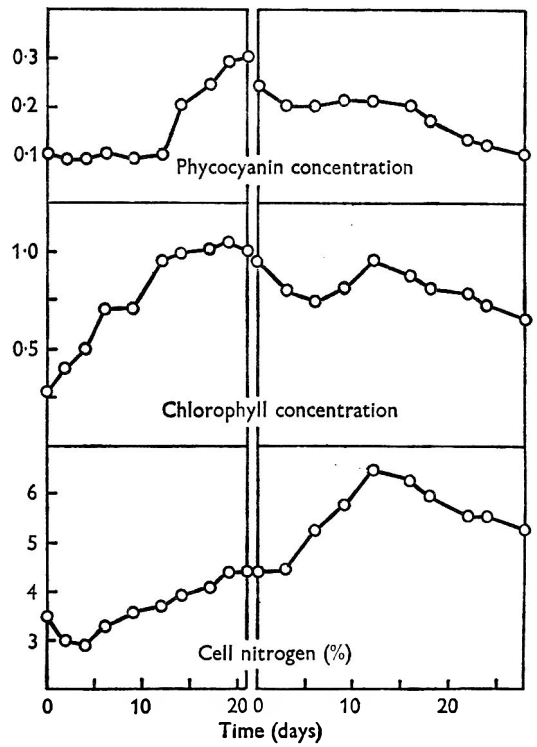

Fig. 4. Changes in cell nitrogen as % of dry wt. of alga and in chlorophyll and phycocyanin concentrations (arbitrary units) in a synchronized culture of *Chlorogloea fritschii*. Left, transformation of stage IV to stage I; right, transformation from stage I to stage IV.

or less synchronous cell divisions. The curves for cell nitrogen and dry weight also show steps but, initially at least, these are out of phase with those in cell number indicating, as one would expect, that synthesis of cell material was most vigorous in the intervals between cell divisions. The cells remained small for the first 6 days then their size increased fairly steadily until at the end of the 27-day period the mean diameter was about twice its initial value. Cell nitrogen/unit dry wt. of organism started at a low value, rose to a maximum at 12 days, then declined somewhat. The amounts of phycocyanin and chlorophyll/unit dry wt. remained at a nearly constant high value so long as the cell nitrogen/unit dry wt. was increasing, but thereafter declined. However, the amount of both pigments per cell remained at an approximately constant value throughout the period of the experiment.

Effects of mutagenic agents on growth and nitrogen fixation

It has so far proved impossible to grow *Chlorogloea fritschii* from single cells on solid media, so that the usual microbiological techniques for recognizing and isolating mutants cannot be applied. Consequently our procedure was to give repeated massive mutagenic treatments with the object of obtaining genetically distinct resistant strains. Kumar (1963) was successful in producing in this way distinct strains of another blue-green alga, *Anacystis nidulans*, which are stable over many subcultures.

The following treatments were given:

(1) 5 successive irradiations with X-rays, of 100,000–150,000r. each time, the alga being subcultured in between irradiations;

(2) 15 successive 10 min. exposures to ultraviolet radiation, the alga being subcultured in between exposures;

(3) 5 successive subcultures in a medium containing 10–20 mg. colchicine/100 ml., followed by another 5 subcultures in medium containing 40 mg. colchicine/100 ml.;

(4) 5 successive subcultures in a medium containing 10–250 mg. urethane/100 ml., followed by another 10 in medium containing 500 mg. urethane/100 ml.

Table 1. *Comparison of nitrogen contents of five strains of Chlorogloea fritschii*

Values per 200 ml. culture suspension (mean values in parentheses)

			N content of alga				Total N	
Strain	Age (days)	Dry wt. of alga (mg.)	mg.	mg./100 mg. dry wt. alga	N content of culture filtrate		mg.	mg./100 mg. dry wt. alga
X-rayed	21	18·5	0·662	3·58 (3·70)	0·043 (0·051)		0·705	3·81 (3·97)
		19·0	0·726	3·82	0·058		0·784	4·12
Control	21	10·2	0·418	4·09 (4·03)	—	—	—	—
		12·6	0·500	3·97				
Control	14	12·5	0·614	4·91 (4·96)	0·022 (0·019)		0·636	5·09 (5·16)
		11·9	0·608	5·02	0·016		0·624	5·24
Ultraviolet irradiated	14	10·3	0·620	6·02 (4·44)	0·052 (0·051)		0·672	6·52 (5·03)
		7·2	0·205	2·85	0·049		0·254	3·53
Colchicine treated	14	13·7	0·561	4·09 (4·32)	0·070 (0·064)		0·631	4·61 (4·88)
		11·4	0·529	4·55	0·058		0·587	5·15
Urethane	14	12·3	0·533	4·33 (4·38)	0·061 (0·060)		0·594	4·83 (4·86)
		12·3	0·545	4·43	0·058		0·603	4·90

Before comparing them, the 4 strains so obtained and the untreated strain were subcultured, without irradiation or other treatment, in basal medium. This was repeated after 7 days and 0·5 ml. portions taken as inocula from these cultures after a further 7 days. The experimental cultures were 100 ml. in volume and were incubated at 35° with occasional shaking. After 14 or 21 days the cultures were harvested and the dry weight of alga and the nitrogen content of cells and medium determined. The results are presented in Table 1. Growth in terms of the total dry wt. of cell material per culture was about the same for the control, ultraviolet-, colchicine- and urethane-treated strains, but growth of the X-ray-treated strain appeared somewhat better than that of the control. However, for 3 treated strains, and perhaps also for the fourth (ultraviolet-treated), the nitrogen content on a dry

weight basis was less and the proportion of extracellular combined nitrogen more, than for the untreated strain.

A strain produced by 40 successive 10 min. exposures to ultraviolet radiation and subculturings was compared with the untreated strain by using the ^{15}N isotope of nitrogen as a means of determining nitrogen fixation. Each strain was grown under optimum conditions; then the cells were suspended in fresh medium and exposed to an atmosphere with 0·2 atm. N_2 enriched with 7 % (v/v) ^{15}N, 0·2 atm. O_2, 0·05 atm. carbon dioxide and 0·55 atm. argon. The cells were incubated at 30° and 3000 metre-candles with continuous agitation by a magnetic stirrer for 3 days. The nitrogen-fixing capacity of the treated and control strains was compared in terms of the atom % excess ^{15}N in the cell material (Table 2). The rate of incorporation of ^{15}N by the ultraviolet resistant strain was slightly less than that by the untreated strain. This agrees with the results reported above obtained by the conventional micro-Kjeldahl method.

Table 2. *Comparison of ^{15}N uptake by untreated and ultraviolet-irradiated strains of Chlorogloea fritschii after 3 days exposure in the light to elementary nitrogen enriched with 7 % ^{15}N*

Sample		Atom % ^{15}N	Atom % ^{15}N excess
Standard $(NH_4)_2SO_4$		0·362	—
Untreated strain	(1)	0·545 (0·543)*	0·183 (0·181)
	(2)	0·541	0·179
U.v.-treated strain	(1)	0·534 (0·536)	0·172 (0·174)
	(2)	0·537	0·175

* Mean values

DISCUSSION

Chlorogloea fritschii was described as a new species by Mitra (1950) and referred to the Entophysalidaceae, order Chroococcales, which includes unicellular or colonial, non-heterocystous, blue-green algae. However, our material (Cambridge Culture Collection, no. 1411/1) when grown in medium free from combined nitrogen, produces distinct filaments and heterocysts at certain stages (Pl. 1, fig. 5). The presence of combined nitrogen is known to suppress heterocyst formation (Fogg, 1949) and the state of affairs in *C. fritschii* resembles that in another filamentous blue-green alga, *Camptylonema lahorense* (Kumar, 1962), which is generally devoid of heterocysts in the early stages of growth in Chu no. 10 medium but which, according to Pande & Mitra (1961), produces them in nitrogen-deficient media. In view of these findings *C. fritschii* can no longer be regarded as a member of the Chroococcales. Dr J. W. G. Lund, who has kindly examined our material, has expressed the tentative opinion that it is a *Nostoc* (personal communication).

This view is supported by the resemblance of the life-cycle of *Chlorogloea fritschii* to that of *Nostoc muscorum* as described by Lazaroff & Vishniac (1961). These authors reported an alternation of coccoid ('aseriate') and filamentous stages, successive cell divisions producing a cluster of cells surrounded by a membrane which later breaks down liberating the hormogonia. When *N. muscorum* is grown in the dark on an organic substrate, the life-cycle is interrupted at the aseriate stage and, by exposing such a culture to low-intensity light, synchronous cell division may

be induced. The main difference between this life-cycle and that of *C. fritschii* is that in the latter the filamentous habit is only evident in the early stages.

The life-cycle of *Chlorogloea fritschii* comprises several cell generations and is more elaborate than that of *Chlorella* spp., which is completed in one division cycle. Nevertheless, comparison with the cycle in Chlorella, as described by Hase (1962) and Tamiya (1963), is worthwhile. Our results give little information about variations in metabolic activity during the division cycle in *C. fritschii* but there seem to be resemblances between certain stages in the life-cycle of this species and stages in the division cycle of Chlorella. In their small size and high pigment content stage II cells correspond to D_a cells of Chlorella. This is the phase of most active nitrogen fixation and, according to Tamiya *et al.* (1953), the nitrogen content of *Chlorella ellipsoidea* is highest in the D_a stage. In their large size, low pigment content and low nitrogen content, stage IV cells of Chlorogloea resemble L cells of Chlorella. As in nearly all studies with synchronous cultures (Maaløe, 1962) there is uncertainty as to how far the changes observed with *C. fritschii* are artefacts due to the treatment used to induce synchronous division. Repetition of the pattern in a second growth cycle would be an indication that it is correlated with the normal growth cycle, but this has not yet been achieved.

It is to be expected *a priori* that mutation is more likely to reduce the efficiency of a complex process such as nitrogen fixation than to enhance it. Mutants of Azotobacter in which nitrogen fixation or associated processes are impaired have been obtained (e.g. Green, Alexander & Wilson, 1953; Mumford, Carnahan & Castle, 1959) but no mutants with increased nitrogen-fixing powers appear to have been described, although here the difficulty of devising a technique for selecting such mutants must be borne in mind. Increase in the relative activity of the fixation process itself will result in a greater amount of nitrogen fixed per unit amount of growth, e.g. increase in dry weight. The four strains of *Chlorogloea fritschii* studied by us all fixed no more, or less, nitrogen per unit dry wt. of organism than the wild type and our results are thus in accordance with expectation. Singh (1957) has reported an X-ray-induced mutant of *Mastigocladus laminosus* with increased nitrogen-fixing capacity but it is not clear whether nitrogen fixation was increased in the above sense. An increase in final yield of cell material without an increase in the relative rate of nitrogen fixation will, of course, lead to a greater total fixation, as it did in the test of our X-irradiated strain (Table 1). We have not investigated this instance further, but it might well depend on some quite trivial circumstance as, for example, the greater production of extracellular materials capable of forming complexes with inorganic ions (Fogg & Westlake, 1955) leading to a greater availability of trace elements and this to a longer period of growth. In any case, conditions affecting rates of growth and yields are likely to be different in laboratory cultures and in the field, so that a strain which shows high nitrogen-fixing capacity or high yields in laboratory culture would not necessarily be of practical use in increasing the fertility of agricultural land. However, an increased production of extracellular nitrogenous products, such as observed with all four of our treated strains, might be of value if, as seems possible from the work of Allen and Arnon (see Arnon, 1958), these products are more or less directly assimilated by crop plants such as rice.

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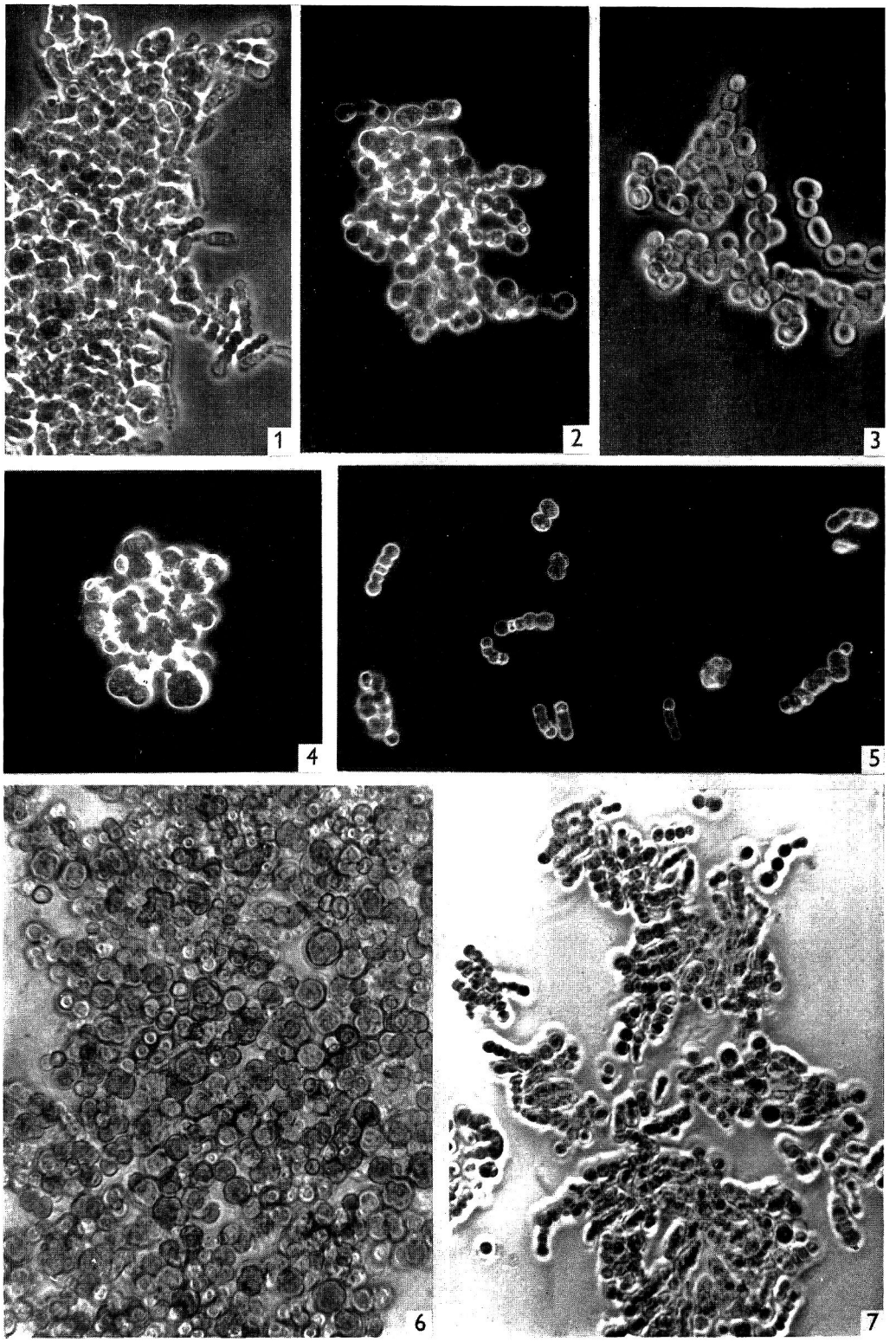
EXPLANATION OF PLATE 1

Phase-contrast photomicrographs of living material of *Chlorogloea fritschii*, illustrating its life-cycle. Fig. 1, stage I; Fig. 2, stage II; Fig. 3, stage III; Fig. 4, stage IV; Fig. 5, filaments with heterocysts; Fig. 6, stage IV starting material for synchronous culture; Fig. 7, synchronized population of stage I cells (all $\times 540$).



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(*Facing p* 360)