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The Occurrence of Polythionates as Intermediates in the Metabolism of Thiosulphate by the Thiobacilli

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SUMMARY

Further evidence for the inclusion of polythionates in the pathway of thiosulphate oxidation by members of the genus *Thiobacillus* is presented. Manometric experiments showed tetrathionate oxidation to be a stage in thiosulphate oxidation by suspensions of *T. thioparus* and *T. thiocyanoxidans*. *Thiobacillus thioparus* accumulated a sufficient concentration of polythionate in the medium to allow chromatograms to be prepared, and the type of polythionate accumulated was influenced by the ratio between the sodium and potassium ions in the medium. The restriction of the oxidation of tetrathionate at the lower concentrations of K^+ may be due to a restriction in the entry of phosphate into the cell during growth; this is discussed.

INTRODUCTION

The first report of polythionate accumulation by a *Thiobacillus* sp. growing in a thiosulphate medium was made by Nathansohn (1902) when he described the accumulation of an incompletely oxidized sulphur compound which did not react with iodine, but which was oxidized to sulphate by bromine. Since that time there have been several attempts to show the accumulation of polythionates by these organisms. Starkey (1935) was unable to detect the formation of polythionates by pure cultures of *T. thioparus* and *T. novellus*. He showed the formation of tetrathionate in cultures of *T. trautweinii*, which he considered was a heterotrophic organism and which has since been reported to be facultatively autotrophic (Parker & Prisk, 1953). Skarzynski & Szczepkowski (1959), by using a chromatographic method, were unable to detect the presence of polythionates in cultures of *T. thioparus*; they concluded that polythionates were not concerned in thiosulphate oxidation. The manometric experiments of Vishniac (1952) suggested that tetrathionate and trithionate were intermediates, whilst Trudinger (1959) showed the incorporation of radioactive ^{35}S into tetrathionate and trithionate, and subsequently into a compound which he was unable to identify. Parker & Prisk (1953) showed the formation of tetrathionate by some *Thiobacillus* spp., but not by *T. thioparus*, and Pratt (1958) showed the formation of polythionate by the strains of *T. thioparus* and *T. thiocyanoxidans* used in this work.

METHODS

Organisms

Thiobacillus thiocyanoxidans. A strain derived from a single rod obtained from a crude culture oxidising thiocyanate.

Thiobacillus thioparus. The original culture was obtained from the National

Collection of Industrial Bacteria—NCIB 8370—described as 'Starkey's original non-motile strain'. This culture was purified by the isolation of a single colony on a thiosulphate agar plate.

Both these organisms have been maintained on thiosulphate agar slopes with weekly subculturing.

Thiosulphate medium

A solution containing: 10·0 g. $Na_2S_2O_3.5H_2O$; 3·5 g. KH_2PO_4; 1·4 g. KOH; 0·1 g. NH_4Cl; in 1 l. glass distilled water was autoclaved at 120° for 15 min., and after cooling, 10 ml. each of the sterile salt solutions B and C were added. Salt solution B contained 1·0 g. $MgSO_4.7H_2O$; 0·2 g. $MnSO_4.4H_2O$; 0·2 g. $FeCl_3$; 3 ml. concentrated hydrochloric acid; glass distilled water to 100 ml. Salt solution C was a solution (1 %, w/v) of calcium chloride in glass distilled water. Both salt solutions were sterilized by autoclaving at 120° for 15 min. The complete medium was at pH 7. Solid media were prepared by adding 4 % (w/v) washed agar to thiosulphate medium prepared at twice the concentration shown above to give a final agar concentration of 2 % (w/v).

Suspensions of organism

The organisms were grown in 2 × 5 l. batches aerated with air containing 5 % (w/v) carbon dioxide at 30°–32° for 3–4 days. Harvesting was carried out with a de Laval centrifugal separator and a refrigerated M.S.E. centrifuge. All the equipment coming into contact with the organisms during harvesting was sterilized before use. Ten l. of thiosulphate medium produced about 1·0 g. wet wt. organism which was suspended in 20 ml. of M/15 phosphate buffer (pH 7·0) made from Na_2HPO_4 and KH_2PO_4.

Manometry

Manometric experiments were carried out in a conventional Warburg apparatus under aerobic conditions at 30°. Carbon dioxide was absorbed with filter paper soaked in 0·2 ml. 20 % (w/v) KOH solution in the centre well. Substrate (0·5 ml. M/50) was put in the side arm, 1·0 ml. of cell suspension in the main compartment with M/15 phosphate buffer (pH 7·0), to bring the total volume of the flask contents to 3·0 ml. The flasks and manometers were equilibrated in the bath for 15 min. before the reaction was started.

Estimation of polythionate

The method used was that described by Starkey (1934) which depends on the conversion of polythionates to thiosulphate and sulphite when heated with KOH.

Chromatography

Identification of the accumulated polythionates was attempted by ascending chromatography, with the solvent isopropanol + acetone + water + potassium acetate (50 + 20 + 30 + 2 g.) described by Pollard, McOmie & Jones (1955). Portions (10 ml.) of culture filtrate was freeze-dried, taken up in 0·5 ml. distilled water, and spotted on acid-washed Whatman No. 1 chromatography paper. After running for 5 hr.

the chromatograms were dried, sprayed with 0·5 N-silver nitrate, and excess silver removed by successive washings with water, 50% (w/v) sodium thiosulphate solution, and water. The markers used for chromatography were sodium thiosulphate (Analar) and the potassium salts of tetrathionate and trithionate prepared by the methods described by Stamm, Goehring & Feldmann (1942).

RESULTS

Manometric experiments

Manometric experiments on the oxidation of thiosulphate showed similar results for suspensions of both organisms. The change in slope of the thiosulphate oxidation curve reported by Vishniac (1952) was observed, although in our experiments this change occurred after an oxygen consumption in excess of the 56 μl. reported by

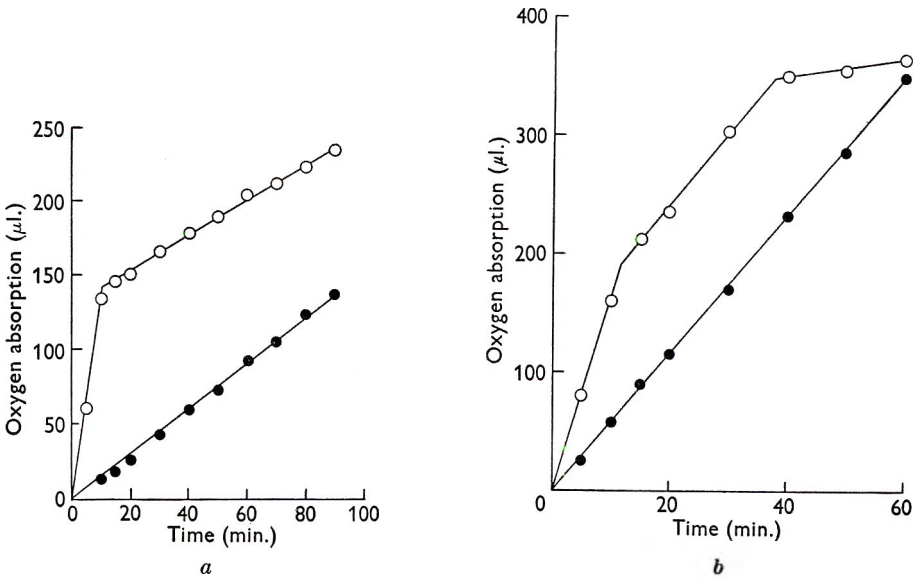

Fig. 1. (*a*) Oxidation of thiosulphate ○, and tetrathionate ●, by suspensions of *Thiobacillus thioparus*. (*b*) Oxidation of thiosulphate ○, and tetrathionate ●, by cell suspensions of *Thiobacillus thiocyanoxidans*.

Vishniac. Figures. 1 *a* and *b* shows the rates of thiosulphate and tetrathionate oxidation by *Thiobacillus thioparus* and *T. thiocyanoxidans*. We endeavoured to obtain evidence of the presence of tetrathionate at the time when the change in slope occurred. To stop the reaction, the reaction mixture from the flasks was tipped into ethanol, the organisms centrifuged down and the supernatant fluid freeze-dried. Chromatograms showed no trace of polythionate.

Accumulation of polythionates

In the standard medium cultures of *Thiobacillus thioparus* accumulated considerable amounts of polythionate in the medium during growth. The chromatogram obtained from cultures of *T. thioparus* grown at 32° in static culture for 67 hr. showed a spot which corresponded to the trithionate marker. With further incubation

another spot running in advance of the tetrathionate marker appeared which we think may be pentathionate. Our attempts to prepare a pentathionate standard with which to make a direct comparison were unsuccessful.

In their review on the thiobacilli, Vishniac & Santer (1957) gave details of a requirement for either phosphate or arsenate to allow complete oxidation of thiosulphate to sulphate by suspensions of *Thiobacillus thioparus*. Experiments were therefore carried out with media containing higher concentrations of sodium potassium phosphate buffer than usual in an attempt to suppress the accumulation of polythionate in cultures of *T. thioparus*. The results of such an experiment are shown in Table 1. The phosphate concentrations used were of the same order as those described by Vishniac & Santer (1957) for their manometric experiments.

Table 1. *The effect of phosphate concentrations upon the utilization of thiosulphate and the accumulation of polythionate* (S_nO_6) *by* Thiobacillus thioparus

Cultures were grown in 250 ml. conical flasks containing 50 ml. of medium. Each culture was inoculated with 0·5 ml. of an aseptically filtered 4-day thiosulphate grown culture.

	Phosphate concentration														
	M/10			M/20			M/30			M/100			M/250		
Time (hr.)	S_2O_3	S_nO_6	pH	S_2O_3	S_nO_6	pH	S_2O_3	S_nO_6	pH	S_2O_3	S_nO_6	pH	S_2O_3	S_nO_6	pH
0	196	2	6·78	201	0	6·78	205	1	6·78	208	0	6·46	198	1	5·97
23	196	6	6·85	192	8	6·84	195	9	6·78	196	10	6·59	190	11	6·23
47	168	4	6·68	162	31	6·74	175	22	6·71	167	37	6·78	137	37	6·55
71	143	3	6·65	153	27	6·71	129	64	6·83	130	75	7·00	78	59	6·39
95	126	2	6·63	117	53	6·78	86	102	6·89	86	90	7·27	2	77	5·86
115	75	2	6·59	60	100	6·75	47	119	6·76	53	124	7·36	0	75	4·07
139·5	79	4·5	6·6	23	109	6·72	18	128	6·74	18	162	7·32	0	83	4·23

Polythionate accumulation was greatly decreased at the highest concentration but in this medium the thiosulphate was not completely utilized. Examination of the chromatograms from these cultures again showed two spots other than that for thiosulphate. The unknown component running in advance of tetrathionate again appeared, but in place of trithionate a spot corresponding to the tetrathionate marker was observed.

The effect of varying the ratio between sodium and potassium ions in the medium was examined. The method used for increasing the phosphate concentration of the medium in the previous experiment increased the ratio between the sodium and potassium ions in the medium. Thus the medium described in the methods section has a ratio of 0·93 g. ion sodium:1 g. ion potassium, whereas in the experimental cultures this ratio varied between 4·2:1 for M/10 phosphate and 21·4:1 for M/250 phosphate. It seemed possible that this increased ratio between the sodium and potassium ions in the medium might be responsible for the change in the type of polythionate accumulated by the organism. By the replacement of some of the potassium salts in the standard medium by the equivalent amounts of the sodium salts, media having ratios between the sodium and potassium ions of 2·4:1, 5·6:1, and 8·7:1 were prepared. The type of polythionate accumulated by the organism growing in these media was compared with that accumulated during growth on the standard medium. A chromatogram was obtained from a culture in a medium having a ratio between the sodium and potassium ions of 5·6:1, and was typical of the

chromatograms obtained from the other two media which had high ratios between the sodium and potassium ions. The chromatogram showed accumulation of tetrathionate instead of trithionate, and also the component which ran in front of tetrathionate.

Further experiments were made to investigate the effect of decreasing the ratio of sodium ion to potassium ion below 0·93:1. Substitution of part of the sodium thiosulphate by the equivalent amount of the potassium salt produced a series of media having ratios of 0·81:1, 0·57:1, and 0·45:1, between the sodium and potassium ions. The type of polythionate accumulated during the growth of *Thiobacillus thioparus* in these media was compared with that accumulated during growth in the standard medium. Typical chromatograms obtained from the cultures grown in the medium having a ratio of Na:K of 0·57:1 showed the accumulation of trithionate and the component which ran in advance of the tetrathionate marker.

DISCUSSION

Evidence has been presented for the inclusion of polythionates in the pathway of thiosulphate oxidation by the thiobacilli and supports that obtained by Vishniac (1952) and Trudinger (1959) that the initial stages in thiosulphate oxidation by the thiobacilli are:

$$S_2O_3 \rightarrow S_4O_6 \rightarrow S_3O_6$$

Manometric experiments with suspensions of *Thiobacillus thioparus* and *T. thiocyanoxidans* showed both organisms to be capable of oxidising tetrathionate. The rate of oxidation of this substrate is similar to that shown by the second part of the thiosulphate oxidation curve (see Fig. 1*a* and *b*). These results are similar to those obtained with *T. thioparus* by Vishniac (1952), except that the change in slope occurred at an oxygen absorption in excess of the 56 μl. observed by Vishniac. Baalsrud & Baalsrud (1954) suggested that in the case of *T. denitrificans* such changes in the rate of thiosulphate oxidation were brought about by damage which the cells sustained when the pH value of the medium decreased during growth. Extending this view to *T. thioparus* and *T. thiocyanoxidans* it seems possible that the disparity between our results and those of Vishniac about the quantity of oxygen utilized before the change in rate of thiosulphate oxidation occurs was caused by this phenomenon.

Although we did not observe the presence of polythionates in the reaction vessels during manometric experiments, *Thiobacillus thioparus* was shown to accumulate considerable amounts of polythionate in the medium during growth on thiosulphate. Cultures grown in the standard thiosulphate medium were shown by chromatographic methods to accumulate trithionate and what may be pentathionate. The work of Vishniac & Santer (1957) showed a requirement by suspensions of *T. thioparus* for phosphate or arsenate to allow complete oxidation of thiosulphate. Increasing the phosphate concentration of the growth medium to M/10 resulted in a considerable suppression of polythionate accumulation. Further experiments which involved altering the ratio between the sodium and potassium ions in the medium indicated that this ratio was also of importance in the oxidation of thiosulphate by growing cultures.

The absorption of potassium in yeast, *Escherichia coli*, '*Bacterium lactis aerogenes*' and some marine bacteria occurs only in the presence of a substance which can pro-

duce energy; phosphate uptake by these species of organisms is stimulated by the presence of potassium, each K^+ ion being associated with the loss of a H^+ ion from the cell and each phosphate being associated with the gain of a H^+ ion by the cell (Rothstein, 1959). Comparable factors may well operate here, the lower concentrations of potassium in some of the media restricting the amount of phosphate entering the cell and thus preventing the further oxidation of tetrathionate.

Our initial experiments on the accumulation of polythionate in the medium were carried out with cultures of *Thiobacillus thiocyanoxidans* and *T. thioparus*; only very small amounts of polythionate were observed in the cultures of *T. thiocyanoxidans*.

From time to time there appear in the literature indications of more complex sulphur compounds as intermediates in the pathway of thiosulphate metabolism. Skarzynski & Szczepkowski (1959) suggested that polythionates were not concerned, and that the sulphur passed through organic intermediates. Santer *et al.* (1960) also suggested the existence of an organic intermediate, and Trudinger (1959) showed the incorporation of radioactive sulphur into an unknown compound after its incorporation into tetrathionate and trithionate. There seems little doubt that polythionates are concerned in thiosulphate metabolism, and perhaps the suggested organic intermediates are concerned in the unknown steps which convert trithionate to sulphate.

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On the Survival of Frozen Bacteria

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SUMMARY

Steadily growing *Aerobacter aerogenes* organisms were largely killed by slow freezing in buffer or by freeze drying. 100% survival was obtained after dropping bacteria suspended in 10% aqueous glycerol into liquid nitrogen and thawing. Ten per cent solutions of diethylene glycol, *i*-erythritol, glucose, sucrose or polyethylene glycol (MW = 10,000) protected equally well; the last three substances did not penetrate the cell cytoplasm. The most lethal medium was dilute NaCl; broth, water or a dilute salt mixture were moderately lethal. These findings are incompatible with the view that the lethal effects of freezing are connected with osmotic shock or that protection from freezing damage requires (*a*) penetration of the protective agent or (*b*) osmotic dehydration of the cytoplasm. Cells frozen and thawed, even with a protective agent, showed a lowered rate of glycerol oxidation and a higher death rate when starved at the optimal temperature and pH value for growth. The storage life of frozen organisms at −20° depended on the protective agent used; only glycerol permitted extended storage.

INTRODUCTION

Rapid chilling of microbial suspensions by dropping them into liquid nitrogen has been used by some workers as a means of disrupting the organisms. Moses (1955) obtained preparations of citric acid cycle enzymes from the mould *Zygorrhynchus moelleri* in this manner; Postgate (1960) used it to obtain a particulate sulphite reductase preparation from *Desulfovibrio desulfuricans*; Wade & Lovett (1961) extracted ribonucleic acid from *Escherichia coli* type B by such a procedure. Paradoxically, however, a considerable volume of reports exists demonstrating that bacteria survive chilling to very low temperatures. Earlier work was reviewed by Luyet & Gehenio (1940), who pointed out that much of the data were not quantitative, but who recorded figures as divergent as 11% kill with *E. coli* in broth frozen to −78° (Sanderson, 1925) to 99·98% kill of *Bacterium typhosum* (*Salmonella typhi*) in broth (Smith & Swingle, 1905) after freezing at −17·8°. Haines (1938) obtained only 5% killing of a staphylococcus by freezing in solid CO_2 and kills of other micro-organisms were small. Wieser & Osterud (1945) recorded modest kills of *E. coli* on freezing (15% at −2°, 50% at −5° or −10°); Straka & Stokes (1959) reported about 10% kills of *E. coli* and pseudomonads on freezing in beef broth; Wood & Rosenberg (1957) recorded 100% survival of yeast frozen in phosphate buffer at −60°. Mazur, Rhian & Mahlandt (1957) reported that slow chilling and rapid warming afforded maximum survival of *Pasteurella tularensis* frozen in 'gelatin saline' to temperatures between −10° and −70°. In all circumstances rather low survivals compared with unfrozen

controls were obtained, but Mazur (1960) mentioned an experiment in which the addition of sucrose or lactose (0·3 M) permitted nearly 100 % survival after slow cooling and slow warming.

Harrison & Cerroni (1956) recorded very slight killing of *Microbacterium flavum* on repeated freezing to −22° and thawing; *Escherichia coli* showed much greater sensitivity though both species were about equally sensitive to mechanical damage. Major, McDougal & Harrison (1955) studied the survival of various bacteria stored frozen in broth at −22° and showed that in most instances the survival was longer the denser the initial population; their data did not include survivals measured immediately after freezing except in the case of *E. coli* frozen in distilled water, which then showed a small (about 10 %) kill and no population effect. Record & Taylor (1953) failed to observe a population effect with *E. coli* frozen in phosphate buffer at −78° and thawed at once (kill 40 %). Harrison (1955) showed that the population effect did not occur with *Lactobacillus fermenti* after a single freeze and thaw (about 90 % kill) but that it was manifest after repeated freezing and thawing with intervals of storage. Aeration influenced the resistance of *E. coli* to freezing in a complex manner. Harrison (1956) studied the influence of environment on freezing three bacterial species at −22° and showed that broth was a more lethal environment for freezing than was distilled water. Suspensions chilled to −22° but prevented from freezing with NaCl (4·6 M) or glycerol (4·1 M) had similar store lives to frozen suspensions.

Hollander & Nell (1954) showed that addition of 15 % glucose to broth permitted 100 % survival of *Escherichia coli*, *Diplococcus pneumoniae*, *Treponema pallidum* and probably of *Rhodospirillum rubrum*. They considered the protective effect of glycerol to be analogous to its action on bull spermatozoa (Polge, Smith & Parkes, 1949) or erythrocytes (Lovelock, 1953). Howard (1956) used a frozen glycerol broth according to Hollander & Nell's prescription to preserve cultures of bacteria; so did Tanguay (1959) and Floodgate & Hayes (1961). Fox & Hotchkiss (1957) used freezing in aqueous glycerol to preserve pneumococci in a receptive state for transforming deoxyribonucleic acid. None of these workers quoted quantitative data on survival after freezing. Squires & Hartsell (1955) reported some quantitative data on the survival of *E. coli* after freezing at −25° and storage at −9°; as an additive 5 % glycerol was superior to gelatin and a vegetable oil for organisms frozen in a buffered broth. Quadling (1960) reported 100 % survival of *Xanthomonas phaseoli* on freezing in strong glycerol broth, though exposure of the organisms to this medium killed about 60 % of the population without freezing.

In the course of other studies we wished to preserve populations of steadily growing *Aerobacter aerogenes* while retaining as nearly as possible 100 % viability, and we performed a number of experiments on deep freezing. These are reported below, since they have some relevance to the question of freezing damage and were performed on a single culture of the same organism in a phase of growth analogous to the logarithmic phase, commonly associated with enhanced sensitivity to stresses of various kinds including freezing (Toyokama & Hollander, 1956); in addition they deal largely with survival in solutions of single pure substances.

METHODS

Aerobacter aerogenes, obtained from Professor Sir Cyril Hinshelwood's laboratory, had been maintained for some 18 months in continuous aerobic culture in a defined (0·2 %, w/v) glycerol medium analogous to that used for *Cloaca cloacae* by Herbert, Elsworth & Telling (1956). We shall describe the cultural conditions and history of the strain in more detail elsewhere; for present purposes it is sufficient to record that the dilution rate was 0·25 vol./hr., pH 7 ± 0·1, 40°; growth limited by glycerol concentration to 1·05 ± 0·05 mg. dry wt. organism/ml. This corresponded to a viable count of about $2{\cdot}4 \times 10^9$ organisms/ml., equal to the total count determined by a combination of microscopy and interferometry (Norris & Powell, 1961).

Viability was determined by slide culture at 37° (Postgate, Crumpton & Hunter, 1961) on the growth medium supplemented with yeast extract (Difco, 0·1 %, w/v), casein hydrolysate (Difco, 0·1 %, w/v), a tryptic meat broth (10 %, v/v) and set with agar (1·5 %, w/v). The nitrogenous supplements should have ensured growth of 'nutritionally injured' organisms of the type demonstrated by Straka & Stokes (1959). Before slide culture, frozen populations were thawed by diluting in 50 or 500 vol. saline phosphate buffer (9 parts 0·137 M-NaCl + 1 part of a 1:5 mixture of 0·066 M-KH_2PO_4 + 0·066 M-Na_2HPO_4; pH 7·40 ± 0·05) at room temperature. In one experiment death curves were measured in a saline 'tris' buffer (9 parts 0·137 M-NaCl + 1 part 0·048 M-2-amino-2-hydroxymethylpropane-1:3-diol ('tris') + 0·316 mM ethylenediaminetetra-acetic acid (EDTA); pH 7·00 ± 0·05). Reagents of analytical grade were used except that *i*-erythritol and D-glucose were 'bacteriological' sugars, diethylene glycol was a re-distilled (b.p. 135–137°) specimen kindly provided by Major L. H. Kent, polyethylene glycol was a well-dialysed, freeze-dried preparation of mean mol. wt. 10,000 kindly provided by Dr B. R. Record. Water was distilled and then de-ionized by treatment with a mixed bed ion exchange resin. Percentages recorded in this paper are w/v except in the cases of glycerol and diethylene glycol when they are v/v.

RESULTS

Sensitivity of the population to cold

Gram-negative bacteria in the logarithmic phase of growth are sometimes killed by cold shock even when no freezing takes place (Sherman & Cameron, 1934; Hegarty & Weeks, 1940). This phenomenon requires a 'toxic' suspending fluid of low osmotic pressure (Meynell, 1958) and is not shown by staphylococci (Gorrill & McNiel, 1960). The population of *Aerobacter aerogenes* used in this work did not show appreciable cold shock of this kind. Organisms harvested from the culture chamber, washed by centrifugation in 0·8 % NaCl and diluted into cold buffer showed 99 % viability in saline phosphate buffer at room temperature, 98 % at 4°, 94 % after squirting on to frozen buffer at its melting point. Suspensions were, however, killed when 10 ml. portions in tubes were frozen in solid CO_2: the initial viability fell from 96 % to less than 1 % in these conditions. Use of *mist. desiccans* (Fry & Greaves, 1951) in place of buffer afforded no protection (93·8 % viable population fell to less than 0·3 % after freezing in *mist. desiccans* with solid CO_2). Effluent from the continuous culture freeze-dried without further treatment fell from about 98 to 5 %; centrifugation and resuspension in *mist. desiccans* provided no significant protection (viability fell to

6 %). The low survivals obtained in these conditions are probably attributable to our use of organisms in what corresponds to a logarithmic phase of growth. Organisms re-suspended in the original medium (containing 0·2 % glycerol) and freeze-dried had the marginally greater viability of 10 %.

Protection from killing by freezing

A thick suspension (equiv. about 50 mg. dry wt. organism/ml.), resuspended in a salt mixture corresponding to the growth medium without glycerol, was allowed to drop from a pipette into liquid nitrogen, so that the suspension froze as discrete beads. These were allowed to thaw to room temperature and showed 35 % viability. This observation suggested that deep freezing might provide a method of preserving the population, and the viability (in various suspending media) after deep freezing with liquid nitrogen was studied. Organisms were harvested from the continuous culture, centrifuged and made up as suspensions equivalent to 1 or 10 mg. dry wt. organism/ml. Within 5–15 min. these were frozen as beads of about 0·05 ml. by dropping from a pipette into liquid nitrogen. This procedure took 1–2 sec. The beads were then removed after 3 to 10 min. with cold forceps and thawed by dropping into 25 or 2·5 ml. saline phosphate buffer at room temperature. This procedure adjusted the population density to a value suitable for slide culture; thawing in these conditions took 2–3 sec.

Table 1 records a representative set of experiments. Suspension in 10 % aqueous glycerol protected all the viable organisms from killing by freezing (1*b*; 2*a*, *b*, *c*); there was no population effect of the kind reported by Major *et al.* (1955) over a 100-fold range of suspension concentrations (1*c*, *d*; 2*a*, *b*, *c*). A tryptic broth of meat allowed considerable killing (4*f*); glycerol as prescribed by Hollander & Nell (1954) protected the organisms from the toxicity of broth (4*g*). Unlike the organisms studied by Major *et al.* (1955), Harrison (1956) and Clement (1961), considerable kills of our bacteria occurred on freezing in distilled water (1*a*); comparable kills were obtained when suspensions equivalent to 1 mg. dry wt. organism/ml. were frozen and thawed in (i) distilled water which had not been treated with resin (viability: 58 %), (ii) water distilled twice, the second time from glass (viability: 67 %), (iii) the distilled and resin-treated water usually used in this work (viability: 68 %) or (iv) our local tap water (viability: 53 %). Sodium EDTA (10 mM) had no significant effect on the kill (viability: 42 % with EDTA, 49 % without). These experiments make it unlikely that impurities in the distilled water or population effects contributed to the kill. Carry-over of salts in the washing procedure likewise did not account for the kill, since a suspension repeatedly centrifuged in distilled water gave 68, 47 and 48 % viabilities after the first, second and third centrifuging; the unfrozen control was 98 % viable after the third centrifuging.

NaCl solutions were the most lethal environments encountered and the kill after freezing and thawing was independent of the salt concentration over a wide range (5*a–e*). Buffered sodium chloride with 'tris' made little difference (5*f*), but phosphate had a marginal protective effect (5*g*) and probably accounts for the lowered kill in the substrate-free medium (1*c*, *d*) which was essentially a phosphate mixture with trace elements. Addition of glycerol to the medium afforded partial protection (1*e*). Harrison (1956) showed that glycerol protected organisms frozen in salt solutions.

Glycerol at 10 % also protected stationary phase organisms harvested from an agar slope (3*a*).

The glycerol concentration could be reduced to 2 %, but not below this value, without losing its protective effect. It could be replaced by 10 % aqueous solution of diethylene glycol (4*a*), glucose (4*b*), *i*-erythritol (4*e*), sucrose (4*c*) and a polyethylene glycol of mean molecular weight 10,000 (4*d*). Though some of these solutions had slight intrinsic toxicities, lowering the initial viability of the population from about 97 to about 90 %, the survivors were wholly protected from freezing. The molarities of these solutions were: glycerol, 1·08 M; diethylene glycol, 0·93 M; *i*-erythritol, 0·82 M; glucose, 0·55 M; sucrose, 0·29 M; polyethylene glycol, 0·01 M.

Table 1. *Viabilities of suspensions of* Aerobacter aerogenes *after a single freeze and thaw*

Aerobacter aerogenes growing at an imposed doubling time of 2·8 hr. in continuous culture were centrifuged, drained and re-suspended in the solutions indicated at about equiv. 10 mg. dry wt. organism/ml. in experiments 1 and 2, equiv. 1 mg. organism/ml. in experiments 3, 4 and 5. Drops (about 0·05 ml.) of suspension were frozen in liquid nitrogen diluted in buffered saline and the % viable organism determined by slide culture.

Experiment	Medium	Viability (%) Control	Frozen
1*a*	Distilled water	97	63
1*b*	Aqueous 10 % glycerol	97	96
1*c*	Basal medium without carbon source	97	39
1*d*	As 1*c* but equiv. 1 mg. dry wt. organism/ml.	97	32
1*e*	Basal medium + 10 % glycerol	97	70
2*a*	10 % glycerol	97	96
2*b*	As 2*a*, but equiv. 1 mg. dry wt. organism/ml.	97	95
2*c*	As 2*a* but 0·1 mg. organism/ml.	97	95
3*a*	10 % glycerol; stationary phase organisms from agar slope	90	89
4*a*	10 % diethylene glycol	97	95
4*b*	10 % glucose	91	88
4*c*	10 % sucrose	98	95
4*d*	10 % polyethylene glycol, mol. wt. about 10,000	92	89
4*e*	10 % *i*-erythritol	97	95
4*f*	Tryptic meat broth	93	48
4*g*	As 4*f* + 15 % glycerol	92	91
5*a*	M-NaCl (5·6 %)	95	12
5*b*	0·4 M-NaCl	97	5
5*c*	0·1 M-NaCl	97	11
5*d*	0·05 M-NaCl	96	25
5*e*	0·005 M-NaCl	96	23
5*f*	'Saline tris buffer'	98	15
5*g*	'Saline phosphate buffer'	99	36

An illusion of high viability could have occurred if freezing had induced lysis of the suspensions before the organisms were spread on the agar films for slide culture. No signs of lysis were observed on the slide cultures, and if such lysis had been significant it should have been detectable as an optical density change on thawing a frozen suspension. This was not observed: a suspension equiv. 2·2 mg. dry wt. organism/ml. 10 % glycerol had an optical density reading (540 mμ) of 0·258 on dilution (1 vol. 10) in 0·85 % NaCl; a portion of the same suspension frozen in liquid nitrogen and thawed before dilution had an optical density of 0·262.

Effect of freezing on physiology

Other work in our laboratory has shown that populations of logarithmic phase *Aerobacter aerogenes* grown as described here have an initially linear death rate at their growth temperature and pH value in a non-nutrient buffer (see Postgate *et al.* 1961; Fig. 1). Glycerol and other substrates which the organisms metabolize affect the death rate, but diethylene glycol, which is not utilized by this strain, had no effect at all up to 0·2 %. The innocuous nature of diethylene glycol enables one to test the effect of deep freezing upon subsequent death. A suspension equiv. 1 mg.

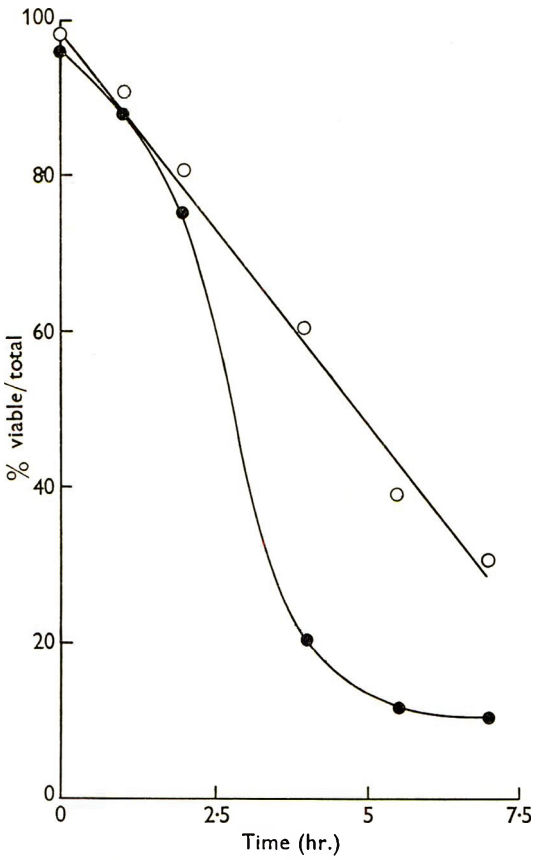

Fig. 1. Death rate of frozen and thawed *Aerobacter aerogenes*. *A. aerogenes* (equiv. 1 mg. dry wt. organism/ml.) grown as in Table 1 were washed by centrifugation, frozen in 10 % diethylene glycol at −200°, thawed, diluted 50-fold and aerated in saline 'tris' buffer (pH 7·00 ± 0·05) at 40° (the growth temperature and pH value). Viabilities were determined by slide culture; the control was a similar suspension not frozen. ○, Control; ●, frozen and thawed cells.

dry wt./ml. 10 % diethylene glycol was divided in two and one portion frozen in liquid nitrogen and thawed. One ml. of each was diluted into 50 ml. non-nutrient buffer (diethylene glycol finally 0·2 %) and incubated with aeration at 40°. Figure 1 shows the viability of samples taken at intervals: the population died more rapidly after deep freezing in the glycol, though the initial viabilities were similar.

Deep freezing might affect the respiratory system. A population (equiv. 10 mg. dry wt./ml.) frozen in 10% glycerol was thawed in 10 vol. saline phosphate and the Q_{O_2} determined manometrically at 40°; it was $-183\,\mu l.\ O_2$/mg./hr. A control treated similarly but stored at −4° in glycerol during the freezing and thawing had a Q value of $-229\,\mu l.\ O_2$/mg./hr. In a second experiment the respective values were −243 and $-325\,\mu l.\ O_2$/mg. hr.

Effect of rate of freezing on survival

Mazur *et al.* (1957) reported that maximum survival of *Pasteurella tularensis* was obtained with slow freezing and rapid warming. In our studies warming was always as rapid as practicable. Some crude experiments were undertaken to seek any effect of freezing rate: 0·2 ml. of a suspension (equivalent to 1 mg. dry wt./ml.) in 10% glycerol was frozen in a deep freezer at −20° (taking 3 ± 1 min. to solidify) beside 2·3 ml. of a similar suspension (which took about 20 min. to solidify). The specimens were deliberately placed where freezing would be slow. No differences among the viabilities were observed: control, 98%; quickly frozen suspension, 98%; slowly frozen, 97%. Freezing in liquid nitrogen by the procedure we routinely used took 1–2 sec.; freezing in isopentane chilled to −190° took much less than 1 sec. and, though exposure to isopentane killed a few bacteria (97% viability reduced to 95%), deep freezing had no subsequent effect (95% viability).

Penetration by protective agents

The substances tested as protective agents against freezing damage were chosen as three likely to penetrate freely into the cell cytoplasm (glycerol, diethylene glycol, erythritol) and three unlikely to do so on a large scale (glucose, sucrose, polyethylene glycol). It was necessary to know whether in fact these materials behaved as expected. Mager, Kuczynski, Schatzberg & Avi-dor (1956) showed that the optical densities of live bacterial suspensions in osmotically active solutions were greater than in distilled water, and interpreted this 'optical effect' as an increase in the refractive index of the cytoplasm consequent on its adjustment to the osmotic pressure of the environment. Materials that penetrate to the cell cytoplasm should be osmotically neutral and should show no such effect. The permeability of the bacteria used in this work was examined by using this principle, after preliminary tests had shown that a maximum optical effect occurred with 0·4 M-NaCl (optical density in 0·4 M-NaCl = 130% that in distilled H_2O). The optical densities of suspensions equivalent to 0·13 mg. dry wt., 95–100% viable bacteria/ml. medium were compared in 0·4 M solutions and in distilled water; the extents of the optical effect were: glycerol, −4%; diethylene glycol, −1·6%; *i*-erythritol, +2%; glucose, +15%; NaCl control, +34%. Some of the optical effect of glucose was doubtless masked by the relatively high molar refractive increment of glucose; with sucrose this property rendered the method inapplicable (optical effect −15%), and its penetration was investigated by using the thick suspension technique of Mitchell & Moyle (1956) and estimating sucrose refractometrically. The sucrose-penetrable space in bacteria centrifuged to constant volume in 0·1 M-sucrose was 19% of their total volume. This value is incompatible with penetration into the cells. Theoretically, the intercellular water between close-packed bacteria should occupy about 26% of the volume and

the inter+intracellular water about 70 % (Mitchell & Moyle, 1956). Penetration by polyethylene glycol was not studied; it was assumed not to penetrate the organisms because of its high mean molecular weight of 10,000.

Storage of frozen populations

Batches of frozen beads of bacterial suspension were transferred to cold, closed test tubes and stored at −20° in a deep freezer to examine their storage lives with various protective agents. The storage lives were dramatically different (Table 2).

Table 2. *Cold storage of frozen suspensions of* Aerobacter aerogenes

Suspensions of *Aerobacter aerogenes* (equiv. 1 mg. dry wt. organism/ml.) grown as in Table 1 were suspended in the 10 % aqueous solutions below, frozen as beads of about 0·05 ml. in liquid nitrogen and stored in closed tubes at −20°. Individual beads were thawed by dropping into 2·5 ml. buffered saline at 18 to 20° and the viabilities of the thawed suspensions measured by slide culture.

Solute		Storage life at −20°							
Glycerol	Days	0	2	6	10	20	27	40	
	Viability (%)	95	92	91	86	92	85	85	
Polyethylene glycol	Days	0	0·7	1·7	4	16			
	Viability (%)	96	80	87	70	65			
Diethylene glycol	Days	0	2	16					
	Viability (%)	98	72	53					
Sucrose	Hours	0	2	5	8	11·7	24		
	Viability (%)	98	78	72	60	25	25		
Glucose	Hours	0	2	4·5	7·5	16·5			
	Viability (%)	92	31	22	19	12			
i-Erythritol	Minutes	0	5	10	15	20	100		
	Viability (%)	94	98	9	3	4	1		
Glycerol*	Days	0	0·7	1·8	4·8	9	15·6	29	61
	Viability (%)	98	94	96	93	94	86	86	78

* In this case the suspension contained equiv. 10 mg. dry wt. organism/ml.

Even after 40 days a majority of those populations frozen in glycerol were viable, whereas after 10 min. the majority of those frozen in erythritol were dead. It is probable that the population taken at 5 min. from the erythritol series had not completely warmed up from the −200° of liquid nitrogen to the −20° of the storage cabinet. Other materials gave intermediate storage lives. The data given in Table 2 are representative of at least two sets of experiments with each storage medium; erythritol was tested three times owing to the exceptionally short storage life observed with it; polyethylene glycol was tested three times since on one occasion death was more rapid than that recorded in Table 2 (viability dropped from 87 to 20 % between the 24th and 40th hours). Beads of glycerol, polyethylene glycol and sucrose took on a damp appearance after 1–3 days' storage; those of erythritol and diethylene glycol did not. None of the beads appeared vitreous after freezing in comparison with a frozen solution of 50 % glycerol. Some indication that the population effect of Major *et al.* (1955) did not occur on storage with glycerol is indicated by the experiment also recorded in Table 2 in which a frozen population equiv. 10 mg. dry wt. organism/ml. survived at −20° in a manner similar to one equiv. 1 mg. dry wt./ml.

DISCUSSION

The mechanism of killing by freezing and of protection of organisms from such injury is complex and has been discussed by several of the workers mentioned in the Introduction. For the particular case of bacteria there is reason to believe that mechanical injury by ice crystals is not responsible (Harrison & Cerroni, 1956). Evidence exists that the major contributory factor is osmotic shock (Harrison, 1956) though Mazur's (1960) findings do not support this view. By analogy with the protective action of glycerol on erythrocytes (Lovelock, 1953), it has been suggested that partial dehydration by penetration of glycerol into the cell cytoplasm is necessary for bacteria to survive freezing (Hollander & Nell, 1954). We shall restrict our discussion to pointing out where certain of our data are relevant to these questions.

The necessity for penetration by the protective substance. With our populations, glycerol, erythritol and diglycol penetrated the bacterial cytoplasm. Glucose and sucrose did not, and it is not likely that the high-molecular weight polyglycol did so. As protective agents all these substances were effective; hence penetration is irrelevant to protection against killing by freezing.

The necessity for partial dehydration. The two classes of protective agent, penetrating and non-penetrating, have in common that they could both dehydrate the cell cytoplasm; one class would do this by osmotic dehydration, the other class would cause a more modest dehydration by physical dilution of the cell cytoplasm. If dehydration were necessary for protection, a high molecular weight substance should have no protective effect owing to its low osmotic pressure in 10 % solution; yet polyethylene glycol protected perfectly well.

Freezing as a form of osmotic damage. The protective action of materials that do not penetrate the organisms yet which were present at initially high molarities (glucose or sucrose) makes it unlikely that concentration of solute during freezing exerted any lethal effect on our populations. In addition, survival in distilled water ought to be maximal if osmotic damage of this kind were relevant, but with our population distilled water was as lethal an environment as broth or the basal medium. This observation conflicts with those recorded by Harrison (1956) and Clement (1961), who found distilled water a relatively innocuous environment for freezing; the difference may be due to our use of a different species, or to the fact that our organisms were in a state corresponding to the logarithmic phase of growth of a batch culture whereas those studied by Harrison and Clement were stationary. The phase of growth is known to influence markedly the sensitivity of bacteria to damage by freezing (Toyokama & Hollander, 1956).

Metabolic damage after freezing and thawing. Though protected populations were still viable after this treatment, it is clear that they sustained some damage since the deep-frozen organisms died more rapidly than controls in starvation conditions and had a reduced rate of substrate oxidation. Squires & Hartsell (1955) reported that freezing and storage altered the lags and growth rates of their bacteria and Straka & Stokes (1959) reported a 'nutritional injury' of frozen bacteria whereby a proportion of the population, after thawing, was found to be nutritionally exacting for materials of a peptide character.

Toxicity of the preserving agent. Though glycerol is widely used as a protective agent, toxic effects at the necessary concentration have been recorded. For example,

Quadling (1960) reported about 39 % survival after exposure of *Xanthomonas phaseoli* to 15 % glycerol broth though the whole 39 % then survived freezing and thawing. Our populations were not affected by 10 or 15 % glycerol (though 30 % aqueous glycerol killed 60 % of the organisms) but in other tests we have observed such toxicity. *Vibrio anguillarum* (NCMB 6) fell in viability from 55 to 40 %; *Chromobacterium violaceum* (NCIB 8182) fell from 92 to 87 %; *Escherichia coli* (Jepp) was uninfluenced (94 % in control, 96 % in 10 % glycerol). With our population of *Aerobacter aerogenes* slight toxicities occurred with certain substitutes for glycerol (Table 1) but, in agreement with Quadling, the survivors of such toxicity were protected from freezing damage.

Distinction between freezing and cold storage. Though several different chemicals protected our organisms against freezing damage, the storage life of a frozen suspension at $-20°$ was very much influenced by the nature of the protective agent; periods of maximum survival ranged from a few minutes in erythritol to many days in glycerol. Speculation about the reasons for these differences would not be fruitful on the data available, but it is obvious by arguments similar to those above that penetration or osmotic dehydration are irrelevant to store life in the frozen state. Our observations suggest that the population effect reported by Major *et al.* (1955) is not involved in the freezing or thawing processes. It is probably a phenomenon that only becomes manifest on storage and even then may not occur in protective environments such as 10 % glycerol.

Effect of freezing rate. In the protective environment of 10 % glycerol we could detect no significant effect of rate of freezing, though our experiments were cruder than those of Mazur *et al.* (1957) in that we knew the freezing rates only roughly. Mazur *et al.*'s experiments were concerned with survival in a non-protective environment, and in such conditions our organism seemed to differ from *Pasteurella tularensis*, since with *Aerobacter aerogenes* slow freezing of 10 ml. samples in buffer in solid CO_2 was more lethal (viability dropped from 95 to 1 %) than rapid freezing of similar samples in liquid N_2 (32–39 % in Table 1).

Record & Taylor (1960) showed that organisms which have been dried in sucrose solutions are subject to internal diffusion pressures on reconstitution due to sugar trapped between cell wall and protoplast membrane. Such diffusion pressures may cause disruption and death of the organisms; high molecular weight substances protect by decreasing the amount of sugar so trapped. During the present work diffusion pressures must have arisen when frozen preparations were in process of thawing, particularly during the experiments involving glucose and sucrose; we attribute the fact that our organisms survived freezing and immediate thawing to our use of solutions which were unlikely to have become as concentrated during this period as they would have done during a treatment that involved drying. The observations reported here help to resolve the paradox mentioned in the introduction. Moses (1955) and Postgate (1960) suspended their organism in very dilute buffer (0·0625 M-KH_2PO_4) and Wade & Lovett (1961) used distilled water containing traces of salts carried over in the washing procedure. These are among the most lethal environments encountered in the present work. Even broth had some protective effect, and the majority of studies in which high survivals have been shown were concerned with organisms suspended in such partially protective environments. Broadly speaking, one might expect maximum destruction of organisms on freezing in

'saline', minimum damage in a 10% solution of a non-electrolyte, intermediate degrees of damage in ordinary bacteriological media, distilled water or tap water.

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A Comparison of Methods for Classifying Rhizosphere Bacteria

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SUMMARY

A comparison was made between the following three methods of classifying bacteria: (1) division on associated characters, (2) identification by the use of Skerman's key, (3) classification by means of Affinity Index. Division on associated characters was of no value in this particular case. Skerman's key was adequate for the identification of the isolates, while the Affinity Index (a slight modification of the Similarity Index of Sneath) gave a comprehensive view of the relationships between isolates. It was considered that a random sample of 43 isolates selected from a collection of 318 rhizosphere bacteria formed a spectrum, rather than a series of groups.

INTRODUCTION

The reviews on rhizosphere bacteria (Clark, 1949; Katznelson, Lochhead & Timonin, 1948; Starkey, 1958) indicate that the successful identification of all the rhizosphere isolates has not yet been achieved. Many workers have devised their own systems of classification, e.g. the nutritional classification (Lochhead & Chase, 1943), morphology and gelatin digestion (Conn, 1948) and morphology, staining and dye tolerance (Clark, 1940). Sperber & Rovira (1959) used Skerman's key (1959) of identification in *Bergey's Manual* (1957) to identify over 80 % of their isolates to generic level. The isolates classified as *Arthrobacter* spp. by these authors were divided into 'arthrobacters' and 'arthrobacter-like-nocardias'. In a preliminary comparison between these isolates and type *Arthrobacter* spp. it became evident that there were certain generalized differences between Arthrobacter and the rhizosphere isolates. The main difference was in the coccal form; *A. globiformis* breaks up completely into perfect spheres while the rhizosphere isolates formed coccobacilli, with some rods in most older cultures. *A. globiformis* had certain of its stages Gram-positive, whereas the rhizosphere isolates did not show this Gram-variable characteristic; also, there were differences in the metabolic patterns. As a consequence of these observations it was decided to re-examine the complete data collected by Sperber & Rovira on their 318 isolates.

The principle of division on associated characters suggested by Sneath (1957*a*) was attempted with the 318 isolates. It was not possible to use the Similarity Index system of Sneath, as no electronic computer was available. In addition to this re-examination of existing results, 43 cultures were selected at random from the Sperber–Rovira collection, and together with 21 known cultures were subjected to a

further series of tests. The results from these tests were used to group and identify the cultures by three methods, the details of which appear in this paper together with the re-examination of the original results.

METHODS

Organisms. The original collection of rhizosphere isolates of Sperber & Rovira (1959) was maintained at 4° and subcultured on yeast extract-peptone-soil extract (YPS) agar (Bunt & Rovira, 1955) at intervals of 6 months. The named cultures from various laboratories listed below were:

Achromobacter A1, *Achromobacter* A2 (University of Melbourne); *Agrobacterium radiobacter* NCIB 8149 (Department of Scientific and Industrial Research, England); *Arthrobacter aurescens*, *A. citreus*, *A. globiformis*, *A. simplex*, *A. ureafaciens* (Department of Agricultural Science Service Laboratories, Ottawa, Canada); *Bacillus subtilis* NCTC 6342, *Flavobacterium* sp. (University of Melbourne); *Micromonospora* sp. (University of Sydney); *Nocardia asteroides* NCTC 6761, *N. rubra* (University of Sydney); *Pseudomonas fluorescens* (University of Adelaide); *P. medicaginis*, *P. syringae* (University of Melbourne); *Rhizobium trifolii* (from D. Norris, C.B. 721 from *Trifolium polymorphum*), *R. meliloti* (Q.A. 867 from Rothamsted), *Rhizobium* sp. (from G. D. Bowen Q.A. 549 from *Centrosema pubescens*); *Streptomyces venezualae* (University of Sydney); *Xanthomonas campestris* (University of Melbourne).

Basal medium. The yeast-extract peptone nitrate broth of Sperber & Rovira (1959), with 15 g. agar/l. for solid medium, was used.

Gelatin agar. Gelatin (0·4 %, w/v) in basal agar. Hydrolysis was observed by flooding the plates with saturated ammonium sulphate following incubation at 26° for 7 days.

Starch agar. Starch (0·2 %, w/v) in basal agar. Hydrolysis was observed by flooding the plates with iodine solution after 7 days at 26°.

Casein agar. Ten ml. of sterile 9 % (w/v) solution of powdered milk were added to every 100 ml. basal agar immediately before pouring plates. Hydrolysis was observed as a clearing round the colony.

High pH *medium.* Difco veal infusion (23 g.) and 15 g. agar/l. water; finally adjusted to pH 8·5.

Nutrient agar. Oxoid nutrient agar.

Apatite agar. As used by Sperber (1958).

Peptone yeast extract (*PY*) *broth.* Peptone (10 g.), Difco yeast extract (5 g.), sodium chloride (5 g.)/l. water. H_2S production was determined with lead acetate filter paper strip in the mouth of the tube.

Methylene blue agar. Basal agar + 1 % (w/v) glucose and 1 ml./l. of 0·5 % (w/v) methylene blue was tubed in 10 ml. lots and inoculated before setting. Those isolates which reduced more than half the depth of the agar were considered positive.

Sucrose mannitol sauerkraut (*SMS*) *broth.* Sucrose (5 g.), mannitol (5 g.), sauerkraut juice (20 ml.), sodium citrate (1 g.), magnesium glycerophosphate (1 g.), Casamino acids (1 g.), ammonium nitrate (0·1 g.), distilled water 1000 ml. (Harris, J. R., personal communication). This medium was used to demonstrate fluorescence when the selected cultures were retested.

Ulrich's milk. The reaction was recorded after 15 days at 26° (Ulrich, 1944).

Glucose broth. Ten g. glucose, 1 g. Difco yeast extract, 0·4 g. K_2HPO_4, 0·5 g. KNO_3, 0·05 g. $MgSO_4.7H_2O$, 0·1 g. NaCl, 0·17 ml. 6 % (w/v) solution of ferric citrate, 4 ml. indicator (equal parts of saturated aqueous bromcresol purple and cresol red) in 1 l. water.

Sucrose broth. As for glucose broth but with 10 g. sucrose. One series of sugar broths was incubated aerobically, the other anaerobically by the steel wool technique of Parker (1955).

Sensitivity to antibiotics. This was tested by surface seeding basal agar plates with cultures and then placing one Oxoid 'Multodisk' (11–14 D)/plate. Each disk contains eight peripheral disks—one for each of the following: chloramphenicol (50 μg.), erythromycin (50 μg.), sulphafurazole (500 μg.), novobiocin (30 μg.), oleandomycin (10 μg.), penicillin (5 units), streptomycin (25 μg.), tetracycline (50 μg.). The zones of inhibition were recorded after incubation for 7 days at 26°.

Examination of original data

The data previously collected by Sperber & Rovira (1959) from a series of 19 tests on 318 rhizosphere isolates were used. The two tests, namely, colour and fluorescence which were performed on two media were recorded as follows: colour 1 was presence of pigment in colonies on apatite agar; colour 2 was presence of pigment on nutrient agar; fluorescence 1 was obtained on yeast extract mannitol agar; fluorescence 2 on nutrient agar.

The results were transferred to edge-punched cards, one card for each isolate. Holes were punched for positive values and left blank for negative values. The total number of isolates positive for each test was counted, and those tests which did not have some positive and some negative isolates were discarded. Three tests were discarded for this reason, namely, the breakdown of cellulose (all negative), the nodulation of subterranean clover (all negative) and growth in peptone water (all positive).

A 2×2 table was set up for each combination of the remaining 16 tests and χ^2 values calculated for each of the 120 tables. When the association was significant at the 1 % level, the degree of association was measured by using Yule's coefficient of association (the Q value). Moroney (1957) set out the methods used in these calculations. Sneath (1957*a*) has pointed out that the associations revealed by these calculations may be due to two expressions of the one character, and when discovered such associations should be removed. For this reason the closely associated characters 'dissolving of apatite' × 'acid production' (Sperber, 1958) and 'colour 1' × 'colour 2' were removed from the calculations.

RESULTS

Examination of original data

The data obtained by Sperber & Rovira (1959) from 16 tests on 318 rhizosphere isolates were examined by the methods set out above. Table 1 shows the Q values for these isolates and the algebraic sum of the Q values for each test. The highest Q value in Table 1, −0·86, was for Gram reaction against growth on high pH medium. Of the 249 isolates which grew on the high pH medium only 19 were Gram-positive. All the other Q values were low, the lowest being only −0·29. The total Q values

(disregarding sign) for ammonia production and colour 1 were similar. Colour 1 was significantly associated with eight other characters (disregarding colour 2), whereas ammonia production was associated with seven other characters. Colour 2 had a much lower total Q value than colour 1. The selection of a character for division was difficult; those characters strongly associated with some characters, namely, Gram-reaction and colour 2, were not associated with enough characters to give them a high total Q value. The characters which were associated with a large number of other characters had high total Q values, even though they had only moderate or low degrees of association.

Table 1. *Q values significant at the* 1 % *level for* 318 *rhizosphere isolates*

Only half the table of Q values is shown, the blank part being the mirror image of the part below. The total number of isolates positive for each test and the algebraic sum of the Q values are also shown. C 1 = colour 1, Ap = solution of apatite, Ac = acid production from glucose, Gr = Gram reaction, H_2S = H_2S production from peptone, NH_3 = NH_3 production from peptone, NO_2^- = NO_2^- production from nitrate, Mo = motility, Ca = catalase, Ge = gelatine hydrolysis, F1 = fluorescence 1, pH = growth on high pH medium, C2 = colour 2, F2 = fluorescence 2, Y = presence of Y forms. Pl = presence of pleomorphic forms.

	Total positives	C1	Ap	Ac	Gr	H_2S	NH_3	NO_2^-	Mo	Ca	Ge	F1	pH	C2	F2	Y	Pl
C1	112																
Ap	126	0·37															
Ac	204	—	—														
Gr	55	−0·32	—	—													
H_2S	117	0·48	—	−0·44	—												
NH_3	238	0·50	0·45	—	—	0·49											
NO_2^-	83	0·33	—	—	—	0·37	0·56										
Mo	197	−0·29	—	−0·39	0·63	—	—	—									
Ca	221	0·50	0·50	—	—	—	0·35	—	—								
Ge	147	—	0·59	0·56	—	—	0·49	—	—	—							
F1	79	−0·49	−0·40	—	—	—	—	—	—	−0·48	−0·39						
pH	249	—	—	—	−0·86	—	—	—	0·51	—	—	—					
C2	206	—	—	—	—	—	0·45	—	−0·40	0·64	−0·63	—	—				
F2	31	—	—	—	—	—	—	—	—	—	—	—	−0·69	—			
Y	174	—	0·39	—	−0·38	—	—	—	—	—	—	—	—	—	—		
Pl	77	—	—	—	—	—	—	—	—	—	—	—	—	—	—	0·53	
Total Q values		3·28	2·70	1·39	2·19	1·78	3·29	1·26	2·22	2·47	2·66	1·76	2·06	2·12	0·69	1·30	0·53

The most important character should have a large number of other characters strongly associated with it, therefore the character which had the highest algebraic sum of its Q values was considered the most important for division. Table 1 shows that ammonia production had the highest total Q value (3·29), so the isolates were divided on ammonia production (see Fig. 1). The two resulting groups, the ammonia-producing group and the non-ammonia-producing group, were treated in exactly the same manner as the original isolates, i.e. all possible associations were tested for significance, and those found significant at the 1 % level measured by the Q test. The resultant Q values were then algebraically summed and the character with the highest algebraic sum of Q values used for the next division. This character was gelatin hydrolysis for the ammonia-producing group, and growth on high pH media for the non-ammonia-producing group. Division was continued in this way until there was no character that was significantly correlated with any other two characters.

The Q value table for the first division (Table 1) is the only one presented here in order to conserve space. The six groups which resulted from this division on associated characters are given in Fig. 1, but a comparison with the identification by Sperber & Rovira (1959) showed that each group contained several genera. In view of the heterogeneity of the groups, the second part of this investigation was undertaken using typed cultures as well as the selected rhizosphere isolates.

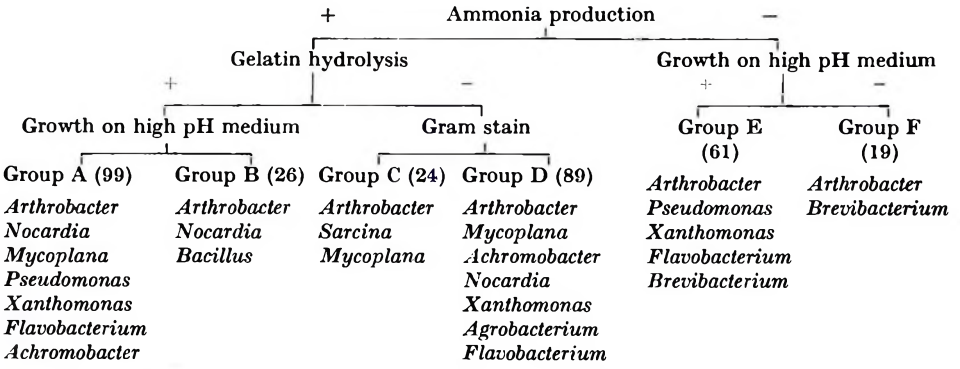


Fig. 1. Division of the 318 rhizosphere isolates using associated characters as shown. Positive reactions are on the left, negative reactions on the right. The number of isolates in each group is shown in brackets and the principal genera as identified by Sperber & Rovira (1959) are listed for each group.

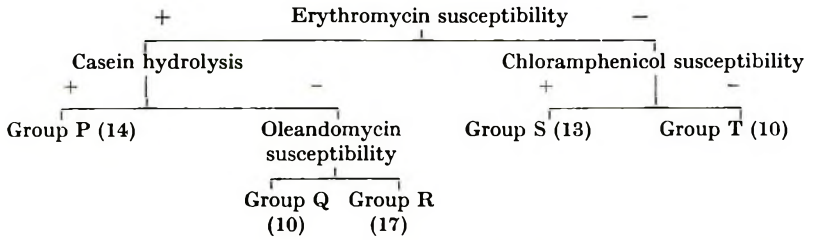
Retesting of selected bacteria

The morphological, cultural, biochemical and antibiotic sensitivity characteristics of 21 named cultures and 43 rhizosphere isolates were studied. Forty-one characters were recorded as positive or negative on edge-punched cards. Of these characters, thirty-one were used to determine the Q values (Table 2). The most notable feature of Table 2 was the small number, 28, of associations significant at the 1 % level, as compared with 35 in Table 1, even though the number of tests had risen from 16 to 31 increasing the number of possible associations from 120 to 535. The decrease in numbers of significant associations at the 1 % level can be attributed to the lower number of organisms used in the second case, making it less likely to obtain significant results. For this reason the significance level was set at 5 % and the Q values of Table 2 have been worked out for associations significant at this level.

A decision had to be made as to which of the associations should be removed in accordance with the principles laid down by Sneath; to conform with Table 1 the associations of colour 1 × colour 2 and dissolving of apatite × acid production were removed. The removal of other associations may be justified, but, as it was considered that inclusiveness was better than exclusiveness no further associations were removed. In comparing Table 1 with Table 2, the only common associations found were colour 1 × colour 2; dissolving of apatite × acid production, and pleomorphism × Y forms. The association of growth at high pH value × Gram reaction, which was the highest in Table 1, does not appear in Table 2, indicating that the association probably was spurious. When this association was disregarded the Q values (ignoring sign) of Table 1 ranged from 0·29 to 0·69 and those in Table 2 from 0·46 to 1·00.

If the levels of significance in both tables had been adjusted so that only Q values above a given figure (say 0·8) were recorded, then there would be more associations in Table 2. The greater range of bacteria and tests used for Table 2 accounted for the greater number of high associations in Table 2 than in Table 1, e.g. the association between Gram-positivity and erythromycin susceptibility could be demonstrated only when there was sufficient Gram-positive organisms and when the test for erythromycin susceptibility was performed.

A system of division was built up for the 64 bacteria by using the methods described for Fig. 1, the only difference being that a 5% level of significance was used. These results appear in Fig. 2. Erythromycin susceptibility had the highest total Q value (6·76) in Table 2, and was used to make the first division in Fig. 2. This is a good character to divide on, as it has a large number of characters strongly associated with it. Pleomorphic forms also had a high total Q value of 5·89. Five groups resulted from the system of division set out in Fig. 2. The fact that three out



Group P	Group Q	Group R	Group S	Group T
Arthrobacter aurescens	*Arthrobacter globiformis*	*Nocardia rubra*	*Rhizobium meliloti*	*Achromobacter* A1
Bacillus subtilis	*A. citreus*	*Rhizobium trifolii*	*Nocardia asteroides*	*Achromobacter* A2
Micromonospora sp.	*A. simplex*	*Flavobacterium* sp.	*Agrobacterium radiobacter*	*Arthrobacter ureafaciens*
Streptomyces venezualae		*Pseudomonas syringae*		*Pseudomonas fluorescens*
Xanthomonas campestris				*P. medicaginis*
				Rhizobium sp.

Fig. 2. Division of 64 bacteria on the basis of associated characters. Positive reactions are on the left, negative reactions on the right. The number of bacteria in each group is shown in brackets. The named bacteria within each group are also shown.

of the four characters used for division were reactions to antibiotics was not entirely due to associations between the various antibiotics. In the group of 23 bacteria that formed the erythromycin-resistant group, chloramphenicol-sensitivity was negatively associated with: dissolving of apatite, acid production in Ulrich's milk, aerobic production of acid from glucose, anaerobic production of acid from sucrose, and fluorescence; and it was positively associated with novobiocin sensitivity. In the group of 27 bacteria sensitive to erythromycin and not attacking casein, oleandomycin sensitivity was positively associated with the breakdown of starch, the solution of apatite and the presence of cocci.

The groups formed in Fig. 2 have been labelled P, Q, R, S, T, to facilitate comparison with the groups obtained from the 318 isolates (Fig. 1). The named cultures have been listed under the groups to which they belonged. In general each group

contained several genera except for group Q, in which only three *Arthrobacter* spp. occurred.

A comparison of the groupings of the 43 rhizosphere isolates common to Fig. 1 and Fig. 2 is given in Table 3. This shows a poor coincidence of groups, e.g. group Q isolates identified as *Arthrobacter* spp. included members from groups A, D, E, and F. Therefore it was impossible to use Fig. 2 to identify any of the groups A to F from Fig. 1.

Owing to the failure of the system of division on associated characters to give satisfactory groupings of the named cultures, the 64 cultures were traced through

Table 3. *A comparison of the groupings of the 43 isolates common to both Fig. 1 and Fig. 2*

Groups from Fig. 2	Groups from Fig. 1						
	A	B	C	D	E	F	Totals
P	6	1	—	1	1	—	9
Q	1	—	—	3	1	2	7
R	3	2	—	4	4	—	13
S	1	—	1	5	3	—	10
T	2	—	—	2	—	—	4
Totals	13	3	1	15	9	2	43

Skerman's key of *Bergey's Manual* (1957); the results are given in Table 4. Of the seven rhizosphere isolates which were considered to be *Arthrobacter* spp. in Fig. 2, only one was identified as such by the key. It would appear that many of the named isolates were incorrectly identified. This was the fault of the testing procedures rather than of the key, e.g. *Rhizobium* spp. fell into the *Pseudomonas-Alcaligenes* group because there was no satisfactory way of identifying *Rhizobium* except on its ability to nodulate legumes, a test which is virtually impossible to perform against all legumes. The lack of a successful routine technique for determining flagellation was also a great handicap.

Although the use of Skerman's key resulted in the naming of organisms, quite different isolates often appeared to belong to the same genus because they were identical only in the characters listed in the key, while they varied in a great number of other characters. This arbitrary nature of the key exists because of the lack of comparative studies with a wide range of organisms. Sneath's method of calculating Similarity Index would seem the ideal way of making such comparative studies. It was therefore decided to modify the method slightly so that it could be used for these isolates without the use of an electronic computer. The modifications made were that negative similarities were considered as important as positive similarities, and all characters which were entirely positive or entirely negative were omitted. Of the forty-one characters recorded there were thirty-nine that could be used on this basis. To avoid confusion the modified index was called the 'Affinity Index' (AI) and it may be expressed as

$$\text{Affinity Index} = \frac{\text{No. of characters in common}}{\text{Total No. of characters considered}} \times 100\,\%.$$

The punch cards which had been prepared for determination of associated characters in the 64 isolate groups were used to determine the Affinity Indices. The denominator

Table 4. *The identification of* 64 *bacteria according to Skerman's key*

Achromobacter	*Arthrobacter*	*Bacillus*	*Brevibacterium*	*Flavobacterium*	*Nocardia*	*Micromonospora*
SR* 60 (ER)	SR 16 (ER)	*B. subtilis*	SR 15 (FQ)	SR 12*b* (BR)	*N. asteroides*	*Micromonospora* sp.
	SR 29 (AQ)		SR 80 (NR)	SR 78 (AP)	*N. rubra*	
	SR 60 (AT)		SR 221 (FQ)	SR 82 (AP)		
	SR 195 (ER)			SR 89*b* (AP)		
	SR 311 (DT)			SR 108*a* (DS)		
				SR 198 (DQ)		
	A. aurescens			SR 231 (DP)		
	A. simplex			SR 242 (DQ)		
	A. globiformis			SR 266 (AP)		
	A. citreus			SR 270 (AQ)		
				SR 281 (DR)		
				SR 315 (DQ)		

Pseudomonas or *Achromobacter*	*Pseudomonas* or *Alcaligines*	*Pseudomonas*, etc. Page 1020, no. 58	*Sarcina*	*Streptomyces*	*Xanthomonas* or *Alcaligines*	*Xanthomonas* or *Flavobacterium*	Not grouped
SR 3 (DR)	SR 139 (EP)	SR 294 (AR)	SR 12*a* (BP)	*S. venezualae*	SR 63*b* (DS)	SR 75 (AP)	SR 46 (AP)
SR 83 (DT)	SR 156 (DR)				SR 127 (AR)	SR 194*b* (AR)	
SR 143*b* (ES)	SR 227 (DS)	*Achromobacter* A 1			SR 202 (DS)		
SR 166 (ER)	SR 232 (AT)	*Achromobacter* A 2			SR 215 (DR)	*Flavobacterium* sp.	
SR 225 (CS)	SR 234 (ES)	*Arthrobacter ureafaciens*			SR 236 (DS)		
		P. fluorescens			SR 245*b* (AS)		
					SR 267*b* (ES)		
Ps. medicaginis	*Agrobacterium radiobacter*						
Ps. syringae	*Rhizobium trifolii*				*X. campestris*		
	R. meliloti						
	Rhizobium sp.						

* Cultures selected from Sperber–Rovira collection are prefixed SR.

Column headings indicate the genus or group of genera to which the bacteria belong. The groupings from divisions in Fig. 1 and Fig. 2 respectively follow the rhizosphere isolate number.

of the expression given above was constant at 39, i.e. the number of tests having some positive and some negative values, and the numerator was determined for each possible pair of isolates. The border of the first card was stained red and compared with the second and subsequent unstained cards. Comparison was made by placing the red card 1 on white card 2 when any test positive (punched) on 1 but negative (not punched) on 2 showed as a white space in a red gap and these spaces were counted. The two cards were then turned over so that white card 2 now lay on red card 1 and any test positive on 2 but negative on 1 showed as a red space filling a white gap. These spaces were counted. The sum of these two totals, which gave the number of tests different between 1 and 2, was recorded. The number of tests in common was the total number (39) less the number of tests which differed. Having obtained the number of tests which differed between cards 1 and 2, card 1 was then compared with card 3 and so on until 1 had been compared with all the cards. Card 2 was then stained and compared with all the unstained cards. In this way over 2000 Affinity Indices were calculated, a difference of four or less characters corresponded to an Affinity Index of 90 %, a difference of five or six characters corresponded to an Affinity Index of 85 % and a difference of seven or eight characters corresponded to an Affinity Index of 80 %. Having obtained Affinity Indices in this way, the results were sorted in the manner suggested by Sneath, the most closely related being put together first and the grouping extended as the relationship decreased. The relationships discovered are shown in a linear manner in Table 5. The name or SR number of the isolate is given in the left-hand column, the code number used in this investigation is shown in the second column, then follows a list of the code numbers of all cultures with an Affinity Index of 80 % or more. Thus *Rhizobium* sp. isolated from *Centrosema pubescens* Benth. was related with an 80 % Affinity Index to SR 225 and to *Rhizobium meliloti*. The cultures at the extreme ends of Table 5 were unrelated to each other or to the cultures in the centre of the table and their order was of no taxonomic significance. The normal way of showing relationships of this type, as used in other papers (Sneath, 1957*b*; Sneath & Cowan, 1958; Hill, 1959) is set out in Fig. 3, in which the squares show those cultures which differ by no more than eight characters and the triangles show those cultures which are dissimilar by twenty or more characters.

A sorting which resulted in a good arrangement was typified by having related isolates close together. When this occurred in Fig. 3 the dark squares representing high affinity lay close to the diagonal. Although this condition was reasonably fulfilled, there was no block of closely related organisms, which indicated that there were no well defined groups of isolates. There was a large group of isolates in the centre, composed mainly of rhizosphere isolates which formed a spectrum of related bacteria rather than a series of related groups. The known bacteria which had the highest affinity to this large spectrum were *Agrobacterium radiobacter* (no. 51), *Xanthomonas campestris* (no. 70), and two *Rhizobium* sp. (nos. 65 and 66). Above the large group was a small group consisting of *Achromobacter* A1 and A2 (nos. 49 and 50), *Flavobacterium* sp. (no. 58), *Pseudomonas fluorescens* (no. 62), *Arthrobacter ureafaciens* (no. 56) and SR 83 (no. 15). The culture which was labelled '*Arthrobacter ureafaciens*' differed in morphology, colony colour, motility, hydrogen sulphide and acid production from the description for this organism given by *Bergey's Manual* (1957). With the exception of *Flavobacterium* sp. which was poorly chromogenic

Table 5. *The relationships between* 64 *cultures according to the Affinity Index*

Column 1 shows the name or number of the culture and column 2 the code number. On the right of the table are shown the code numbers of all the bacteria having 80 % or more affinity with each of the 64 cultures. Italic figures indicate 85 % affinity, bold figures 90 % affinity.

Name or no.	Code no.	
SR 60	8	
Arthrobacter aurescens	52	
Rhizobium sp.	68	32, 66
SR 294	45	23, 44
SR 311	47	51
Achromobacter A1	49	**50**
Achromobacter A2	50	**49**, 56, 62
Flavobacterium sp.	58	*62*, 56
Pseudomonas fluorescens	62	**56**, *15*, *58*, 50
Arthrobacter ureafaciens	56	**62**, *15*, 50, 58
SR 83	15	*56*, *62*, 63
SR 245B	40	33
Pseudomonas syringae	64	33
SR 225	32	3, 20, 66, 68
Arthrobacter citreus	53	13
A. globiformis	54	**55**
A. simplex	55	**54**, 23
Pseudomonas medicaginis	63	10, 15
SR 69	10	**23**, 65, 13, 22, 44, 63
SR 166	23	**10**, *65*, *22*, 1, 26, 55, 45, 66
Rhizobium trifolii	65	*23*, 10, 13, 22, 66, 1, 5, 19, 36
R. meliloti	66	*5*, 36, 37, 65, 23, 32, 68
SR 80	13	*5*, 53, 65, 10, 54
Agrobacterium radiobacter	51	*22*, *30*, *42*, 17, 33, 5, 20, 37, 47
SR 156	22	*30*, *51*, *23*, *33*, 1, 42, 65, 10, 21, 70
SR 215	30	*22*, *33*, *51*, 70, 1, 5, 9, 42, 43, 46
SR 227	33	*20*, *30*, *21*, *22*, 1, 36, 40, 42, 43, 51, 64
SR 134B	20	*33*, *3*, *43*, 1, 5, 19, 9, 32, 36, 42, 51
SR 301	46	*44*, *43*, 14, 16, 34, 70, 1, 3, 11, 12, 19, 30
SR 281	44	*43*, *46*, 1, 3, 19, 20, 46, 33, 48, 5, 9, 26, 30
SR 270	43	*44*, *1*, *3*, *19*, *20*, *46*, 33, 48, 5, 9, 26, 30
SR 127	19	*3*, *43*, 5, 9, 20, 26, 46, 65
SR 12B	3	*5*, *19*, *20*, *26*, *43*, 32, 44
SR 195	26	*3*, 23, 1, 5, 9, 19, 37, 42, 43, 48
SR 3	1	*5*, *37*, *43*, 22, 23, 33, 44, 61, 3, 20, 26, 30, 36, 42, 46, 65
SR 16	5	**37**, *13*, *66*, *1*, *3*, *42*, 6, 19, 20, 26, 30, 36, 43, 51, 53, 65
SR 236	37	**5**, **42**, *17*, *1*, 25, 66, 3, 26, 36, 48, 51
SR 267	42	**37**, *17*, *5*, *51*, 22, 25, 33, 70, 1, 3, 20, 26, 30, 35, 61
SR 108A	17	*37*, *42*, *70*, 35, 51, 21, 25
SR 232	35	*21*, 17, 70, 42
SR 139	21	*35*, *33*, *70*, 17, 22
Xanthomonas campestris	70	*16*, *17*, *21*, 30, 35, 42, 46, 14, 22, 34
SR 89B	16	**14**, *34*, *70*, 41, 46
SR 82	14	**16**, *34*, 46, 70
SR 231	34	**14**, *16*, 11, 12, 46, 44, 70
SR 234	36	33, 66, 1, 5, 20, 37, 65
SR 63B	9	28, 19, 20, 26, 30, 43, 3
SR 194B	25	37, 42, 17, 69
SR 75	11	**12**, 34, 46
SR 78	12	**11**, 34, 46
SR 315	48	39, 43, 26, 27, 37, 44
SR 242	39	4, 48, 44

Table 5 (*cont.*)

Name or no.	Code no.	
SR 15	4	31, 39, 44, 27
SR 221	31	4, 44
SR 198	27	4, 48
SR 266	41	14, 16, 44, 59
SR 29	6	5
Nocardia rubra	61	1, 42
Streptomyces venezuelae	69	25
SR 202	28	9, 33
Micromonospora sp.	59	41
SR 12A	2	
Bacillus subtilis	57	
Nocardia asteroides	60	

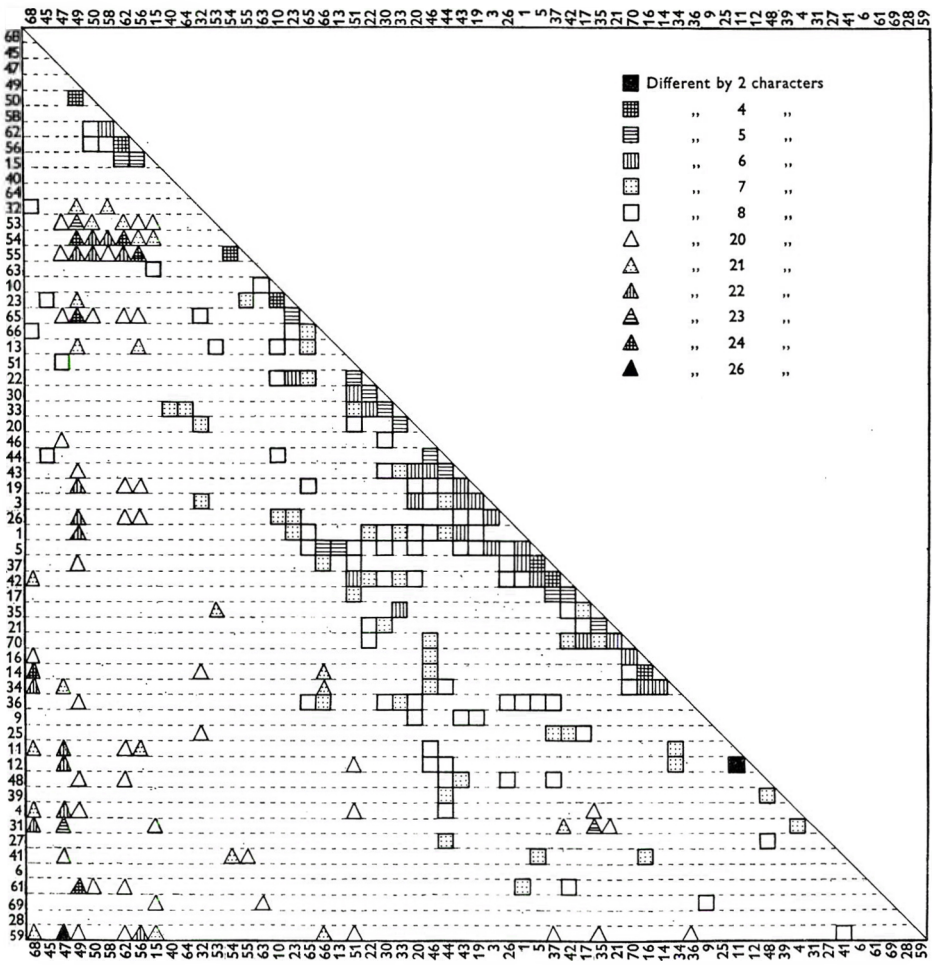


Fig. 3. Diagram showing the relationship between 64 cultures. The shading of squares indicates the number of characters by which similar cultures differ. The triangles indicate the number of characters by which dissimilar cultures differ. Intermediate relationships are not shown. The key for the culture numbers or names is given in Table 5.

and non-proteolytic all other named cultures corresponded closely with the descriptions given by *Bergey's Manual* (1957). Thornley (1960) reported that *Pseudomonas* could be differentiated on the basis of the anaerobic breakdown of arginine. All 64 cultures were tested for anaerobic arginine breakdown and of those which were Gram negative, penicillin resistant, and produced acid from glucose aerobically only six gave a positive arginine test. Those cultures were *Pseudomonas fluorescens*. *Pseudomonas medicaginis*, *Achromobacter* A1 *and* A2, *Arthrobacter ureafaciens*, and the rhizosphere isolate RS 83. Thus, according to Thornley, the so-called *Arthrobacter ureafaciens*, *Achromobacter* cultures, and SR 83 are really members of the genus *Pseudomonas*. It is interesting to note that in Table 5 these cultures have been grouped together on the basis of Affinity Index quite independently of the arginine test and showed a very poor relationship to the majority of the rhizosphere isolates.

DISCUSSION

The system of division on associated characters proved to be of no value in this particular case, firstly because of the lack of distinct groups within the range of bacteria considered, and secondly because of the somewhat complex nature of the analytical procedure. While it is true that where groups differ in many characters these characters will be associated, it is not necessarily true that where there are associated characters there will be taxonomic groups. It is considered that it is only rarely that the elaborate computation of associations as performed in this paper will be necessary. A simpler process would be to obtain groups by using the Affinity Index and to find differences between groups by inspection.

The best system for identifying the isolates is undoubtedly Skerman's key especially when the type cultures of the genera most likely to be encountered are included with the isolates. The key has several deficiencies. Firstly, it is based on *Bergey's Manual* and any deficiencies in the *Manual* are reflected in the key. Secondly, there is the difficulty in the standardization of testing procedures, which should largely be solved by using *A Guide to the Identification of the Genera of Bacteria* (Skerman, 1959). The present paper arose out of this difficulty of technique standardization. Sperber & Rovira (1959) demonstrated the presence of branching forms in many cultures which would normally be considered to have unbranched forms. This gave rise to their large groups of 'Arthrobacter' and 'Arthrobacter-like-Nocardias', which upon re-examination and comparison with known cultures of these genera proved to be incorrectly identified. Nevertheless, the prevalence of branching amongst the rhizosphere cultures was an important observation and a further study of this phenomenon with type cultures seems necessary.

The relationships between bacteria are well shown by the use of Sneath's Similarity (or Affinity) Index. The limitations of this method lie in the sorting procedures and the selection of tests or characters to be used. A close examination of Fig. 3 shows that several cultures have in fact been missorted, and correction would require a rearrangement of the figure. However the authors believe this is not justified as a perfect arrangement is not possible. As there are only two positions equidistant from any organism in the linear order, a compromise must be used if there are more than two organisms with an equal relationship to any other organism. The presentation of the data as in Table 5 simplifies the sorting procedures as the relation-

ships are more easily seen than in Fig. 3. The other limitations of this method lie in the selection of tests or characters to be used. If the natural groupings of the bacteria considered differ from one another in ten characters, then the chances of determining the groups will depend on how many of the ten good tests are selected and how many poor tests are added to the good tests. The larger the number of poor tests the more the result will tend towards one diffuse group. In discussing test selection for taxonomic purposes, Krassilnikov (1959) pointed out that a good test must be a repeatable test. He suggested that the loss of a character was not as important as the spontaneous gain of a character, and also emphasized the importance of morphology in taxonomy. Cowan (1955) posed the question that bacteria form a spectrum of gradually merging forms. In trying to determine whether this is the case for the rhizosphere bacteria, the two factors to be borne in mind are that the inclusion of too many poor tests would tend to give this result and that the demonstration of a range of bacteria is not sufficient to say that bacteria do not occur as natural taxonomic groups. If any of the types in the range is rare then this would be the place to make a taxonomic division. Thus a 'spectrum' implies not only a continual slight change in the type of bacteria, but also that the numbers of bacteria either remain constant for each type, or the numbers fall off slowly from some central type. The vast numbers of bacteria in the rhizosphere make measurement of the numbers of various types an extremely difficult task, liable to very large sampling errors. Conditions in the rhizosphere favour a large number of organisms from a great range of species, with common features such as being predominantly Gram-negative rods with a fast growth rate and the ability to rapidly utilize organic supplements (Lochhead, 1940; Rovira, 1956; Rouatt & Katznelson, 1957; Katznelson & Rouatt, 1957; Zagallo & Katznelson, 1957).

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RNA Synthesis and Degradation during Antibiotic Treatment and its Relation to Antibiotic-induced Lag

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SUMMARY

When growing cells of *Escherichia coli* were treated with chloramphenicol or erythromycin for 1 hr. and then suspended in antibiotic-free medium, there was a 45 min. lag before growth resumed. By eliminating growth factors or other essential nutrients during antibiotic treatment, it was possible to show that the lag occurred only when ribonucleic acid (RNA) synthesis could take place and did not require the synthesis of deoxyribonucleic acid (DNA). This antibiotic-induced RNA was apparently abnormal and was degraded when the antibiotic was removed. This degradation is a hydrolytic process and does not require the presence of a complete growth medium. During the recovery from the antibiotic-induced lag, DNA and protein synthesis did not occur, but RNA synthesis occurred, even though this new RNA synthesis was not required for the lag to be overcome. When antibiotic-treated cells were suspended in phosphate buffer, a decrease in optical density of the suspension occurred which resembled a lytic process, but lysis apparently did not occur. Although these results clarify considerably earlier observations on antibiotic-induced lag, they leave unsolved the question of why the antibiotic-induced RNA is abnormal, and how it brings about the lag.

INTRODUCTION

Several years ago there were independent reports from several laboratories of accumulation of RNA and its subsequent degradation in chloramphenicol-inhibited cells (Hahn *et al.* 1957; Neidhardt & Gros, 1957; Horowitz, Lombard & Chargaff, 1958). The first two groups of workers showed that cells which had been treated with antibiotic, and had accumulated RNA, exhibited a lag before growth resumed when they were suspended in antibiotic-free medium. Although these workers had indirect evidence for the relation of this antibiotic-accumulated RNA to the subsequent lag, they supplied no direct evidence for this hypothesis.

The present work was begun when the authors (Brock & Brock, 1959*a*) discovered that erythromycin and chloramphenicol were similar in action and began to compare a number of facets of erythromycin action with those of chloramphenicol. It was readily shown that erythromycin also brought about RNA accumulation, and erythromycin-treated cells also exhibited a lag when resuspended in antibiotic-free growth medium. The availability of a mutant of *Escherichia coli* 15 T^-U^- (Barner &

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Cohen, 1958), which requires thymine + uracil for growth, provided us with a tool for controlling RNA and DNA synthesis during antibiotic treatment and recovery, to show directly the involvement of RNA in antibiotic-induced lag. A preliminary report of this work has been presented (Brock & Brock, 1960).

METHODS

Organisms. Escherichia coli strains 15 T^-U^- and $T^-A^-U^-$ were kindly provided by Dr S. Cohen; *E. coli* B_4 (prolineless) was obtained from Dr L. Frank, and *E. coli* ML35, from Dr J. Monod. A methionine-requiring mutant of strain 15 T^-U^- was isolated by the authors and is designated $T^-U^-M^-$.

Medium and growth conditions. A defined medium of the following composition was used (g.): K_2HPO_4, 6·0; KH_2PO_4, 3·0 NH_4Cl, 0·5; $MgSO_4.7H_2O$, 0·1; glucose, 4·0; distilled water to 1 l. Thymine and uracil were added to give final concentrations of 8 and 40 μg./ml. except in the radioactivity experiments. Arginine and methionine were added to final concentrations of 50 μg./ml., and proline to 100 μg./ml. except in the radioactivity experiments.

Growth was followed by measuring optical density (O.D.) at 420 mμ on a Lumetron colorimeter.

Organisms were grown overnight in defined medium at 37° on a rotary shaker, diluted into fresh medium to an optical density of 0·035–0·050, and dispensed 100 ml./500 ml. flask. After logarithmic growth had been re-established, at approximately O.D. 0·150, the suspensions were chilled and centrifuged, then washed twice with cold buffer of the following composition (g.): K_2HPO_4, 6·0; KH_2PO_4, 3·0; distilled water, 1 l. The organisms were then resuspended in fresh medium with and without various supplements at 37° to O.D. 0·150 and replaced on the shaker for antibiotic treatment. This same procedure for washing suspensions was repeated after the antibiotic treatment.

Antibiotics. Chloramphenicol was purchased from Parke, Davis and Co., and erythromycin was a gift from Eli Lilly and Co. Stock solutions of these antibiotics were prepared in 50 % (v/v) aqueous ethanol, and these were diluted 1/100 upon addition to suspensions of organisms. Chloramphenicol was used at a final concentration of 50 μg./ml. and erythromycin at 1000 μg./ml.

Assays. For chemical assays of deoxyribonucleic acid (DNA), ribonucleic acid (RNA) and protein, 10 ml. samples were removed and chilled in an ice bath at 4°, centrifuged in the cold for 5 min. at 13,000 rev./min., and washed in succession with 8 ml. volumes of cold 0·5 N-$HClO_4$, 95 % (v/v) ethanol in water, and anhydrous ether. The residue was then air dried and extracted with 4 ml. of 0·5 N-$HClO_4$ at 90° for 15 min. The extracts were allowed to cool to room temperature and were then centrifuged. A 1 ml. sample of this supernatant was used for assay of DNA by the method of Burton (1956). A 1 ml. sample was diluted with 2 ml. of 0·5 N-$HClO_4$ for assay of RNA by the method of Chargaff & Davidson (1955). For assay of protein by the method of Lowry (Lowry, Rosebrough, Farr & Randall, 1951) the residue from the hot $HClO_4$ extraction was suspended in 10 ml. of N-NaOH and heated to 100° for 1 hr.; a 1 ml. sample was used. Commercial preparations of DNA, RNA, and lysozyme were used as standards.

Radioactivity experiments. Thymine-2-^{14}C (Volk Radiochemical Co., specific

activity 1·0 mc./mM) was added to a concentration of 2 μg./ml., $1{\cdot}15 \times 10^4$ c.p.m./ml. Uracil-2-^{14}C (Volk Radiochemical Co., specific activity 3·2 mc./mM) was added to a concentration of 10 μg./ml., $1{\cdot}95 \times 10^5$ c.p.m./ml. Proline (Schwartz Bioresearch, Mt Vernon, New York; specific activity 100 μc./mM) was added to a concentration of 45 μg./ml., $3{\cdot}88 \times 10^4$ c.p.m./ml. Sodium sulphate-^{35}S (Oak Ridge National Laboratories, carrier-free) was added at $8{\cdot}64 \times 10^4$ c.p.m./ml.

For measurement of incorporation of the radioactive compounds, washed organisms were resuspended in the appropriate medium, the radioactive compound added, and samples taken. In the case of uracil, 0·1 ml. samples were taken, cooled in an ice bath, and 0·9 ml. of cold 0·5 N-$HClO_4$ was added. For thymine, proline and sulphate, 1·0 ml. samples were taken, cooled, and 9·0 ml. of cold 0·5 N-$HClO_4$ added. After standing for 30 min. in the cold, the suspensions were filtered on membrane filters (Millipore type HA, 0·45 μ), washed twice with equal volumes of cold 0·5 N-$HClO_4$, and the filter disks glued to stainless steel planchets (Roberts *et al.* 1957).

In experiments in which the RNA was labelled with radioactive uracil during or before antibiotic treatment, uracil of a specific activity of 0·96 mc./mM was added at a concentration of 10 μg./ml. Samples (0·2 ml.) were taken and mixed with 1·8 ml. cold 0·5 N-$HClO_4$ and processed as above.

Counts were made with a Nuclear Chicago gas flow counter. No corrections for self-absorption were made. The results are reported as counts/min. above background.

β-Galactosidase assay. To 1·0 ml. samples of cell suspensions or supernatant fluids of ML 35 grown in salts + glycerol medium was added 4 ml. of M/600 *o*-nitrophenyl-β-D-galaetoside (ONPG) in 0·1 M-Na_2HPO_4 buffer (pH 7). After incubation at 37° for sufficient colour development, the reaction was stopped by adding 5 ml. of M-Na_2CO_3. Following centrifugation the optical density at 420 mμ was determined; *o*-nitrophenol was used as a standard (Brock & Brock, 1959*b*).

Viable counts. Viable counts were made by the pour plate method with nutrient agar; counts were made after incubation for 24 hr.

RESULTS

Antibiotic-induced lag occurs only when RNA synthesis can take place

In the following work, chloramphenicol and erythromycin gave essentially the same results, so for simplicity results are presented for chloramphenicol only. When chloramphenicol (50 μg./ml.) or erythromycin (1000 μg./ml.) was added to growing cultures of *Escherichia coli*, protein synthesis was almost completely inhibited, while RNA and DNA synthesis continued. When the organisms were washed several times to remove antibiotic and then resuspended in growth medium, there was a lag of 45–60 min. before growth resumed. This lag did not occur when the organisms were not treated with antibiotic, nor did it occur when growth was inhibited by withholding an essential amino acid or other nutrient such as a nitrogen, carbon or phosphorus source, rather than by antibiotic inhibition. It was not due to the killing of a portion of the population. Since the RNA synthesized in the presence of chloramphenicol differs in a number of ways from normal RNA (Horowitz *et al.*

1958; Pardee *et al.* 1957), it seemed possible that the lag was due to the prior synthesis of this abnormal RNA (Hahn *et al.* 1957). To test this point, a mutant of *E. coli* was used which requires thymine + uracil for growth, since Barner & Cohen (1958) had shown that withholding uracil prevented RNA synthesis without preventing DNA synthesis, and withholding thymine prevented DNA synthesis without affecting RNA synthesis. When growing cultures of this mutant were treated with antibiotic for 2 hr. in the presence or absence of either of these growth factors, and then tested for lag by resuspending them in antibiotic-free medium containing both growth factors, the following results were obtained: (1) With thymine + uracil present during antibiotic treatment there was a 1 hr. lag on recovery. (2) With thymine only present and uracil absent there was no lag. (3) With uracil only present and thymine absent there was a 1 hr. lag on recovery. (4) With neither thymine nor uracil present there was no lag. RNA synthesis did not take place in the absence of uracil, and DNA synthesis did not take place in the absence of thymine. These results indicate that, for the subsequent lag to occur, the cells must be able to synthesize RNA during antibiotic treatment.

As a further test of this point, uracil was added or withheld during the first or second hour of antibiotic treatment, or during both hours, or neither, and the amount of RNA synthesized was measured and correlated with the subsequent lag. These results are shown in Table 1, in which the lag is given as the length of time

Table 1. *Synthesis of RNA and induction of lag during chloramphenicol treatment in the presence or absence of uracil*

Escherichia coli 15 T^-U^-, in salts + glucose medium, thymine and chloramphenicol present in all.

	RNA* synthesized during chloramphenicol treatment (μg./ml.)	Lag† (min.)
No uracil	0	0
Uracil, 2 hr.	46	55
Uracil, 1st hr.	33	45
Uracil, 2nd hr.	38	30

* Initial RNA, 35 μg./ml.

† Lag measured after reincubation in salts + glucose + thymine and uracil, given as time for O.D. to double minus the time for O.D. of logarithmically growing cells to double.

for the culture to double in optical density after resuspension, after subtracting the doubling time of a logarithmically growing culture (Lockhart, 1960). It can be seen that the lag was longer when uracil was present for 2 hr. than when it was present for 1 hr. Further, uracil present during the first hour induced a longer lag than uracil present during the second hour, even though approximately equal amounts of RNA were synthesized under both conditions. DNA synthesis occurred in all four treatments.

As a further correlation between RNA synthesis and lag, two mutants were used which required amino acids in addition to thymine and uracil. One of these was *Escherichia coli* 15 $T^-A^-U^-$ of Kanazir, Barner, Flaks & Cohen (1959) which was isolated by them from *E. coli* 15 T^- by an apparent one-step mutation to both

arginine and uracil requirements, although the blockage in synthesis of both of these compounds is not complete. When arginine was withheld in the presence of uracil and thymine, no RNA synthesis occurred, apparently because of the obligatory coupling of RNA and protein synthesis under these conditions. However, when chloramphenicol or erythromycin was present, RNA synthesis occurred in the absence of arginine, even in arginine-starved cells. This is at variance with the observations of Pardee & Prestidge (1956) and of Gros & Gros (1958) that RNA synthesis did not occur in the absence of an essential amino acid, even in the presence of chloramphenicol. However, since this *E. coli* mutant $T^-A^-U^-$ can form some arginine, this may be enough to support the synthesis of antibiotic-induced RNA even though it will not support normal RNA synthesis. When growing organisms of this mutant were treated with antibiotic in the absence of arginine, a 45 min. lag was induced, while 21 μg./ml. of RNA were synthesized (Table 2). When both arginine and uracil were withheld, there was no antibiotic-induced RNA synthesis and no lag. This is further support for the idea that antibiotic-induced lag is due to the synthesis of abnormal RNA, since in this mutant, in the absence of arginine, only antibiotic-induced RNA can accumulate, and this accumulation leads to a subsequent lag.

A methionine-requiring mutant of *Escherichia coli* 15 T^-U^-, designated $T^-U^-M^-$,

Table 2. *Synthesis of RNA and induction of lag during chloramphenicol treatment; arginine and uracil present or absent during antibiotic treatment*

Escherichia coli 15 $T^-A^-U^-$, in salts + glucose medium, thymine present in all during treatment; thymine, arginine and uracil present in all during recovery.

Conditions during treatment	RNA* synthesized during treatment (μg./ml.)	Lag† (min.)
Arginine + uracil	55	0
Arginine + uracil + chloramphenicol	21	45
Uracil, no arginine	3	0
Uracil + chloramphenicol, no arginine	21	45
No uracil, no arginine	0	0
No uracil, no arginine, chloramphenicol present	2	0

* Initial RNA, 35 μg./ml. † Generation time of controls, 65 min.

Table 3. *Synthesis of RNA and induction of lag during chloramphenicol treatment; methionine present or absent during antibiotic treatment*

Escherichia coli 15 $T^-U^-M^-$, in salts + glucose medium, thymine and uracil present in all during treatment; thymine, uracil and methionine present in all during recovery.

Conditions during treatment	RNA* synthesized during treatment (μg./ml.)	Lag† (min.)
Methionine	93	0
Methionine + chloramphenicol	24	30
No methionine	7	0
No methionine, chloramphenicol present	40	40

* Initial RNA, 58 μg./ml. † Generation time of controls, 70 min.

was isolated by the authors. This mutant could also synthesize some RNA in the absence of methionine, when chloramphenicol was present, and a lag was found on recovery (Table 3). A proline-requiring mutant, *E. coli* B_4, did not synthesize antibiotic-induced RNA in the absence of proline and did not exhibit any antibiotic-induced lag. It can therefore be concluded that antibiotic-induced lag occurs only when antibiotic-induced RNA is synthesized, and the lag may be related to the synthesis of some abnormal RNA. The results with the arginine- and methionine-requiring mutants show clearly that it is an antibiotic-induced RNA which is responsible for the lag, and not an excess of a normal RNA.

Ability to synthesize new RNA is not a prerequisite to recovery from antibiotic-induced lag

Growing cultures of *Escherichia coli* 15 T^-U^- were treated with antibiotic for 1 hr. in glucose salts medium with thymine + uracil present. The organisms were then washed and resuspended in antibiotic-free medium from which uracil, thymine, glucose, ammonium chloride or phosphate was omitted. Ordinarily, organisms treated as above and resuspended in complete medium began to divide only after incubation for 45 min. The requirement for various nutrients during this lag period was determined by withholding the nutrient for 45 min., then adding it back, and determining whether the organisms started growing immediately (i.e. with no additional lag) or exhibited a further lag before growing. In each experiment, a control flask of antibiotic-treated organisms had the nutrient present during the lag. The lag was not increased in length significantly by withholding any of the nutrients essential for the synthesis of RNA, DNA or protein, namely, glucose, phosphate, ammonium salt, uracil or thymine. Since normal organisms do not grow or synthesize macromolecules when any of these nutrients is withheld, this seems to indicate that recovery from the antibiotic-induced lag is essentially a degradative process, probably involving the destruction of abnormal RNA. Since the degradative enzymes can operate in non-growing organisms (Horiuchi, 1959; Mandelstam, 1958), this interpretation seems justified. It also seems unlikely that the lag is due to a deficiency in any of the small molecules produced by the cell, since the addition of 0·5 % (w/v) yeast extract during the recovery period did not shorten the lag.

Synthesis of DNA and protein does not begin until the lag is over; but RNA synthesis can begin immediately

Preliminary measurements by chemical methods of RNA, DNA and protein during the recovery period revealed that synthesis of these macromolecules did not begin until the lag was over. However, chemical measurements would not indicate any turnover of macromolecules, such as degradation and resynthesis. Therefore, radio-isotope methods were used to follow these processes. Since the organism used, *Escherichia coli* 15 T^-U^-, has absolute requirements for both thymine and uracil, it was possible to measure synthesis of DNA and RNA by determining the incorporation of radioactive thymine and uracil into cold perchloric acid-insoluble material in whole organisms. Preliminary experiments comparing the isotope method with the chemical method indicated a close correspondence when logarithmically growing cultures were used. Presumably none of the radioactive thymine enters RNA, and

only a small fraction of the radioactive uracil enters DNA, by way of cytosine. In every experiment a no-glucose control used to check for non-specific absorption always gave a negligible reading. As a measure of protein synthesis, the incorporation of ^{14}C-proline and $Na_2{}^{35}SO_4$ was determined.

Figure 1 shows the results of the incorporation studies with these isotopes. With thymine, proline or sulphate, there was a lag in the incorporation of isotope in chloramphenicol-treated organisms, and this lag paralleled the lag in growth. However, incorporation of ^{14}C-uracil differed from the other isotopes in one important

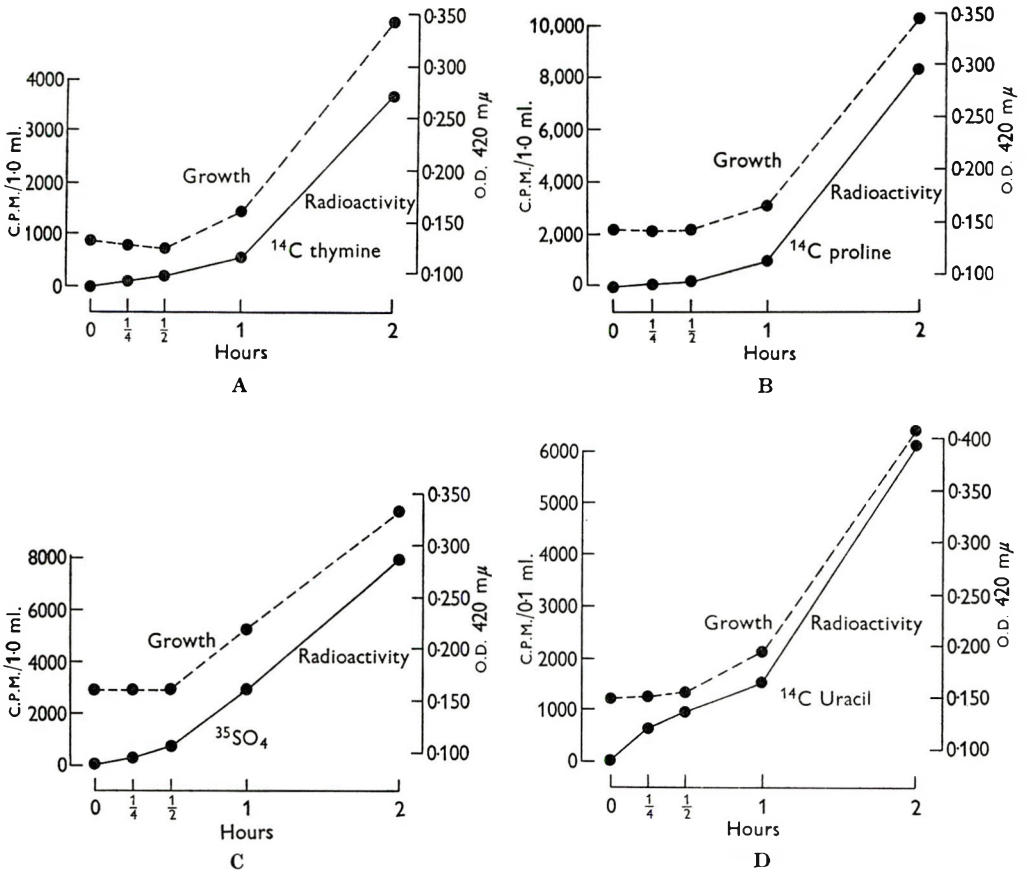

Fig. 1. Growth of *E. coli* 15 T⁻U⁻ and incorporation of: (A) radioactive thymine, (B) radioactive proline, (C) radioactive sulphate, and (D) radioactive uracil during chloramphenicol-induced lag. – – – –, Growth; ——, radioactivity.

respect. There was an initial rapid increase in uracil incorporation, followed then by a slower rate, and this was followed by the normal rate of incorporation after growth had resumed. Although brief, this initial rapid increase was reproducible. The interpretation is advanced that the antibiotic-treated organisms, although unable to synthesize DNA or protein during the lag, were able to synthesize RNA, and that the initial rapid increase in incorporation was due to synthesis of new RNA. However, during the antibiotic treatment the organisms have accumulated RNA which is being degraded, and soon this degradation provides sufficient non-radio-

active RNA precursors to dilute out the isotopic uracil, and the synthesis of new RNA probably then occurs predominantly from the non-radioactive sources. Only after all of this RNA was degraded, and the lag was overcome, was radioactive uracil again incorporated at a maximum rate. This interpretation supports the idea advanced by others (Kjelgaard, Maaløe & Schaechter, 1958) that when there is a shift in growth rate from low to high, RNA synthesis occurs in advance of this shift and apparently provides the higher concentrations of RNA necessary in the cell to support this new growth rate. It is concluded therefore that the synthesis of RNA can begin immediately in the non-growing cells, whereas DNA and protein synthesis can occur only after the lag is overcome.

Loss of antibiotic-accumulated RNA may occur under some conditions

In earlier work on antibiotic-induced lag it was reported (Hahn *et al.* 1957; Neidhart & Gros, 1957; Horowitz *et al.* 1958) that the RNA which accumulated was degraded and excreted before growth resumed. It was suggested by these workers that this excretion was essential to the recovery process. In the present experiments, when the antibiotic-treated organisms were resuspended in complete growth medium, there was no evidence that the degradation of RNA was followed by the excretion of RNA precursors during the lag. However, when the organisms were suspended in non-growth medium, such as phosphate buffer, there was a marked loss of RNA from the cells during the recovery period. This observation is not completely at variance with earlier work, since Neidhardt & Gros (1957) and Horowitz *et al.* (1958) only studied excretion under non-growing conditions, and Hahn *et al.* (1957) used much longer periods of antibiotic treatment which may have brought about more drastic metabolic derangements. Horiuchi, Sunakawa & Mizuno (1958) also found in *Escherichia coli* B that antibiotic-induced RNA was excreted under non-growing conditions but not under growing conditions.

In the present work it was noted that whenever there was a loss in RNA there was a sharp decrease in optical density. This decrease in optical density seemed to indicate that antibiotic-treated organisms were lysing when they were not growing and not lysing when they were growing, and that excretion of RNA degradation products was not occurring, but that a complete lysis and release of all of the cell contents occurred in some of the organisms. More detailed studies showed this idea to be wrong; the results to be presented below show that antibiotic-induced RNA was degraded and the degradation products excreted under non-growing conditions, whereas under growing conditions they were immediately incorporated into new RNA. The decrease in optical density was not due to lysis but to some other change in the morphology of the cells.

It was found that the membrane filter technique did not distinguish between high-molecular weight RNA within cells or in lysates. Thus, when cells were labelled with ^{14}C-uracil in the absence of antibiotic, lysed with lysozyme and ethylene diamine tetracetic acid, and the lysates diluted into cold perchloric acid and filtered, all of the radioactivity was retained by the filter, and the counts obtained were the same as when unlysed organisms were used. Treatment of lysates for 30 min. with 0·1 N-NaOH at 0° almost completely degraded the RNA of these lysates, so that the radioactivity then passed through the filter. These results showed that the mem-

brane filter technique would withhold high molecular weight RNA whether within cells or free, whereas low molecular weight materials passed through the filter.

Two series of experiments were set up. In the first, the RNA of cells was labelled by growth for three generations in radioactive uracil in the absence of chloramphenicol. The organisms were then washed and suspended in growth medium with chloramphenicol and cold uracil for 1 hr., then washed again to remove antibiotic and suspended in phosphate buffer or growth medium with antibiotic. The optical density of the suspension was measured periodically, and samples were taken for membrane filtration. It can be seen in Fig. 2*a* that in growth medium there was the usual lag on recovery but no loss of radioactivity. In phosphate buffer there was a marked decrease in optical density in the suspension of antibiotic-treated organisms but no loss in radioactivity. This shows that preformed RNA was not degraded during recovery, although the decrease in optical density seems to indicate some antibiotic-induced change in the organisms.

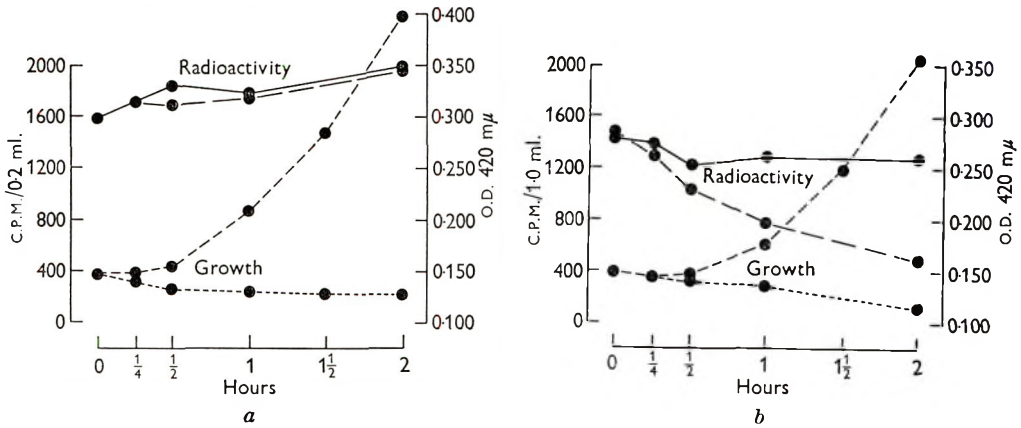

Fig. 2. Growth of *Escherichia coli* 15 T^-U^- and changes in radioactivity after treatment with chloramphenicol. (*a*) Cells labelled with ^{14}C-uracil before chloramphenicol treatment, then 1 hr. chloramphenicol treatment, then recovery in antibiotic-free salts + glucose medium or phosphate buffer. (*b*) Cells labelled with ^{14}C-uracil during 1 hr. chloramphenicol treatment, then recovery in antibiotic-free salts + glucose medium, or phosphate buffer. Graphs represent growth and retention or loss of radioactivity. ——, Radioactivity, cells suspended in salts + glucose; — —, radioactivity, cells suspended in phosphate buffer; – – – –, growth, cells suspended in salts + glucose; ------, growth, cells suspended in phosphate buffer.

In the second series of experiments, the RNA was labelled by having ^{14}C-uracil present during a 1 hr. chloramphenicol treatment and the organisms then washed and resuspended in growth medium or in phosphate buffer and the optical density and loss of radioactivity determined. In Fig. 2*b* it can be seen that in growth medium there was the usual lag on recovery but only a slight and transitory loss in RNA. In phosphate buffer there was a definite decrease in optical density and a marked loss of radioactivity. These results show that antibiotic-accumulated RNA was degraded to low molecular weight material and lost from the organisms when they were suspended in non-growing conditions. Under growing conditions there was only a negligible loss in radioactivity, so the degraded material must have been immediately incorporated into new RNA. This immediate re-incorporation is in

agreement with the results above which showed that RNA synthesis began immediately, even during the lag period. Thus antibiotic-accumulated RNA was degraded during recovery, whereas normal RNA was not degraded even in antibiotic-treated organisms.

The decrease in optical density of suspensions of antibiotic-treated organisms in phosphate buffer seemed to indicate that lysis occurred in these organisms. However, viable counts of control and antibiotic-treated organisms of samples taken at intervals up to 2 hr. in phosphate buffer indicated no loss in viability which would be expected to accompany lysis (Table 4). As a more sensitive measure of possible lysis the hydrolysis of ONPG by a β-galactosidase-constitutive, permease-less mutant of *Escherichia coli* (ML35) was measured (Brock & Brock, 1959*b*). In addition, the enzyme activity of supernatant fluids from such organisms was measured. No increase in rate of hydrolysis was noted in antibiotic-treated organisms as compared to control organisms after 1 hr. in phosphate buffer, although most of the radioactivity was lost during this 1 hr. treatment, as shown in Fig. 2*b*. Thus there was no evidence that the decrease in optical density was due to frank lysis, or to a markedly altered permeability of the cells brought about by the antibiotic treatment. An alternative explanation is that the decrease in optical density was due to an increase in pool materials because of an accumulation of RNA degradation products. This would result in an increased osmotic pressure in the cell, bringing about an increase in diffusion of water into the cells, leading to a swelling of the cells and a decrease in optical density. This latter explanation seems the more likely.

Table 4. *Viable counts*

Escherichia coli 15 T^-U^-, previously treated for 1 hr. with chloramphenicol in salts + glucose + thymine + uracil, resuspended in phosphate buffer and viable counts determined during incubation at 37°.

Time in buffer	Viable count $\times 10^6$	O.D.
0	263	145
30 min.	262	133
1 hr.	243	130
2 hr.	247	120

DISCUSSION

The results of the present work help to clarify the problem of antibiotic-induced lag and antibiotic-induced RNA synthesis in *Escherichia coli*. It appears that the lag occurs only under conditions where antibiotic-induced RNA synthesis occurs, and when this antibiotic-induced RNA synthesis is prevented by any means (such as deprivation of a nutrient essential for its synthesis) no subsequent lag occurs. Interesting in this regard are the results with the arginine- and methionine-requiring mutants which did not synthesize normal RNA but which synthesized antibiotic-induced RNA in the absence of added arginine and methionine, respectively, and which exhibited a subsequent lag after this synthesis. A proline-requiring mutant which did not synthesize chloramphenicol-induced RNA in the absence of proline exhibited no lag on recovery.

The observations of Borek & Ryan (1958) are pertinent to this discussion. They found that in one particular methionine-requiring mutant of *Escherichia coli*, RNA

was synthesized in the absence of methionine, and the RNA-rich organisms exhibited a lag after methionine was added before growth was resumed. (No antibiotic was added in this experiment.) This lag may have been due to the accumulation of abnormal RNA which had to be degraded before growth could be resumed. The results of Borek & Ryan may be analogous to the present results concerning antibiotic-induced lag.

Since no requirements for energy-yielding or essential nutrients were found, recovery from the antibiotic-induced lag appears to be a degradative process in which hydrolytic enzymes cleave abnormal RNA. During the recovery period, DNA and protein synthesis did not occur, but the organisms appeared to be able to synthesize RNA during the lag while RNA was being degraded, provided that energy sources and the RNA precursors were supplied. This new RNA synthesis is probably not essential for recovery, since recovery occurred in the absence of many nutrients essential for new RNA synthesis. However, the fact that RNA synthesis occurred during the lag (even though not required) may be a further indication of the hierarchy of macromolecular syntheses for growing organisms (Kjelgaard *et al.* 1958).

Excretion of the degradation products is not a requirement for recovery, since when the cells were suspended in growth medium, these degradation products were immediately incorporated into new high molecular weight RNA. The degradation products apparently accumulated both within the cell and in the supernatant fluid under non-growth conditions (Neidhardt & Gros, 1957). The accumulation within the organisms was probably responsible for the decrease in optical density of suspensions that took place in organisms under non-growth conditions, since there was no evidence of lysis.

An unanswered question from this and earlier investigations is: why does the lag occur? It is not a sufficient explanation to state that this is abnormal RNA and thus prevents growth. Why is this RNA abnormal? As yet there is no answer to this question, since so much is lacking about our understanding of the macromolecular strategy of normal growth processes.

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Some Properties of a Cytopathogenic Bovine Orphan Virus (Van den Ende Strain)

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SUMMARY

A cytopathogenic bovine orphan (CBO) virus isolated from a bovine with lumpy skin disease appears unrelated to any of the three groups of viruses now associated with this condition. The virus multiplies efficiently in cultures of whole chick embryo tissue and may be titrated by plaque counting methods. Tissue culture fluids contain infective particles of at least three sizes, with sedimentation constants of 460, 72 and 20 S. The intermediate particle, if spherical and lipid free, would be comparable in size to the virus of foot and mouth disease. Heterogeneity of the virus has also been demonstrated by plaque morphology, electrophoresis and chromatography on DEAE cellulose. It is adsorbed to fresh but not to receptor-destroying enzyme (RDE) treated red cells of chicken and goose, but haemagglutination has not been observed with cells of any of the species tested. The virus lacks enzyme activity comparable to that of influenza virus.

INTRODUCTION

While investigating the etiology of bovine lumpy skin disease, Van den Ende, Alexander, Don & Kipps (1948) and Van den Ende, Don & Kipps (1949) reported the isolation in eggs of a virus from a skin nodule and a lymph gland of a calf which had died while the disease was still active. This virus produced unique and characteristic lesions in chick embryos. By common usage and in affection for the late Director of the Virus Research Unit, this virus has come to be known throughout the Union of South Africa as the 'Van den Ende Virus' but it is better called the Van den Ende strain of a cytopathogenic bovine orphan (CBO) virus. What appeared to be a successful transmission of the disease to bovines by inoculation of material from the 66th egg passage (Van den Ende & Turner, 1950), led to the assumption that this virus was in fact the cause of lumpy skin disease (Polson & Turner, 1954). Further studies, however, did not confirm this association and the earlier apparently successful transmission was recognized in retrospect as a natural infection in the laboratory herd.

The etiology of lumpy skin disease is now well known, and Alexander, Plowright & Haig (1957) reported the isolation of three groups of viruses from bovines suffering from the disease; of these viruses the prototypes Allerton virus and Neethling virus are representative of the responsible etiological agents. Although numerous isolations of these two viruses have been made, neither of them bears any relationship to the virus described in this paper. Attempts to re-isolate CBO virus from

bovines with lumpy skin disease have all been unsuccessful. An enteric cytopathogenic bovine orphan (ECBO) virus isolated during this investigation was found to possess some of the characters of the Van den Ende strain but was antigenically distinct. The present paper describes some of the physical and biological properties of the Van den Ende strain of CBO virus. The effect of inhibitors present in normal animal sera upon this virus is discussed in another communication (Turner, Kipps, Polson & Van den Ende, 1961).

METHODS

Virus

After its isolation, CBO virus was at first maintained by periodic amniotic or allantoic passage in chick embryos. Later it was found to be stable after drying from the frozen state, and active lyophilized reference stocks of the 16th egg passage have been kept for many years.

Propagation in tissue culture

CBO virus grows readily in cultures of whole chick embryo tissue. Cell suspensions were prepared by tryptic digestion of 9–10 day chick embryos and grown in a medium consisting of 0·5 % (w/v) lactalbumin hydrolysate in Hanks balanced salt solution with 20 % (v/v) horse serum. In the maintenance medium the serum was omitted.

Virus stocks for the majority of the experiments were harvested from Roux flask cultures when cytopathic changes were complete, approximately 3 days after inoculation. After light centrifugation to remove tissue debris, the supernatant fluid was stored in sealed glass ampoules at $-70°$. There was little loss of infectivity over a period of many months.

Titration

The titration of the virus in eggs has been described (Van den Ende & Turner, 1950). Titrations in tissue culture were carried out by the plaque techniques of Dulbecco (1952) and Cooper (1955). Chick embryo cultures were prepared in 4 cm. diameter Petri dishes using not less than 1×10^7 cells/dish in 3 ml. nutrient medium and duplicate dishes were seeded with 0·2 ml. volumes of virus suspension or serum-virus mixture. The plates were incubated at 37° in air containing about 5 % (v/v) CO_2 for 2 days, after which they were flooded with 1/5000 neutral red in Earle's saline. The dye was removed after 2 hr. when the plaques could be easily counted. Repeat counts were made next day. Titres were expressed as plaque forming units (p.f.u.).

Antisera

Immune sera were produced in fowls and rabbits. Adult hens were inoculated with graded intramuscular doses of emulsions of infected embryos. The intravenous route was used for the rabbits. Blood was collected 10–12 days after the last of a course of 7–8 injections given twice weekly. The blood was allowed to clot and the separated serum heated at 56° for 30 min. and stored at $-20°$.

Neutralization tests

Two methods were used. In the first, a single concentration of virus known to produce about 60 plaques/plate was used. Unit volumes of tenfold dilutions of serum were mixed with equal volumes of the virus dilution, the mixtures allowed to stand at room temperature for 1 hr. and each mixture then used to inoculate duplicate agar suspension cultures of chick embryo cells (Cooper, 1955).

In the second method serial tenfold dilutions of both serum and virus were used so that the titre of the virus in the presence of different concentrations of antibody or inhibitor could be measured. The time of contact and the method of plating were the same as in the method employing a single concentration of virus.

Zone electrophoresis

The technique of Polson & Cramer (1958) was used. The virus suspension was mixed with phenol red and rabbit haemoglobin as reference substances. Electrophoresis was allowed to proceed at room temperature under a voltage gradient of 3·5 V./cm. and a current of 15 mA. through a sucrose density gradient (35 to 0 %) in borate buffer (pH 8·6) until the phenol red reached a level of 16 cm. from the origin. The gradient column between the origin and the phenol red was divided into 16 equal fractions by removal through a fine capillary at the bottom of the column. Each fraction was titrated for infectivity in agar suspension cultures of chick embryo cells (Cooper, 1955).

Ultracentrifugation

Sedimentation coefficients were determined by the methods of Polson & Van Regenmortel (to be published) using the S.W. 39 rotor of the model LH Spinco centrifuge at temperatures below 4°. In these methods convection was prevented by a steep sucrose concentration gradient below the layer of virus suspension. In a series of tubes the length of the 'effective virus column' was kept constant at 1 cm. After centrifugation the whole of the fluid above the sucrose gradient was removed and titrated. In this way the conditions of centrifugation necessary to cause sedimentation of the virus through a measured distance were accurately maintained. Series of tubes prepared as indicated were centrifuged either at constant rotor velocity for different time intervals, or for constant periods of time at various rotor velocities.

The sedimentation constants of the particles under investigation were calculated from the following equations:

$$S_{20} = 3{\cdot}50 \frac{\eta_T}{\eta_{20}} \frac{\log X}{N^2 t}, \tag{1}$$

where η_T and η_{20} are the viscosities of the dispersion medium at the temperature of centrifugation and of water at 20° respectively, N the rotor velocity in rev./min. and t the time of centrifugation in minutes,

$$X = \frac{x + l}{x + l\,(C_t/C_0)}$$

in which x is the distance from the upper meniscus to the centre of rotation, l is the effective column (i.e. the distance between the upper meniscus and the upper limit

of the sucrose gradient), and C_t/C_0 the ratio of average virus or protein concentration in the 'effective column' after and before ultra-centrifugation.

DEAE chromatography

The CBO virus in infected tissue culture fluid was concentrated by pervaporation and centrifugation at 30,000 rev./min. for 2 hr. The final pellet was suspended in 2 ml. distilled water and applied to a 10 cm. column of DEAE cellulose (Peterson & Sober, 1956), containing 0·7 g. of adsorbent which had been equilibrated with 0·01 M-phosphate buffer (pH 7·2). A gradient prepared from 50 ml. of the buffer and 50 ml. of M-NaCl was used for elution. Twenty-five 2 ml. fractions were collected and titrated individually in chick embryo tissue culture by the plaque technique.

Enzymes

Dried commercial receptor-destroying enzyme (RDE; Philips-Roxane) was reconstituted to the volume recommended by the manufacturer in pH 6·0 buffer containing 100 units penicillin and 100 μg. streptomycin/ml. Active influenza B virus (Lee) propagated in eggs according to well known standard techniques was used as a source of neuraminidase.

RESULTS

Adaptation of virus to tissue culture

Virus passaged in chick embryos grew well in chick embryo tissue cultures and produced plaques. The ratio of p.f.u./ml. to the titre in chick embryo was relatively low in extracts of infected embryos and in fluid obtained from the first two or three passages in tissue culture, but the ratio increased rapidly to a value which remained almost constant after many passages in tissue culture (Table 1). The typical lesions are still regularly observed in chick embryos inoculated with the tissue culture adapted virus.

Table 1. *The adaptation of CBO virus to chick embryo tissue culture and the ratio of p.f.u./ml. to the titre in eggs*

Virus	p.f.u./ml.	Titre in eggs (LD50/ml.)
Suspension of virus-infected egg	$10^{3\cdot8}$	$10^{6\cdot2}$
Suspension from plate tissue culture after 1 TC passage	$10^{4\cdot4}$	$10^{7\cdot2}$
Suspension from plate TC after 3 TC passages	$10^{6\cdot1}$	$10^{7\cdot3}$
Suspension from plate TC after 4 TC passages	$10^{6\cdot2}$	$10^{7\cdot1}$

Plaque morphology

After incubation at 37° for about 48 hr. two types of plaque were encountered. One was well defined and 1–2 mm. in diameter while the other, appearing in roughly equal numbers, was less well defined and measured 2–5 mm. in diameter. Incubation for another 24 hr. resulted in the enlargement of existing plaques and the appearance of many secondary plaques. On subculture, virus from either type of plaque yielded a mixture of the two.

Neutralization tests

The concentration of the virus clearly has an important bearing on the apparent neutralizing titre of the serum (Table 2). Most CBO fowl immune sera in dilutions of 10^{-3} showed significant neutralization. These sera did not neutralize the Allerton and Neethling strains of lumpy skin disease virus.

Titration of antibodies to CBO virus in bovine sera of both normal animals and those convalescent from lumpy skin disease were found to be unreliable owing to the non-specific neutralization by inhibitors in both groups of sera. Neutralization tests with CBO antisera produced in rabbits were similarly complicated by the presence of high titre non-specific inhibitors. These inhibitors were found in many other animal sera in varying concentration (Turner *et al.* 1961).

Table 2. *Neutralization of the virus by fowl immune serum in agar suspension cultures of chick embryo cells*

c, confluent plaques; sc, semi-confluent plaques.

Virus dilution	Average number of plaques/plate serum dilution					Control without serum
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	
10^{-2}	0	12	c	—	—	c
10^{-3}	0	1	58	c	—	c
10^{-4}	0	0	35	sc	sc	c
10^{-5}	0	0	0	22	37	37
10^{-6}	0	0	1	3	6	5

Animal inoculation

Numerous attempts were made to develop a pathogenicity test in animals but the CBO-virus consistently failed to cause detectable symptoms or lesions when inoculated by various routes into rabbits, guinea pigs, adult and suckling mice, ferrets, sheep and bovines, as well as young chicks and adult domestic fowls.

Electrophoretic mobility of CBO virus particles

Three different electrophoresis experiments were done (Fig. 1). The two peaks indicate that the infective particles are electrophoretically inhomogeneous. These diagrams are quite different from those obtained by the same technique from the three groups of viruses isolated by Alexander *et al.* (1957) from bovines with lumpy skin disease (Polson, unpublished). No further characters have been ascribed to these two electrophoretically distinct components of the CBO virus.

Ultracentrifugation

Five experiments were done to determine the relationship between virus titre and the speed of centrifugation. The results (Fig. 2) show the presence in infected tissue culture fluid of virus particles having sedimentation constants of 460 S and 72 S. Experiments with fluid from which the 460 S particles had been removed by centrifugation at 10,000 rev./min. for 90 min. revealed, in addition to the 72 S particles, a small proportion of very light infective units which were not completely sedi-

mented during prolonged centrifugation at 30,000 rev./min. and had a sedimentation constant less than 20 S.

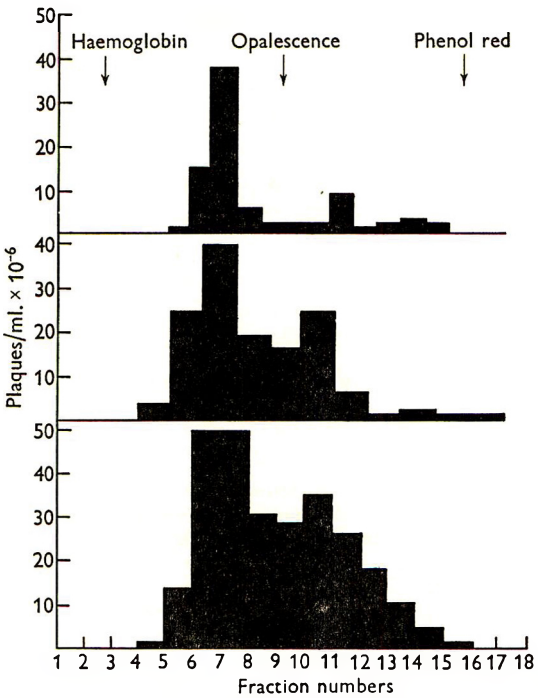

Fig. 1. Zone electrophoretograms of CBO virus in a sucrose density gradient at pH 8·6, 3·5 V./cm. and 15 mA.; phenol red and rabbit haemoglobin were added to indicate the progress of electrophoresis. The two peaks indicate inhomogeneity of the virus.

Chromatography on DEAE cellulose

Most of the virus was eluted at NaCl molarities between 0·2–0·3 (Fig. 3). About 10 % of the virus appeared to be eluted at a slightly lower salt concentration than the component in the main peak.

Adsorption of CBO virus to red cells

Attempts were made to agglutinate various washed red cells with virus obtained from chick embryo tissue culture. These experiments were conducted at 4°, at room temperature and at 37° in a range of pH values from 6·0 to 7·6. All were negative. Virus suspensions concentrated by centrifugation to a titre of 4×10^8 p.f.u./0·2 ml. failed to agglutinate fowl and goose red cells even when followed by CBO fowl antiserum. However, adsorption of the virus by goose and fowl red cells was indicated by centrifuging mixtures of cells and virus after 3 hr. contact at 4° and titrating the supernatant fluid and the lysed sediment brought to the original volume (Table 3). The virus was eluted when the cells were lysed in distilled water. Since many normal sera were known to contain inhibitors capable of neutralizing the CBO virus, the effects of enzymes on the sites of adsorption of the virus particles on the red cells were tested. RDE effectively decreased the number of receptor sites on fowl cells for the CBO virus (Table 4). The virus itself, however, lacked enzyme activity of the

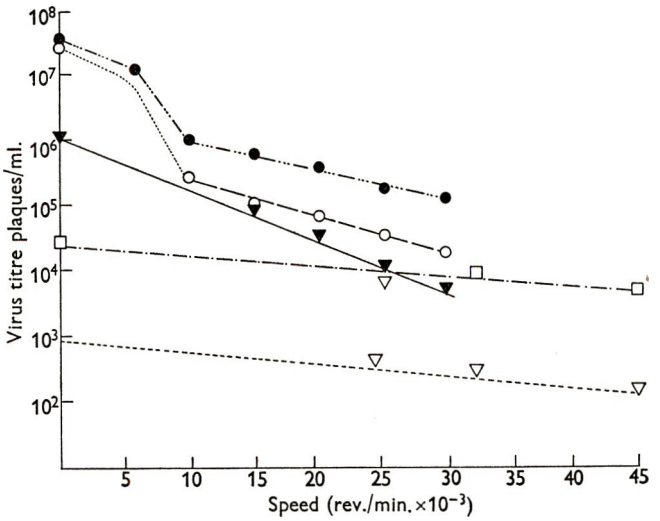

Fig. 2. Sedimentation diagrams of CBO virus. The infective titre of the fluid in the 1 cm. column above a level 7·1 cm. from the centre of rotation is plotted against the speed of centrifugation. The duration of centrifugation was 90 min. in all cases. ●—···—···●, Titre of original virus suspension spun at the speeds indicated and showing the presence of rapidly and slowly sedimenting components; ○— —○, repeat of first experiment (dotted line imaginary); ▼—▼, titre of virus suspension after centrifugation of the material from which the rapidly sedimenting components had been removed by 10,000 rev./min. for 90 min.; □—·—·—□, titre of virus suspension after centrifugation of the material from which the fast and the slower sedimenting components had been removed by 30,000 rev./min. for 90 min.; ▽- - - -▽, titre of virus suspension after centrifugation at 25,000 rev./min. for 90 min. to remove the particle of 20S = 72 and spun again at the velocities indicated.

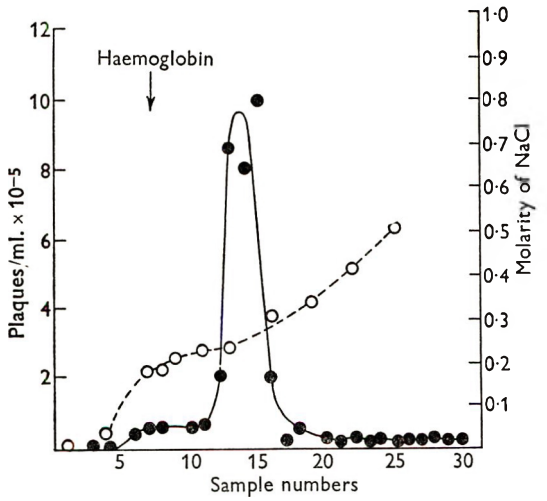

Fig. 3. Chromatography of CBO virus on DEAE cellulose. The bulk of the virus is eluted at concentrations between 0·2–0·3 M-NaCl, but about 10% of the virus elutes at slightly lower salt concentrations. The arrow marks position at which rabbit haemaglobin elutes. ●, Virus titre; ○, molarity of NaCl.

type exhibited by influenza virus, since adsorbed CBO virus did not elute at 37° and treatment of fowl cells with CBO virus did not reduce their agglutinability by influenza B virus (Lee).

Table 3. *The adsorption of CBO virus to goose and fowl cells*

Adsorption 3 hr. at 4°; 2 ml. packed cells and 0·5 ml. virus suspension.

Sample	Virus titre (p.f.u./ml.)
(1) Original untreated virus suspension	$2{\cdot}0 \times 10^6$
(2) Virus suspension after adsorption with goose RBC's	$8{\cdot}0 \times 10^4$
(3) Laked goose RBC's used for adsorption	$3{\cdot}5 \times 10^6$
(4) Virus suspension after adsorption with fowl RBC's	$1{\cdot}7 \times 10^5$
(5) Laked fowl RBC's used for adsorption	$5{\cdot}0 \times 10^6$

Table 4. *Adsorption of CBO virus to fowl red cells after treatment of cells with RDE*

2 ml. packed cells + 10 ml. RDE at 37° for 2 hr. washed × 5 in saline then 2 ml. packed cells + 0·5 ml. virus; 3 hr. at 4°.

Sample	Virus titre (p.f.u./ml.)
(1) Original untreated virus suspension	$4{\cdot}0 \times 10^6$
(2) Virus suspension after adsorption with buffer treated cells	$2{\cdot}0 \times 10^5$
(3) Virus suspension after adsorption with RDE treated cells	$6{\cdot}0 \times 10^6$

DISCUSSION

The findings of Alexander *et al.* (1957) make it improbable that the CBO virus has any etiological relationship to bovine lumpy skin disease. This virus, isolated from a skin nodule of a calf in the acute stages of the disease, produced characteristic lesions in chick embryos which have been previously described (Van den Ende *et al.* 1948, 1949). CBO virus was readily adapted to growth in cultures of chick embryo cells, and produced well defined plaques in agar suspension cultures of chick embryo cells (Cooper, 1955). Tissue culture fluid frequently contained more than 10^6 p.f.u./ml. Physical studies of this virus facilitated by the plaque count technique showed that it was inhomogeneous. Plaques of two sizes were always seen. Infective particles of at least three sizes were detectable by centrifugation, but the finding that one infective particle has a sedimentation constant of 20 S or less is of some interest. The slow sedimentation of this component may be due to a high degree of asymmetry (a filamentous particle perhaps?) or to a diminished particle density owing to the presence of lipid. The particle of 72 S corresponding to 19 mμ may be compared with the virus of foot and mouth disease which has a similar sedimentation constant (Bradish & Brooksby, 1960). We have not been able to relate the differences in plaque morphology with differences in sedimentation constants. If all the virus particles carried the same surface charge density irrespective of size, all should migrate together in an electric field. Electrophoresis of the virus, however, revealed the presence of at least two electrophoretic components. Whether these correspond with the two larger components separated by ultracentrifugation and which account for nearly all the infectivity, has not been determined. The behaviour of the virus

on electrophoresis was, however, quite different from that of any of the virus strains associated with bovine lumpy skin disease (Polson, unpublished). Inhibition of the infectivity of CBO virus by normal sera of several animal species has complicated serological studies, and investigation of this inhibitor is the subject of another paper (Turner *et al.* 1961). Although the virus is adsorbed by fowl and goose red cells, all attempts to demonstrate haemagglutination either directly or indirectly, were unsuccessful; nor was any evidence obtained of enzyme activity similar to that of the influenza viruses. The characters of the ECBO viruses are not yet sufficiently defined to be able to decide whether the CBO virus belongs to this group but some points of similarity make it a possibility.

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Neutralization of a Cytopathogenic Bovine Orphan Virus in Tissue Culture by Heat Stable Francis Type Inhibitors in Normal Animal Sera

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SUMMARY

A heat stable inhibitor present in the serum of normal animals was found to neutralize the infectivity of a cytopathogenic bovine orphan (CBO) virus. Its distribution and some physical, chemical and biological properties were examined. These properties suggest that the inhibitor for the virus infectivity is similar to, but not identical with Francis type inhibitors in normal animal sera.

INTRODUCTION

Many non-specific inhibitors of virus activity have been described in the tissues, body fluids or sera of normal animals. These inhibitors have been differentiated principally by the viruses which they affect, by their sensitivity to heat, enzymes or chemical reagents and by their ability to inhibit haemagglutination or to neutralize infectivity. Only a few have been obtained in pure or almost pure state. During an investigation of the properties of the Van den Ende strain of a cytopathogenic bovine orphan (CBO) virus (Kipps, Turner & Polson, 1961), it was noticed that the sera of normal animals of several species caused striking inactivation of the virus, shown by a decrease of the infectivity in chick embryos or the plaque-forming capacity in chick embryo tissue cultures. Further study revealed that the inactivating agent was relatively resistant to heat and resembled in many respects the heat stable serum components inhibiting haemagglutination by myxoviruses collectively known as the Francis, or α, inhibitor. The present paper describes some of the properties of the CBO virus inhibitor and its relationship with other known inhibitors.

METHODS

The viruses, the preparation of antisera, the propagation and titration of this CBO virus and the methods of neutralization are described in the preceding paper (Kipps *et al.* 1961).

Normal sera. Sera were obtained from clotted blood and heated to 56° for 30 min. to inactivate heat labile inhibitors. When serum samples were not used immediately they were stored at −20°.

Mucoproteins. Samples of meconium and of urinary and sheep salivary mucoprotein were obtained from the Walter and Eliza Hall Institute, Melbourne,

Australia. Egg white inhibitor was prepared according to the method of Sugihara, McDonell, Knight & Feeney (1955). Ovomucin from allantoic fluid was concentrated by dialysis of the fluid against pH 4·3 buffer at 4°. The resultant precipitate was redissolved in a minimal volume of 0·066 M-phosphate buffer (pH 8·2).

Haemagglutination inhibition (HI). Inhibition of haemagglutination was estimated by standard methods using 4 agglutinating doses (AD) of heated influenza B virus (Lee) as indicator.

Electrophoresis. Two methods were used for the separation of inhibitor in normal rabbit sera. One made use of the method of Polson (1952) allowing the simultaneous analysis of two solutions, and the other the technique of Svensson & Valmet (1955) in a vertical column of modified cellulose (Peterson & Sober, 1956).

Preparation of materials. Crystalline trypsin (Armour) was dissolved in phosphate buffer (pH 8·2) immediately before use. Commercial receptor-destroying enzyme (RDE, Phillips Roxane) was reconstituted according to the maker's instructions. Solutions of potassium periodate of various molarities were freshly prepared in distilled water. Zymosan and properdin-free sera were prepared by the method of Pillemer *et al.* (1954).

Buffer solutions. Dilute acetate buffer (pH 5·2) contained 2 g. NaOH and 4 ml. glacial acetic acid/l. Phosphate buffers were prepared by mixing 0·066 M-Na_2HPO_4 and 0·066 M-KH_2PO_4 to give the required pH value; pH 4·3 buffer contained 2·5 g. citric acid and 2·5 g. Na_2HPO_4/l.

RESULTS

Neutralization of CBO virus by normal sera

The apparent neutralizing titre of the serum depends on the virus concentration (Table 1), but with 60–100 plaque-forming units (p.f.u.) most rabbit sera diluted 10^{-3} caused a 50 % reduction in the plaque count. For this reason normal rabbit

Table 1. *Neutralization of CBO virus by normal rabbit serum*

Virus dilution	Serum dilutions 10^{-1}	10^{-2}	10^{-3}	10^{-4}	Control without serum
10^{-2}	65	c	c	c	c
10^{-3}	15	sc	sc	c	c
10^{-4}	3	42	85	sc	sc
10^{-5}	1	11	34	60	60
10^{-6}	0	0	5	12	13

Numbers = average plaque count from duplicate plates; c, confluent plaques; sc, semi-confluent plaques.

serum was used for most of the experiments. Normal rabbit serum was also tested for its neutralizing activity in chick embryos, mice or monkey kidney tissue cultures against the viruses of influenza (PR 8), Newcastle disease, poliomyelitis (Mahoney), rabies (Flury), Rift Valley fever (pantropic and neurotropic), African horse sickness, yellow fever, West Nile and Semliki Forest viruses. In no instance was significant inhibition of infectivity demonstrated. The concentration of inhibitor in the heated sera of a variety of normal animals was determined against 60 p.f.u. of CBO virus. In order to allow as far as possible for individual variations, pooled sera from a

group of each species were used. The neutralizing effect of human, rabbit and guinea-pig sera was high, that of rat, bovine, horse and ferret sera somewhat lower, while fowl and mouse sera had little if any activity (Fig. 1).

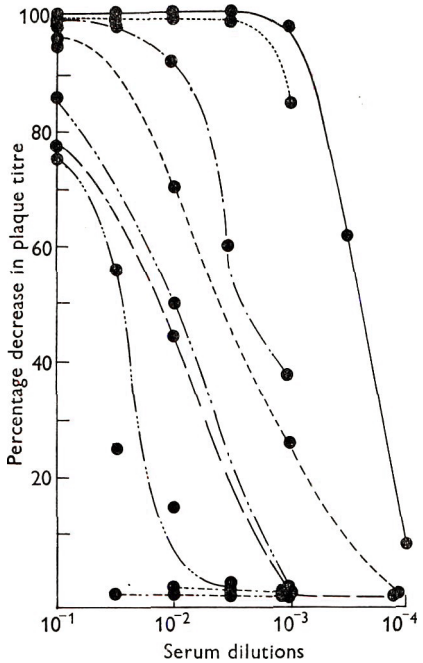

Fig. 1. The inhibitory effect of various normal animal sera against 60 p.f.u. of virus. Sera: ●—●, rabbit; ●···●, human; ●—·—·●, guinea-pig; ●– – –●, rat; ●–·–·–●, bovine; ●— —●, horse; ●—···—···●, ferret; ●–·–·–·–●, mouse; ●–·–·–●, fowl.

Relation between inactivation of CBO virus and haemagglutination inhibition

The neutralizing powers of the pooled sera against CBO virus were compared with their potency as inhibitors of haemagglutination by heated influenza B virus (Lee) (Table 2). Although there was some parallelism between the neutralizing and HI

Table 2. *Haemagglutination-inhibition and CBO virus neutralization by normal animal sera*

Normal* animal sera	Reciprocal of dilution of serum causing inhibition of haemagglutination by 4 AD Lee B indicator virus	Reciprocal of dilution of serum causing 50 % reduction of plaque count with 60 p.f.u. CBO virus
Mouse	10	< 10
Ferret	2580	10–100
Fowl	80	< 10
Guinea-pig	320	100–1000
Human	1280	> 1000
Horse	5	100–1000
Bovine	5	100
Rabbit	80	> 1000

* Heated at 56° for 30 min.

powers of the different sera (in the cases of human and mouse sera for example), ferret serum was relatively more effective as an inhibitor of haemagglutination and rabbit serum more effective as a neutralizer of CBO virus. Mucoproteins from sources other than serum, though highly active as inhibitors of haemagglutination, failed to neutralize CBO virus (Table 3).

Table 3. *Haemagglutination-inhibition and CBO virus neutralization by mucoproteins*

Mucoprotein from	Concentration	Dilution causing inhibition of haemagglutination by 4 AD Lee B indicator virus	Inhibition of 60 p.f.u. CBO virus by undiluted mucoprotein
Meconium	10 mg./ml.	10^{-4}	nil
Sheep salivary mucin	1 mg./ml.	10^{-6}	nil
Urinary mucin	1 mg./ml.	10^{-4}	nil
Egg white in saline	20 % (v/v)*	$10^{-1\cdot8}$	nil
Allantoic fluid	20 × conc.*	$10^{-1\cdot8}$	nil

* See text, p. 416.

Concentration of the CBO inhibitor from rabbit serum

The precipitates formed by bringing the normal serum to half saturation with ammonium sulphate contained much of the virus neutralizing activity. Dialysis overnight against acetate buffer pH 5·2 was as effective. Little activity remained in the supernatant fluid and most of it could be recovered from the precipitate by extraction with phosphate buffer of pH 8·4 (Table 4). The sera of fowls which had been immunized against CBO virus contained neutralizing antibody which, however, was not precipitated at pH 5·2, the activity remaining in the supernatant fluid (Table 5). The agent was not sedimented by centrifugation of the serum at 33,000 rev./min. for 3 hr. in the no. 40 rotor of a Spinco Model L centrifuge, nor was it present in the lipid layer which separated during this treatment. When the serum was shaken with ether and the mixture centrifuged the agent remained in the aqueous phase.

Table 4. *Plaque count in presence of serum fractions diluted* 1/10

Sample	Plaque count
(1)* Globulins	$2\cdot0 \times 10^3$
(2) Albumin	$1\cdot1 \times 10^6$
(3) Virus alone	$4\cdot8 \times 10^6$
(4) pH 5·2 ppt	$2\cdot0 \times 10^4$
(5) pH 5·2 SNF	$5\cdot0 \times 10^7$
(6) Virus alone	$4\cdot0 \times 10^7$

* (1) Precipitate at half saturation with $(NH_4)_2SO_4$ redissolved in distilled water and dialysed against saline.

(2) Supernatant fluid from (1) dialysed to remove $(NH_4)_2SO_4$.

(4) Serum dialysed overnight at 4° against a large volume of dilute acetate buffer (pH 5·2). The precipitate redissolved in phosphate buffer (pH 8·4) dialysed against buffered saline and brought to the volume of the original serum.

(5) The supernatant fluid (SNF) from (4) dialysed against buffered saline.

Table 5. *Fractionation of CBO neutralizing agent in fowl immune serum by precipitation at* pH 5·2

The sera (normal or immune) were dialysed at 4° overnight against acetate buffer (pH 5·2). The precipitates were redissolved in phosphate buffer (pH 8·4). The supernatant fluids and the redissolved precipitates were dialysed against buffered saline and, after correction of their volumes to those of the original sera, were titrated against CBO virus in agar suspension cultures of chick embryo cells.

	Dilution of sample causing 50 % decrease in plaque count		
	Original serum	pH 5·2 precipitate	pH 5·2 supernatant fluid
Normal fowl serum	10^{-1}	N.D.	N.D.
Immune fowl serum	$10^{-3\cdot8}$	$10^{-1\cdot8}$	$10^{-3\cdot5}$

N.D. = not done.

Electrophoresis of the CBO inhibitor

Normal rabbit serum which had been heated at 56° for 30 min. was submitted to electrophoresis in phosphate buffer (pH 8·2, ionic strength 0·1). The apparatus (Polson, 1952) allowed simultaneous analysis of two solutions and convenient sampling after electrophoresis. The progress of separation (descending boundaries only) was observed by the Lamm scale method. After electrophoresis at 4·5 V./cm. and 1·5° for about 4 hr., successive layers were removed (Polson, Joubert & Haig, 1946), serially diluted and tested for neutralizing activity against CBO virus on agar suspension cultures of chick embryo cells (Cooper, 1955). Photographs of the scale taken before sampling and after removal of each fraction enabled the electrophoresis diagram and the relation thereto of the samples to be accurately determined. The electrophoresis diagram of the serum (Fig. 2) shows the position of the samples tested and relative neutralizing power. Most of the inhibitor migrated to a position between the albumin and α globulin.

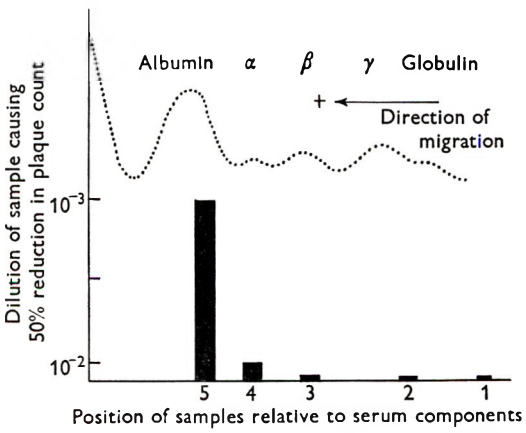

Fig. 2. Electrophoresis of normal rabbit serum at pH 8·2, 4·5 V./cm. and 1·5° for 4 hr. showing the position of the virus inhibitor.

Partially purified CBO inhibitor was also submitted to zone electrophoresis at pH 8·6. The fraction of normal rabbit serum precipitated at pH 5·2 was redissolved in phosphate buffer (pH 8·6) and centrifuged at 12,000 rev./min. for 15 min. The sediment and a small lipoid layer which collected at the surface were discarded. The solution, which was faintly blue, was dialysed against borate buffer (pH 8·6) and submitted to electrophoresis at 46–47 mA. for 19 hr. in a vertical column of modified cellulose (Peterson & Sober, 1956) in the apparatus of Svensson & Valmet (1955) adapted to permit continuous recirculation of buffer through the electrode vessels. Buffer streams leaving the anode and cathode compartments were mixed and returned to an overhead supply vessel by a small pump. The current through the apparatus and the pK value of the buffer then remained constant throughout a run. After electrophoresis the column contents were displaced by buffer and collected fractionally. The fractions were tested for ultraviolet absorption at 275 mμ, neutralizing activity against CBO virus and haemagglutination inhibition against heated Lee B virus (Fig. 3).

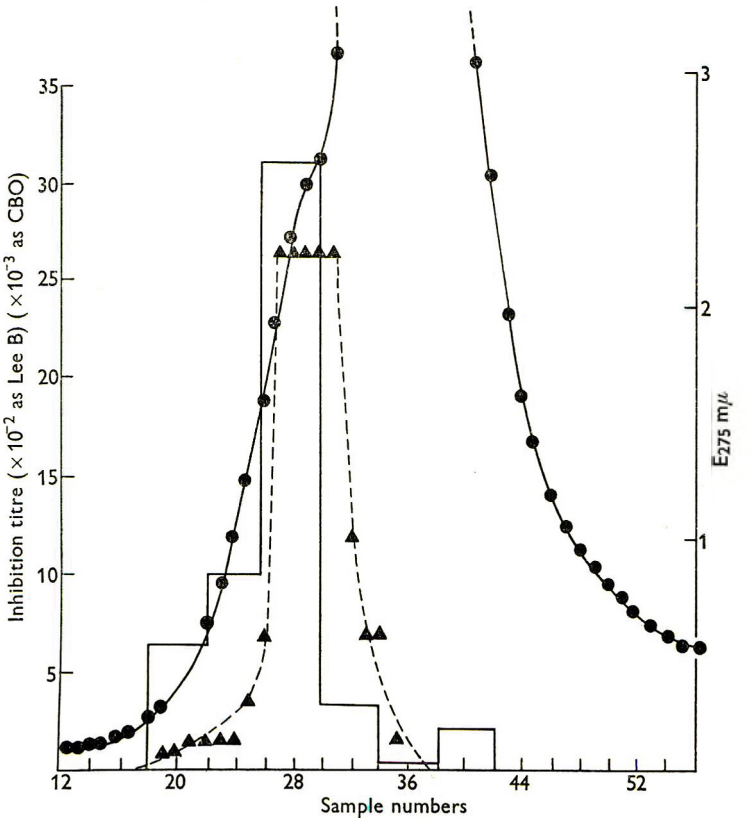

Fig. 3. Column electrophoresis of partially purified inhibitor. ●—●, Light absorption; ▲--▲, inhibition of 4 AD Lee B indicator virus; ⊓, 50 % inhibition of CBO virus.

The effect of heat on the CBO inhibitor in normal rabbit and human sera

The neutralizing power of normal rabbit serum against CBO virus was not significantly decreased by heating for 1 hr. at 56°. Portions of normal rabbit serum diluted

1/5 in saline were heated in a water-bath for 30 min. at various temperatures above 56° and assayed for haemagglutination inhibition (HI) of Lee B virus and neutralization of CBO virus. In the temperature range 65°–100° the neutralizing activity was more sensitive to heat than the HI activity; both were, however, still detectable in the sample heated at 100° for 30 min. (Fig. 4).

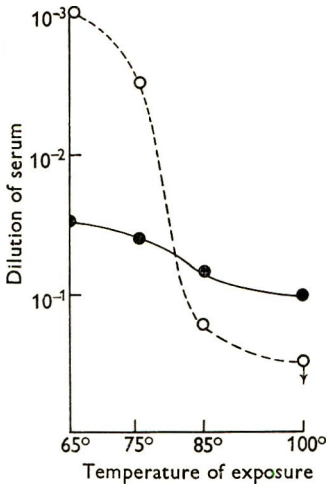

Fig. 4. The effect of heat on the inhibitors in normal rabbit serum to CBO virus and influenza (Lee B) virus. ○----○, Serum dilutions causing 50 % decrease in plaque count of CBO virus; ●—●, serum dilution causing inhibition of 4 AD of influenza virus (Lee B).

Zhdanov, Hamburg & Svet-Moldavsky (1959) showed that the HI activity of normal human serum towards certain strains of Type A_2 influenza virus was greatly increased by heating at 85°. A sample of normal human serum heated at this temperature for 15 min. was found to have lost most of its neutralizing activity against CBO virus whereas its HI titre when tested against influenza virus A_2/Cape Town/59 had increased from 60 to more than 2560.

The effect of receptor-destroying enzyme (RDE) and active influenza B virus (Lee) on the CBO inhibitor in normal rabbit serum

One ml. portions of normal rabbit serum were treated either with 5 ml. RDE solution or 5 ml. saline, incubated for 18 hr. at 37°, heated for 1 hr. at 56° and assayed for neutralization of CBO virus and HI of influenza B indicator. Similarly, 1 ml. amounts of normal rabbit serum were treated with 1 ml. volumes of active influenza B virus (Lee) purified according to Burnet (1948) and containing 400 AD. The mixtures were incubated at 37° for 18 hr., heated to 65° for 30 min. and diluted for assay. Controls were prepared in the same way except that active Lee B virus was replaced by heat inactivated influenza B virus or by tap water. The CBO inhibitor resembled the haemagglutination inhibitor in normal rabbit serum in its sensitivity to the enzymes in RDE and active influenza B virus (Table 6).

The effect of trypsin on the CBO inhibitor in normal rabbit serum

One ml. portions of normal rabbit serum were treated either with 1 ml. of buffer (pH 8·2) containing 8·0 mg. crystalline trypsin or 1 ml. buffer without trypsin. The mixture was incubated at 37° for 20 hr., treated with 3 ml. saline containing 12 mg.

Table 6. *The effect of RDE and active Lee B influenza virus on the inhibitor in normal rabbit serum*

For details see text.

Normal rabbit serum treated with	% decrease of inhibitory activity of samples of serum against: CBO virus	Heated Lee B virus
(1) Nil	0	0
(2) Saline	2	5
(3) RDE	85	94
(4) Heated Lee B virus	4	25
(5) Water	4	25
(6) Active Lee B virus	85	94

soya-bean trypsin inhibitor, and assayed for CBO virus neutralizing activity and HI of influenza B (Lee) indicator virus. The enzyme caused considerable reduction in both types of inhibitory activity (Fig. 5).

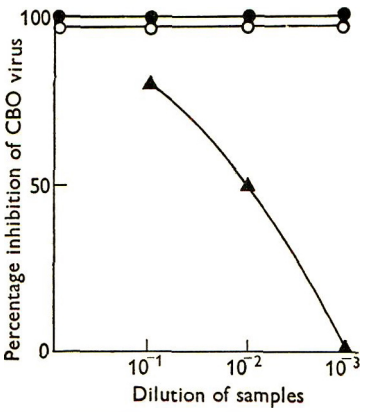

Fig. 5. Effect of trypsin on the inhibitor to CBO virus in normal rabbit serum. ●—●, control samples without trypsin (HI titre with heated Lee B virus = 160). ▲—▲, trypsin-treated sample (HI titre with heated Lee B virus < 5).

The effect of potassium periodate

Portions of normal rabbit serum were mixed with equal volumes of either saline or of one of a series of dilutions of a freshly prepared solution of potassium periodate. The mixtures were held at 37° for 1 hr., treated with glycerol (0·02 ml.) to destroy excess reagent and assayed for their effect on CBO and Lee B viruses. Both types of activity were about equally sensitive to periodate treatment (Fig. 6).

Differentiation of the CBO inhibitor from properdin

Treatment of freshly drawn normal rabbit serum with a concentration of zymosan which had proved sufficient to remove the C3 component of complement in normal rabbit serum had no effect upon its neutralizing activity against CBO virus. Normal

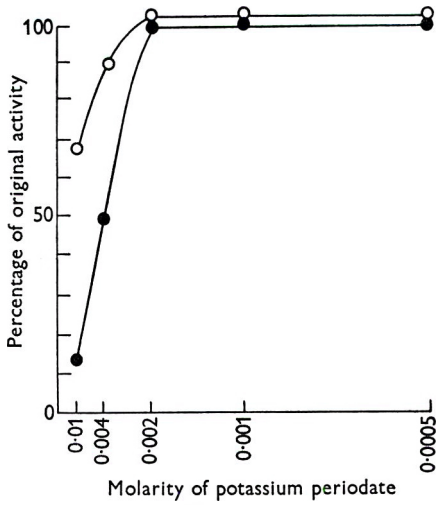

Fig. 6. Effect of periodate on CBO and Lee B inhibitors in normal rabbit serum. O—O, against heated Lee B virus; ●—●, against CBO virus.

human serum containing 5·7 units properdin/ml. and a sample of RP (Pillemer *et al.* 1954) prepared from it by removing all detectable properdin with zymosan, gave identical results in the neutralization test against CBO virus.

The effect of RDE and active influenza B virus (Lee) on the virus + inhibitor complex

During the experiments recorded above it was noticed that RDE and active influenza B virus (Lee) were capable of liberating active CBO virus from the inactive complex which it forms with the inhibitory agent. A mixture of undiluted normal rabbit serum and suitably diluted CBO virus was allowed to stand at room temperature for 1 hr. and divided into six portions. To one portion was added 1 ml. of active influenza B virus (Lee), to the second heat-inactivated influenza B virus (Lee) (56° for 30 min.), to the third 5 ml. of active RDE and to the fourth 5 ml. of heat-inactivated RDE (100° for 15 min.). Hanks solution (5 ml.) was added to portions 5 and 6. Portions 1–5 were incubated overnight at 37° and portion 6 was held at 4°. Two control mixtures of CBO virus and Hanks solution were similarly allowed to stand at room temperature for 1 hr. They were then diluted with 5 ml. Hanks solution and one was incubated overnight at 37° and one held at 4°. On the following morning RDE, where present, was inactivated by the addition of sodium citrate, and all the solutions were diluted to 10 ml. and assayed for HI activity with heated influenza B virus (Lee) and for active CBO virus. CBO virus was only slightly inactivated during incubation at 37° and sufficient inhibitor was supplied in the rabbit serum to inactivate the CBO virus completely. No reactivation of virus occurred as a result of the incubation or dilution, but active CBO virus was released from combination with the neutralizing agent of rabbit serum by RDE and active influenza B virus (Lee), both of which destroyed the HI activity of the serum against

heated influenza B virus (Lee) (Table 7). The recovery of active virus was about 50 % when RDE was used, about 10 % with active influenza B virus (Lee).

Table 7. *The effect of RDE and active influenza B virus (Lee) on the CBO virus + inhibitor complex*

CBO virus + inhibitor complex treated with	Titre of CBO virus p.f.u./ml.	Inhibitor content against 4 AD of Lee B indicator virus
(1) Active Lee B*	$1{\cdot}3 \times 10^3$	< 10
(2) Heated Lee B	Nil	40
(3) RDE	$6{\cdot}7 \times 10^3$	< 10
(4) Heated RDE	Nil	40
(5) Hanks at 4°	Nil	40
(6) Hanks at 37°	Nil	40
(7) CBO virus control at 4°	34×10^3	—
(8) CBO virus control at 37°	$12{\cdot}5 \times 10^3$	—

* Active Lee B did not produce plaques on agar suspension cultures of chick embryo cells.

DISCUSSION

The infectivity of the Van den Ende strain of CBO virus, demonstrable by the formation of plaques in chick embryo cell monolayer cultures, cytopathic effect in chick tissue cultures or mortality in chick embryos, appeared to be almost completely neutralized by normal rabbit sera heated to destroy inhibitors of the heat labile type. Such sera had no effect upon the infectivity of all the other viruses tested in this investigation with the exception of one strain of enteric cytopathogenic bovine orphan virus (ECBO).

In the normal sera examined, the species distribution of the CBO virus inhibitor differed considerably from that of the Francis type of inhibitor of influenza B (Lee) indicator virus. This difference was most marked in rabbit and ferret serum. This lack of parallelism in inhibitory activity has been demonstrated by Ananthanarayan & Paniker (1960) who showed that among the myxoviruses considerable variations occurred in the susceptibilities of different strains to the same or similar inhibitors. Conversely, it may be true that haemagglutination inhibition is not due to one substance but to a number of serum mucoproteins of varied activity towards different strains of virus.

The electrophoretic behaviour of the α inhibitor in animal sera was examined by Tyrrell (1954), Harboe, Raenaas & Oppedal (1958) and Levy, Norman & Wagner (1959) and when allowance is made for different techniques, their results indicate that the active substances have mobilities approximating to that of the α globulin fraction. The CBO inhibitor in normal rabbit serum has a similar mobility. The peaks of inhibitory activity for haemagglutination by Lee B virus and for the infectivity of CBO virus were coincident.

Confirmation of the association of the inhibitor with the globulins was obtained when sera were half saturated with ammonium sulphate. It is not clear whether the precipitation of the inhibitor at pH 5·2 is a specific property of the substance or whether it was due to adsorption to other serum constituents precipitated at this

pH value. It provided, however, a further method of eliminating albumin and distinguishing it from immune γ globulin. In addition, the inhibitory activity was shown to be independent of properdin.

The heat stability and the sensitivity of the inhibitor to periodate and trypsin suggest that it is a mucoprotein and the presence of sialic acid may be inferred from its sensitivity to neuraminidase. Mucoproteins from other sources having high haemagglutination inhibition activity for the influenza B (Lee) indicator virus were without effect upon CBO virus infectivity, suggesting that CBO virus is more restricted than some of the influenza viruses in the range of mucoproteins with which it can combine.

The experiments with the virus + inhibitor complex show that, in contrast to active influenza virus, the complex is not dissociable by time or temperature, nor is it dissociated on simple dilution. The adsorption of CBO virus to certain red cells which are not agglutinated (Kipps *et al.* 1961), and to the inhibitory substance in normal animal sera, can be reversed by RDE and active influenza B virus (Lee), implying that while the CBO virus combines with inhibitor in a manner similar to the influenza viruses it lacks the necessary enzyme for its own elution.

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Titration of Trachoma Virus with Observations on Yolk-sac Infection and Sensitivity to Oxytetracycline, using the Single-dilution method

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(*Received* 17 *March* 1961)

SUMMARY

The T'ang strain of trachoma virus was titrated in chick embryo yolk sac by a single-dilution method (Golub, 1948), using 3–6 eggs/dilution. The limitations incurred by the use of small numbers of eggs are discussed. The method was used to make observations on the fate of virus after inoculation into the yolk sac, and to study the sensitivity of virus to oxytetracycline. The method was found to be unsuitable for titration of neutralizing antibodies in fowl serum.

INTRODUCTION

The experiments described in this paper were carried out for two reasons. Firstly to examine the efficacy of Golub's (1948) single-dilution method of titration when applied to the T'ang strain (T'ang, Chang, Huang & Wang, 1957) of trachoma virus, using smaller numbers of fertile eggs than Golub employed for psittacosis virus; secondly, to use this titration method to make some observations on the behaviour of the virus in the chick embryo and yolk sac.

METHODS

Virus. The T'ang strain of virus was used throughout. It was received as twenty-sixth yolk-sac passage material from Dr L. H. Collier (M.R.C. Trachoma Research Unit, Lister Institute, London). Virus stocks were prepared by homogenization of infected yolk sacs made up to a total volume of 10 ml. with sucrose + potassium glutamate (SPG) solution (Bovarnick, Miller & Snyder, 1950). The crude homogenate was distributed in 0·5 or 1·0 ml. amounts in ampoules, rapidly frozen, and stored at −70°. Fresh stocks were made about every 2 months.

Inoculation of yolk sacs. Except when stated otherwise, virus was injected in 0·2 ml. quantities into the yolk sacs of eggs after 7 days' incubation at 37°. The eggs were then incubated at 35° and candled daily. Embryos dying within 3 days of inoculation were discarded. After the first deaths had occurred eggs were candled twice daily. Smears were made of yolk sacs of all dead embryos and stained with 1/5 (v/v) Giemsa stain (G. T. Gurr, London) in pH 6·8 buffer for 30 min. From more than a hundred such smears made in the early stages all but a few contained elementary bodies; therefore embryos found dead with negative smears were not included in the results. All dilutions of virus were made in sucrose + potassium glutamate solution.

Oxytetracycline. Soluble tablets of oxytetracycline hydrochloride (Pfizer) were use to prepare fresh solutions within 1 hr. of injection into eggs. The tablets were dissolved in phosphate buffered saline (pH 7·2). Sensitivity determinations were made by inoculating into 7-day yolk sacs 0·2 ml. of a mixture of stock virus and oxytetracycline solution in which the final concentration of virus was 1/10 and the amount of oxytetracycline was as shown (Tables 5 and 7).

Antiserum. Antiserum was prepared by injecting 0·5 ml. of stock virus intraperitoneally into a hen at weekly intervals for 5 weeks. Blood was collected by heart puncture before the first injection and 1 week after the last injection. The second serum gave a titre of 1/128 in an indirect complement-fixation test against a suspension of virus partly purified by extraction with ether followed by differential centrifugation.

Neutralization test. Virus and fowl antiserum were mixed to give the final dilutions shown in Table 8. After incubation at 35° for 15 min. 0·2 ml. of each mixture was inoculated into three 7-day-old eggs which were incubated at 35° and candled daily thereafter.

RESULTS

Assessment of the single-dilution method of titration

Golub (1948) described a single-dilution method of titrating the infectivity of psittacosis virus, using 10–30 fertile eggs per dilution, and estimating the mean survival time after inoculation of virus. It was found possible to titrate the T'ang strain by this method, with fewer eggs than Golub used, and a consequently diminished accuracy. For some purposes, however, 3–4 eggs per point enable limited conclusions to be drawn from the mean survival times. The regression of survival times on $\log_{10}$ LD50 inoculated was examined by inoculating 4–6 eggs with tenfold dilutions of stock virus. Eggs were incubated for a further 14 days, and the LD50 calculated by Thomson's Method of Moving Averages (Thomson, 1947). Embryos surviving for the whole 14 days were considered to be uninfected. The mean values of the survival times, even for such small groups of eggs, lay fairly close to a straight line drawn by eye (Fig. 1), which supported the idea that the relationship of survival time to infectivity of the inoculum was the same as that described by Golub for psittacosis virus.

An estimate of the number of elementary bodies in one LD50 was made by spreading 0·02 ml. of stock virus evenly over a ruled area of 4 cm.^{2} on a slide, staining with Giemsa, and counting the number of bodies in five microscope fields at a magnification of 1700. Two values for the LD50 obtained in this way were 7600 and 554 elementary bodies. Several similar counts were performed during these experiments on yolk sac homogenates prepared from embryos dying between the fifth and tenth days after infection. These gave values of 10^9 to 10^{10} elementary bodies per yolk sac at death. The multiplication, in terms of elementary bodies, of an inoculum which killed embryos 14 days after infection was therefore of the order of 10^6 to 10^7 times.

An assessment of the sensitivity of the method is given in Table 1, taking the regression of survival time on dilution of virus inoculated as linear. A difference in infectivities equivalent to a dilution of less than about one in thirty would not be expected to show as a significant difference between mean survival times.

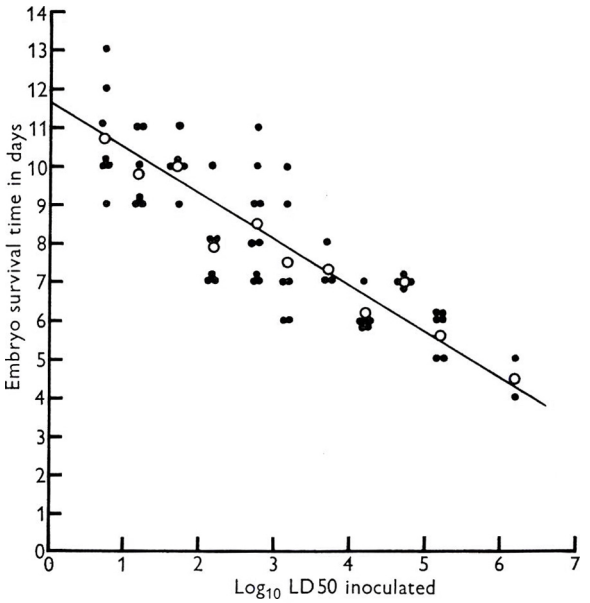


Fig. 1. Regression of survival time of embryos on $\log_{10}$ LD 50 of Trachoma virus (T'ang strain) inoculated into the yolk sac. ○, mean survival times.

This application of the single-dilution method of Golub is therefore extremely coarse, but it is economical and will enable useful conclusions to be drawn provided that the limitations are borne in mind. To avoid the additional sources of variation involved in determining the LD 50, the results of titrations are expressed throughout this paper as the mean survival time (MST) of a group of 4–6 embryos after injection

Table 1. *Sensitivity of method of titration*

$\log_{10}$ dilution of virus inoculated	Survival times of embryos (days)	
	1	2
0	4, 5	7, 7, 7, 7
1	5, 5, 6, 6, 6	7, 7, 8
2	6, 6, 6, 6, 6, 7	7, 7, 7, 9, 10
3	6, 6, 7, 7, 9, 10	7, 7, 7, 9, 10
4	7, 7, 7, 8, 8, 10	10, 10, 11, 13
5	9, 9, 9, 10, 11, 11	—

If Mean Survival Time (MST) = $K \times \log_{10}$ dilution of inoculum, 1 day = about 1 $\log_{10}$ unit (Fig. 1). Variance of single observation = about 1 day. Variance of MST of 4 embryos = about $\frac{1}{4}$ day. Variance of difference between MST values in groups of 4 embryos = $\frac{1}{2}$ day, and S.D. = $1/\sqrt{2}$ day. Therefore difference will be significant at 95 % level if it exceeds $2 \times$ S.D. = about $\sqrt{2}$ = about 1·4 days.

on the seventh day of incubation of 0·2 ml. of virus suspension. The abbreviations in brackets after the abbreviation MST indicates the source of the material inoculated, for example, MST(YS), means that the figure given is the mean survival time of a group of eggs after the injection of yolk-sac suspension; in the text this may be expressed as the MST of a suspension. Embryos surviving the whole

14-day period are treated for quantitative purposes as though they died on the fourteenth, so that an MST of 14 days implies that all the embryos survived. This is necessary to deal with such a result as the death of one embryo in a group before 14 days, and the survival of the others.

Observations on growth of the virus

Effect of infection on embryo and yolk-sac development

A possible explanation for the relationship between survival time and inoculum is that the growth rate is independent of the size of the inoculum and that the embryo will die when a certain number of cells has been irreparably damaged. There could also be a slow toxic action of the material inoculated. The increase in weight of small numbers of infected embryos was not greatly different from that of uninfected embryos (Table 2). This is not the kind of result to be expected of some toxic effect beginning soon after inoculation.

Table 2. *Increase in weight of infected and uninfected embryos*

Days after injection of 0·2 ml. virus suspension* into 8-day eggs	Weights (g.) of infected embryos	Weights (g.) of uninfected embryos
0	—	0·7, 0·7, 0·9, 0·9, 1·2
1	1·3, 1·5, 1·8	1·3, 1·4, 1·5
2	1·4, 1·6, 1·6	1·8, 1·9, 2·0
3	2·5, 2·6, 2·6	2·0, 2·2, 2·6
5	4·5, 5·8	3·7, 4·9, 5·0
6	5·6, 6·0, 6·1	6·1, 7·0, 7·9

* Stock virus diluted 1/10. Survival time 6–7 days.

In another experiment yolk sacs harvested at intervals after infection increased in weight in the same way as uninfected yolk sacs until a day or two before death of the embryo, at which time they actually fell in weight (Fig. 2).

Distribution of virus after inoculation of yolk sac

The infectivity of yolk after inoculation with virus was determined by removing the shell over the air-space and aspirating a quantity of yolk from the yolk sac which was easily visible beneath the shell membrane. The aspired yolk was used to inoculate other eggs in 0·2 ml. amounts, without further dilution (Table 3). Suspensions for the titration of yolk-sac infectivity were prepared by washing the sac carefully in two changes of phosphate buffered saline and homogenizing it in a total volume of 10 ml. of sucrose + potassium glutamate solution. When the homogenate was not to be inoculated into eggs at once it was rapidly frozen and stored at $-70°$ until required. Storage of virus in this way caused some loss of infectivity (Table 3). 0·2 ml. of homogenate was inoculated into the eggs used for titration. In one experiment embryos were titrated for infectivity in the same way as yolk sacs (Table 3). When the volume of yolk is taken as 20 ml., the dilution of the inoculum after injection is 1/100, so that the infectivity of yolk immediately after inoculation

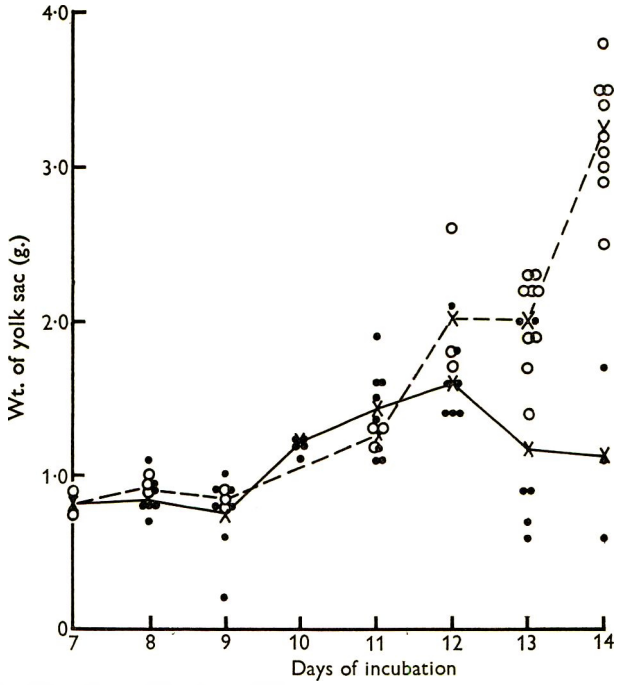


Fig. 2. Growth of normal yolk sacs and yolk sacs infected on the seventh day of incubation. ○, Normal yolk sacs; ●, infected yolk sacs; ×, mean weights.

would be expected to show an MST 2–3 days greater than the inoculum and this expectation is borne out by the figures (Table 3). One day after injection infectivity could still be recovered from the yolk in quantities similar to that present immedi-

Table 3. *Infectivity of yolk, yolk sacs, and embryos at intervals after inoculation of virus*

Expt. no.	MST of inoculum	Infectivity of	Days of incubation at 35° after inoculation						
			0	1	2	3	4	5	6
28*b*	6·4	Yolk*	—	14	—	—	—	—	—
		Yolk sac*	—	14	9·0	7·0	7·0	5·0	5·0
		Embryos*	—	14	14	11·0	—	6·3	8·0
56	5·4	Yolk sac*	—	13·5	7·5	—	6·0	6·8	—
82*a*	6·0	Yolk	8·0	7·0	—	—	—	—	—
		Yolk sac	—	9·5	—	—	—	—	—
82*b*	6·0	Yolk	7·0	7·0	—	—	—	—	—
		Yolk sac	—	11·0	—	—	—	—	—
78	9·0	Yolk	11·0	10·0	—	—	—	—	—
		Yolk sac	—	10·0	—	—	—	—	—

* Stored at −70° for 1 week before titration.

ately after injection. Infective virus could also be recovered from washed homogenized yolk sacs 1 day after inoculation. The presence of significant amounts of infective virus in the yolk even 1 day after inoculation implies that absorption of virus by entodermal cells may continue for too long after injection to make this system a useful one for studies on the kinetics of virus growth.

Phagocytosis of Indian ink by yolk sac

In order to test the possibility of dispersal of virus throughout the whole egg contents after inoculation a 10 % (v/v) suspension of 'Pelikan' ink was injected into the yolk sacs of several 7-day-old eggs which were incubated at 35°. The contents of these eggs were examined at intervals after injection. Fifteen minutes after injection washed yolk sacs showed a faint grey band (Pl. 1, fig. 1). Over the next 24 hr. this band increased in density and slightly in width, and at this time ink was present only in the yolk-sac contents. Histological sections showed widespread uptake of ink by entodermal cells with a heavy concentration in the position of the band. There may therefore not be much dissemination of inoculated virus throughout the egg, and phagocytosis may play at least some part in the entry of virus into cells.

Effect of oxytetracycline on infection

The embryos were protected by 20 μg. of oxytetracycline against all the doses of virus used. Below this amount of oxytetracycline the sensitivity was inversely related to the dose of virus (Table 4). For example, 5 μg. oxytetracycline failed to

Table 4. *Effect of size of inoculum of virus on sensitivity to oxytetracycline (OT)*

MST of inoculum (days)	...	5·8	7·2	8·8
MST after OT (days)	20 μg.	14	14	14
	10 μg.	11·8	—	14
	5 μg.	6·8	14	14
	2·5 μg.	5·8	9·3	11·3

protect against an inoculum whose MST was 5·8 days, whereas the same dose protected against an inoculum whose MST was 7·2 days.

The phenomenon of delayed death shown in the first column of Table 4 was observed several times in this system, and a similar effect was reported for this and other antibiotics acting on the psittacosis-lymphogranuloma group by Katz (1956) and Jawetz & Hanna (1960*b*). It must be due to one of three causes, decay of antibiotic, retardation of virus growth, or appearance of a resistant variant. The third possibility was examined by testing the sensitivity of the virus recovered from the yolk sacs in the eggs which showed a delayed death after 10 μg. oxytetracycline. No increase of resistance was detected.

Table 5. *Effect of time of addition of oxytetracycline (OT) on its ability to protect infected embryos*

Days after infection on which 50 μg. OT added	0	1	2	3	4	5	No OT 6·5
MST	14	14	14	13·4	10·0	8·2	6·5

It was interesting to know at what time after inoculation death could no longer be prevented by antibiotic treatment. This method was used very successfully by Keppie, Smith & Harris-Smith (1955) in studying infection of guinea-pigs with *Bacillus anthracis*. After inoculation of virus, groups of 4–6 eggs were injected at

24 hr. intervals with 0·2 ml. oxytetracycline solution containing 50 μg. antibiotic. Embryos were completely protected up to 2 days after infection, almost completely after 3 days, and with decreasing efficiency after 4 and 5 days (Table 5). Several determinations were made of the amount of infectivity that could be recovered from yolk sacs some days after the administration of a protective dose of oxytetracycline (Table 6). Infective virus could be recovered from the yolk sac 14 days after injection when oxytetracycline was injected more than 2 days after virus injection.

Table 6. *Survival of virus in yolk sac after oxytetracycline (OT)*

Days after injection on which OT given	Infectivity (MST) of inoculum	μg. OT injected	Infectivity (MST) of infected yolk sacs at these intervals (days) after inoculation of virus						
			2	3	4	6	8	10	14
0	7·0	100	14	—	—	—	—	—	14
1	6·5	100	—	—	—	—	14	—	—
2	6·5	100	—	—	—	—	14	—	14
3	6·5	100	—	—	—	—	7·7	—	8·5
3	7·9	50	—	11·8	—	—	—	—	14
3	7·9	50	—	12·0	—	—	—	—	14
3	6·0	50	—	(7)*	—	10·0	9·0	9·3	(8)*
4	6·5	100	—	—	—	—	7·5	—	—
5	6·5	100	—	—	—	—	5·0	—	9·0

* Survival time of one embryo.

Table 7. In vitro *effect of oxytetracycline (OT)*

Virus 0·5 ml. + yolk 10 ml. + OT 1 mg. at 35°/24 hr. 0·2 ml. of 1:10 dilution (containing 2μg. OT + stock virus 1/200) injected into yolk sac.

Duration of exposure of virus to OT	MST of virus/OT mixture (days)
0 min.	10·0
24 hr.	10·6

There was some decrease in the degree of infectivity which could be due to thermal inactivation, metabolic activity of the cells, or mechanical removal of virus. A slow viricidal effect of oxytetracycline cannot strictly be excluded, but this is made unlikely by the absence of a viricidal effect in 24 hr. incubation of virus in the presence of 100 μg. oxytetracycline/ml. (Table 7), a concentration many times greater than that to which intracellular virus would have been exposed. These experiments provided no evidence against the idea that oxytetracycline exerts a virustatic effect on this strain of trachoma virus.

Table 8. *Neutralization of T'ang virus (MST's of serum/virus mixtures)*

Figures in parentheses = % 'neutralization'.

Final virus dilution	Final antiserum dilution			Normal serum
	1/2	1/10	1/100	1/2
10^{-1}	8·0 (99·3)	5·5	5·5	5·5
10^{-2}	9·0 (99·7)	7·0	5·6	6·0
10^{-3}	10·0 (97·8)	8·3	8·0	8·0

Neutralization of virus

Fowl antiserum diluted 1/2 produced a prolongation of mean survival time of 2–3 days with all three dilutions of virus. There was no prolongation with the higher dilutions of serum (Table 8).

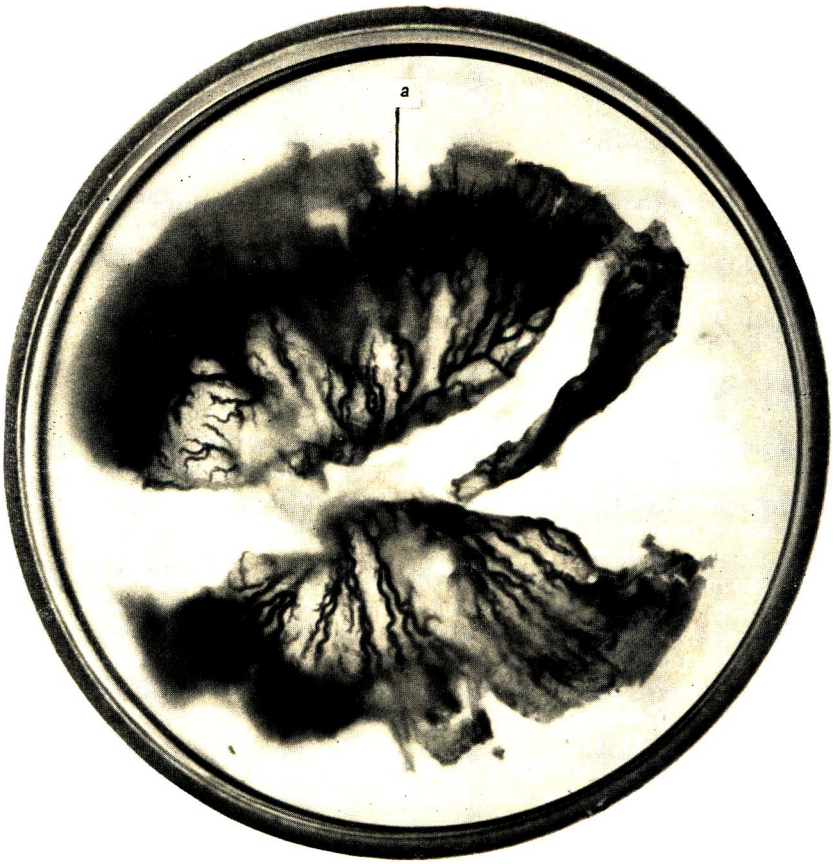
DISCUSSION

Dependence of survival time upon the amount of trachoma virus inoculated into yolk sacs was shown by Jawetz & Hanna (1960*a*) who used three strains isolated in the United States. They found that 10^5 LD50 doses killed embryos in 5–6 days, and 10 LD50 killed in 10–12 days, which agrees with the survival times recorded here for the T'ang strain. Since appreciable amounts of infectious virus were found in embryos 3 days after infection (Table 3), and embryos could survive for 2 weeks after inoculation of virus when they were protected by the virustatic action of oxytetracycline injected 3 days after infection (Table 6), it is unlikely that the virus kills embryos by a toxic action of the kind described in mice by Bell, Snyder & Murray (1959). The loss of weight of infected yolk sacs (Fig. 2) may have been due to the fact that a high proportion of the weight of the yolk sac consists of materials in transit from yolk to embryo (Romanoff, 1960); it may be that when infection has damaged cells beyond a certain point they are no longer able to take up yolk material, but can still pass on, or metabolize, what they already contain. On this hypothesis lack of nutrients contributes to the death of the embryo. Such fatal damage to the entodermal cells must occur a short time before the death of the embryo, since infected embryos weighed the same as uninfected ones, and the growth curve of infected yolk sac followed that of uninfected until a day or two before death (Fig. 2).

Katz (1956) found that feline pneumonitis virus was inhibited by 31 μg. tetracycline or oxytetracycline. Jawetz & Hanna (1960*b*) found that two of their strains were inhibited by 2 μg. tetracycline/ml. (that is, 1·0 μg. in the inoculum). The T'ang strain was inhibited completely by 20 μg. oxytetracycline.

A possible explanation of the inverse relationship of sensitivity to virus dose below 20 μg. oxytetracycline (Table 4) may be that an intracellular threshold concentration of oxytetracycline is attained after injection of less than 20 μg. which can inhibit the development of one infective particle/cell, such as may occur after a small inoculum, but not of two or more, which would be present in many cells after a large inoculum. Katz (1956) found that the activity of tetracycline on feline pneumonitis virus was independent of the inoculum. His figures suggest that feline pneumonitis virus is more lethal for chick embryos than trachoma virus. For example 10^4 LD50 doses of feline pneumonitis virus gave a mean survival time of 5·2 days, whereas the same dose of trachoma virus killed in 7·0 days. It is therefore possible that at a dosage level of 10^5 LD50 doses of feline pneumonitis virus most infected cells contained one particle only. If the hypothesis is correct that an intracellular threshold amount of antibiotic is required to inhibit one intracellular particle, the difference between the findings of Katz and those in Table 4 can be explained.

The failure of a fowl antiserum to neutralize the T'ang strain completely is disappointing. Even the slight prolongation of survival time may have been due, for example, to agglutination rather than neutralization. The system itself is not very



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suitable for demonstrating neutralization, since a decrease of infectivity to less than one-thirtieth of the initial value must be attained before a significant difference can be shown in the mean survival times (Table 1). If the figures in Table 8 are taken at their face value, to indicate neutralization, and the MST values converted to approximate LD50 dose by using Fig. 1, the percentage neutralization is seen to be of the same order with all three dilutions of virus. This recalls the Percentage Law of Andrewes & Elford (1933).

I am grateful to Dr L. H. Collier for supplying the T'ang strain, Dr M. Bulmer for the statistical analysis, Mr S. Buckingham for the photograph, Miss Christine Court for drawing the graphs, and Mr R. Vivian for technical assistance.

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EXPLANATION OF PLATE 1

Fig. 1. 7-day yolk sac 1 hr. after injection of 'Pelikan' ink into the yolk. *a*, Band of heavy uptake of ink.

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Influence of Certain Sterols and 2:4-Dinitrophenol on Phosphate Accumulation and Distribution in *Tetrahymena pyriformis*

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SUMMARY

Orthophosphate accumulation by *Tetrahymena pyriformis* W was enhanced by stigmasterol, β-sitosterol or cholesterol and depressed by 2:4-dinitrophenol (DNP) to an extent determined by the concentration of DNP and hydrogen ion in the suspension medium. The depression of phosphate accumulation by DNP was mitigated by the addition of stigmasterol, the degree of annulment being dependent in part on the stigmasterol concentration. The distribution of accumulated phosphate was not significantly altered by the addition of sterol and/or DNP under the conditions used, indicating that these compounds may influence entry of orthophosphate by affecting a membrane phenomenon.

INTRODUCTION

The interaction of sterols and 2:4-dinitrophenol (DNP), colchicine and several steroids in the growth of *Tetrahymena pyriformis* has been reported (Conner, 1957; Conner & Nakatani, 1958; Conner, 1958, 1959*a*, *b*). The growth inhibitors represent a wide variety of compounds that have been reported to interfere with phosphate metabolism. Colchicine (Lettre, 1951) was reported to inhibit ATPase activity; steroids have been shown to interfere with oxidative phosphorylation and to stimulate 'latent' ATPase activity in mitochondria (Wade & Jones, 1956*a*, *b*), as does 2:4-dinitrophenol (Loomis & Lipmann, 1948; Hunter, 1951). DNP is believed to lead to a loss of some of the energy which is normally available to cells through oxidative processes; accordingly, energy-dependent growth and maintenance become diminished. The lowering of phosphate accumulation in Tetrahymena in the presence of DNP was reported by Conner, Goldberg & Kornacker (1961). Van Wagtendonk & Wulzen (1951) showed that guinea-pigs fed a diet deficient in plant sterols ('anti-stiffness' factor) developed a syndrome most readily explained in terms of a disturbance of phosphate metabolism. These findings, coupled with the DNP inhibition of phosphate accumulation in Tetrahymena, led to a study of the relationship of sterols and DNP to phosphate accumulation and distribution in this ciliated protozoan.

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METHODS

The organism used was *Tetrahymena pyriformis* W; it was grown in a proteose peptone medium at pH 6·5 for the phosphate accumulation studies. For the distribution experiments the organism was grown in a modification of the chemically defined medium previously used for growth studies (Conner, 1959*b*). In the present study the phosphate concentration was decreased by a factor of ten and the Tween 80 was omitted.

Stigmasterol was added to the suspension medium as an aqueous emulsion, prepared by dissolving the sterol in absolute ethanol and injecting the ethanolic solution into distilled water (at about 90°) with a fine-tipped pipette. An ethanol blank was prepared at the same time and an equal volume added to all flasks to which the sterol emulsion was not added. The dilutions used gave final concentrations of ethanol in the suspension medium of not greater than 0·05 % (v/v).

The ciliates were incubated with the sterol emulsion or ethanol blank for 1 hr. before the addition of DNP; labelled orthophosphate (^{32}P) was added 15 min. after the DNP, and the organisms removed from the suspension medium 2 hr. after the phosphate addition. The experimental procedures used and the method of calculation were as described by Conner *et al.* (1961).

RESULTS

In all experiments accumulation of orthophosphate by *Tetrahymena pyriformis* W was augmented by the addition of stigmasterol, lowered by DNP, and the DNP effect was mitigated by the stigmasterol (Figs. 1, 2). The increase in orthophosphate accumulation in the presence of a given concentration of stigmasterol was variable, ranging from 2 to 14 % in a series of sixteen experiments. The ethanolic solution had no effect on phosphate accumulation.

The influence of DNP concentration with regard to orthophosphate accumulation is shown in Figs. 1 and 2. At pH 6·5 a concentration of $2{\cdot}5 \times 10^{-5}$M-DNP inhibited approximately 68 % of the phosphate accumulation while at pH 7·5 a DNP concentration of 2×10^{-4}M inhibited to about the same extent. The concentration of undissociated DNP at pH 6·5 is $8{\cdot}25 \times 10^{-8}$M and at pH 7·5 is $6{\cdot}6 \times 10^{-8}$M.

The use of a single concentration of DNP and different amounts of stigmasterol resulted in considerable variation in the degree of protection afforded by the stigmasterol, indicating that one or more factors were not adequately controlled in these seven experiments. Figure 3 shows the results of an experiment which most clearly indicated an effect of a sterol concentration gradient. Little difference either in augmentation or in annulment of the DNP inhibition of phosphate accumulation was noted when cholesterol or β-sitosterol were used in place of stigmasterol.

The results of experiments determining the distribution of the accumulated phosphate are given in Table 1. Within a given experiment, the distribution of radiophosphate in the three fractions in terms of percentages remained constant in the presence of DNP and/or stigmasterol, even though an alteration of total radiophosphate accumulation was brought about by these substances. Experiments were performed in duplicate as a gauge of the reliability of the method used; solutions and animals were taken from the same stocks, and time factors were

Table 1. *Inter-experimental range of percentage accumulation and percentage of radiophosphate recovery in trichloroacetic acid (TCA)-insoluble, TCA-soluble organic and inorganic fractions following treatment of Tetrahymena with DNP* (1×10^{-4} M) *and stigmasterol* (1×10^{-5} M) *at* pH 7·0 (8 *experiments*)

	Accumulation (%)		Phosphate distribution: TCA-insoluble (%)		TCA-soluble organic (%)		Inorganic (%)	
Flask	Mean ± S.D.*	Range	Mean ± S.D.*	Range	Mean ± S.D.*	Range	Mean ± S.D.*	Range
Control	86 ± 5	78–90	20 ± 4	15–28	11 ± 4	8–13	69 ± 4	64–76
Stigmasterol (1×10^{-5} M)	91 ± 5	82–95	22 ± 5	15–30	11 ± 4	8–14	67 ± 5	60–75
DNP (1×10^{-4} M)	44 ± 9	31–53	15 ± 8	5–28	17 ± 4	11–23	68 ± 6	58–77
DNP + stigmasterol	64 ± 5	56–72	17 ± 6	12–27	15 ± 3	10–22	68 ± 3	63–72

* S.D. = Standard deviation.

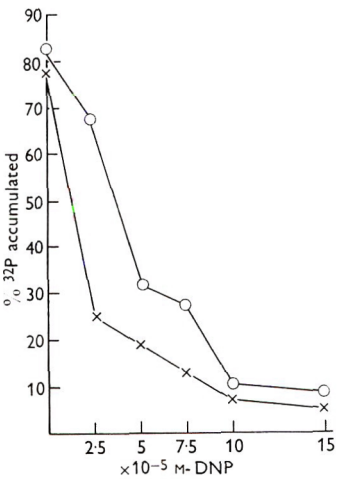


Fig. 1

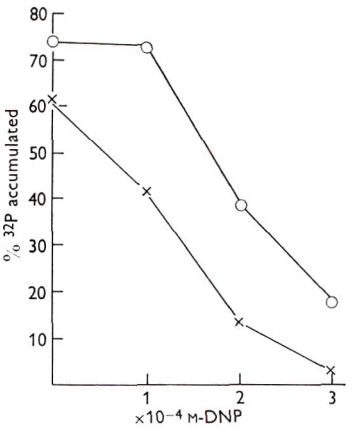


Fig. 2

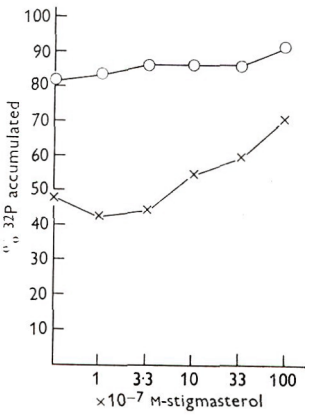


Fig. 3

Fig. 1. The influence of DNP and stigmasterol on phosphate accumulation in Tetrahymena. One ml. of organism suspension at an optical density of 0·5 as determined by a Lumetron colorimeter using a 650 μ filter was added in a final volume of 10 ml. suspension at pH 6·5. Suspension medium containing DNP (×—×). Suspension medium containing DNP and 1×10^{-5} M-stigmasterol (○—○).

Fig. 2. The influence of DNP and stigmasterol on phosphate accumulation in Tetrahymena. Two ml. of organism suspension at optical density of 0·5 in a final volume of 10 ml. Suspension medium at pH 7·5; with DNP (×—×), with DNP and 1×10^{-6} M-stigmasterol (○—○).

Fig. 3. The influence of stigmasterol and 1×10^{-4} M-DNP on phosphate accumulation in Tetrahymena. Two ml. organism suspension at optical density 0·5 in a final volume of 10 ml. suspension medium at pH 7·5. Stigmasterol (○—○); stigmasterol and 1×10^{-4} M-DNP (×—×).

Table 2. *Determination of accumulation and distribution of radiophosphate in Tetrahymena in the presence of DNP* (1×10^{-4}M) *and/or stigmasterol* (1×10^{-5}M) *at* pH 7·0, *in duplicate experiments*

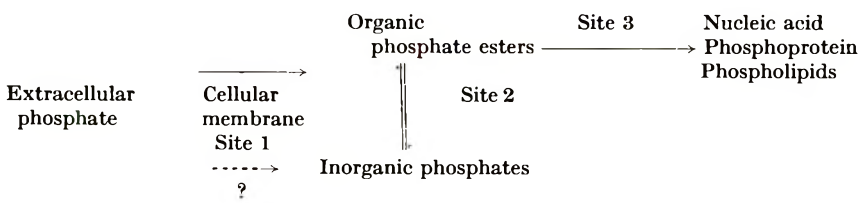
Exp. no.	Flask contents	% removed from medium	TCA insoluble*	TCA soluble*	Inorganic*
1*a*	Control	87 ± 1·0	22 ± 1·0	10 ± 1·0	68 ± 1·0
	Stigmasterol	95 ± 1·0	30 ± 1·0	8 ± 1·0	62 ± 1·0
	DNP	42 ± 2·0	22 ± 2·5	11 ± 3·0	66 ± 2·0
	DNP + stigmasterol	66 ± 1·5	23 ± 2·0	11 ± 2·0	65 ± 2·0
1*b*	Control	89 ± 1·0	28 ± 1·5	8 ± 1·5	64 ± 1·5
	Stigmasterol	95 ± 1·5	29 ± 1·0	12 ± 1·0	59 ± 1·5
	DNP	48 ± 1·0	28 ± 2·0	14 ± 2·0	58 ± 2·5
	DNP + stigmasterol	72 ± 1·5	27 ± 2·5	10 ± 3·0	63 ± 3·0
4*a*	Control	79 ± 1·5	19 ± 1·5	12 ± 1·0	69 ± 1·5
	Stigmasterol	84 ± 1·5	23 ± 1·0	13 ± 1·0	64 ± 1·5
	DNP	32 ± 1·5	16 ± 3·0	14 ± 2·0	70 ± 5·0
	DNP + stigmasterol	60 ± 1·5	15 ± 1·5	16 ± 1·0	67 ± 2·0
4*b*	Control	78 ± 2·0	19 ± 1·5	14 ± 1·0	67 ± 1·5
	Stigmasterol	82 ± 2·0	19 ± 1·5	12 ± 1·0	69 ± 1·5
	DNP	31 ± 2·0	20 ± 3·5	16 ± 5·0	63 ± 5·0
	DNP + stigmasterol	56 ± 2·0	17 ± 2·0	16 ± 2·0	67 ± 3·0

* Given as percentage of phosphate accumulated.

controlled as closely as possible. Representative duplicate experiments (Table 2) revealed an uncertainty up to 6 % in phosphate accumulation and 8 % in distribution (see also Kamen & Spiegelman, 1948).

DISCUSSION

In light of available information about phosphate metabolism it is reasonable to propose the following pathway in *Tetrahymena pyriformis* W:



A diminution of phosphate accumulation by DNP can be pictured as a disruption of phosphate metabolism at one or more sites.

Extrapolating from the effects of DNP on phosphate metabolism in mitochondria, lowered ATP production via the oxidative phosphorylation reactions and stimulation of ATPase activity, a decrease in organic phosphate esters and TCA insoluble compounds might be expected, perhaps accompanied by an increase in inorganic phosphate. Loughman & Russell (1957) observed inhibition of radiophosphate accumulation in young barley roots treated with DNP; upon fractionation, they observed greatest diminution in the organic phosphate esters, specifically in the nucleotides, and an increase in orthophosphate. Styrett (1958), working with Chlorella, has reported the ATP concentration within the cells to be decreased by high concentrations of DNP.

Similarly, it was thought that stigmasterol might produce an alteration in the pattern of distribution of phosphate which could be interpreted to indicate a site of action. In context of the sterol involvement in the guinea-pig stiffness syndrome, Van Wagtendonk & Wulzen (1951) suggested the steroid might act as a prosthetic group for the enzyme responsible for adenylic acid phosphorylation or possibly as a restraint to ATPase activity.

The pattern found in the Tetrahymena experiments shows *no* disturbance in the radiophosphate distribution when the animals are subjected to 1×10^{-4}M-DNP and 1×10^{-5}M stigmasterol at pH 7·0, either singly or in combination. This pattern, too, has an interpretation in terms of the foregoing scheme; DNP acts to prevent transfer of environmental orthophosphate across the cellular membrane to the intracellular 'pool' and apparently the sterols oppose the DNP effect.

It is not possible on the basis of the lack of change in the distribution of accumulated phosphate in the presence of 2:4-dinitrophenol and/or the stigmasterol to distinguish, in Tetrahymena, between a direct surface or membrane phenomenon and a general involvement of energy-requiring processes. However, regardless of which mechanism proves to be correct, it is necessary to postulate the sterol and DNP act in a related system. The sterol may act in the process of phosphate accumulation at the level of the cellular membrane or may aid in supplying the energy necessary for accumulation.

Orthophosphate entry seems to many investigators to be connected with metabolic processes at the cell surface. Helder (1952) and Lundegårdh (1955) have proposed mechanisms for surface adsorption in plants. Kamen & Spiegelman (1948), supported by Rothstein & Dennis (1953), argue for a cell-surface esterification as the mechanism for radiophosphate permeation in yeast.

Conner (1961) suggested that phosphate entry in Tetrahymena is an active process. DNP could influence this process in one of three ways: (1) by lowering the utilization or increasing the intracellular concentration of orthophosphate; (2) interference with an active accumulation mechanism involved in phosphate entry, directly by combining with a 'carrier'; or (3) indirectly by decreasing the supply of energy required for active transport (Conway, 1955).

Further elucidation of the site and mode of DNP depression and sterol elevation of phosphate accumulation is being attempted by noting if there is sterol interaction with DNP with regard to respiratory elevation (Hamburger & Zeuthen, 1957), respiratory quotient elevation (inhibition of the Pasteur effect) and glycogen loss. An interaction between the sterol and DNP in all of these phenomena would favour a generalized energy hypothesis or interaction in the energy supply mechanisms, while a lack of sterol influence on these DNP-induced disturbances of metabolism would favour a membrane site of action.

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The Type A Phages of *Salmonella typhimurium*: Observations on Temperate Phage and Lysogenesis

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SUMMARY

When a standard suspension of *Salmonella typhimurium* Q1, an indicator strain sensitive to the type A phages recovered from lysogenic strains of this organism, was exposed to concentrations of these phages giving a phage:bacteria ratio of approximately 1:10 (thus ensuring that, with rare exceptions, bacteria became infected with single phage particles) it was found that definite percentages of the bacteria were either lysogenized or productively infected (lysed). These percentages were constant for each particular phage type, but varied widely in the 11 members of the group. It was concluded that each phage consisted of a mixed population of particles, some capable, as single infections, of producing lysogenization (α particles) and others lacking this property (β particles). An increase in the phage:bacteria ratio, resulting in multiple infections of single bacteria, led to an increase in lysogenization at the expense of productive infection. The number of α particles present in any particular concentration of phage was calculated from the figures determined at low phage:bacteria ratios (limit dilution). With some phages, when bacteria became infected with more than one particle by exposure to rising phage concentrations, the number lysogenized was compatible with the hypothesis that α particles are dominant over β particles, and that every bacterium infected by an α particle is lysogenized. This hypothesis was however invalidated by the fact that, with other phages, either the number of bacteria lysogenized was in excess of the available α particles, indicating that bacteria had been lysogenized by infection with two or more β particles, or the number of α particles was grossly in excess of the number of bacteria lysogenized. The implications of these findings are discussed.

Very high concentrations of these temperate phages produced lysis-from-without, and in some cases appeared to induce lysis of bacteria which had first been lysogenized. In all cases in which such experiments were carried out, exposure to low temperatures (20°), to high temperatures (42°), to the salts of certain organic acids, and to anaerobiosis had no significant effect on lysogenization.

INTRODUCTION

The type A phages of *Salmonella typhimurium* (Boyd & Bidwell, 1957) have been studied by us over a number of years, and various details of their lysogenizing properties have been observed, some of which are in agreement with accepted ideas, while others are not. As these closely related phages were all isolated from strains of *S. typhimurium* recovered from infected human beings or contaminated food,

and are therefore members of a natural group, and not artificially produced mutants, the findings are considered of sufficient interest to be placed on record. They may serve to focus attention on the fact that, while remarkable progress has been made in the investigation of the biochemical and biophysical properties and of the genetic aspects of certain 'laboratory' strains of phage, little or no work of a systematic nature has been done in identifying and correlating the phages found, in prophage form, in different species of bacteria. As a consequence, knowledge of bacterial viruses as they occur in nature is fragmentary and elementary, and a vast source of information is virtually untapped.

METHODS

In general, the materials and methods are those which have been described in previous papers (Boyd, 1950; Boyd & Bidwell, 1957). The media used were standardized nutrient agar and Lemco broth, though occasionally, in an endeavour to repeat the results of others, the special media recommended by them were employed. The indicator strain used throughout was *Salmonella typhimurium* Q1 (Boyd, 1956) and the phages were 11 of the 12 types classified by cross-immunity tests (Boyd & Bidwell, 1957) all of which have been adapted to and are maintained and propagated on Q1. Type A4 was not investigated, as repeated and prolonged attempts have failed to recover from this temperate phage the virulent mutant necessary for the experiments. Concentrated suspensions of phage particles—10^{11}/ml. and over—were prepared by the method described by Liu (1958). All incubations which involved accurate timing were carried out in a Grant waterbath. The EEL nephelometer was used to estimate the turbidity of cultures and suspensions of bacteria. The methods of counting free phage particles in a suspension, which are fundamental to the work, are described in detail in the first part of the paper.

A bacterium is said to be 'lysogenized' when the invading temperate phage is converted to prophage, and 'productively infected' when the phage particle multiplies at the expense of the bacterium to produce a brood of daughter particles. The latter process is for brevity referred to as a 'burst'. Throughout the paper the bacterial indicator is designated Q1, the different phages A1*a*, A1*b*, etc., and the lysogenic bacteria Q1 (A1*a*), Q1 (A1*b*), etc. The phage particles of a temperate phage are for convenience differentiated by the symbols suggested in an earlier publication (Boyd, 1953). α particles are those which as single infections of a sensitive bacterium produce lysogenesis. β particles are those which, as single infections, produce, not lysogenesis, but a burst. The outcome of such bursts is, of course, a temperate phage-population consisting of both α and β particles. Permanent virulent mutants (Boyd, 1951*a*) which produce only virulent (lytic) particles (except for an extremely rare back-mutation giving rise to a β particle) are designated γ particles.

RESULTS

Preliminary observations

Counts of bacteria

To make a reliable assessment of the multiplicity of infection, i.e. the number of phage particles adsorbed to each bacterium, accurate counts of bacteria and of the phage particles are required. All bacterial cultures contain non-viable organisms in

numbers which vary according to the technique of preparation. In the experiments recorded in this paper bacterial suspensions were prepared as follows. A nutrient agar slant was inoculated from a stock culture and incubated overnight at 37°. The following morning the required number of tubes of broth was inoculated from this culture and kept for 3 hr. at 37°, by which time the organisms were well advanced in the logarithmic phase of growth. These cultures were then pooled and diluted with broth to an opacity shown by previous trials to contain approximately 10^8 viable bacteria/ml. when further diluted with 1/10 of its volume of broth or phage concentrate. In every experiment a control count of viable bacteria was carried out.

Using suspensions prepared in this way, counts of all the bacteria, viable and non-viable, were made by means of a Thoma haemocytometer, 0·02 mm. in depth, while counts of viable bacteria were calculated from colony counts on agar plates which had been flooded with 0·5 ml. of a diluted suspension. The results, shown in Table 1,

Table 1. *Comparison of total counts and viable counts of bacterial suspensions*

Expt.	Total bacteria* per ml.	Viable bacteria† per ml.	% viable	% non-viable
1	$1{\cdot}9 \times 10^8$	$1{\cdot}04 \times 10^8$	52·5	47·5
2	$2{\cdot}5 \times 10^8$	$1{\cdot}45 \times 10^8$	57·5	42·5
3	$1{\cdot}74 \times 10^8$	$9{\cdot}9 \times 10^7$	57·0	43·0
4	$1{\cdot}32 \times 10^8$	$9{\cdot}2 \times 10^7$	69·0	31·0
5	$1{\cdot}51 \times 10^8$	$9{\cdot}1 \times 10^7$	60·0	40·0
6	$1{\cdot}47 \times 10^8$	$1{\cdot}04 \times 10^8$	70·0	30·0
7	$1{\cdot}57 \times 10^8$	$1{\cdot}17 \times 10^8$	74·0	26·0
8	$1{\cdot}51 \times 10^8$	$1{\cdot}05 \times 10^8$	69·0	31·0
9	$1{\cdot}79 \times 10^8$	$1{\cdot}14 \times 10^8$	64·0	36·0
Average	$1{\cdot}7 \times 10^8$	$1{\cdot}08 \times 10^8$	63·5	36·5

* Total counts were made by means of a Thoma haemocytometer, 0·02 mm. in depth, using dark-field illumination.

† Viable counts were made from suitable dilutions of the bacterial suspension flooded in 0·5 ml. quantities over the surface of at least 3 plates of nutrient agar.

reveal the fact that roughly one-third of the bacteria are non-viable. It is not possible to separate viable and non-viable bacteria in order to assess their respective adsorptive properties, but it may be assumed that the adsorptive capacity of non-viable bacteria will not differ greatly from that of bacteria killed by minimal heat. In Table 2 the adsorption of phage by a living culture is compared with that of a culture heated to 58·5° for 30 min. There is little significant difference. The slightly decreased adsorption shown by the dead bacteria may be explained by the fact that they are non-motile and hence may have made slightly fewer collisions with the phage particles. This suggests that viable and non-viable bacteria in a culture are likely to have similar adsorptive properties.

The presence of relatively large numbers of non-viable bacteria in the suspensions used in the experiments to be described does not in fact vitiate the results. As identical suspensions were used in counting the phage particles, it follows that an equivalent proportion (approximately one-third) of the phage particles were 'lost' in the counting process by becoming adsorbed to non-viable bacteria. The phage:bacteria ratio and similar calculations are therefore correct when given in terms of the viable bacteria in a suspension, provided a suspension prepared in the same way was used in counting the phage particles. The total bacterial count and

the total phage count are in fact half as much again as the figures indicate, but this can be disregarded, for both sides of the equation are equally affected. The practical point which emerges is that each experiment on lysogenization or lysis must be controlled, not by a total count of bacteria by whatever means calculated, but by a count of the viable bacteria in the suspension used in the particular experiment.

Method of counting virulent (γ) phage particles

Phage particles which develop only by the 'productive' or lytic cycle, and do not produce lysogenesis, were counted, not by the usual pour-plate method (Hershey, Kalmanson & Bronfenbrenner, 1943, slightly modified by Adams, 1959), but by the

Table 2. *Comparison of the adsorptive properties of living and heat-killed suspensions of Q1*

The bacterial culture gave a count of viable organisms of approximately 10^8/ml. Phage was added to give the ratios indicated, and the mixtures incubated for 15 min. at 37°. Thereafter the mixtures were at once diluted to prevent further adsorption, the living bacteria killed by heating to 70° for 30 min. and counts made of the plaque-forming free phage particles.

Phage:bacteria ratio	...	15:1	1·5:1	0·15:1
Percentage of phage particles adsorbed by	Culture of living bacteria	67·5	94	98·3
	Bacteria killed by heating to 58·5° for 30 min.	65·5	92	95·2

Table 3. *Comparison of plaque-counting techniques*

Decimal dilutions of concentrated phage A1*b* were counted by three methods. Each figure in the table is an average of 2 counts. The surface count, measuring drops with the Agla micrometer syringe, is consistently higher than Adams's pour-plate method using 0·7 % agar for the 'layer'. The third method, using 1·2 % agar, gives an even lower count and shows that, the greater the viscosity of the agar, the greater is the loss caused by the mixture sticking to the tube from which it is poured.

	Surface count	Adams's pour-plate (0·7 % agar layer)	Pour-plate (1·2 % agar layer)
		Plaques/ml.	
1	$1{\cdot}25 \times 10^{11}$	$1{\cdot}0 \times 10^{11}$	$8{\cdot}1 \times 10^{10}$
2	$1{\cdot}23 \times 10^{10}$	$1{\cdot}17 \times 10^{10}$	$8{\cdot}15 \times 10^{9}$
3	$1{\cdot}21 \times 10^{9}$	$1{\cdot}02 \times 10^{9}$	$9{\cdot}0 \times 10^{8}$
4	$1{\cdot}28 \times 10^{8}$	$1{\cdot}24 \times 10^{8}$	$8{\cdot}55 \times 10^{7}$

surface-count method (Boyd, 1950). After a preliminary rough titration, the phage concentrate was accurately diluted to a point where it contained, in terms of the preliminary titration, 10^3 particles/ml., and, with an Agla micrometer syringe, 5 drops of exactly 0·01 ml. of this diluted concentrate were placed at different points on a 'lawn' of Q1 (i.e. the dried surface of a 10 cm. Petri dish of nutrient agar previously flooded with a broth culture of Q1). The drops were spread, but not to the point of coalescence, by gently rocking the plate, which was then incubated overnight. In the morning the plaques were counted, and from this count, multiplied by the appropriate figure to correct the dilution, the number of particles in the original concentrate was calculated. This method is simpler and quicker than the pour-plate method, and gives counts which are about 10 % higher (Table 3). This is

because, in the pour-plate method, bacteria and phage are mixed with soft agar in a tube before being poured on to a plate of agar of normal consistency. Inevitably some of the agar mixture sticks to the sides of the test tube, and so is lost to the count, whereas when using the Agla syringe, a drop of the exact size is ejected from the square-cut needle without loss.

Method of counting temperate phage particles

When bacteria are mixed with temperate phage diluted to such an extent that each organism is infected by only one phage particle, the infected bacteria are either rendered lysogenic (by α particles) or are productively infected (by β particles). If such a phage-bacteria mixture, suitably diluted, is spread on a lawn of indicator bacteria, the productively infected bacteria will produce turbid plaques of normal size, but, as will be seen later, only a relatively small number of the lysogenized bacteria will produce plaques, most of which will be of small size. To make an accurate count it is therefore necessary to use a technique which will reveal both lysogenized and productively infected bacteria.

The α particles in a temperate phage were counted as follows. A preliminary count of the plaque-forming particles was made by the method just described for γ particles. (For convenience this preliminary count of a temperate phage, which is used as a starting point in certain of the experiments, is called a plaque count. In a high-titre concentrate the plaque count was approximately 10^{11}/ml.) The concentrate was then accurately diluted to give 10^8 plaque-forming particles/ml. One ml. of this dilution was added to 9 ml. of a suspension of Q1, giving a final count of 10^7 plaque-forming particles and approximately 10^8 bacteria/ml., i.e. a ratio of about 1:10. This is regarded as 'limit' dilution, the dilution at which each phage particle will in general infect a single bacterium. The phage-bacteria mixture was immediately placed in a waterbath at 37° for 10 min. to allow adsorption to take place, and thereafter rapidly diluted $10^{-5} \times 1/5$ in broth. Volumes of 0·5 ml. of this dilution were flooded on to plates of nutrient agar which had been impregnated with 10^9 particles/ml. of the virulent (γ) mutant of the phage under investigation. On such a medium only bacteria which had been lysogenized by the temperate phage multiplied and formed colonies: productively infected bacteria disintegrated, and all non-lysogenized (sensitive) bacteria were destroyed. After overnight incubation, the average number of colonies per plate $\times 10^6$ gave the number of lysogenizing particles per ml. of the phage-bacteria mixture, and a further calculation in terms of the preliminary dilution gave the count in the original concentrate. The accuracy of this method of estimating lysogenized bacteria has been tested by plating equal quantities of phage-bacteria mixture on (*a*) nutrient agar and (*b*) nutrient agar impregnated with virulent phage. Each colony developing on (*a*) was tested independently for lysogenesis. The totals of the lysogenic colonies on (*a*) and (*b*) in a series of experiments with graded phage concentrations are shown in Fig. 1. If anything, the percentage of lysogenization revealed by the impregnated plate technique was lower than in the control, a finding which indicates that multiple infection with γ particles does not produce lysogenization. In this and all subsequent experiments, the percentage of bacteria lysogenized—or lysed—was calculated in terms of the count of viable bacteria in the control culture which was included in every experiment.

The second half of the count—the estimation of the number of particles which give productive infection—was a continuation of the same experiment. Another portion of the phage-bacteria mixture was diluted in broth $10^{-7} \times 1/16$, and distributed in quantities of 0·4 ml. in 200 small sterile test-tubes. This gave an average of 1 bacterium per 4 tubes, a distribution which made it improbable that more than one bacterium would find its way into any one tube. As, at the phage dilution used, only

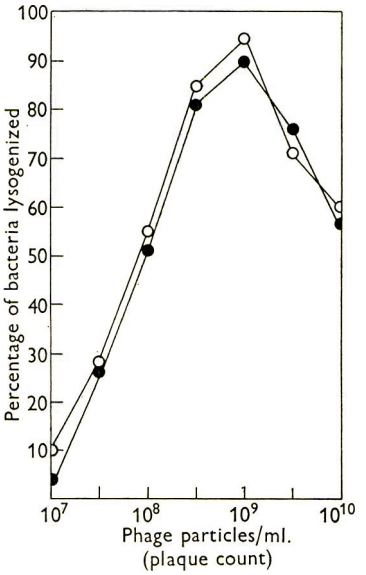

Fig. 1. Comparison of two methods of estimating lysogenization. Q1, at a concentration of 10^8 bacteria/ml., was exposed to graded concentrations of phage A1*b*, and incubated at 37° for 10 min. The preparations were then diluted $10^{-5} \times 1/5$ and equal quantities from each plated on (1) nutrient agar and (2) nutrient agar impregnated with 10^9 particles/ml. of A1*b*γ. All colonies which developed on (1) were tested for lysogenesis. Only lysogenic colonies developed on (2). ○, Percentage of bacteria lysogenized, calculated from (1); ●, percentage of bacteria lysogenized, calculated from (2).

about 1 in 10 of these bacteria was phage-infected, the chance of more than one productively infected bacterium being in any one tube can be disregarded. The tubes were then incubated at 37° for 90 min. Thereafter a loopful from each was placed on a numbered section of a lawn of Q1, and incubated overnight. The presence of multiple plaques on any one section indicated a burst. If a section showed only one plaque, which might be produced by a residual free phage particle, a larger sample was tested. With the above technique and dilutions, the number of tubes showing bursts multiplied by 2×10^6 gave the number of β particles/ml. of phage-bacteria mixture.

Lysogenization and productive infection at limit dilution in the Type A phages

The results of a series of counts of phages A1*b* and A2*d* by the methods described are recorded in Tables 4 and 5. These are two phages used in many of the experiments to be described, as they are representative types having respectively high and low lysogenizing properties. In both cases the sum of the α and β particles,

which will be taken as the true count, was higher than the plaque count, though this difference was more marked in A1*b* than in A2*d*. Counts of particles carried out in this way at limit dilution gave relatively constant and repeatable results, both in the same batch and in different batches of any one phage. These relative proportions of α and β particles appear therefore to be a stable character of each particular phage when prepared and tested by the methods described.

Table 4. *Comparison of counts of particles in temperate phage A1b made by enumerating lysogenic bacteria and bursts, and by plaque count*

The counts of lysogenic bacteria and bursts were made from a standard phage-bacteria mixture containing $1{\cdot}1 \times 10^7$ particles/ml. (as estimated by plaque count).

Expt.	Lysogenic bacteria	Bursts	Sum of lysogenic bacteria and bursts	Plaque count
1	$8{\cdot}25 \times 10^6$	$1{\cdot}0 \times 10^7$	$1{\cdot}825 \times 10^7$	—
2	$9{\cdot}5 \times 10^6$	$6{\cdot}0 \times 10^6$	$1{\cdot}55 \times 10^7$	—
3	$9{\cdot}0 \times 10^6$	$4{\cdot}0 \times 10^6$	$1{\cdot}3 \times 10^7$	—
4	$9{\cdot}25 \times 10^6$	$8{\cdot}0 \times 10^6$	$1{\cdot}725 \times 10^7$	—
Average	$9{\cdot}0 \times 10^6$	$7{\cdot}0 \times 10^6$	$1{\cdot}6 \times 10^7$	$1{\cdot}1 \times 10^7$

Note. The discrepancy between the plaque count and the bursts is attributable to the fact that newly lysogenized bacteria are unstable, and frequently, after 3 or 4 divisions, produce daughter cells in which productive development occurs. The liberation of free phage in this way produces, in the fully incubated plate, plaques of a size related to their time of origin, early ones being large and late ones small.

Table 5. *Comparison of counts of particles in temperate phage A2d made by enumerating lysogenic bacteria and bursts, and by plaque count*

The counts of lysogenic bacteria and bursts were made from a phage-bacteria mixture containing $1{\cdot}0 \times 10^7$ particles/ml. (as estimated by plaque count).

Expt.	Lysogenic bacteria	Bursts	Sum of lysogenic bacteria and bursts	Plaque count
1	$1{\cdot}85 \times 10^6$	$9{\cdot}6 \times 10^6$	$1{\cdot}15 \times 10^7$	—
2	$1{\cdot}95 \times 10^6$	$1{\cdot}1 \times 10^7$	$1{\cdot}2 \times 10^7$	—
3	$1{\cdot}15 \times 10^6$	$1{\cdot}34 \times 10^7$	$1{\cdot}5 \times 10^7$	—
4	$1{\cdot}3 \times 10^6$	$1{\cdot}0 \times 10^7$	$1{\cdot}13 \times 10^7$	—
5	$1{\cdot}1 \times 10^6$	$1{\cdot}2 \times 10^7$	$1{\cdot}31 \times 10^7$	—
Average	$1{\cdot}47 \times 10^6$	$1{\cdot}12 \times 10^7$	$1{\cdot}26 \times 10^7$	$1{\cdot}0 \times 10^7$

Counts of all 11 types of A phage were carried out by these methods at limit dilution. The average of several counts—at least two—is given in Fig. 2, which shows the relative proportions of α and β particles. The wide range of variation is a notable feature.

Observations on multiplicity and lysogenization

It has been pointed out (Boyd, 1951*b*) and confirmed (Lwoff, Kaplan & Ritz, 1954) that when a sensitive culture is exposed to temperate phage, the percentage of bacteria which becomes lysogenized increases when the bacteria are infected by more than one phage particle. Such multiplicity of infection and subsequent lysogenization is governed partly by the proximity of the bacteria and phage particles,

and partly by the availability of phage particles. Table 6 records an experiment showing the extent of lysogenization when a culture of Q1 containing 10^8 organisms/ml. was exposed to widely spaced concentrations of A1*b*. The peak of lysogenization was reached when the concentration of phage was 10^9 particles/ml., a phage:bacteria ratio of 10:1.

The phage:bacteria ratio is not in itself the dominant factor. Decreasing the number of bacteria, and so increasing the phage:bacteria ratio does not increase the degree of lysogenization (Table 7).

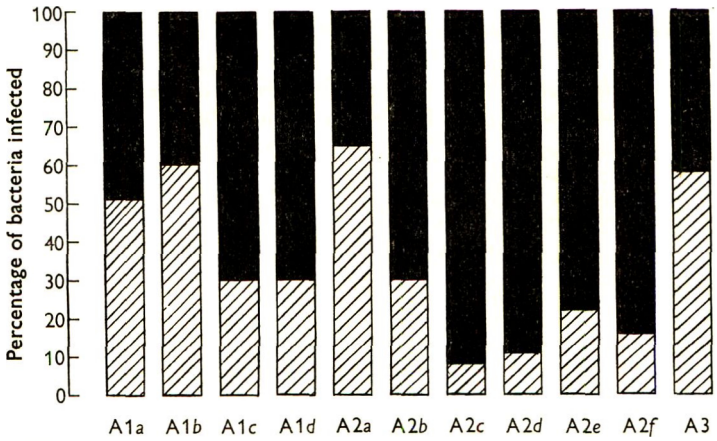

Fig. 2. Relative proportions of α and β particles in the A phages. The α (lysogenizing) and β (productive infection) particles in each phage were counted, at limit dilution, by the method described. ▨, α particles; ■, β particles.

Table 6. *Relationship of phage concentration and lysogenization* (Q1 *and* A1*b*)

Mixtures of Q1 and A1*b* were prepared by the methods described, and incubated at 37° for 10 min. to allow adsorption to occur. Thereafter they were appropriately diluted and plated on agar impregnated with A1*b* γ. The percentage of bacteria which had become lysogenized was calculated from the number of colonies which developed. The figures given are the average of several experiments.

Concentration of phage particles/ml.	$1{\cdot}0\times10^7$	$3{\cdot}1\times10^7$	1×10^8	$3{\cdot}1\times10^8$	$1{\cdot}0\times10^9$	$3{\cdot}1\times10^9$	$1{\cdot}0\times10^{10}$
Phage:bacteria ratio (approximate)	0·1:1	0·31:1	1:1	3·1:1	10:1	31:1	100:1
Percentage of bacteria lysogenized	5·0	18·0	38·0	77·0	87·0	77·0	65·5

On the other hand, when the bacterial concentration is lowered, lower phage concentrations, within limits, give an equally high degree of lysogenization, as the bacteria have the opportunity to come in contact with and adsorb a greater number of particles (Table 8).

These results emphasize the need for a standard technique, if consistent and comparable results are to be obtained. It is for this reason that bacterial suspensions containing approximately 10^8 viable organisms/ml. have been used in all experiments.

Lysogenesis and productive development at different phage concentrations

Phage A1 b

Experiments were carried out in which Q1 was exposed to different concentrations of A1 *b*. Thereafter estimations were made of the percentage of bacteria lysogenized, the percentage of bacteria which underwent productive development, the percentage which escaped phage infection, and the percentage 'unaccounted for' (in low phage concentrations a small and variable number attributable to unavoidable experimental error). Figure 3 gives in graphic form the results of a series of experiments with A1*b*, using widely spaced phage concentrations, and Fig. 4, the results of a more closely spaced series.

Table 7. *Lysogenization with constant phage concentration and decreasing bacterial concentration*

Decreasing concentrations of Q1 were exposed to a constant concentration of temperate A1*b*, giving an increasing phage:bacteria ratio. This produced no significant increase in the number of bacteria lysogenized.

Phage particles/ml.	$7{\cdot}5\times10^8$	$7{\cdot}5\times10^8$	$7{\cdot}5\times10^8$	$7{\cdot}5\times10^8$	$7{\cdot}5\times10^8$	$7{\cdot}5\times10^8$	$7{\cdot}5\times10^8$
Viable bacteria/ml.	$9{\cdot}3\times10^7$	$4{\cdot}65\times10^7$	$2{\cdot}325\times10^7$	$1{\cdot}162\times10^7$	$5{\cdot}81\times10^6$	$2{\cdot}9\times10^6$	$1{\cdot}45\times10^6$
Phage:bacteria ratio	7·5:1	15:1	30:1	60:1	120:1	240:1	480:1
Lysogenized bacteria/ml.	$7{\cdot}5\times10^7$	$3{\cdot}93\times10^7$	$2{\cdot}05\times10^7$	$9{\cdot}46\times10^6$	$4{\cdot}42\times10^6$	$2{\cdot}4\times10^6$	$1{\cdot}1\times10^6$
% lysogenized	80	84	88	81	76	82	77

Table 8. *Lysogenization when bacterial concentration is low*

Against lower bacterial concentrations, lower phage concentrations produce a higher percentage of lysogenization.

	Phage particles/ml. (A1*b*)			
	10^7	10^8	10^9	10^{10}
Bacteria/ml.	% bacteria lysogenized			
10^8	8·5	51	85	55
10^6	40·0	84	83·5	58·5

The first column in Fig. 3, in which the phage:bacteria ratio is 1:10, represents limit dilution, and shows the respective proportions of α and β particles in this particular sample of phage. Lysogenization increased at the expense of productive development up to a phage concentration of 4×10^8, and maintained a high level in subsequent concentrations up to 10^9. Beyond this there was a progressive increase both in the percentage of bacteria which ceased to be viable for reasons other than productive development of phage, and, unexpectedly, in the percentage of bacteria in which phage underwent productive development. These increases were balanced by a decrease in the percentage of lysogenized bacteria and by the absence of unaffected bacteria.

Except at limit dilution, the phage:bacteria ratio does not give any exact indication of the number of particles adsorbed by the infected bacteria. Although in ratios of 1:1 and under, over 95 % of the added phage was adsorbed before the calculation was obscured by phage production from lysing bacteria (Fig. 5) the number of bacteria infected was less than the number of phage particles available (Figs. 3, 4),

and it can be assumed that the excess phage was shared by the infected bacteria in accordance with the Poisson distribution. Thus in ratios as low as 0·31 : 1 some of the bacteria adsorbed more than one particle.

Phage A2*d*

As A2*d*, when examined at limit dilution, has been found to contain a lower percentage of α particles and a higher percentage of β particles than A1*b* (Fig. 2), the action of this phage on Q1 at different concentrations was investigated in the

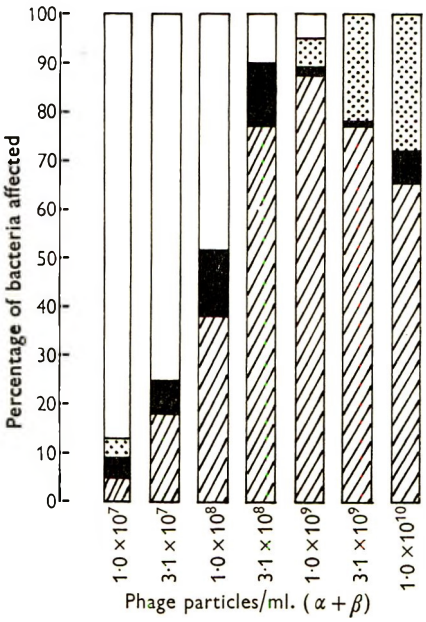

Fig. 3

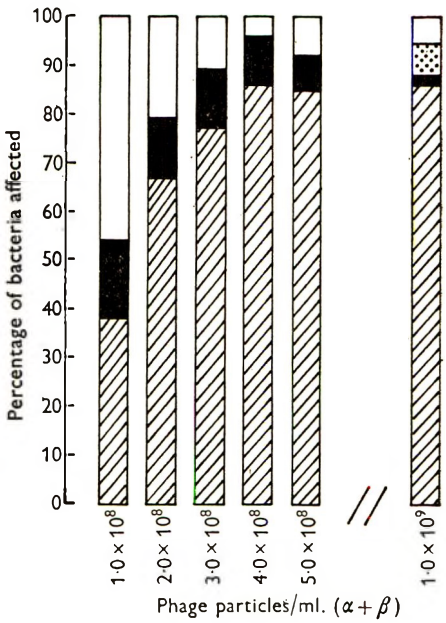

Fig. 4

Fig. 3. Action of widely spaced rising concentrations of A1*b* on Q1. Q1 (10^8 bacteria/ml.) was exposed for 10 min. at 37° to the phage concentrations indicated. Thereafter the percentage of lysogenized bacteria and the percentage of bursts was determined by the methods described. The percentages of 'bacteria uninfected' and bacteria 'lost' were calculated from the viable count of a control bacterial suspension to which no phage was added. In this figure, the phage concentrations are based on true counts, i.e. the sum of α and β particles calculated at limit dilution. Each column is the average of 2 or more experiments. ▨, Lysogenics; ■, bursts; ▩, bacteria lost; □, bacteria uninfected.

Fig. 4. Minimum concentration of A1*b* needed for maximum lysogenization of Q1. A similar experiment to that recorded in Fig. 3, but with closely spaced phage concentrations. Same key as for Fig. 3.

same way as was A1*b*. The results are shown graphically in Fig. 6, and can be seen to conform to the same general pattern. Maximum lysogenization, which however was at a lower level than with A1*b*, occurred at a similar phage concentration and increased in the same way at the expense of productive infection. The percentage of bursts was higher at all concentrations than in the case of A1*b*.

Relationship of the number of α particles to the number of bacteria lysogenized when exposed to different concentrations of temperate phage

Suspensions of all 11 type A phages were prepared at limit dilution and in accurately measured ascending concentrations thereof. Standard suspensions of Q1 were exposed to these phages, and the number of bacteria lysogenized in a given unit (actually 0·5 ml. of a $10^{-5} \times 1/5$ dilution of the original mixture) were estimated in the usual way. The number of bacteria lysogenized at limit dilution revealed the

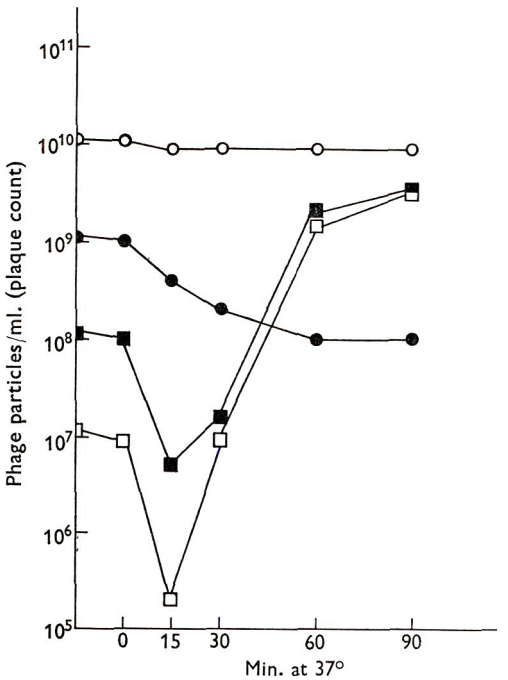

Fig. 5. Adsorption of A 1*b* on Q 1. A 1*b* was added to suspensions of Q 1 (10^8 bacteria/ml.) to give the concentrations indicated. Samples were removed at intervals and immediately heated to 70° for 30 min. to kill the bacteria. The curves are plaque counts of free phage particles. Phage concentration: ○, 10^{10}/ml.; ●, 10^9/ml.; ■10^8/ml.; □ 10^7/ml.

number of α particles in this particular unit. From this the number of 'available' particles in units of the higher concentrations was calculated. In Fig. 7 the number of bacteria actually lysogenized is shown in relation to the number of α particles available. It will be seen that in some cases the α particles were in excess of the bacteria lysogenized, while in other cases the reverse held good. These results will be analysed in the Discussion.

The well-marked variations in lysogenization resulting from exposure to the different phages were not due to a smaller number of bacteria becoming phage-infected because of defective adsorption. Experiments on a more limited range of concentrations, in which both lysogenization and productive development were estimated (Table 9), showed that, while variations occurred in the total number of bacteria infected, these were not related to the degree of lysogenization. Thus A2*b*

produced a relatively low rate of lysogenization associated with a high percentage of infection, while A2*c* had an opposite reaction in the top ratio, namely a higher degree of lysogenization with a much lower percentage of infection. The exceptionally low percentage of infected bacteria in the 10:1 ratio of A2*c* was associated with a high percentage of 'bacteria lost', and may be attributable to unusually active 'lysis-from-without'.

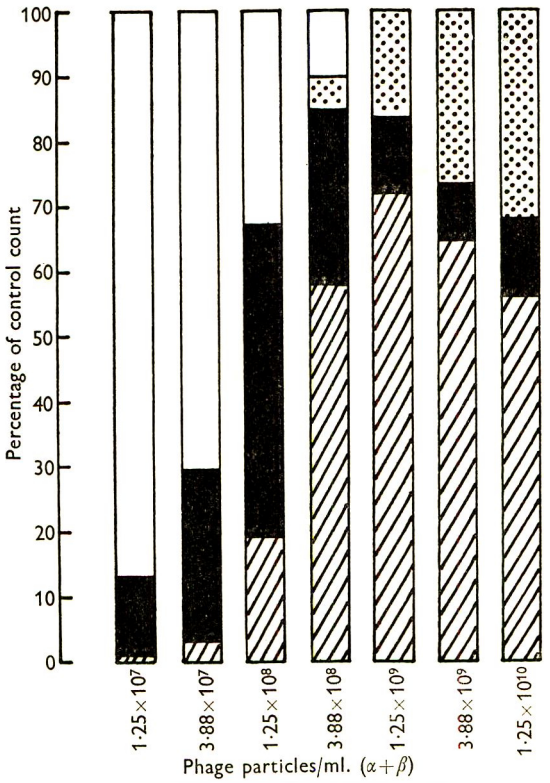

Fig. 6. Action of widely spaced concentrations of A2*d* on Q1. A similar experiment to that recorded in Fig. 3, but with A2*d*. ▨, Lysogenics; ■, bursts; ▩, bacteria lost; □, bacteria uninfected.

Lethal action of high concentrations of temperate phage

Andrewes & Elford (1932) drew attention to the fact that an excess of virulent phage brought about destruction of a number of bacteria without any increase of free phage—a phenomenon which they called 'lysis-from-without'. This phenomenon has frequently been described in relation to virulent phage. Lieb (1953) mentions results which suggest similar action in the case of temperate phage R, on *Escherichia coli* K 12 S. It can be seen from Fig. 3 that, in one of the lower phage concentrations, a small percentage of bacteria is 'lost'. This is a not uncommon experimental error attributable to minor variations in the viable counts of the control and the test suspensions. In higher concentrations—from 10^9 onwards—there is a progressive increase in the number of 'missing' organisms (Figs. 3, 6). Although there is no

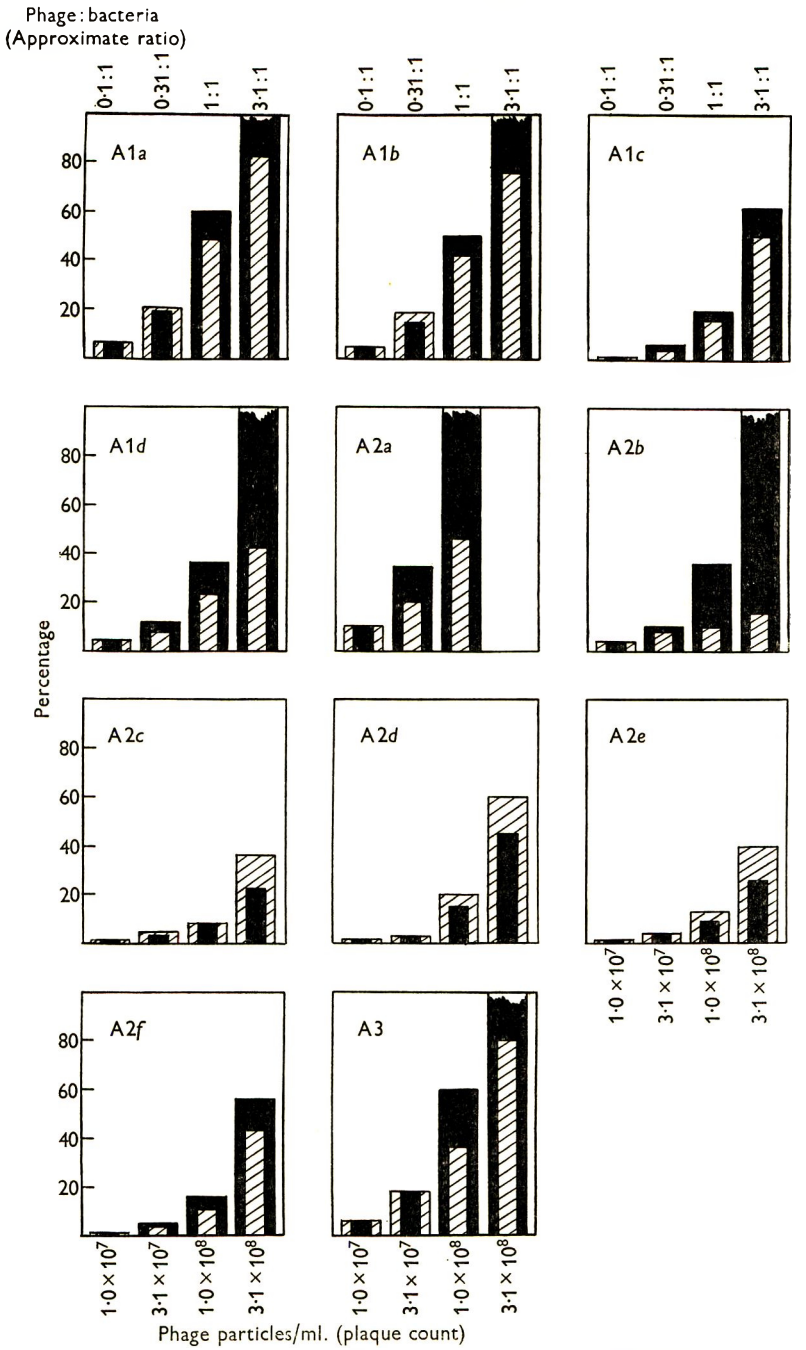

Fig. 7. Relationship of α particles to lysogenesis. Suspensions of Q1 (10^8 bacteria/ml.) were exposed to graded concentrations of the phages and the percentage of bacteria lysogenized estimated by the usual technique. The percentage of α particles 'available' in each phage concentration was calculated from the figure obtained at limit dilution. For convenience in comparing results the lower figure in each column is shown within the higher. ■, α particles in this phage concentration; ▨, bacteria lysogenized.

definite proof that these bacteria were destroyed by lysis-from-without, the picture resembles so closely that found in similar experiments with virulent phage that it leaves little doubt that the bacteria have been killed in this way. A control experiment using corresponding concentrations of the original phage lysate which had been filtered through a gradacol membrane to remove phage particles gave negative results.

Table 9. *Percentage of bacteria infected at different ratios*

The percentage of lysogenics and bursts at different phage:bacteria ratios was estimated by the standard technique. The ratios are in terms of the plaque count. Except in the case of A2*c*, there are no gross variations in the total number of bacteria infected at the different ratios.

Phage:bacteria ratio	%	Phages containing high proportion of α particles		Phages containing medium proportion of α particles		Phages containing low proportion of α particles	
		A1*b*	A2*a*	A1*d*	A2*b*	A2*c*	A2*d*
0·1:1	Lysogenics	7·9	8·7	3·6	3·6	0·8	1·25
	Bursts	5·5	4·6	8·8	8·5	9·3	11
	Total infected	13·4	13·3	12·4	12·1	10·1	12·25
1:1	Lysogenies	49·25	41·25	22·3	15·7	10	19
	Bursts	14·75	30	36·0	58·0	48	48
	Total infected	64·0	71·25	58·3	73·7	58	67
10:1	Lysogenies	89	80·5	70·0	41·5	61·0	72
	Bursts	1·5	17·7	20·0	51·0	7·3	12
	Total infected	90·5	98·2	90·0	92·5	68·3	84

Table 10. *Lysis-from-without produced by A1b γ*

Q1 was exposed to A1*b* γ in the concentrations recorded, incubated at 37° for 10 min., diluted $10^{-7} \times 1/16$, and distributed in 0·4 ml. quantities in 200 tubes. At the same time appropriate quantities were plated to estimate viable survivors. After the tubes had been incubated at 37° for 2 hr., a loopful from each was spotted on a lawn of Q1 to ascertain the number of tubes in which bursts had occurred.

Phage particles/ml.	Percentage of Bursts	Surviving bacteria	'Missing' bacteria
10^7	10·6	85·4	4·0
10^8	62·5	23·6	13·9
10^9	82·0	1·3	16·7
10^{10}	63·0	1·0	36

Confirmatory evidence of the lysis-from-without phenomenon was provided by experiments in which Q1 was exposed, not to temperate phage A1*b*, but to its virulent mutant, A1*b* γ, which does not produce lysogenesis. The effect of different phage concentrations on the percentage of bacteria in which the phage underwent productive development was investigated by the usual technique. The results of one series of experiments are given in Table 10. The percentage of bacteria which supported productive development was approximately the same as the percentage of bacteria which were lysogenized by temperate phage, and at high phage concentrations there was a similar decrease in the number of infected bacteria, presumably due to lysis-from-without.

Productive infection in high concentrations

An unexpected and constant finding in experiments with A1*b*, which was present but less obvious in the case of A2*d*, was that productive infection reached its minimum at a phage concentration of around 10^9 particles/ml. and tended to increase in the higher concentrations (Figs. 3, 6). It would therefore appear, in a heavy multiple infection, either that lysogenesis is not established, and productive development supervenes, or that the immunity which lysogenesis affords breaks down. This problem was explored by exposing an established strain of Q1 (A1*b*) to the action of different concentrations of A1*b*, and examining for bursts in the usual way. With a phage concentration of 10^9 particles/ml., no productive infection occurred, while at $10^9 \times 5$, 6 %, and at 10^{10}, 8 % of the bacteria showed productive infection.

In these experiments there was no evidence of lysis-from-without, although A1*b* is as freely adsorbed to Q1 (A1*b*) as to Q1. Counts of viable bacteria made before and after exposure to the phage showed no significant variation other than the decrease attributable to the bursts.

Q1 (A2*d*) is immune to A1*b* in concentrations of 10^9 particles/ml. When Q1 (A2*d*) was exposed to 10^{10} particles A1*b*/ml. some 6 % of the cells burst, liberating particles of A1*b* and A2*d* in approximately equal numbers. Thus it would seem that, although Q1 (A2*d*) is immune to low concentrations of A1*b*, a high multiple infection with A1*b* induces Q1 (A2*d*). The significance of these results will be discussed later.

Influence of temperature on lysogenization and productive development

Bertani & Nice (1954) found that incubation of infected bacteria at a temperature of 20° for 2 hr. blocked the productive development of phage P1, and also of its virulent mutant, whereas the proportion of infected bacteria which became lysogenized was much higher at a temperature of 20° than at 37°. Temperate phage P2 did not react in this way. This observation was investigated, burst experiments being carried out with A1*b* at a phage:bacteria ratio of 0·75:1. One portion was incubated at 37°, the other at 20°. In one the percentage of bursts was 17·2 and in the other 18. Thus there was no significant difference and no suppression of productive development as found in the case of P1. Similar results were obtained in further experiments using different phage concentrations, in which both lysogenesis and productive development were estimated. Like P2, A1*b* is therefore unaffected in this way by incubation at low temperature.

Lieb (1953) reports that, in the case of *Escherichia coli* and phage λ, the temperature to which the bacteria are exposed during the first 1 to $1\frac{1}{2}$ hr. after infection has a predominant influence on the proportion of lysogenics in clones, i.e. in the progeny of what are believed to be single cells. This is a different type of phenomenon from that described by Bertani, but it was considered of interest to find out if exposure to a high temperature affected the lytic or productive response of Q1 infected with A1*b*. The standard technique was used and the results are given in Table 11. The higher temperature reduced the number of bacteria which showed the effects of phage infection, presumably due to restricted bacterial growth, but the relative proportions of bacteria lysogenized and bacteria productively infected did not vary significantly from the control.

Influence of organic acids on lysogenization

Lwoff *et al.* (1954) using A1*a* and *Salmonella typhimurium* 1404 as indicator, report that specified concentrations of the potassium salts of certain organic acids (citric, malic, oxaloacetic, malonic, pyruvic, lactic, and pyrophosphoric) increase productive infection from 3 to 5 times, presumably at the expense of lysogenic infection. Early experiments carried out with the same phage and indicator some years ago failed to confirm this claim, the only significant action observed, with the methods and media then in use, being some interference with adsorption and consequent slight reduction in the number of bacteria which became phage infected.

Table 11. *Influence of high temperature on lysogenization*

The A1*b*:Q1 ratio in all cases was 0·75:1. Lysogenization and productive infection were ascertained by the standard technique.

	Temperature of incubation	
	37°	42°
% lysogenized	35·2	24*
% productively infected	13·5	9·3

* The figures under 42° are the average of 2 experiments.

Table 12. *Influence of citrate and malonate on lysogenization*

Lwoff's synthetic medium was used throughout. The concentration of phage A1*b* in all experiments was $7{\cdot}5 \times 10^7$/ml. Lysogenesis and productive infection were ascertained by the standard technique.

	% of bacteria	
	Lysogenized	Productively infected
Synthetic medium control	25·5	15·5
Synthetic medium + potassium citrate 10^{-2} M	31·5	12·1
Synthetic medium + potassium malonate 2×10^{-2} M	34	12·2

The action of potassium citrate and potassium malonate has been re-investigated with A1*b* and Q1, but using Lwoff's synthetic medium instead of Lemco broth. The results confirmed those given by the earlier experiments. There was no gross variation in productive development, and only a slight increase in lysogenization (Table 12). Lwoff's synthetic medium was used throughout and in the control the degree of lysogenization was less than is found in Lemco broth. The concentration of A1*b* in all experiments was $7{\cdot}5 \times 10^7$/ml. In further experiments with A1*a* and 1404 at a 5:1 ratio, in which only lysogenization was estimated, there was a slight reduction in the percentage of organisms lysogenized (41·5 % control, 36·5 % with potassium citrate M/10^{-2}, 33·25 % with potassium malonate M/2×10^{-2}). In view of the results given by the early experiments, this can be attributed to some interference with adsorption. In the absence of a marked decrease in lysogenesis, no significant increase in productive infection can have occurred.

Influence of anaerobiosis on lysogenization

It has been stated that anaerobiosis decreases the proportion of lysogenic responses (Lwoff, 1953). No such phenomenon was observed in experiments with A1*b* and A2*d* (Table 13). Two sets of plates were inoculated with aliquots of phage-infected bacterial suspensions prepared by the standard technique, and one set was incubated aerobically, the other anaerobically. The colonies which developed on the plates incubated anaerobically were considerably smaller than those which grew under aerobic conditions, but numerically there was no difference. Two further experiments similar to the above were carried out, in which anaerobiosis was maintained at all stages of the experiment, and not merely during incubation of the plates. Once again the only difference was in the size of the 'anaerobic' colonies, which were much smaller than those in the control.

Table 13. *Influence of anaerobiosis on lysogenization*

The assessment of lysogenization was made by the standard technique. The controls, which are not shown in the table, were normal.

	% of bacteria lysogenized	
	Incubated aerobically	Incubated anaerobically
A1*b*:Q1 = 0·75:1	29	28·3
A2*d*:Q1 = 1:1	12	12

Influence of chloramphenicol on lysogenization

In contrast to these negative results, chloramphenicol had a well-marked influence on lysogenization. This is a reaction of considerable complexity which it is hoped to make the subject of a separate paper.

DISCUSSION

An essential prerequisite for the study of the processes of lysogenization is a reliable technique for estimating the percentage of bacteria lysogenized and the percentage productively infected when a culture of sensitive bacteria is exposed to the action of temperate phage. The methods which have been elaborated for this purpose are given in detail in the first part of this communication, and do not require further discussion. They are considered to be reliable and accurate, and have been found to give repeatable results. The method of estimating the numbers of productively infected bacteria by counting plaques ('lytic centres') has proved unreliable with the systems we are investigating. We have found that newly lysogenized Q1 is relatively unstable, and may, after a few divisions, throw off a daughter cell in which the phage undergoes productive development. If this occurs before the bacteria in the sensitive lawn have multiplied to any appreciable extent, the free phage thus liberated forms a plaque which is smaller than but otherwise indistinguishable from the plaque produced by an organism productively infected *ab initio*. We have not used as a routine the method described by Levine (1957) turning on the use of gal$^+$ and gal$^-$ strains of *Salmonella typhimurium*, as the gal$^-$ indicator strains available at the time this work was done were found to contain B

phages (Boyd, 1950) and were insensitive to several of the type A phages of the series.

When Q1 was exposed to one of these temperate phages at a concentration sufficiently low to ensure that only single infection occurred it was found that some of the infected bacteria were lysogenized and others were productively infected. The proportions of lysogenized and productively infected bacteria were, within the limits of experimental error, constant in repeated tests of the same batch of phage and in other batches of the same phage made by the same technique but at a different time. Thus it can be concluded (*a*) that a temperate phage consists of a mixture of particles, some (α particles) endowed with the property of producing lysogenesis, and others (β particles) incapable, as single infections, of doing so and going on instead to productive development, and (*b*) that the two types of particle, under standard conditions, are present in relatively constant proportions in each temperate phage.

Eleven of the 12 type A temperate phages (Fig. 2) were investigated in this way (type A4 had to be excluded as no virulent mutant of this phage has been isolated). The proportions of α and β particles varied greatly from phage to phage, though each type behaved consistently. In some the α particles were equal in number to or even more numerous than β particles, in others they constituted as little as 8% of the total. These accurate estimates of the two varieties of particle in any one temperate phage, and the demonstration of their presence in varying proportions in the 11 members of the series, provide a useful means of studying certain of the problems of lysogenization.

It has been suggested (Parry & Edwards, 1953) that the decision towards lysogenesis or productive development of the phage turns on the interplay of two factors, namely varying degrees of virulence on the part of the phage particles and varying degrees of resistance to lysis on the part of the bacteria. However, in view of the wide variation in the pattern of lysogenesis and lysis shown by the 11 phages when tested against the same bacterial suspension, it is obvious that under these conditions bacterial resistance plays a secondary role in determining the outcome of phage infection. Clearly the dominating factor is in the phage particle, and is a constant feature of each particular type. This point is well demonstrated by the results given by A1*b* and A2*d* (Figs. 3, 6), in which the differences recorded have been confirmed in experiments repeated at long intervals of time and with different batches of reagents.

In previous papers (Boyd, 1951*b*, 1953), the existence of α and β particles was deduced from less convincing experimental evidence, and it was suggested that the explanation of the multiplicity phenomenon might lie in a dominance of α particles over β particles. When bacteria were exposed to rising concentrations of phage, increasing numbers would be infected with several particles, some with β particles only, some with α and β particles. In the latter case the pattern of development, i.e. lysogenesis, would be imposed by the dominant α particle, thus producing a higher degree of lysogenization. This idea has been independently explored by Prell & Prell (1959). Working with phage P22, which is derived from the same lysogenic bacterium as A1*b* and gives similar reactions, Prell & Prell found that with low phage:bacteria ratios (0·3:1) the probability per phage is 0·57 for lysogenization and 0·43 for lysis. This is in good agreement with the finding recorded in Fig. 3.

Prell & Prell develop the speculative theory outlined above, and conclude "in multicomplexes the immunity induced by one of the superinfecting phages is superimposed upon the one-hit lytic infection causing the percentage of lysogenization increasing with multiplicity".

Bertani (1960) suggests that calculations of the numbers of bacteria lysogenized by single hits may be complicated by the presence of 'doublets' in the culture—doublets being 'physiologically independent, but incompletely separated, sister bacteria'. He points out that, in a stationary culture, if one half of a doublet is hit by a particle which multiplies productively, the second half will be heavily infected when the first half bursts, and so will run a good chance of being lysogenized by this multiplicity. He points out rightly that this is more likely to increase the proportion of bacteria lysogenized when the phage:bacteria ratio is low. In our experiments the bacteria were actively motile throughout. In the case of A1*b* the proportion of lysogenics and lytics is such that any part played by doublets in increasing lysogenization would be difficult to assess. But from the results given by A2*c* and A2*d*, where at limit dilution approximately 10 % or less of the particles give lysogenesis, while 90 % or more give lysis, it can be seen that, with the technique used, the occurrence of late lysogenesis resulting from the lytic infection of one half of a doublet must be relatively rare. As the bacterial suspensions in all these experiments were prepared by standard methods, and therefore contained the same proportion of doublets, it would appear that our results have not been significantly affected by this possibility.

The results recorded graphically in Figs. 3, 4 and 6 show that with rising phage concentrations and hence rising phage:bacteria ratios, resulting in multiple infection of bacteria by phage particles, the maximum degree of lysogenization is reached in phage concentrations between 4×10^8 and 10^9 particles/ml., at which concentrations two-thirds or more of the phage is adsorbed in 15 min. (Fig. 5). (In the case of A1*b*, calculating from the protocols from which Fig. 5 was constructed, at a concentration of 10^7 (ratio 0·1:1) over 98 % of the phage was adsorbed; at 10^8 (ratio 1:1) 95 % and at 10^9 (ratio 10:1) 67·5 %. Similar experiments with other phages gave results of the same order.)

At limit dilution (for convenience, a plaque count:bacteria ratio of 1:10) the relative proportions of α and β particles per unit of phage of known concentration are revealed (Fig. 7, Table 9). These figures enable the actual number of α and β particles in the accurately measured higher phage concentrations to be calculated. In these higher concentrations the total number of infected bacteria (i.e. both lysogenized and lysed) is less than the total number of phage particles which have been adsorbed. It follows therefore that some infected bacteria have taken up more than one phage particle: the expected distribution can be calculated from the Poisson formula. The total number of bacteria infected by different members of the series and at different concentrations shows some variation (Table 9), but this variation bears no obvious relationship to the proportions of bacteria lysogenized or lysed.

These principles have been employed in constructing the graphs in Fig. 7. The starting point in each case is the first column, which shows the number of bacteria lysogenized by, and hence the number of α particles in, a unit of the phage at limit dilution. The figures recorded in the subsequent columns show the results given by

identical units of accurately measured rising concentrations of the same phage. The hatched columns show the bacteria lysogenized, while the solid columns show the number of α particles in the unit, calculated from the figure given at limit dilution. To make the results obvious at a glance the lower column of the two is enclosed in the higher.

Three different types of response are revealed in Fig. 7.

(1) The number of lysogenized bacteria exceeds the number of available α particles. This reaction is seen in the second column (phage concentration $3{\cdot}1 \times 10^7$) of A1*a* and A1*b*, and throughout in A2*c*, A2*d*, and A2*e*.

(2) The number of lysogenized bacteria falls significantly below the number of available α particles. This is well marked in the case of A2*b*, less so in A1*d* and A2*a*.

(3) The number of lysogenized bacteria, though lower than the number of α particles, represents approximately the number of bacteria which, in terms of the Poisson distribution, would have been hit by one or more α particles. This is seen in certain concentrations of A1*a*, A1*b*, A1*c*, A2*f* and A3.

It must be reiterated that as the same bacterium (Q1) was used throughout these experiments, and as the different phages were adapted to and propagated on this organism, such variations as occur are attributable only to the phage, and thus as far as bacterial resistance is concerned, the different experiments act as controls, one for the other.

The results given by the phages listed under (1) disprove the speculative hypothesis (Boyd, 1951*b*) that lysogenesis results only when an α particle infects a bacterium, either as a single infection or in association with β particles. In the higher concentrations of A2*c*, A2*d*, and A2*e*, the number of bacteria which were lysogenized exceeded the number of α particles in the unit of phage to which they were exposed. On the other hand, in these higher phage concentrations a number of bacteria were infected by two or more particles, which in most cases were β particles. It can be assumed that single infections with a β particle will produce the same result (a burst) in high as in low phage concentrations, and that the only significant difference between infected bacteria in the high phage concentrations and infected bacteria in the limit phage concentrations was that some of the former were infected by more than one particle. It therefore would appear that in these systems multiple infection, irrespective of whether the particles are α or β, can bring about lysogenesis, and that when two or more β particles enter one bacterium, they act together in some way to produce the conditions essential for lysogenization. A possible but speculative explanation is that α particles possess a component which determines conditions leading to lysogenization: that in individual β particles this component is incomplete, but that when the infective material of two or more β particles enters a bacterium, the effect is additive, and the necessary conditions for lysogenization are produced.

This finding has something in common with observations made by Kaiser (1957) and Levine (1957). Kaiser studied independently occurring clear plaque-forming mutants of phage, among which he recognized three different phenotypes. Mixed infection with a pair of phenotypically different mutants, each of which lysogenized poorly or not at all, produced a high frequency of lysogenization characteristic of infection with the wild type. The surviving bacteria were lysogenic for one or both of the infecting types of phages. Levine worked with clear plaque-forming mutants

of P22 (A1*b*). He found that, when sensitive bacteria were exposed to a mixture of a virulent mutant, which of itself gave no lysogenesis, and a very weak temperate phage, which gave a very low degree of lysogenization, these two phages combined in multiple infections produced a high degree of lysogenization. These of course are true mutants with genetic deficiencies: if kept uncontaminated, they reproduce their own type indefinitely. β particles are not mutants in this sense, as the progeny of single infection with a β particle is a mixed brood of α and β particles in the standard proportions characteristic of the parent type.

In the case of the phages listed under (2), the position is reversed. At limit dilution the percentage of α particles is average to high. When bacteria are exposed to higher phage concentrations, the number of lysogenized bacteria falls below, and in some cases far below, the number of available α particles, even when 'doubling up' is taken into consideration. This is not due to defective adsorption (Table 9). In the case of these phages, multiplicity would appear to antagonize lysogenization instead of increasing it. Whether or not this is due to the presence of a restraining component in the β particles or to some external factor, so far unrecognized, is a question which cannot at present be answered.

The phages listed under (3) give results which are mainly in conformity with the original theory that lysogenization follows infection with a dominant α particle, and if no other phage types but these had been examined (the type on which the theory was based was A1*a*) a convincing case could have been built up.

An alternative explanation of these findings which has been propounded is that each phage consists, not of a mixed population of particles with differing properties of lysogenization, but of a uniform population in which there is a certain probability that any one phage particle can give rise to a lysogenic clone: that this probability is a constant for each phage: and that the probability is doubled in a doubly infected cell, trebled in a trebly infected cell and so on. When multiplicity values are calculated according to the Poisson formula, and adjusted to the probability revealed by the percentages of lysogenesis and lysis at limit dilution, a set of theoretical figures can be constructed. The results set out in Fig. 7 show a considerable degree of agreement with these theoretical figures (much better agreement than the discarded 'dominant α particle' theory) but in all cases show some deviation, and in one or two cases gross deviation.

Apart from the by no means perfect fit of theoretical figures and results this hypothesis is open to criticism on at least two scores. What are the factors which determine the certain probability that any one particle can give rise to a lysogenic clone, bearing in mind that this probability varies widely in these closely related but nevertheless distinct phage types? The bacteria are prepared by standard methods, and while individual organisms may have varying characters they present the same front in all the experiments. The varying degrees of lysogenesis and lysis produced by infection with the different phage types cannot therefore be attributed to the bacteria. Before either lysis or lysogenesis occurs, the hazards of adsorption and penetration lie behind: these cannot explain the phenomenon. The 'certain probability' must therefore result from events inside each bacterium after it has been penetrated by the infective material of a phage particle. The bacteria, although they may vary individually, e.g. in age in terms of the last division, are *en masse* alike in all the experiments. The infective material from each phage particle, according

to this hypothesis, is uniform for each phage type. When A1*b* is the infecting phage, an average of six out of ten of these identical units succeed in circumventing some undefined barrier, and become integrated in the bacterial chromosome: four of the ten fail to do so. In the case of A2*d*, only one out of ten of the invading particles reaches this goal. As all the particles in each phage are held to be alike, it follows that all, both A1*b* and A2*d*, are capable of producing lysogenesis. The varying number of successful lysogenizations must therefore be attributed to the chance evasion of some barrier or inhibiting mechanism within the bacterium, a chance evasion in which the different phage types have varying but consistent degrees of success. When the issues involved are examined in this way, the probability hypothesis becomes unconvincing, the more so as the existence of a mechanism capable of preventing lysogenization is speculative and its nature is undefined.

The second criticism of the probability theory lies in the fact that none of the experiments, admittedly limited in number, involving environmental changes of the bacterium-phage complex, made any significant difference to the percentages of lysogenesis or lysis.

On balance, the conception of a phage population, mixed in the sense that its members, though genetically identical, are endowed to a varying degree with some factor essential for lysogenization, is more in keeping with the available experimental evidence.

The fallacy of drawing conclusions of a general nature from the results given by a single system is heavily underlined by the results of these experiments, and, as a corollary, the necessity for widening the field of investigation is clearly demonstrated. Apart from showing that in certain cases lysogenization can result from multiple infection with β particles, these findings are of interest mainly because they show the complexity of the multiplicity phenomenon. They provide no solution capable of general application.

The experiments with A1*b* and A2*d* (Figs. 3, 6) demonstrate two other responses in bacteria exposed to very high concentrations of these temperate phages which are worthy of brief mention.

The first is a lethal response corresponding to the phenomenon which has been observed in the case of virulent phages and has been described as lysis-from-without (Andrewes & Elford, 1932). The possibility of this reaction being produced by a weak bactericide which is lethal only when in high concentration cannot be altogether disregarded, although rendered improbable by the fact that the lethal property is removed by passing the lysate through a gradacol filter with pores sufficiently small to hold back the phage particles. The alternative explanation, a weakening of the bacterial wall by the tail-enzymes of the phage particles (Puck & Lee, 1954) is in keeping with the accepted basis of this phenomenon in other systems.

The second is the occurrence of an increasing number of bursts, particularly in the case of A1*b* (Fig. 3). This observation prompted an experiment in which an established strain of Q1 (A1*b*) was exposed to a high concentration of its homologous temperate phage, A1*b*, to which in low concentration it is immune. A number of bursts resulted. An established strain of Q1 (A2*d*) was then exposed to a high concentration of the heterologous temperate phage, A1*b*, to which in low concentrations it is immune. Again a number of bursts occurred, the resulting free particles being of both types, A1*b* and A2*d*. With this evidence that a high concentration of

temperate phage can 'induce' a lysogenic bacterium which is immune to the same phage in lower concentrations, it seems possible that the bursts under consideration arise from a two-stage process—an early lysogenization followed by induction.

With the exception of chloramphenicol, various chemical and physical agencies which have been found by other workers to affect lysogenesis have shown no specific or significant action on the systems which we have tested. Neither heat, nor cold, nor anaerobiosis, nor organic acids have had any effect other than a non-specific action attributable to interference with bacterial growth and multiplication.

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The Effect of *N*-Ethylmaleimide on the Radiation Sensitivity of Bacteria

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SUMMARY

The sensitization of *Escherichia coli* strain B/r to γ radiation was shown to take place within a few minutes of the addition of *N*-ethylmaleimide. Sensitization was demonstrated with as little as 0·0001 M-*N*-ethylmaleimide, but a much higher concentration (> 0·002 M) was necessary for any bactericidal effect. The *N*-ethylmaleimide had to be present during irradiation in order to be effective. *Staphylococcus aureus* and a *Pseudomonas* sp. but not spores of *Bacillus subtilis*, were also sensitized by *N*-ethylmaleimide. The *Pseudomonas* sp. was only sensitized under anoxic conditions; this may indicate that, with this organism, *N*-ethylmaleimide and oxygen acted competitively in enhancing radiation damage. Possible mechanisms of sensitization are discussed.

INTRODUCTION

Nitric oxide and oxygen both enhance radiation damage when present in suspensions of the vegetative forms of bacteria during irradiation (Howard-Flanders, 1957; Hollaender, Stapleton & Martin, 1951). Oxygen is also effective with spores, although nitric oxide has been found to have a net protective effect on them (Powers, Webb & Kaleta, 1960). Until recently little attention had been paid to other substances which might sensitize bacteria to radiation. Bridges (1960) reported that *N*-ethylmaleimide (NEM), when present during irradiation, was able to increase the lethal action of γ radiation on *Escherichia coli*, an effect more pronounced under anoxic conditions. The present paper records further study of the action of NEM on certain micro-organisms.

METHODS

Chemical. *N*-ethylmaleimide (NEM) was obtained from the Aldrich Chemical Co. Inc. (Milwaukee, Wisconsin, U.S.A.). It was dissolved in sterile 0·067 M-phosphate buffer (pH 7) to make a 0·01 M-solution which was kept at 3–5° and diluted as required. Sterilization of this solution was found to be unnecessary.

Micro-organisms. *Escherichia coli* strain B/r (in the sequel this strain will be referred to as *Escherichia coli* B/r), obtained in 1958 from Miss Tikvah Alper (Hammersmith Hospital), was used throughout this work. The *Pseudomonas* sp. was isolated from a chicken carcass by Dr Margaret Thornley (Low Temperature Research Station, Cambridge), and *Staphylococcus aureus* was no. 7447 of the National Collection of Type Cultures. Cultures (20–24 hr.) on nutrient agar slopes

were washed off and shaken with 10 ml. 0·067 M-phosphate buffer (pH 7) and 1 ml. of this suspension added to 9 ml. of the test solution before irradiation. Spores of *Bacillus subtilis* were obtained by allowing 24 hr. cultures on nutrient agar plates to stand on the bench for several days. The growth was then washed off with buffer, centrifuged, resuspended in buffer, and the suspension heated in an 80° water bath for 10 min. The spore suspensions were shaken with glass beads to break up clumps and were used within a few hours of preparation. NEM was added 4–6 min. before irradiation except where otherwise stated.

Estimation of radiation damage. The ability of bacteria to form visible colonies on the surface of nutrient agar (Oxoid) plates incubated at 30° was used as the criterion of viability. Colonies usually appeared within 24 hr. although after treatment with a high concentration of NEM subsequent growth was slow and colonies continued to appear for up to six days.

Irradiation technique. A ^{60}Co γ radiation source giving a dose rate of 300 krad./hr. was used at room temperature. Samples (10 ml.) of test suspension were irradiated in glass vessels, and air or oxygen-free nitrogen was bubbled vigorously through the suspensions during the irradiation. With nitrogen, bubbling was begun 6 min. before irradiation, to remove dissolved oxygen.

RESULTS

Sensitization of Escherichia coli *B/r*

The survival curves for *Escherichia coli* B/r in the presence and absence of 0·001 M-NEM are shown in Fig. 1; it can be seen that a pronounced sensitization occurred. Where the shape of survival curves of sensitized and control bacteria is the same, the extent of any sensitization may be described by the 'dose modifying factor' (d.m.f.) which is the ratio of the dose of radiation needed to cause a given degree of damage in the absence of the sensitizer to that required in its presence. For example, the presence of oxygen in an air-saturated suspension results in a d.m.f. of 2·6 with *E. coli* B/r. Under anoxic conditions, 0·001 M-NEM was equivalent to a d.m.f. of 2·0, but sensitization was not so marked under aerated conditions where the d.m.f. was 1·3. There was no enhancement of radiation damage when NEM (to 0·001 M) was added immediately after irradiation in buffer (Fig. 1). Bacteria initially incubated with 0·0005 M-NEM for 5 min. and then diluted 100-fold or treated with

Table 1. *The effect of removing* N-*ethylmaleimide* (*NEM*) *immediately before irradiation on the radiation sensitivity of* Escherichia coli *strain B/r*

NEM was used at 0·0005 M; irradiations were performed under anoxic conditions.

		Mean survival after 30 krad. (%)
Untreated controls	—	33·1
NEM added 10 min. before irradiation	(*a*) Not removed	16·1
	(*b*) Removed by addition of excess cysteine 5 min. before irradiation	31·6
	(*c*) Removed by 1/100 dilution 5 min. before irradiation	32·8

excess (0·001 M) cysteine to remove any unreacted NEM were not sensitized to subsequent irradiation in absence of oxygen (Table 1).

The greater effect of NEM under anoxic conditions might have been due to a requirement for a period of incubation with the compound before irradiation, since under anoxic conditions there was always nitrogen bubbling for 6 min. before each incremental dose of radiation. Various times of pre-incubation under aerobic conditions were therefore tried. It can be seen from Fig. 2 that a definite sensitization was observed with pre-incubation for as little as 1 min. There was no increase in sensitization when the pre-incubation period was increased from 5 to 15 min. The difference in pre-incubation time does not therefore explain the difference in effect as between aerated and anoxic conditions. The rapidity with which sensitization was established would seem to exclude the possibility that any gross changes in the physiological state of the cells before irradiation were involved although it does not exclude the possible involvement of the reaction with cellular –SH groups.

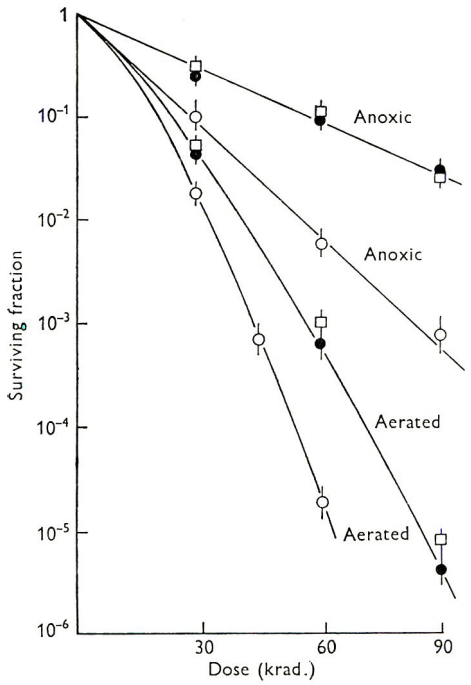

Fig. 1

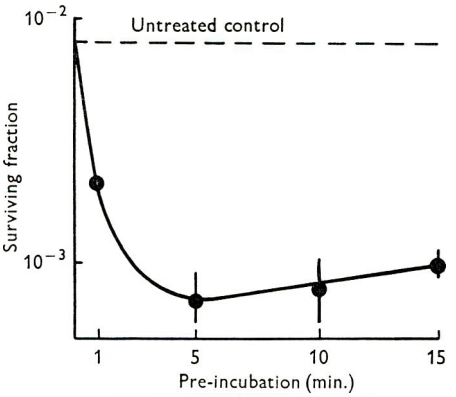

Fig. 2

Fig. 1. Effect of 0·001 M-NEM on the radiation sensitivity of *Escherichia coli* strain B/r. ●, No NEM; ○, NEM present during irradiation; □, NEM added immediately after irradiation.

Fig. 2. Effect of pre-incubation with 0·001 M-NEM on surviving fraction of *Escherichia coli* strain B/r after 45 krad. under aerated conditions.

Toxicity of NEM

Figure 3 shows the toxicity of NEM at varying concentrations. As judged by the ability of the treated organisms to form visible colonies on nutrient agar, NEM was non-toxic up to 0·001 M, while at 0·01 M there was an appreciable lethal effect. Although treatment with 0·001 M-NEM for 1 hr. did not prevent the subsequent

formation of colonies, they appeared more slowly than the controls. Colonies appeared at the normal rate, however, when 0·001 M-homocysteine was present in the plating medium. The presence of this sulphydryl compound did not decrease the toxicity of NEM or the degree of sensitization.

Effect of concentration

The influence of concentration on sensitization under anoxic conditions is given in Fig. 4; a similar curve was obtained under aerated conditions. There appears to be a threshold at about 0·00005 M-NEM, below which no significant sensitization was observed. Above this concentration the effect increased rapidly and reached a maximum at 0·0003 M. Above this there was apparently a very slight decrease in sensitization.

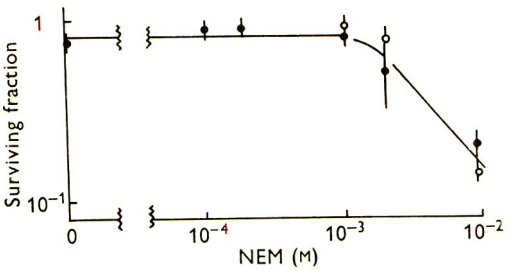

Fig. 3

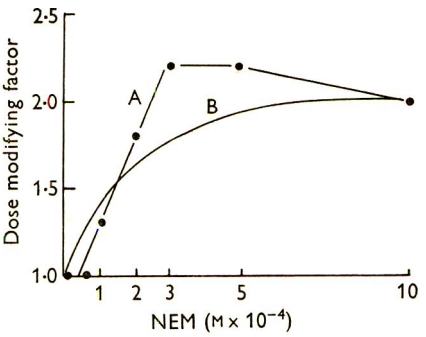

Fig. 4

Fig. 3. Effect of incubation with NEM for 1 hr. on viability of *Escherichia coli* strain B/r. ●, Plate counts on nutrient agar; ○, Plate counts on nutrient agar containing 0·001 M-homocysteine.

Fig. 4. Effect of concentration of NEM on surviving fraction of *Escherichia coli* strain B/r after 60 krad. under anoxic conditions. A, experimental curve; B, theoretical curve according to equation of Alper & Howard-Flanders (1956) where $m = 2{\cdot}2$, $K = 175$ μM.

Alper & Howard-Flanders (1956), in studying the oxygen effect, represented the effect of concentration by the following formula:

$$\text{d.m.f.} = \frac{m[O_2]+K}{[O_2]+K},$$

where m is the maximum d.m.f. and K is the concentration of oxygen at which half the maximum sensitization is attained. Applying these terms to NEM under anoxic conditions, m may be assumed to be 2·2 and K, 175 μM. A curve plotted from these values in such an equation is shown in Fig. 4; the curve does not characterize the experimental points. The effect of the concentration of NEM appears therefore to be significantly different from that of oxygen.

Experiments with other organisms

The effect of 0·001 M-NEM on the radiation sensitivity of *Staphylococcus aureus* is shown in Fig. 5. It was essentially similar to that obtained with *Escherichia coli* B/r, the d.m.f. values being 1·55 under anoxic conditions and 1·17 in air. The *Pseudomonas* sp. differed from *S. aureus* and *E. coli* B/r in that although no sensitization was produced by 0·001 M-NEM under aerated conditions, there was a very marked effect

under anoxic conditions where the d.m.f. was 2·0 (Fig. 6). It is of some interest that the effect of oxygen on radiation sensitivity was large with this pseudomonad, the slope of the survival curve under air being 4·25 times as great as that under nitrogen. Spores of *Bacillus subtilis* were not sensitized to radiation by 0·001 M-NEM under anoxic or aerated conditions. This might have been due to the failure of NEM to penetrate the spore wall but no experiments were carried out to verify this. NEM was not toxic to any of the above organisms at 0·001 M.

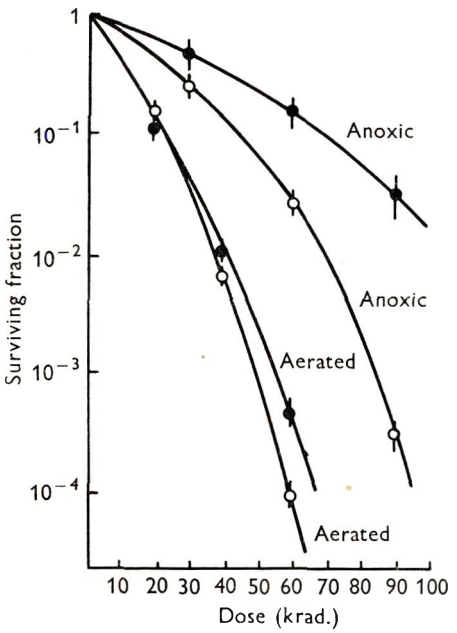

Fig. 5

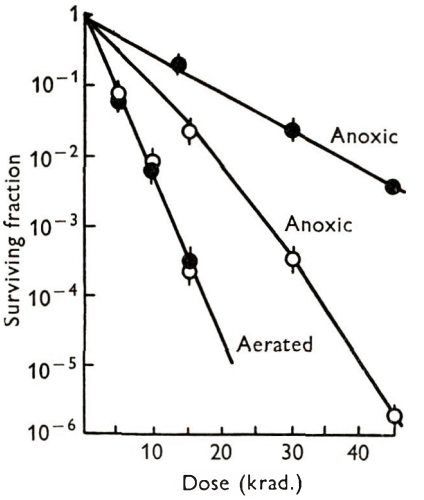

Fig. 6

Fig. 5. Effect of 0·001 M-NEM on the radiation sensitivity of *Staphylococcus aureus*. ●, Control; ○, NEM.

Fig. 6. Effect of 0·001 M-NEM on the radiation sensitivity of *Pseudomonas* sp. ●, Control; ○, NEM.

DISCUSSION

Sensitization is apparently dependent upon the simultaneous action of NEM and radiation since organisms incubated with 0·0005 M-NEM for 10 min. and then diluted 1/100 or treated with excess cysteine (to 0·001 M) to remove unreacted NEM, were not sensitized. This eliminates the two most obvious explanations of the mechanism of sensitization, i.e. that NEM combines with cellular –SH groups and in some way renders them more sensitive to the action of radiation, or that NEM combines with naturally occurring –SH protective agents. Sulphydryl groups on biological molecules have been classified as 'freely reacting', 'sluggish' and 'masked' (Barron, 1951). All the freely reacting –SH groups are likely to react with NEM within a very few minutes of its addition (Roberts & Rouser, 1958), and since the reaction is irreversible they will lose their biological activity. That some physiological damage does occur seems indicated by the finding that the endogenous respiration of *Escherichia coli* B/r is 70% inhibited by 0·001 M-NEM (Bridges & Marples, un-

published observation). This damage to cellular –SH groups is obviously not lethal since the organisms are able to form colonies when diluted out and plated. Although colony formation is slower than usual it may be speeded by up the addition of –SH groups to the medium in the form of homocysteine. Such a treatment does not, however, overcome the sensitization.

The fact that a certain amount of NEM reacts with cellular –SH groups probably accounts for the threshold observed in the concentration curve. Presumably a certain concentration is required before there is sufficient uncombined NEM remaining to cause sensitization. The organisms used in these experiments were not washed and might be expected to have had a fairly high concentration of –SH compounds arising from the nutrient medium, in addition to those normally present within the organism.

A possible explanation of the data is that the NEM reacts with some molecule which is in a short-lived reactive state induced by the radiation. This molecule, in the absence of NEM, stands a certain chance of being restored or changed to an inocuous form. A similar model has been proposed for the enhancing effect of oxygen (Alper & Howard-Flanders, 1956; Alper, 1958). In this case the oxygen is envisaged as undergoing a 'metionic' reaction with a free radical centre, probably on a carbon atom, resulting in the formation of a peroxide radical. It is quite possible that NEM could react across its double bond with a carbon-free radical centre. Such reactions are known in the radiation-induced cross-linking of rubber which is catalysed by dimaleimides (Vale & Roberts, 1960). A further possibility is that NEM reacts with –SH groups (or possibly –S-free radicals) which result from the radiation-induced breakage of –S–S– bonds, necessary for the functional state of some proteins and other molecules of biological importance. Combination of NEM with either of the sulphur atoms could prevent the –S–S– bond reforming and result in the loss of the biological integrity of the molecule.

Since oxygen partially (completely, with the *Pseudomonas* sp.) inhibited the action of NEM, it is conceivable that both compete for the same free radical centres. The study of sensitizing agents such as NEM may therefore be of use in understanding the oxygen effect and, ultimately, the nature of the lethal damage which radiation causes within the cell.

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The Nature of the Widespread Soil Fungistasis

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SUMMARY

Fungistasis of Conover loam, muck, and hardwood forest soils to conidia of *Glomerella cingulata*, *Fusarium oxysporum* f. *lycopersici*, and *Penicillium frequentans* was demonstrated by indirect methods such as agar disks, double agar layer plates, and cellophan folds, as well as by direct placement of spores on or in soils. All attempts to extract toxic substances from soil with water or organic solvents failed. Fungistatic volatile substances could not be demonstrated in soil. Redox, pH, and osmotic conditions were not responsible for soil fungistasis. Various lines of evidence led to the conclusion that the so-called widespread soil fungistasis as observed by indirect methods is the result of production of antibiotics by soil microbes growing on the surface of the assay media, and is not due to a reserve of toxic substances in soils. The possibility is suggested that individual fungus spores serve as nutrient microsubstrates in soil and stimulate the rapid growth of soil microbes on their surface or in their immediate vicinity, and that this results in the production of sufficient fungistatic substances to prevent spore germination. Preliminary evidence in support of this suggestion are results with concentrated 50 % ethanol washings from teliospores of *Ustilago zeae* which markedly stimulated growth of mixed soil microbes and of pure cultures of *Streptomyces* sp. and *Pseudomonas* sp., known antibiotic producers, in agar media.

INTRODUCTION

Spores of fungi which are able to germinate readily in distilled water or nutrient substrates fail to germinate in natural soils. Since the work on this problem by Dobbs & Hinson (1953), many authors have demonstrated fungistasis in different soils by using spores of numerous fungi (Chinn, 1953; Hessayon, 1953; Jefferys & Hemming, 1953; Jackson, 1958*a*; Lockwood, 1959; Stover, 1958). This apparently widespread fungistasis has been attributed to diffusible toxic factors in soil, and appears to coexist with biological activity since soils sterilized by heat or fumigants are not inhibitory (Dobbs & Hinson, 1953; Jackson, 1958*b*; Lockwood, 1959; Stover, 1958). Toxicity to fungi has been restored to autoclaved soil by inoculating with a specific bacterium (Park, 1956*a*) or with various actinomycetes (Lockwood, 1959). Fungistasis has been decreased by addition of plant residues (Chinn & Ledingham, 1957), or organic compounds such as glucose (Dobbs & Hinson, 1953; Jackson, 1958*a*) to soil.

Many attempts have been made to elucidate the precise nature of soil fungistasis.

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On the basis of the similar inhibitory spectrum of various soils, Jackson (1958*b*) speculated that the inhibitors are likely to be a simple mixture of a few components, probably of microbial origin. Some workers have reported unidentified fungistatic substances in soil extracts and diffusates (Dobbs & Hinson, 1953; Hessayon, 1953; Jefferys & Hemming, 1953; Stover, 1958). Except for extraction of fungitoxic lignin-like material from soil (Lingappa & Lockwood, 1960) these results have usually been inconclusive, and the factors responsible for fungitoxicity have not been isolated and identified.

Special kinds of soil fungitoxicity such as those of the highly calcareous Newborough sands (Dobbs & Bywater, 1959) and the very acid (pH 2·8) Charnwood forest soils (Jackson, 1958*b*) seem to differ from the general fungistasis expressed by almost all soils. There is need to explain the nature of the widespread soil fungistasis. In the present investigation, the methods used by previous workers were repeated, and work was extended to include additional controls and new experiments. Attempts to determine whether the results from indirect assay methods in common use might lead to erroneous interpretation of soil fungistasis were of particular interest.

METHODS

Conidia from 4- to 7-day-old cultures of *Glomerella cingulata*, *Fusarium oxysporum* f. *lycopersici* and *Penicillium frequentans* maintained on potato dextrose agar were used. Conidia of *G. cingulata* do not germinate well in distilled water, but do so on water agar, whereas those of *F. oxysporum* f. *lycopersici* and *P. frequentans* germinate readily in distilled water and on water agar. It was necessary to wet spores of the latter with a $1/10^5$ dilution of polyoxyethylene sorbitan monooleate (Tween 80) to obtain uniform dispersion. A *Streptomyces* sp. and a *Pseudomonas* sp. which produced wide antibiotic inhibition zones on agar media were grown in yeast extract agar (Difco) and nutrient agar (Difco), respectively. All agar media used in these experiments contained 2 % (w/v) Bacto agar (Difco). This material contains, according to the manufacturer, about 0·4 % total protein (calculated from total N), 0·04 % lipids (ether extractable), and 3·86 % mineral content (ash). Bacto peptone (Difco) was used in 0·05 % (w/v) concentration unless specified otherwise.

The soils used were Conover loam, muck, and a hardwood forest soil, all at 50–60 % of saturation, from East Lansing, Michigan, U.S.A. Talc, diatomaceous earth (Celite), kaolin, bentonite, decolorizing carbon (Norit A), cellulose filter paper, or fibre glass filter paper were sometimes used in place of soil.

Many of the methods used by previous workers for assaying soil fungistasis were repeated in this laboratory. These included: (*a*) cellophan packets or folds (Dobbs & co-workers, 1953, 1957, 1959); (*b*) double agar layer method (Lockwood, 1959); (*c*) buried slide coated with seeded agar (Chinn, 1953); (*d*) water or peptone agar disks (2 mm. thick; Jackson, 1958*a*, *b*); (*e*) a modification of the method of Stover (1958) in which a layer of seeded agar was poured over a sterilized cellophan dialysis membrane. All these methods were used in Petri dishes filled to a depth of $\frac{1}{4}$ in. with soil and with the surface smoothed. Dishes were sealed with rubber bands and incubated at 28°. Assay media, except for buried slides, were pre-incubated on soil for 4–8 hr. (7 days for double agar layer method) followed by application of spores, then by continued incubation for another 16 hr. before counting germination. Three

microscope fields in each of 3 plates and a total of about 300 spores for each treatment were counted.

Direct methods of observing spore germination in soil were also used. About 50,000 conidia of the test fungi in 0·2–0·4 ml. of water were placed in a $\frac{1}{4}$ in. diameter area on moist soil in Petri dishes. Samples of these spores were removed after 16 hr. at 28° by lightly pressing 7 mm. diameter water agar disks on the demarked area. Disks and adhering spores were inverted on a glass slide for observation. In another method microscope slides containing a suspension of conidia of *Glomerella cingulata* were air dried to fix the spores to the glass surface, then were buried in soil and treated like those of Chinn (1953). In an extract method $\frac{1}{2}$–1 million conidia of *G. cingulata* were mixed with 1 g. soil. After incubation for 16 hr. the soil samples were shaken with 10 ml. of 50 % (v/v) glycerol in water containing 0·5 % household detergent (Tide), and centrifuged for 2–20 min. at 1200 rev./min. (160 *g*); 70–80 % of the spores remained in the supernatant fluid which was free from all but colloidal soil particles. Spores were counted with a haemocytometer. Only ungerminated spores were recovered by this method. By comparison of the recovery from natural and autoclaved soils with controls refrigerated to prevent germination, estimates of germination could be calculated.

Several types of soil extracts were made. (1) Samples (100 g.) of loam or muck were extracted twice by shaking with 200 ml. of various C.P. grade solvents. (2) Drainage water from a tilled agricultural field of Conover loam of about 40 acres was collected from man-holes during dry weather when seepage was slow, and also after heavy rainfall; 16 l. samples were collected and passed through coarse filter paper. (3) Nearly 1000 lb. (about 450 kg.; 8·5 cu.ft.) of Conover loam soil in a greenhouse bench was periodically irrigated with excess water and up to 8 l. of drainage water collected each time in a polyethylene sheet. (4) Loam soil to a depth of 1 in. was placed on a $\frac{1}{2}$ in. deep bed of Norit A in glass trays and irrigated for 24, 48 or 72 hr. The recovered Norit A was extracted with various organic solvents. Water extracts of soils, or drainage water from the greenhouse bench were also treated with Norit A which was subsequently recovered and extracted with organic solvents.

Water extracts larger in volume than 1 l. were evaporated to 1/5 volume in 20 min. at 40°–50° in a Precision Evaporator (Precision Scientific Co., Chicago, Illinois, U.S.A.). The resultant extracts, water extracts less than 1 l., and those of organic solvents were evaporated to a small volume under vacuum at 28–30° in a flash evaporator and assayed in seeded agar by direct incorporation or in filter paper disks ($\frac{1}{2}$ in. diameter).

Measurements of pH value and redox potential were made with a Beckman Model H2 pH meter.

All experiments were in triplicate and were repeated at least once. Sterile methods were used. In the case of natural soil, experiments were limited to 18 hr. in length when sterility was a consideration, due to the contaminating effects of motile soil microfauna.

RESULTS

Comparison of assay methods

Soil fungistasis was demonstrated by various methods, both indirect and direct. Some of the methods and typical results are given in Table 1. In addition, the modified method of Stover (1958) and the buried slide method of Chinn (1953) also

showed fungistasis in soil. Muck soil and hardwood forest soil gave inhibition of germination similar to that of Conover loam. Loss of fungistasis in autoclaved soils was shown by all methods; in these treatments fungi overgrew the entire plates. Factors such as thickness of agar, and temperature and duration of incubation on soil influenced the intensity of soil fungistasis as measured by the agar methods. By standardizing these factors, all three soils expressed a similar spectrum of inhibition of the test fungi in the order: *Penicillium frequentans* > *Glomerella cingulata* > *Fusarium oxysporum* f. *lycopersici*; *P. frequentans* was most readily inhibited.

Table 1. *Fungistasis of Conover loam soil assayed by various methods*

Treatment	Soil	Mean % germination of conidia*		
		G. cingulata	*F. oxysporum*	*P. frequentans*
Directly on soil	Natural	0	0	0
	Autoclaved	100	100	100
Glycerol extraction	Natural	0	—	—
	Autoclaved	94	—	—
Double agar layer	Natural	12	26	0
	Autoclaved	90	95	92
Cellophan packets	Natural	0	0	0
	Autoclaved	100	100	100
Water agar disks	Natural	0	0	0
	Autoclaved	98	100	100
Buried slide	Natural	0	0	0
	Autoclaved	100	92	92
Water agar control		98	100	90

* Mean of nine microscope fields with a total of approximately 300 conidia.

Table 2. *Fungistatic effects of different soils and substrates*

Soil or substrate		Mean % germination of conidia of *G. cingulata**	
		Direct†	Agar disk‡
Conover loam	Natural	0	6
	Autoclaved	100	100
Muck	Natural	0	12
	Autoclaved	96	100
Forest soil	Natural	0	0
	Autoclaved	96	95
Sand	Natural	4	16
	Autoclaved	40	100
Fibre glass filter paper	Autoclaved	0	90
Cellulose filter paper	Autoclaved	0	90
Charcoal (Norit)	Autoclaved	0	10
Bentonite	Autoclaved	6	98
Kaolin	Autoclaved	0	90
Celite	Autoclaved	0	90
Talc	Autoclaved	0	95

* Mean of nine microscope fields with a total of approximately 300 conidia. Results were similar with conidia of *P. frequentans* and *F. oxysporum* f. *lycopersici*.

† Spores placed directly on soil surface.

‡ Spores placed on agar disks after disks were preincubated for 4 hr. on soil or substrate.

Fungistatic effects of substrates other than soil

There are reports that substances such as kaolin, alumina, slate dust (Dobbs & Hinson, 1953) and a calcareous sand (Dobbs & Bywater, 1959) decrease germination of fungus spores. Autoclaved cellulose filter papers, washed fibre glass filter papers, activated charcoal, bentonite, kaolin, talc, sand and diatomaceous earth (Celite) were tested for fungistatic effects by placing spores directly on them, and by the agar disk method. Results were similar with all three test fungi. The results for *Glomerella cingulata* (Table 2) show that these materials were fungitoxic when autoclaved and assayed by direct methods, but the fungistatic substances were apparently not diffusible, with the exception of those from sand and charcoal. Since fungistasis by soils was removed by autoclaving and the fungistatic substances were diffusible, it seems unlikely that the widespread soil fungistasis is due to clays or other colloidal or mineral materials.

Soil extracts

From experimental results similar to those in Table 1, previous workers have suspected the existence of diffusible fungistatic substances in natural soil. Following their lead, 100 g. samples of Conover loam soil were extracted with 50, 70 and 95 % (v/v) ethanol in water, *n*-butanol, methanol, acetone, chloroform, chloroform + methanol (1 + 1 by vol.), light petroleum ether, ethyl ether or water. When the concentrated extracts were assayed in water agar no inhibition of spore germination was observed; instead, stimulation of germination and growth was often observed. Likewise, concentrated drainage water from 8·5 cu.ft. of soil in the greenhouse, and from 40 acres of field soil did not inhibit germination. However, a more elaborate extraction of washed and defatted muck or mineral soil with 95 % (v/v) dioxane or ethanol in water yielded a fungistatic extract in the lignin-like fraction (Lingappa & Lockwood, 1960), This work will be discussed elsewhere (Lingappa & Lockwood, to be published).

The presence of stimulatory substances in soil extracts suggested that the expression of fungistatic substances might be masked. Concentrated extracts were chromatographed on filter paper strips in an attempt to separate inhibitory from stimulatory fractions. Chromatograms were developed in ethanol + acetic acid + water (12 + 6 + 1 by vol.), and in *n*-butanol + acetic acid + water (4 + 1 + 5 by vol.) and assayed on water agar seeded with *Glomerella cingulata*. Localized areas of stimulation of growth but no inhibition of spore germination were detectable in the assay plates. Strips of filter paper were also inserted into moist soil and diffusible materials from the soil allowed to accumulate at the tips of the hanging wicks (Wilson, 1958). After 16 hr. the strips were sterilized by immersing the buried end in 70 % (v/v) ethanol in water until the solvent reached the tip. When assayed on seeded agar no inhibitory substances were shown; again, areas of growth stimulation were evident.

Tests for volatile fungitoxic substances

The production of volatile antibiotic substances by micro-organisms has been reported (Bilai, 1956), and Dobbs & Bywater (1957) suspected that in some of their soils volatile fungitoxic substances might be present. Therefore it appeared possible

that our failure to extract fungistatic substances from soil might be due to loss of such volatile materials during the evaporation of solvents. Experiments were designed to detect such substances in natural soil. A coverglass coated on one side with a thin layer of water agar was inverted over a Van Tieghem ring and sealed with Vaseline. The rings were pressed into moist soil so that only a few mm. of space was left between soil and agar surface. Agar so exposed for 12–18 hr. at 28° was streaked with spores of the three test fungi and incubated again over soil or in moist chambers. Exposure for longer periods was not considered desirable because of inevitable contamination. In other experiments agar seeded with *Glomerella cingulata* was exposed to the soil atmosphere in Petri dishes for 16 hr. No inhibition of spore germination was observed in any of these tests. Absence of volatile fungitoxic materials in soil is also suggested by observations of Dobbs & Hinson (1953) that conidia on buried slides had germinated in pockets of condensed water. Similar observations were made in the spaces of buried Nylon gauze (Waid & Woodman, 1957). It was also our observation that complete contact of cellophan or agar disks with soil was necessary to avoid islands of spore germination over the air pockets. For these reasons it is believed that natural soil fungistasis is not caused by volatile substances from soil.

pH *value and redox potential*

The well known effects of pH value and redox potential of the medium on spore germination and growth of micro-organisms suggested the examination of these factors in natural soil fungistasis, although there appears to be no evidence that the pH of soils functions in this regard (Dobbs & Hinson, 1953; Jackson, 1958*a*). Table 3

Table 3. *Relation of* pH *value and redox potential of soil to fungistasis*

Substrate	Redox potential (mv)*	pH* value	% germination†
Natural Conover loam	216	7·9	−
Autoclaved Conover loam	198	7·5	+
Natural muck	192	6·5	−
Autoclaved muck	176	7·2	+
Natural Conover loam + 0·5 % peptone	−120	7·6	−
Natural muck + 0·5 % peptone	−36	7·5	−
Natural Conover loam + 0·5 % sucrose	−420	6·2	−
Water agar	276	5·8	+
Water agar in contact with soil 1 day	276	7·5	−

* Measurements made 16 hr. after amendments added, at time of taking spore germination data.
† Assays were by direct addition of conidia of *G. cingulata*, *F. oxysporum* and *P. frequentans* to soil. + = 20–100 % germination; − = 0–19 % germination.

shows that addition of small amounts of nutrients to soil did not annul fungistasis although drastically lowering the redox potential, whereas autoclaving soils annulled fungistasis while decreasing redox potential only slightly. Water agar developed strong fungistasis when incubated in contact with soil for 24 hr., but the pH did not change to an unfavourable value and the redox potential did not change at all. The soils in all treatments were at pH values favourable for spore germination, yet such soils were fungitoxic. For these reasons the pH and redox conditions of normal soil do not appear to be the cause of soil fungistasis.

Annulment of fungistasis

Since various workers have reported a decrease in fungistasis when soil was treated with plant residues or glucose (Dobbs & Hinson, 1953; Chinn & Ledingham, 1957), or when glucose was added to soil extracts (Stover, 1958) or agar disks (Jackson, 1958*a*), it was of interest to know whether spores already inhibited could be stimulated to germinate by nutritional supplementation. It has been shown that inhibited spores were viable (Lockwood, 1959). Spores of test fungi which had lain dormant for 5 days on the inhibitory bottom layer of double agar layer plates were removed with uniform disks of agar and transferred to separate Petri dishes. Known amounts of chemical solutions (0·2 ml. of 0·01 M solutions) were placed on these disks. Of the chemicals used only L-cysteine HCl and L-methionine prevented germination of conidia in controls. Similar results were obtained with all three test fungi. Those for *Glomerella cingulata* (Table 4) show the great effectiveness of peptone, even in small amounts (0·05 %, w/v), in annulling fungistasis. Glucose failed to induce germination of inhibited spores under the conditions of these tests. Various amino and organic acids and water were largely ineffective in annulling fungistasis. Osmotic gradients created by solutions of glucose, sorbitol, mannitol or polyglycols of different molecular weight did not annul fungistasis nor did they interfere with spore germination in controls. Therefore lack of osmotic shock probably would not explain failure of fungal spores to germinate in soils.

Table 4. *Annulment of fungistasis of* Glomerella cingulata *conidia*

Chemical*	% germination†	Chemical*	% germination†
Glycine	0	Succinic acid	15
L-Histidine	0	Glucose (0·1 M)	0
L-Cystine	8	Peptone (0·5 %, w/v)	90
L-Cysteine HCl‡	0	Peptone (0·05 %, w/v)	84
L-Methionine‡	0	Mannitol	10
DL-Phenylalanine	0	Mannitol (0·1 M)	12
L-Tyrosine	0	Sorbitol	0
L-Aspartic acid	30	Polyglycols (200, 300, 400, 600 mol. wt.)	0
L-Glutamic acid	12	Water	0
p-Aminobenzoic acid	0	Untreated	0

* All are 0·01 M unless otherwise indicated; 0·2 ml. placed on 1·5 mm. thick × 8 mm. diam. water agar disks.

† Average of nine microscope fields with a total of 300 spores. The spores were inhibited for 5 days by the double agar layer method before reversal. Results were similar using conidia of *P. frequentans* and *F. oxysporum* f. *lycopersici*.

‡ Inhibited spore germination in controls.

Destruction of conidia in soil

The conidia of at least some fungi are destroyed rather rapidly in contact with soil, a fate not revealed by indirect methods of observations. Conidia of *Glomerella cingulata*, *Fusarium oxysporum* f. *lycopersici* and *Penicillium frequentans* were placed directly on soil. After 3, 6 and 9 days of incubation, direct microscopical observation revealed large numbers of empty or decomposing conidia of *G. cingulata* and *P. frequentans*. It was difficult to detect the microconidia of *F. oxysporum* f. *lycopersici* because of their small size. By plating these soils after 6 days, large numbers

of colonies of *P. frequentans*, a few of *F. oxysporum* f. *lycopersici*, and none of *G. cingulata* developed. After 9 days the number of colonies of *P. frequentans* was decreased and none of *F. oxysporum* f. *lycopersici* developed. No detectable growth of micro-organisms could be seen on the conidia of these fungi. In autoclaved soil the fungi had overgrown the plates as profusely sporulating mycelia. Lysis of spores by soil was also observed by Subramanian (1946, 1950) and by Park (1955).

Source of fungistasis as assayed by indirect methods

When agar methods were used for assaying soil fungistasis, inhibition occurred more rapidly when 0·05 % (w/v) or more peptone was present than with water agar alone. The bottom layer of double agar layer plates became inhibitory in 3 days when the top agar layer contained 0·5 % (w/v) peptone, but without peptone 7–8 days were required. Similarly, peptone agar disks became fungistatic in 4 hr. when pre-incubated on soil at 28°, but 8 hr. were required for water agar disks to become similarly fungistatic. At 21° water agar disks pre-incubated for 4 hr. gave 60 % germination of conidia of *Fusarium oxysporum* f. *lycopersici*, 20 % of *Glomerella cingulata*, and none of *Penicillium frequentans*. Peptone agar disks similarly treated caused complete inhibition of all three fungi.

Table 5. *Effect of pre-incubation temperature on the expression of soil fungistasis by indirect methods*

Method†	Pre-incubation time	Substrate	Pre-incubation temperature: 1° (% germination of *G. cingulata* conidia*)	Pre-incubation temperature: 28° (% germination of *G. cingulata* conidia*)
Double (water) agar layer	5 days	Natural soil	96	6
	14 days	Natural soil	92	0
	14 days	Autoclaved soil	98	96
Water agar disks on cellophane	8 hr.	Natural soil	98	0
	16 hr.	Natural soil	96	0
	48 hr.	Natural soil	92	0
	48 hr.	Autoclaved soil	92	96
Water agar disks	24 hr.	Fungistatic agar layer	0	0

* Average of nine microscopic fields with a total of 300 spores.

† Agar disks or layers were pre-incubated on Conover loam soil or fungistatic agar layer for various time intervals, then streaked with conidia of *G. cingulata* and incubated for 16 hr. at 28°.

These observations raised the question as to whether fungistasis was caused by substances present in soil, or whether fungistatic substances were generated on the surface of the assay medium and then diffused into it during incubation. Since very little or no growth of micro-organisms takes place at 1°, pre-incubation of assay media at this temperature was tested to investigate this point. The double agar (water) layer and agar (water) disk methods were used, and a layer of sterilized cellophan was interposed between soil and agar. Table 5 shows that the agar media were not toxic to spore germination by *Glomerella cingulata* when pre-incubated for extended periods of time on soil at 1°, then removed and incubated with fungal spores at 28°. The soil did not lose the capacity to express fungistasis by exposure to

cold temperatures, since subsequent pre-incubation of new agar disks on the same soils at 28° caused inhibition of spores subsequently applied to the disks. To test the possibility that fungistatic substances might not diffuse at 1°, double agar layer plates were pre-incubated with soil for 12 days at 28°, thus making the bottom layer fungistatic. The fungistatic layers were then covered with sterilized cellophan. Water agar disks were placed on the cellophan and incubated at 1° for 24 hr. the disks then being removed and placed on water agar. Spores of test fungi applied to these disks and incubated at 28° for 16 hr. did not germinate (Table 5). This indicated that fungistatic substances which had accumulated in agar were indeed able to diffuse into assay disks at 1°. Results were the same with all three fungi, strongly indicating that fungistasis as assayed by the indirect methods (agar or cellophan) was due to production of fungistatic substances by soil microbes growing on the contact surface of assay medium and soil, rather than to the presence of a reservoir of such substances in soil.

Table 6. *The effect of amount of soil on expression of fungistasis by indirect methods*

Substrate or method*	Thickness of soil	% germination of *G. cingulata* conidia†
Double (water) agar layer	1 cm.	6
	1 mm.	8
(Water) agar disks	6 cm.	2
(Water) agar disks	< 0·1 mm.	12
(Peptone) agar disks	< 0·1 mm.	0
Cellophan	1 cm.	0
Cellophan	< 0·1 mm.	0
Mixed soil organisms assayed with water agar disks‡	0	0
Natural soil-direct	1 cm.	0
Autoclaved soil-direct	1 cm.	88

* Pre-incubation was 6 hr. except for double agar layer which was 7 days.
† Average of nine microscope fields with a total of 300 spores.
‡ Diluted soil suspension was mixed with water agar or peptone agar, incubated 3 days, and assayed with water agar disks.

This possibility was examined further by using different amounts of soil in contact with assay media. Fungistasis of *Glomerella cingulata* conidia (Table 6) was expressed equally by agar disks incubated on a soil layer < 0·1 mm. thick as on a soil layer 60 mm. thick. Bottom layers of double agar layer plates incubated for 7 days at 28° became equally fungistatic whether 16 g. soil, 10 mg. soil, or a thin layer of a 1/1000 dilution of soil in water was placed on the top layer. The amount of soil did not affect the time required for the bottom layer to become fungistatic. Furthermore, 1/1000 dilutions of soil incubated for 3 days on peptone or water agar, then assayed with agar disks or cellophan strips gave the usual fungistatic effects. Similarly, mixed cultures of soil microbes isolated from soil dilution plates and grown for 3 days on peptone agar caused water agar disks placed on them to become fungistatic. Peptone agar seeded with an antagonistic *Streptomyces* sp. or *Pseudo-*

monas sp. also produced fungistasis in agar disks. Inhibition, as compared with *G. cingulata*, was somewhat greater with *Penicillium frequentans*, and slightly less with *Fusarium oxysporum* f. *lycopersici*, in these tests.

The possible production of fungistatic substances on the assay medium surface was further tested by incorporating antibacterial antibiotics into agar disks, pre-incubating them on soil for 2 hr., then removing them to moist fibre glass filter papers which were soaked with the antibiotics, and assaying the generation of fungistasis in these disks. Results were similar with all three test fungi. Those for *Glomerella cingulata* (Table 7) indicated a decrease in soil fungistasis by these methods. The presence of antibiotic-resistant organisms in soil no doubt prevented complete suppression of fungistasis. These results support the view that soil fungistasis as assayed by indirect methods is due to growth of micro-organisms on the assay medium.

Table 7. *Effect of antibacterial antibiotics on soil fungistasis as assayed with peptone or water agar disks**

Antibiotic	% germination of *G. cingulata* conidia		
	Water agar disks	Peptone agar disks	Water agar disks (no soil)
K Penicillin G	40	90	98
Streptomycin sulphate	90	20	95
Neomycin	67	25	100
Vancomycin	70	90	92
Chloramphenicol	30	75	96
Control (no antibiotic)	12	0	—

* Agar disks containing antibiotics (1 mg./ml.) were pre-incubated directly on soil for 2 hr., then transferred to fibre glass filter paper moistened with the same antibiotic.

The above results were further supported by microscopical observation of the surface of assay media. Water agar or peptone agar disks, or sterile cellophan strips were placed on a smooth moist soil surface and pressed so as to make complete contact. After 8, 12, 24 and 48 hr. they were carefully lifted, inverted on a microscope slide, stained with phenol + rose bengal and washed gently in water. Such preparations revealed extensive colonization of the surface in contact with soil by bacteria within 8 hr. (Pl. 1, figs. 1, 2), and colonization by actinomycetes in 24 hr. (Pl. 1, figs. 3, 4). In this connexion, water agar has been found to be a good substrate for development of soil actinomycetes (Y. Lingappa & Lockwood, 1961).

Antibiotic and nutritional substances from fungal spores and their possible relation to fungistasis in soil

Freshly collected teliospores of the maize smut fungus *Ustilago zeae* did not germinate when placed directly on natural loam soil, but 40 % germinated on water agar in 16 hr. Germination and production of aerial sporidia occurred on autoclaved soil. Samples (5 g.) of air-dry smut teliospores were washed twice by shaking for 10 min. at a time with 100 ml. 50 % (v/v) ethanol in water. Another 5 g. sample was

spread on the surface of a layer of 20 g. natural soil, moistened, and incubated for 4 days at 24°, after which the spores were scraped off and extracted similarly. When the washings from the air-dry spores were evaporated on filter paper disks and assayed by the agar diffusion method against the organisms arising from a diluted soil suspension, they showed a strong antibiotic zone against these organisms as well as against *Fusarium oxysporum* f. *lycopersici*, *Penicillium frequentans*, *Glomerella cingulata*, *Pseudomonas* sp., and the *Streptomyces* sp. A very wide zone of stimulation occurred outside the zone of inhibition. On the other hand, washings from spores incubated in soil for 4 days showed no zone of inhibition, and only a slight zone of stimulation, suggesting exhaustion or biological breakdown of these materials. On the basis of these scanty observations with one fungus, the possibility is suggested that individual spores might behave as microsubstrates in the soil. Nutrients released by fungus spores could stimulate growth of antagonistic microflora in the vicinity of the spore, preventing its germination. The release of antibiotics from the spore could prevent or delay colonization of the actual spore surface.

DISCUSSION

In spite of numerous attempts to obtain fungistatic substances from soil, a characterized fungistatic principle has not been isolated with the exception of the lignin-like preparation of Lingappa & Lockwood (1960). In the work reported in the present paper, soil extracts were stimulatory to the growth of fungi. In spite of the failure to extract fungistatic substances, the fact that soil extracts are often stimulatory, as reported here and by others (Dobbs & Hinson, 1953; James, 1958; Park, 1956*b*), and the fact that spores of fungi which germinate readily in water are inhibited by soil (Dobbs & Hinson, 1953) show clearly that soil fungistasis cannot be the result of insufficient nutrients in soil.

A possible group of suspected fungistatic substances in soil includes the antibiotics. On the basis of researches to date (Brian, 1957), the production and accumulation of detectable quantities of antibiotics from natural unamended and uninoculated soil is considered to be unlikely. The present workers carried out very extensive and intensive extractions of natural soils and failed to demonstrate any antifungal antibiotics. While the accumulation of antibiotics in natural soil has not been demonstrated, evidence generally favours the production of antibiotics on fresh organic substrates in micro-environments of the soil (Brian, 1957). As the amounts produced are likely to be extremely small, when the bulk of the soil is considered, their demonstration has been by necessity indirect. The addition of a fresh organic substrate, such as agar or cellophan, might supply a source of nutrients and support microbial growth and antibiotic production. Such a possibility is supported by the short generation time of many bacteria and the very large number of micro-organisms in soil. An average soil containing 10 million individual micro-organisms/g. would provide an initial and enormous inoculum of up to 100,000 microbes in contact with a 1 cm. square agar block placed on it. Present information (Brian, 1957) permits us to assume that 40–50 % of the soil micro-organisms produce antibiotics. The present work and that of others (Y. Lingappa & Lockwood, 1961) shows that antibiotics are produced by actinomycetes growing on deficient media such as water agar, and that fungistatic substances are produced as well from mixed cultures as from single cultures of antibiotic-producing micro-organisms.

This explanation of the inhibition of fungal spore germination on assay media in contact with soil was supported by five lines of evidence. (1) Peptone agar became fungistatic more rapidly than water agar in contact with natural loam soil. (2) When agar media were pre-incubated on soil at 1°, a temperature which permits diffusion of fungistatic substances but largely prevents microbial growth, no fungistasis occurred. (3) The same degree of fungistasis was obtained with agar disks pre-incubated with thick layers of soil, minute amounts of soil, mixed cultures of soil microbes, or pure cultures of *Streptomyces* sp. or *Pseudomonas* sp. (4) Decrease of fungistasis was observed when high concentrations of antibacterial antibiotics were added to agar disks used for assaying soil. (5) Microscopic observations showed dense growth of micro-organisms within short periods on the contact surfaces of agar and cellophan with soil.

These indirect techniques, then, do not demonstrate the presence of fungistatic substances in soil, but provide a substrate for growth of micro-organisms which produce antibiotic substances, thus rendering the assay media fungistatic. Nevertheless, failure of fungal spores to germinate in natural soil is indeed a fact as shown by direct methods of observation. Understanding of the action of the indirect assay methods may provide some insight about the mechanism of inhibition involved. The introduction of spores into soil can create, qualitatively, as much a nutrient substrate as a piece of water agar, cellophan or straw. The experiments with smut spores showed that these liberated diffusible substances which stimulated growth of a variety of micro-organisms. If speculation is extended to individual spores in soil one might expect, as a result of nutrients diffusing from the spores, stimulation of surrounding microflora and resultant inhibition of fungal spore germination. An analogous situation is the reported stimulation of vegetative growth of the fungus *Fusarium oxysporum* f. *lycopersici* and the production of the antibiotic fusaric acid in the vicinity of the root surface of tomato plants growing in unsterilized soil or sand (Kalyanasundaram, 1958). Observations of various workers (Subramanian, 1946, 1950; Park, 1955; Chinn, 1953; Lockwood, 1959) and the present work showing that fungus mycelium and conidia are destroyed ultimately by soil, and reports of the colonization of the surface of fungal structures in soil (Mitchell, Hooton & Clark, 1941; Subramanian, 1946, 1950; Waid & Woodman, 1957; Stover, 1958) support the assumption that growth of soil micro-organisms on or in the vicinity of fungal structures is involved in the over-all fungitoxicity. Lysis of fungi appears to be the end result of extended microbial activities in the vicinity of spores and mycelium in soil.

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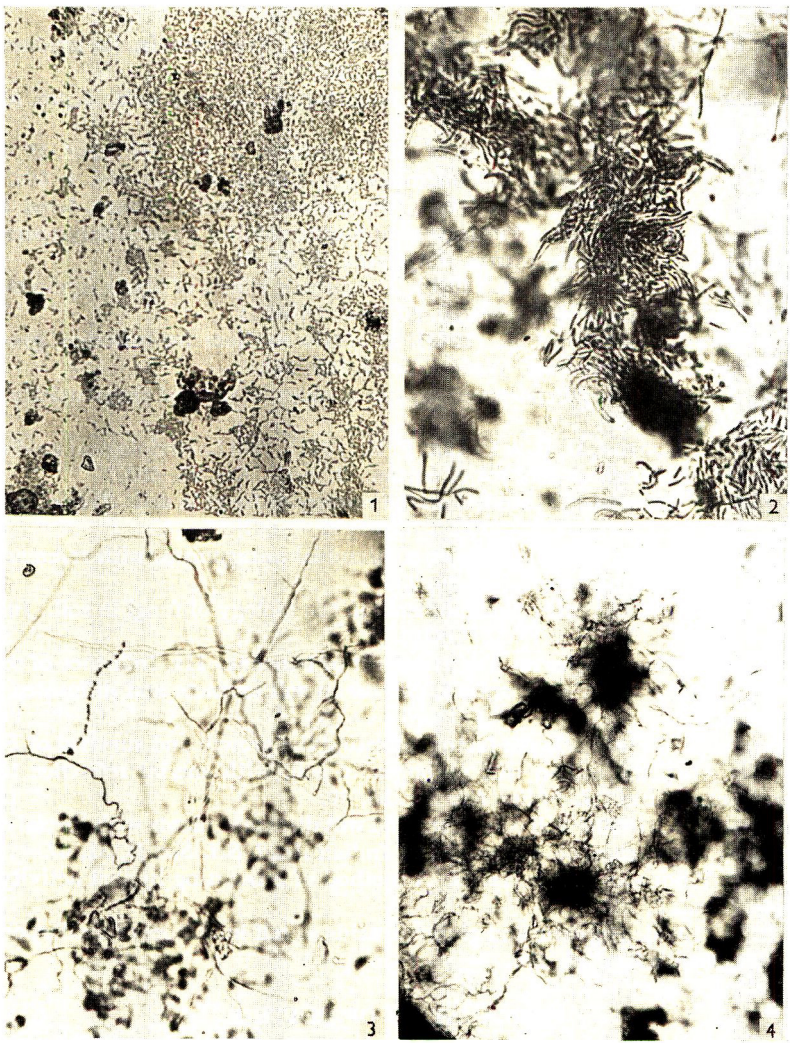
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EXPLANATION OF PLATE 1

Fig. 1. Bacteria colonizing water agar disks after incubation on moist Conover loam soil for 8 hr. at 28°. ×400.

Fig. 2. Bacteria colonizing cellophan after incubation on moist Conover loam soil for 8 hr. at 28°. ×800.

Fig. 3. Actinomycetes colonizing water agar disks after incubation on moist Conover loam soil for 24 hr. at 28°. ×800.

Fig. 4. Actinomycetes colonizing water agar disks after incubation on moist Conover soil for 24 hr. at 28°. ×400.

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Physiology of the Conjugation Process in the Yeast *Hansenula wingei*

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SUMMARY

The yeast *Hansenula wingei* is a favourable organism for study of the physiology of the conjugation process (cell fusion). Microscopic observations on fusion are presented which reveal that the mating cells in contact fuse by a softening of the cell wall, followed by formation of a conjugation tube, dissolution of the cross-walls between them, and formation of a new bud at the point of juncture of the two cells. A simple technique for studying fusion in a liquid medium is described. Up to 80 % of the cells will fuse in 5 hr. at 30° in a medium containing an energy source, $MgSO_4$ and potassium phosphate, under conditions in which no growth or budding of unmated cells would occur. Synthesis of new protein is required for fusion as shown by inhibition by amino acid analogues. The precursors for this new protein come from the amino acid pool. Both mating types must be able to function for conjugation to occur. It is postulated that each mating type produces an inducer which diffuses into the opposite type. Each inducer brings about the synthesis of a wall-softening enzyme which acts upon the cell producing it. Cell fusion is viewed as an extension of the normal budding process.

INTRODUCTION

The physiology of reproduction in fungi was reviewed by Hawker in 1957. It is clear from this review how meagre is knowledge of the biochemical processes involved in reproduction. In particular, there has been no analysis of the physiological processes involved in cell fusion, the first step in the mating cycle in fungi and most other organisms. As Hawker emphasized, the higher fungi are not suitable for such an analysis, partly because they are difficult to handle and partly because differentiation of cells into various types, some involved in mating and some not, makes an analysis at the cellular level difficult.

The yeasts would seem to be more favourable material for such studies. In a sexually reproducing yeast each cell may function either in vegetative growth or in mating. In the heterothallic yeasts, two mating types occur, genetically determined, which can be kept in culture indefinitely as haploid strains. Mating can then be induced at will by mixing the two mating types.

The only earlier work on mating in yeast is that of Nickerson & Thimann (1941, 1943). The present work is an outgrowth of studies on the nature of the mating reactions in *Hansenula wingei*. This yeast, isolated by Wickerham (1956), is unusual

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in that cells of the two mating types exhibit an extremely strong attraction for each other, so that when mixed in mass culture, a marked agglutination occurs. Analysis of the nature of the attractive forces (Brock, 1959*a*, *b*) showed that one mating type, strain 5, possesses a specific carbohydrate on its cell wall, whereas the other mating type, strain 21, possesses a specific protein. Mating agglutination is due to a combination between these complementary macromolecules, analogous to a reaction between an antibody and antigen. Because of the strong attraction between the mating types, cells can be mixed and remain in contact throughout extensive aeration and other physiological manipulation, and during this time cell fusion occurs. It has thus been possible to examine some of the factors involved in the fusion process. Preliminary accounts of this work have been given (Brock, 1959*c*, *d*).

METHODS

Strains used. The yeasts used in this study were supplied by Dr L. J. Wickerham, U.S. Department of Agriculture, Peoria, Illinois, U.S.A. They were *Hansenula wingei* NRRL Y-2340, strains 5 and 21, which were the agglutinative mating types.

Growth of cells. The cells were grown in liquid medium of the following composition: glucose 30 g., yeast extract Difco (Detroit, Michigan, U.S.A.) 7 g., KH_2PO_4 5 g., in 1000 ml. distilled water. This medium was dispensed in 100 ml. amounts into 500 ml. Erlenmeyer flasks, autoclaved 15 min., 121°, and inoculated with cells from stock agar slopes. The flasks were then placed on a reciprocating shaker and incubated for 18 hr. at 30°. The cells were harvested by centrifugation, washed twice with distilled water and suspended in distilled water at the same cell concentration as in the growth medium.

Cell fusion assay. Mating was carried out by mixing together 5 ml. amounts of suspensions of each cell type with 0·1 % (w/v) $MgSO_4.7H_2O$ and centrifuging for 5 min. The packed cells became intimately mixed and agglutinated strongly. The supernatant fluid was then decanted and the packed cells resuspended in 10 ml. of conjugation medium (glucose, 1 %, w/v; potassium phosphate (pH 5·7); $MgSO_4.7H_2O$, 0·1 %, w/v). Although the cells resuspended poorly because of the agglutination, the mixture was made as homogeneous as possible. This suspension was then poured into a 1 in. diameter shell glass vial which was then placed on a reciprocating shaker at 150 rev./min. Usually the vials were shaken for 5 hr. at 30°. At this time the suspension, which was still quite agglutinable, was decanted into a centrifuge tube and sedimented. The supernatant fluid was discarded and the cells suspended in 10 ml. 8 M-urea. The suspension was placed in an autoclave for 10–15 min. at 120° to deagglutinate the cells, the 8 M-urea serving to keep the cells from re-agglutinating when they cooled down. Although this treatment effectively converted the clumped cells to a fine suspension, the conjugated cells remained together when fusion had occurred. It was then possible to take small samples of these cells, stain them with dilute crystal violet, and examine them under oil immersion at × 950 magnification. The number of conjugants and the total number of cells were counted in a number of microscope fields. From these data the % conjugation was calculated by the method of Nickerson & Thimann (1943), counting each conjugating pair as two cells and each single cell as one cell. The data presented here report the

% of conjugants. This procedure assumes that there is no lysis or destruction of cells during the autoclaving and 8 M-urea treatment. There was no evidence that such destruction did occur.

RESULTS

Microscopical observations of conjugation

Cells of each strain grown for 24 hr. in glucose yeast-extract KH_2PO_4 broth on the shaker were mixed in equal parts in the same medium containing 20 % (w/v) gelatin, the mixture placed on a coverslip on a hanging-drop slide, ringed with paraffin wax and observed under a Leitz phase microscope at room temperature. At the cell concentrations used about one-half of the cells were single, and the other half occurred

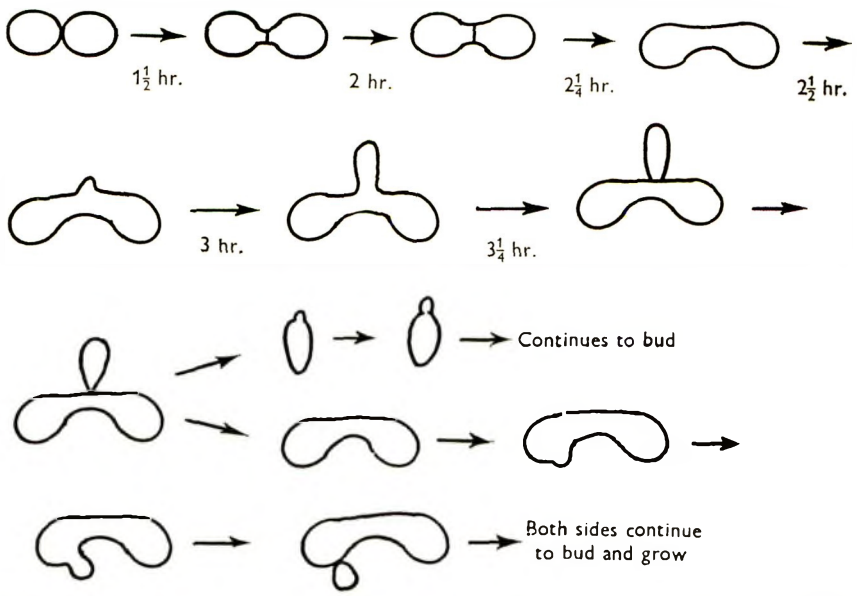

Fig. 1. Details of conjugation process in *Hansenula wingei*. Diagrammatic representation of observations with phase microscope of conjugating cells in glucose + yeast extract + phosphate broth + 20 % (w/v) gelatin at room temperature.

in pairs touching each other. The single cells budded but never exhibited any attraction for adjacent cells. Pairs of cells in contact were observed over a period of 4–5 hr. for conjugation behaviour. The results of the observations are shown in the diagram (Fig. 1). In the first 1–1½ hr. the cells in contact showed no visible behaviour. By 2 hr. many of the paired cells showed conjugation tubes. These tubes were observed to form by an extension of the portion of each cell at the point where it touched its mate. It should be emphasized that at no time were conjugation tubes observed to be formed by growth together of two separated cells, but always by an extension of each of the two cells in contact. In effect, the formation of the conjugation tube was due to each cell pushing its main body away from its mate. The conjugation tube appeared to result from a softening of the cell wall. In *Saccharomyces cerevisiae* Levi (1956) observed conjugation processes between cells not in contact, implying that some extracellular substance induced the process. There was no evidence for such a situation with the organism used in the present work.

At first the cross-walls between the two cells remained intact. At about 2 hr. the cross-walls disappeared. This occurred during a short period of time and was often missed. In one case the cross-walls disappeared within a 10 min. period.

Shortly after the cross-walls disappeared, a small bud formed at the mid-point of the conjugation tube and at a right angle to it. It is assumed that the bud formed here because this was the softest part of the wall. This small bud grew rapidly and by 3 hr. appeared to be fully grown. Shortly after 3 hr. a wall between this bud and the parent cells was quickly formed, often being complete in 5–10 min. This new bud always seemed longer than either of the parent cells, often appearing moderately filamentous. After enlarging fully this first bud could then form new buds, usually from the tip. Later, this bud on the conjugation tube might become completely detached from the parents.

Later, the parent cells often budded individually from locations far removed from the conjugation tube. These buds were morphologically similar to the parents, rather than elongated like the conjugation tube bud. Nothing is known about the nuclear phenomena in the mating process, and no bodies which could definitely be called nuclei were seen. It is assumed that after the cross-wall in the conjugation tube dissolves, nuclei from each parent can move into the tube and either fuse or remain separate. In either case there must be nuclear migration into the bud which forms off the conjugation tube, and this new bud must acquire nuclear material from both parents. Preliminary genetic evidence (Herman, 1959) suggests that the hybrids from matings possess characteristics of both strains but may be heterocaryons instead of true diploids.

In the physiological studies which follow, the only process under consideration is cell fusion and the formation of conjugation tubes. Later stages of growth of the mated cells are not considered and probably do not occur, since the medium used in most studies is inadequate for growth.

Physiology of conjugation

A preliminary experiment was made to determine the time course of conjugation under the conditions selected. In this experiment the conjugation medium was the growth medium. As shown in Table 1, conjugation began after about $1\frac{1}{2}$ hr. and reached about 50 % after 4–5 hr. In later experiments with other conjugation media the % conjugation was somewhat higher. A time of 5 hr. was selected for all subsequent experiments.

Table 1. *Time course of conjugation in* Hansenula wingei

Cells of strains 5 and 21 were mixed and suspended in conjugation medium (glucose + yeast extract + phosphate) and shaken for various times at 30°; samples removed and assayed microscopically for % conjugants as described in Methods.

Time (hr.)	Conjugants (%)	Time (hr.)	Conjugants (%)
0	0	2·5	40
0·5	0	3·0	47
1·0	0	4·0	47
1·5	8·5	6·0	57
2·0	32		

The effect of various nutrient factors on conjugation was studied next, and the results are in Table 2. In all cases except with water the cells remained firmly clumped throughout the incubation period on the shaker; the cells in water, however, no longer remained agglutinated. This is in line with previous observations that cations are necessary for agglutination. Therefore, in all subsequent experiments 0·1 % (w/v) $MgSO_4.7H_2O$ was present in the conjugation medium. Further data in Table 2 indicate that no conjugation occurred unless glucose was present; neither yeast-extract nor casein hydrolysate replaced glucose. An added nitrogen source was not necessary for conjugation.

Table 2. *Nutrient requirements for conjugation in* Hansenula wingei

Cells of strains 5 and 21 were mixed and suspended in the media indicated, shaken 5 hr. at 30°, then assayed microscopically for % conjugation as described in Methods.

Composition of conjugation medium	Conjugants at 5 hr. (%)
Water	< 1
0·1 % (w/v) $MgSO_4.7H_2O$	< 1
0·05 M-phosphate (pH 5·7) + 0·1 % (w/v) $MgSO_4.7H_2O$	2
1·0 % (w/v) glucose + 0·05 M phosphate + 0·1 % (w/v) $MgSO_4.7H_2O$	55
1·0 % yeast extract + 0·05 M phosphate + 0·1 % (w/v) $MgSO_4.7H_2O$	13
1·0 % casein hydrolysate + 0·05 M phosphate + 0·1 % (w/v) $MgSO_4.7H_2O$	10
0·5 % NH_4NO_3 + 0·05 M phosphate + 0·1 % (w/v) $MgSO_4.7H_2O$	4
1·0 % glucose + 0·5 % (w/v) NH_4NO_3 + 0·05 M phosphate + 0·1 % (w/v) $MgSO_4.7H_2O$	58
1·0 % glucose + 1·0 % casein hydrolysate + 0·05 M phosphate + 0·1 % (w/v) $MgSO_4.7H_2O$	51
3 % (w/v) glucose + 0·7 % (w/v) yeast extract + 0·5 % (w/v) KH_2PO_4 (growth medium)	54

Table 3. *Effect of energy source on conjugation in* Hansenula wingei

Cells of strains 5 and 21 were mixed and suspended in conjugation media containing 0·05 M phosphate buffer (pH 5·7) + 0·1 % (w/v) $MgSO_4.7H_2O$ + energy source as indicated, then shaken 5 hr. at 30° and assayed microscopically for % conjugants as described in Methods.

Energy source (%, w/v)	Conjugation at 5 hr. (%)	Energy source (%, w/v)	Conjugation at 5 hr. (%)
Glucose, 1·0	69	Sucrose, 0·5	40
Glycerol, 2·0	51	Lactose, 0·5	2
Ethanol, 4·0	17	Trehalose, 0·5	15
Mannose, 1·0	50	Na citrate, 1	0
Galactose, 1·0	4		

A study of energy sources for conjugation was carried out with the results shown in Table 3. Only substances which had been reported by Wickerham (1956) to be utilized for growth by this organism were useful as energy sources for conjugation. In all subsequent experiments the conjugation medium used was: 1·0 % (w/v) glucose, 0·1 % (w/v) $MgSO_4.7H_2O$, 0·05 M-potassium phosphate buffer (pH 5·7).

No growth occurred in this simple medium as shown by the following experiment. Cells of each strain were suspended separately in the conjugation medium and incubated for 5 hr., then samples removed and examined under the microscope. The number of single cells, budding cells, unequal pairs and equal pairs were counted (Campbell, 1957) to indicate whether growth had occurred; the total count was also

determined. The results after 5 hr. were compared with results obtained with unincubated cells. Table 4 shows that there was neither an increase in total numbers of cells nor an increase in number of budding cells. The number of buds and pairs decreased, while the number of single cells increased. The increase in number of single cells indicates that cell separation was the main process which occurred in this non-growth medium.

Table 4. *Budding and growth of cells of* Hansenula wingei *during 5 hr. incubation in conjugation medium*

Cells not mated, but suspended separately in conjugation medium (glucose + $MgSO_4$ + phosphate), shaken for 5 hr. at 30°, then examined microscopically and buds enumerated.

	Strain 5		Strain 21	
	0 hr.	5 hr.	0 hr.	5 hr.
Total count × 10^{-8} ml.	6·8	6·6	4·1	5·0
Single cells (%)	67	82	59	75
Budding cells (%)	12	4	10	9
Unequal pairs (%)	14	7	13	8
Equal pairs (%)	7	7	18	8

Both cell types must function in the conjugation process; this was shown in several ways. The antibiotic cycloheximide (Actidione; The Upjohn Co., Kalamazoo, Michigan, U.S.A.) completely inhibited conjugation at concentrations which inhibited growth. The wild-type cells were completely inhibited by cycloheximide 0·5 μg./ml. Antibiotic-resistant mutants were isolated from both mating types by periodic transfer to agar containing increasing concentrations of cycloheximide until mutants resistant to 100 μg./ml. were obtained. (These strains were isolated by Miss Alberta Herman and Mr D. Hunt.) The resistant mutants agglutinated and conjugated normally, both with each other and with wild-type cells. Table 5 shows the results when these mutants were mated with wild types in all combinations, with and without cycloheximide. Conjugation occurred in the presence of cycloheximide only when both strains were resistant, indicating that both strains must function for conjugation to occur.

Table 5. *Conjugation of cycloheximide-resistant mutants and wild-type cells of* Hansenula wingei *with and without antibiotic*

Cells of the two strains were mixed and suspended in conjugation medium (glucose + $MgSO_4$ + phosphate), shaken for 5 hr. at 30°, then % conjugants determined microscopically as described in Methods.

		% Conjugation	
Strain 5	Strain 21	No antibiotic	Cycloheximide 1 μg./ml.
Wild	Wild	72	0
Wild	Resistant	57	0
Resistant	Wild	59	0
Resistant	Resistant	63	71

This same conclusion can be drawn from the action of ultraviolet (u.v.) radiation on the conjugation process. Washed cells of both strains were irradiated under a General Electric germicidal lamp at a distance of 15 cm. The cell suspensions were

5 ml. amounts in flat-bottom Petri dishes and were agitated continually during irradiation. These irradiated samples were then mated with unirradiated cells of the opposite type and conjugation allowed to occur. All operations were carried out in subdued light or in darkess to avoid photo-reactivation. As can be seen from Table 6, conjugation was quickly affected by short exposures to u.v. radiation. Viable counts of these irradiated cells indicated that ability to form colonies decreased at roughly the same rate as did ability to conjugate. These results also indicate that both types must function in the conjugation process.

Table 6. *Conjugation of ultraviolet-irradiated cells with unirradiated cells of opposite type of* Hansenula wingei

Cells of each strain were irradiated separately, then mixed with unirradiated cells of opposite mating type, shaken for 5 hr. at 30° in conjugation medium (glucose + $MgSO_4$ + phosphate), then assayed microscopically for % conjugants as described in Methods.

Time of exposure to u.v. radiation (sec.)	% Conjugation	
	Strain 5 irradiated	Strain 21 irradiated
0	68	68
5	56	52
10	38	45
20	21	33
30	20	19
60	2	2

The action of a number of metabolic inhibitors is presented in Table 7, showing that the usual metabolic inhibitors prevented conjugation.

Table 7. *Inhibition of conjugation in* Hansenula wingei *by metabolic inhibitors*

Cells of two mating types were mixed and suspended in conjugation medium (glucose + $MgSO_4$ + phosphate) containing various additions as indicated, shaken 5 hr. at 30°, then assayed microscopically for % conjugants as described in Methods.

Treatment	% Conjugation at 5 hr.	Treatment	% Conjugation at 5 hr.
Control	76	Dinitrophenol, 0·001 M	4
KCN, 0·05 M	4	Na azide, 0·001 M	0
Na arsenate, 0·001 M	63	Boric acid, 0·025 M	0
Na arsenite, 0·001 M	0	Nystatin, 10 μg./ml.	0

Because of the marked morphological changes which occur during conjugation, it seems reasonable to assume that in each cell type there is the synthesis of an enzyme(s) which softens and eventually dissolves the wall between the cells. If enzymes are synthesized for this process, they might be inducible and might be synthesized *de novo* from constituents of the cell. It was shown by Spiegelman & Halvorson (1953) that induced enzyme synthesis in Saccharomyces makes use of the free amino acid pool of the cells for the materials needed for protein synthesis. Several experiments were therefore made to see whether the free amino acid pool is necessary for conjugation in *Hansenula wingei*. Although no quantitative determinations were made, this organism was found to possess an extensive amino acid pool which was extractable by hot water and which reacted with ninhydrin. This pool

was diminished effectively by starvation of the cells by aeration in 1 % (w/v) glucose +0·05 M-phosphate buffer (pH 5·7). After aeration for 4 hr. there was no more ninhydrin-reacting material extractable by hot water remaining in the cells. Table 8 shows qualitatively the amino acid pool of these starved cells and the ability of starved cells to conjugate. These results show that starvation to decrease the free amino acid pool also decreased the number of conjugants, although the pool seemed to diminish faster than did the ability to conjugate. Spiegelman & Halvorson (1953) showed that a considerable degree of induced enzyme synthesis occurred even after there was no detectable amino acid pool.

Table 8. *Conjugation and amino acid pools of starved cells of* Hansenula wingei

Cells starved in glucose + phosphate for various times as indicated. Samples removed for extraction of amino acid pools with hot water, and amount of pool determined quantitatively by reaction with 0·2 % (w/v) ninhydrin in acetone at 100° for 10 min. Remaining cells of each type mixed, suspended in conjugation medium (glucose + $MgSO_4$ + phosphate), shaken for 5 hr. at 30°, then assayed microscopically for % conjugants as described in Methods.

Treatment	% Conjugation	Ninhydrin reaction	
		Strain 5	Strain 21
None	64	++	+++
1 hr. starvation	68	+	+
2 hr. starvation	61	+	+
4 hr. starvation	50	±	−
7 hr. starvation	8	−	−

Another experiment which confirmed the above results made use of the amino acid analogues *p*-fluorophenyl-alanine and ethionine which are specific antagonists of phenylalanine and methionine, respectively. Table 9 shows the effect of these substances.

Table 9. *Effect of amino acid antagonists on conjugation in* Hansenula wingei

Cells of two mating types mixed and suspended in conjugation medium (glucose + $MgSO_4$ + phosphate) containing various additions as indicated, shaken 5 hr. at 30°, then assayed microscopically for % conjugants as described in Methods.

Treatment	% Conjugation
None	66
Fluorophenylalanine, 0·02 M	0
Fluorophenylalanine, 0·02 M + phenylalanine, 0·02 M	75
Phenylalanine, 0·02 M	68
Ethionine, 0·02 M	24
Ethionine, 0·02 M + methionine, 0·02 M	67
Methionine, 0·02 M	69

From a consideration of these results, the following hypothesis seems tenable. When the two mating types are brought together, there is a reciprocal induction in each type by its opposite mating type of an enzyme which softens and eventually digests its own cell wall. Thus the two cells form a conjugation tube between them, and eventually their cytoplasms mix and fusion is completed. The enzyme which is induced may be similar to, or identical with, the normal budding enzyme responsible

for the small hole in the yeast wall through which the bud develops (Nickerson & Falcone, 1956). The only difference is that in the present case the enzyme does not act randomly on the wall, but always at the point where the two cells touch. Support for this hypothesis is shown in the following experiment, where one cell type stimulated budding in the opposite type under conditions in which fusion could not take place. U.v.-irradiated cells of one type were mixed with non-irradiated cells of the opposite type and incubated for 5 hr. This experiment was performed with cells irradiated for 60 sec., since this irradiation time completely inhibited conjugation. After incubation the cells were de-agglutinated as usual, and the number of budding cells counted and compared with the number of budding cells in controls incubated unmixed. In the results given in Table 10, the % of buds was calculated from only those cells which showed young buds indicative of new bud formation. It is impossible to know which buds are on strain 5 cells and which are on strain 21 cells, but since little bud formation occurs when normal or irradiated cells are incubated separately, or when irradiated cells of both types are incubated together, it seems clear that when irradiated cells of one type are incubated with unirradiated cells of the opposite type, the unirradiated cells are stimulated to bud. This might be due to induction of the conjugation (or budding) enzyme by contact with cells of the opposite mating type.

Table 10. *Number of buds on mated ultraviolet-treated cells of* Hansenula wingei

Cells were irradiated 1 min. (sufficient to diminish conjugation to less than 1 %), then mixed with irradiated or non-irradiated cells of opposite type as indicated, then suspended in conjugation medium (glucose + $MgSO_4$ + phosphate) and shaken 5 hr. at 30°. Cells then processed in the same way as in standard conjugation assay, and the % of budding cells determined microscopically. No detectable conjugation was seen.

	Buds (%)
Strain 5, u.v. + Strain 21, u.v.	8
Strain 5, u.v. + Strain 21, normal	29
Strain 5, normal + Strain 21, u.v.	24

DISCUSSION

These studies have revealed a number of new points about the process of conjugation. In the following discussion, conjugation is defined as the process of cell fusion and implies nothing about nuclear interactions or other related events.

In *Hansenula wingei* conjugation occurs only when the two cells of the mating pair are in contact with each other. Conjugation will occur in the absence of growth, in a medium containing only an energy source, magnesium sulphate and potassium phosphate. The addition of nitrogenous compounds is of no benefit to the fusion process. Inhibitors which prevent energy metabolism prevent the fusion process.

Both cell types must function in the fusion process, and fusion will be inhibited when either of the two cells of the mating pair is treated with u.v. radiation or with cycloheximide. The process therefore differs from mating in *Escherichia coli* (Wollman, Jacob & Hayes, 1956) which appears to require only one of the mating pairs, the DNA donor, to be active.

Synthesis of new protein is required for fusion to occur, and this synthesis of protein can be inhibited by amino acid analogues. The precursors for the synthesis of

this new protein probably come from the free amino acid pool. It seems reasonable to assume that the new protein synthesized is a wall-softening enzyme, the synthesis of which is induced in each cell by contact with its mate. Evidence for this enzyme has been advanced by showing that when a cell is in contact with an u.v.-treated cell of the opposite type, the untreated cell is induced to bud, whereas new buds do not normally occur in the conjugation medium. The hypothesis is that the wall-softening enzyme presumed to be involved in cell fusion is similar to, or identical with, that involved in normal budding, and that during conjugation it is synthesized in large amounts, its action being localized at the point of contact of the mating cells. Thus conjugation can be viewed as an extension of the normal budding process. The fact that the first bud of the conjugant always occurs at the point of the conjugation tube midway between the two cells, where the wall is presumably the softest, is in keeping with this hypothesis (see Fig. 2).

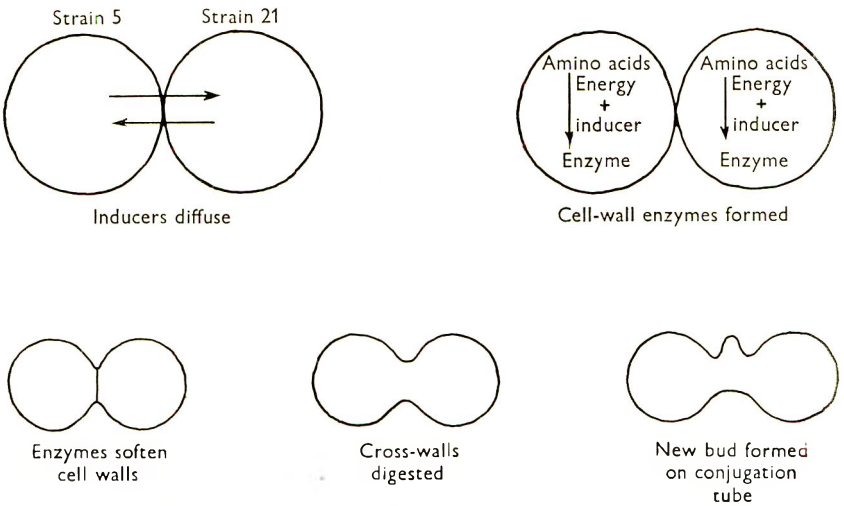

Fig. 2. Hypothetical explanation of physiological processes involved in cell fusion.

Hansenula wingei is an ideal organism for studying the physiology of conjugation, since the strong attraction between cells of opposite mating types makes it possible to aerate agglutinated cells extensively and to handle them easily in physiological experiments.

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Illegitimate Mating in *Paramecium bursaria* and the Basis for Cell Union

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SUMMARY

Cells from stock Wu-67 of *Paramecium bursaria*, syngen 1, can be induced to conjugate *inter se* (self) if they have made brief but transitory contact with cells of a complementary mating type; in mixtures consisting of marked cells of Wu-67 and cells of a complementary mating type both interclonal and intraclonal (Wu-67 × Wu-67) conjugations were recognized. Unmixed cultures of Wu-67 never self nor could selfing be induced in other ways. In contrast to normal conjugation the intraclonal matings are illegitimate because they occur between cells expressing a common mating-type specificity. These and other facts are considered in light of Weiss's hypothesis about cell unions; it is postulated that a primary specific surface reaction dependent upon mating-type complementarity serves to elicit a secondary non-specific ('holdfast') reaction leading to the completion of conjugation. In cells from Wu-67 the sites of secondary reaction are held to be precociously or more readily activated than in normal cells and, when two such activated cells are apposed, illegitimate mating may occur. Under certain circumstances then, cellular adhesion and conjugation may be independent of primary mating-type complementarity and dependent instead upon the availability of secondary non-specific attachment sites.

INTRODUCTION

In Paramecium conjugation takes place when sexually mature cells of complementary mating types are brought together under appropriate environmental conditions. Although cells which belong to a common mating type do not normally conjugate among themselves, and reproductive isolation is the primary basis for the definition of physiological species (syngens or varieties), exceptions to these rules have been noted by various workers. Such findings are of intrinsic interest but they also seem pertinent to more general problems such as the basis for adhesions among somatic cells and the nature of sexuality—how do artificially dissociated somatic cells become properly reoriented and how are cellular changes leading to meiosis and fertilization induced? The observations and experiments reported in the present paper are concerned with illegitimate (intraclonal) conjugations in a mutant clone of *Paramecium bursaria*. The data are consistent with an hypothesis put forward by Weiss (1960), for they suggest that the cellular pairing, which leads to conjugation, is normally a consequence of a primary specificity and a secondary non-specific interaction of cell surfaces.

Previous investigations reported by Sonneborn (1942), Metz (1954) and Hiwatashi (1955) led to the conclusion that the mating reaction in Paramecium involves two

distinct and separable mechanisms. In analyses of the primary specific mating reaction mechanism, Metz and co-workers and Hiwatashi showed that cells belonging to a single clone can mate amongst themselves after some of the individuals have made transient contacts with cells of a complementary mating type. The most recent and provocative work along these lines is that of Hiwatashi (1958) and Miyake (1956, 1958, 1960) who induced conjugations among normally incompatible cells of *Paramecium caudatum*, *P. aurelia* and *P. multimicronucleatum* by the use of chemical agents. In all of these studies, the question arises as to whether or not contact with a complementary cell or chemical treatment causes a specific change in mating type. If a change in mating type does occur, induced intraclonal matings may occur between animals of complementary mating types and should be termed legitimate. One contribution of the present analysis is the resolution of this question for a particular case. A brief report of these results has appeared in abstract form (Siegel & Larison, 1960).

METHODS

Standard clones of *Paramecium bursaria*, representing each of the four complementary mating types (designated A, B, C, D) known for syngen 1 of this species and described in detail elsewhere (Siegel, 1958; Siegel & Larison, 1960) were used in the present work. Stocks Wu-67, JP-55 and NC-64 were kindly furnished by Professor T. T. Chen. In *P. bursaria*, each animal cell normally carries several hundred chlorella cells in hereditary endosymbiosis giving the paramecium a green appearance. When cultures of *P. bursaria* are allowed to reproduce rapidly in darkness the fission rate of the paramecia will exceed that of the symbiotic algae, and the number of algae/animal will rapidly diminish until sublines lacking algae are produced (Siegel, 1960). For convenience we shall designate such cells as 'white'. Separate cultures consisting of parasitized (green) and of asymbiotic or chlorella-less (white) paramecia, readily distinguishable from one another, were prepared for each stock and used in the detection of legitimate interclonal and illegitimate intraclonal conjugations. When a culture of green cells of one mating type is mixed with a culture of white cells of another mating type, green × white pairings are interclonal whereas green × green and white × white pairings are intraclonal. The sexuality of the paramecia is known to be independent of the presence of endosymbionts (Ehret, 1953). The origins and properties of the stocks and their derived sublines are listed in Table 1.

It should be emphasized that these stocks will conjugate only when mixed with sexually reactive cells of a complementary type. The capacity of stock Wu-67 to undergo illegitimate (Wu-67 × Wu-67) as well as legitimate (interstock) conjugation following its mixture with a clone of a complementary mating type is described in a later section. The occurrence of illegitimate pairing was found to be independent of the symbiotic association.

Phase-contrast microscopical examination of living paramecia from stock Wu-67 showed them to be cytologically normal, containing a single micronucleus and a single macronucleus, two contractile vacuoles, gullet, etc. (see Wichterman, 1953).

Methods for cultivating and handling *Paramecium bursaria* as already described (Jennings, 1939; Ehret, 1953; Siegel, 1960) were used; they follow closely the standard procedures for *P. aurelia* (Sonneborn, 1950). Experimental cells were

obtained from mass cultures previously grown at 25° and subjected to diurnal periods of artificial white light (100 ft. candles). Under these conditions the cells reached a peak of sexual reactivity at noon (Ehret, 1953) and matings were initiated at that time. The mixtures were kept at 25° and observed for conjugations at 4 p.m. of the same day.

Mating types of unknown cultures were determined by testing separate samples of each with cells of the four standard mating types. The unknown was classified as a particular type if it failed to mate with the standard of that type, but was able to mate with cells of the remaining three types.

Table 1. *The properties, origins and designations of the stocks of* Paramecium bursaria

Stock	Natural source	Mating type	Endosymbiotic chlorella	Designation
8	Malibu Lake, California	A	Present	8A-g
			Absent	8A-w
Wu-67	Shanghai, China	A	Present	Wu-67A-g
			Absent	Wu-67A-w
25	Malibu Lake, California	B	Present	25B-g
			Absent	25B-w
32	Malibu Lake, California	C	Present	32C-g
			Absent	32C-w
34	Fish Canyon, California	C	Present	34C-g
			Absent	34C-w
JP-55	Sapporo, Japan	C	Present	JP-55C-g
			Absent	JP-55C-w
3	Malibu Lake, California	D	Present	3D-g
			Absent	3D-w
NC-64	North Carolina	D	Present	NC-64D-g
			Absent	NC-64D-w

RESULTS

The initial observation on conjugations involving cells from stock Wu-67 demonstrated their unique ability to mate among themselves. Table 2 shows the results of mixing representative stocks of syngen 1 in all possible combinations of twos; in each combination, one of the cultures consisted of green cells while the other consisted of white cells; thus the origin of each paired cell could be ascertained. As expected, in mixtures of cultures belonging to a common mating type no pairing occurred, while in mixtures of cells of complementary mating types numerous pairs formed, each pair consisting of a cell from each of the two complementary stocks. In striking contrast mixtures of cells from stock Wu-67 with cells of a complementary mating type always yielded exceptional pairs, both mates originating from stock Wu-67, in addition to interstock pairs. Such illegitimate pairing never occurred in unmixed control cultures of stock Wu-67.

Detailed observations were made on those mating reactions which led to the formation of Wu-67 × Wu-67 pairs. The cells of stock Wu-67 were readily distinguished from those of the complementary mating type by the presence of chlorellae. Upon mixing cultures of complementary mating types, the usual swimming movements serve to bring two individuals into accidental contact; the cells immediately

'stick' if they belong to complementary types. These initial unions involve any part of the ciliated surface and so are irregular; they were easily disrupted by picking up and then expelling the cells with the aid of a micropipette. In agreement with the observations of others, a third and fourth cell were seen to adhere to the two initial cells and this continued until, a few minutes after mixture, large masses containing 100 or more individuals were formed. When the clumps were artificially broken up soon after they had formed, many loosely joined pairs were observed. Since neither green × green nor white × white unions were found, it must be con-

Table 2. *The occurrence of* interclonal *and* intraclonal *conjugation among stocks representing the four mating types of* Paramecium bursaria, *syngen 1. Cells were 'marked' by the presence (green) or absence (white) of symbiotic algae*

			Cells contain chlorellae—'green'							
			A		B	C			D	
Cells lack chlorellae—'white'	Mating type	Stock	Wu-67A-g	8A-g	25B-g	32C-g	34C-g	JP-55C-g	3D-g	NC-64D-g
	A	Wu-67A-w	—	—	GW	GW	GW	GW	GW	GW
			.	.	WW	WW	WW	WW	WW	WW
		8A-w	–	–	GW	GW	GW	GW	GW	GW
	B	25B-w	GG	.	.	.	.	.	.	.
			GW	GW	–	GW	GW	GW	GW	GW
	C	32C-w	GG	.	.	.	.	.	.	.
			GW	GW	GW	–	–	–	GW	GW
		34C-w	GG	.	.	.	.	.	.	.
			GW	GW	GW	–	–	–	GW	GW
		JP-55C-w	GG	.	.	.	.	.	.	.
			GW	GW	GW	–	–	–	GW	GW
	D	3D-w	GG	.	.	.	.	.	.	.
			GW	GW	GW	GW	GW	GW	–	–
		NC-64D-w	GG	.	.	.	.	.	.	.
			GW	GW	GW	GW	GW	GW	–	–

'WW' indicates the presence of pairs consisting of two white cells, hence intraclonal conjugation.
'GG' indicates the presence of pairs consisting of two green cells, hence intraclonal conjugation.
'GW' indicates the presence of pairs consisting of one white cell and one green cell, hence interclonal conjugation.
' – ' indicates an absence of pairs, hence stocks belong to the same mating type.

cluded that only cells of complementary types adhered within clumps. During the first 2 hr., the events of the mating reaction followed the normal pattern detailed by others (see Jennings, 1939; Wichterman, 1953). About 2 hr. after the initiation of the mating, the cells began to lose their ciliary stickiness and the large clumps disintegrated into pairs, each cell tightly joined to its mate along the opposing oral surfaces. Now for the first time illegitimate (green × green) as well as legitimate (green × white) pairs were observed. Unlike those formed earlier, these pairs were joined by 'holdfast unions' (Metz, 1947) and most of them could not be forced apart. The point to be stressed is this: legitimate interclonal conjugation proceeds from loose initial loose ciliary agglutinations to the formation of tightly joined and properly oriented pairs. However, intraclonal mating (Wu-67 × Wu-67) apparently occurs directly, omitting the stage of initial ciliary agglutinations; as will be shown

later, it is dependent only on prior *inter*clonal cell unions. Although cells of a given mating type are frequently packed next to one another during clumping, loose and randomly oriented intraclonal unions were not observed. Since the interaction of cells expressing complementary mating types results in loose ciliary unions and since no such unions were observed between two cells of stock Wu-67, these intraclonal conjugations might be interpreted as illegitimate. The two experiments next to be described provide further evidence for this conclusion.

In the first experiment, a sexually reactive culture of Wu-67-g was mixed with a chlorella-less culture of a complementary type. The Wu-67 × Wu-67 (green × green) pairs were isolated and immediately forced apart by repeatedly expelling the pair from a micropipette (Sonneborn, 1950). The viable members of such split pairs were re-isolated, allowed to multiply to form large cultures and subsequently tested for mating type. (As mentioned before, many pairs are firmly united and cannot be forced apart successfully; thus a certain amount of selection enters into the choice of cells to be tested.) All such cultures were found to consist exclusively of cells of mating type A. Thus there was no evidence for an hereditary change of mating type associated with the induction of selfing.

In the second experiment split pairs were obtained in the manner described above, but immediately after the pairs had been disjoined mating-type tests on each member of the pair were begun. Each uncoupled cell was placed first in a culture of sexually reactive chlorella-less testers of mating type B. As soon as an initial mating reaction (ciliary agglutination) was observed between the green (Wu-67) member of the split pair and a white cell (or cells) of the complementary mating type, the Wu-67 cell was removed and placed in another drop, this containing white cells of type C. When the Wu-67 cell reacted with cells of type C, it was again removed and placed with cells of type D, and finally with cells of type A. Such a series of four tests was completed within 20 min. after the original Wu-67 × Wu-67 pair was split. The results appear in Table 3. Some cells could not be fully tested. Several died, apparently due to injury incurred during the process of separation. In other cases a cell from a split pair failed to react with two of the standard mating types, but this was not unexpected because the original pairs were obtained from clumps which were disintegrating due to the gradual loss of initial ciliary mating reactivity. These incomplete tests (split pairs numbered 6–10) provided no data contrary to those furnished by the cells which gave complete sets of tests. None of the cells showed a mating reaction with cells of type A; therefore none can be classified as belonging to a mating type other than A.

The frequency of contact between cells of stock Wu-67 and individuals of a complementary mating type was varied by altering the cell population density (but not their total numbers) in reaction mixtures. The data in Table 4 show that both legitimate and illegitimate conjugations were favoured by higher cellular concentrations. However, in denser populations about 12 % of all conjugations were illegitimate, while less than 2 % of the pairs were illegitimate in more sparse populations. In another experiment, the ratio of Wu-67 cells to those of a complementary type (32C-w) was varied while the total population density was kept constant. It was immediately apparent that in mixtures wherein cells of one mating type are present in excess numbers, the masses of clumped cells which formed soon after mixture contained an excess of individuals of that type. When there was an

Table 3. *The mating types of cells from uncoupled Wu-67 × Wu-67 pairs. Each ex-pair member (I and II) was tested against standard types A, B, C, D*

(Key: '+' = mating reaction; '−' = no reaction; '0' = cell died or was lost.)

Split pair	Cell	Reaction with testers of the indicated mating type			
		A	B	C	D
1	I	−	+	+	+
	II	−	+	+	+
2	I	−	+	+	+
	II	−	+	+	+
3	I	−	+	+	+
	II	−	+	+	+
4	I	−	+	+	+
	II	−	+	+	+
5	I	−	+	+	+
	II	−	+	+	+
6	I	0	+	+	+
	II	0	+	+	0
7	I	−	+	+	+
	II	−	+	+	−
8	I	0	+	+	0
	II	0	+	0	0
9	I	−	+	−	−
	II	−	+	−	+
10	I	−	−	+	+
	II	−	+	+	−

excess of 32 C-w over Wu-67 cells, the frequency of contacts between *given* individuals of Wu-67 and cells of the complementary mating type was greater than that occurring in mixtures set up with an excess of Wu-67 cells. The results summarized in Table 5 are consistent with the idea that the effect of more frequent contacts with complementary cells, brought about by the mating reaction, was to increase the incidence of illegitimate conjugations.

Table 4. *The effect of population density on the frequency of illegitimate conjugation (Wu-67 × Wu-67 pairs)*

No. cells per ml. in mixtures Wu-67 A-g × 25 B-w	Expt.	Number of pairs	
		Illegitimate (green × green)	Legitimate (green × white)
340	1	5	76
	2	5	45
	3	14	56
	4	8	54
	Total	32	231
170	1	1	25
	2	0	30
	3	1	38
	4	0	36
	Total	2	139

All attempts to induce illegitimate Wu-67 × Wu-67 conjugations by breis or cell-free fluids from cultures of complementary types were unsuccessful. Nor could we find evidence for the existence of an 'inducing substance' released by sexually-reacting cells. Sexual reactivity is a requisite for induced selfing; unreactive Wu-67 animals could not be induced to self, and unreactive cells of a complementary type did not induce illegitimate mating. Finally, when reactive cells of Wu-67 were mixed with reactive cells of a non-complementary mating type (mating type E, syngen 2), neither interstock nor Wu-67 conjugations occurred.

Genetic analyses of stock Wu-67 were virtually ruled out by the unfortunate fact that crosses involving this stock yield extremely high proportions of non-viable progeny (see Table 6).

Table 5. *The effect of population composition on the frequency of illegitimate conjugation (Wu-67 × Wu-67 pairs)*

Approx. no. of cells mixed		Number of pairs		
Wu-67 A-g	32 C-w	Illegitimate (green × green)	Legitimate (green × white)	Illegitimate pairs (%)
1200	200	0	165	0
700	700	6	147	4
200	1200	5	58	8

Table 6. *The viability of exconjugant clones from legitimate and illegitimate matings involving stock Wu-67*

Mating	No. clones studied	No. clones viable
Wu-67 A-g × 25 B-w	282	3
Wu-67 A-g × 32 C-w	84	0
Wu-67 A-g × 34 C-w	110	1
Wu-67 A-g × 3 D-w	88	0
Wu-67 × Wu-67	180	2

DISCUSSION

Stock Wu-67 is the only stock of *Paramecium bursaria* known to form pairs *inter se* following contacts with cells of complementary mating types. These intraclonal pairings can be termed illegitimate, for they evidently occur between cells expressing a common mating type. In sharp contrast, the intraclonal conjugations previously reported for *P. bursaria* (Jennings, 1941), *P. aurelia* (Kimball, 1939; Sonneborn, 1947), *P. multimicronucleatum* (Sonneborn, 1957) and *Tetrahymena pyriformis* (Nanney & Caughey, 1955) always resulted from the differentiation of individuals of complementary mating types within the selfing clone. Such conjugation does not differ in principle from the more familiar interclonal matings and may be termed legitimate. (Certain selfing clones of Tetrahymena described by Elliott & Nanney, 1952, do not conform to this pattern.)

Weiss's (1960) model for cell specificity and selectivity appears to account for both legitimate and illegitimate conjugations in Paramecium. This model is based on a dualistic principle of action involving: (1) a specific primary reaction which serves to expose sites of reactivity by alteration of the cell surface; (2) a non-specific

secondary reaction at the level of the exposed sites. The model assumes that the cell surface contains a network of protein molecules which acts as a 'barrier' to cell union, transport and transmission. However, specific end groups on the protein molecules react with complementary groups on an extraneous carrier (particle or cell) and thus are reoriented from a tangential position to one radial to the cell surface. This reorientation causes 'breaches' in the barrier allowing the passage of materials or currents. Metz (1954) showed that cells of complementary mating types possessed specific and complementary sex substances on their surfaces whose union or interaction led to the initial adhesion of potential conjugants and provided a basis for the observed specificity of the primary mating reaction. Weiss's model suggests the next events of conjugation. The interaction of the specific sex substances results in their reorientation thereby exposing secondary non-specific holdfast sites; then, in accord with many observations, cells united at holdfast sites normally proceed through conjugation.

Weiss's model provides an explanation for the well-documented fact that paramecia which unite in conjugation belong to complementary types, for the initial intimate contact brought about by the specific interactions of surface molecules serves to insure heterotypic holdfast unions. By assuming that the non-specific holdfast sites of cells from stock Wu-67 are more readily (or earlier) exposed by ciliary contact with a legitimate prospective mate, illegitimate unions will be expected to occur when two Wu-67 cells are fortuitously pressed together during the mass clumping phase of mating.

The hypothesis seems to account in a satisfactory way for the facts concerning stock Wu-67. Firstly, a mating reaction involving cells of Wu-67 and a complementary type is a necessary condition for illegitimate conjugation. Secondly, cells of Wu-67 which have begun to conjugate illegitimately are unchanged with respect to mating type specificity (all are type A). Thirdly, Wu-67 × Wu-67 pairs first appear when clumps disintegrate, and the mates are always united by one or more of the three holdfast sites known for Paramecium. The fact that Wu-67 × Wu-67 pairs are more frequent in mixtures in which there is an excess of cells of a complementary type may mean that Wu-67 cells must be in repeated or prolonged contact with cells of a complementary type in order to precociously uncover the holdfast site.

The immediate value of the hypothesis is that it appears to account for a number of observations concerning irregular mating reactions in ciliates. For example, the hypothesis predicts that a block might occur between the primary specific reaction and the secondary non-specific reaction. As Sonneborn (1942) and Metz (1954) stressed, the behaviour of a mutant stock of *Paramecium aurelia* indicates the existence of discrete steps in the reactions leading to conjugation. A block CM (= 'can't mate') evidently occurs after the primary interaction of mating-type substances. Thus cell agglutination proceeds normally but the CM cells are unable to complete conjugation because the secondary non-specific sites are effectively unavailable.

Other observations reveal the fact that the initial cellular agglutinations which are brought about by mating-type specificity differences are not inevitably followed by the formation of tightly joined pairs and so are consistent with the notion of separable primary and secondary interactions in conjugation. For example, both nutritive condition and life-cycle stage are factors known to determine whether or

not the loose cellular aggregates characteristic of the early phase of mating will yield tightly joined pairs which complete conjugation.

Hiwatashi (1958) and Miyake (1958, 1960) showed that chemical agents can induce pair formation and conjugation in Paramecium irrespective of mating-type specificity. Particularly striking was the demonstration that chemicals induced matings between individuals of separate syngens and species, as well as intraclonal selfing. Following chemical treatment pairs were formed directly, the mates attached by their holdfast sites; since the initial stages of normal mating reactions (loose ciliary unions and the formation of clumps of reacting cells) are omitted, it does not seem likely that such conjugation is a consequence of specific mating-type changes. Chemical treatment may remove the barrier formed by molecules concerned with primary specific mating-type complementarity and so permit the secondary non-specific mating-type holdfast unions to occur directly.

Although the available data seem to fit Weiss's hypothesis, other kinds of explanations are not excluded. Dr T. M. Sonneborn in a personal communication, suggested that the primary specific mating reaction serves to trigger another *internal* cellular reaction which leads to the non-specific receptivity of the cortical surface of the cell; firm cell attachments and conjugation occur when the activated cortical areas of cells are brought together. On this view the secondary reaction is not a direct mechanical consequence of the primary reactions of complementary molecules as Weiss's model would hold. Future research should be directed towards an understanding of the relationship between the primary and secondary mating reactions.

Finally, it must be pointed out that the mechanism proposed here for illegitimate pairing in *Paramecium bursaria*, and for the chemical induction of selfing described by Hiwatashi and by Miyake for other species, may not be applicable to the induction of pseudo-selfing reported by Sonneborn and by Metz. The latter workers were able to show that cells which have been temporarily united with individuals of a complementary mating type behaved as if they had acquired that mating-type specificity in the course of contact. Thus a transitory shift of mating type may occur in these cases. But in *P. bursaria* both mates need to be 'activated' by contact with cells expressing a complementary type in order that illegitimate conjugation may occur; this is certainly not the case for the pseudo-selfers of Sonneborn and Metz. Although the phenomena may appear superficially similar they probably call for quite different explanations.

Induced selfing of the kind reported for stock Wu-67 is apparently extremely rare in *Paramecium bursaria*. No evidence for this phenomenon was observed among at least 100 clones studied by Jennings (see Sonneborn, 1947) and others (Ehret, 1953; Siegel, 1960; Wichertman, 1953). Therefore homotypic matings are presumably unimportant in the evolution and genetic analyses of this organism.

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Resistance and Cross-resistance of *Escherichia coli* Mutants to Antitumour Agent Mitomycin C

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SUMMARY

The cross-resistance patterns are described for 96 mutants selected in one step from *Escherichia coli* strain S for resistance to Mitomycin C. The test agents used were ultraviolet radiation, seven radiomimetic and two non-radiomimetic compounds. Seven different types of mutants could be selected in one step from the parent. Five of these were radioresistant; two were chemoresistant. Of the radioresistant types two were identical with types previously isolated using other radiomimetic agents for selection; three of the types were new. One of the new types was reactivated by plating medium following ultraviolet irradiation differently from all other radioresistant mutants of *E. coli* S. One of the chemoresistant types was resistant only to Mitomycin C; the other displayed a low degree of cross-resistance to nitronitrosoguanidines and to penicillin. Also described are the cross-resistance patterns of mutants selected in five consecutive steps for increasing resistance to Mitomycin C. Beginning with a first step radioresistant mutant it was possible to select four additional steps, up to 325-fold, in resistance to Mitomycin C. One of these steps appeared to be a shift from one radioresistant type to another.

INTRODUCTION

This paper describes the cross-resistance patterns of mutants of *Escherichia coli* strain S, selected for resistance to Mitomycin C, an antibiotic with antitumour activity (Usubuchi *et al.* 1957). Mitomycin C has radiomimetic properties: in bacteria it is a mutagen (Szybalski, 1958) and like radiation it selectively inhibits the synthesis of deoxyribonucleic acid (Shiba, Terawaki, Taguchi & Kawamata, 1959). Mutants of *E. coli* S selected for resistance to other radiomimetic agents were cross-resistant to Mitomycin C (Mandell, Woody & Greenberg, 1961; Woody, Mandell & Greenberg, 1961; Greenberg, Mandell & Woody, 1961). Furthermore, Oboshi (1959) found that tumours selected for resistance to nitrogen mustard N-oxide (nitromin) were also resistant to Mitomycin C.

In earlier work, 13 first-step mutants of *Escherichia coli* S were selected for resistance to 1-methyl-3-nitro-1-nitrosoguanidine (Mandell *et al.* 1961), to nitrogen mustard and to nitromin (Woody *et al.* 1961), to azaserine, to 1-chloropropyl- and 1-chloroethyl-3-nitro-1-nitrosoguanidine (Greeenberg *et al.* 1961). Each mutant was characterized by its cross-resistance pattern, i.e. its degree of resistance to ultraviolet radiation, seven radiomimetic and two non-radiomimetic agents (penicillin

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and 6-diazo-5-oxo-L-norleucine). The cross-resistance patterns of the 13 mutants are shown in Table 1. Three of the mutants (S/Ni 1*a*, S/As 1*a* and S/Cp 1*b*) were significantly resistant only to the selecting agent or to closely related chemicals. Such mutants have been termed 'chemoresistant' to indicate that resistance is to a specific chemical structure and not to the class of radiomimetic agents. The other ten mutants were resistant to all radiomimetic agents and to ultraviolet radiation but not to the non-radiomimetic agents. These have been called 'radioresistant' mutants (with the limitation that the effect of ionizing radiations on these mutants has not yet been tested). Four different cross-resistance patterns were observed among the 10 radioresistant mutants; these have been designated R_1, R_2, R_3 and R_4. R_1 and R_2 patterns were observed once each; R_3 and R_4 patterns occurred in mutants selected for resistance to three and five, respectively, different chemical agents. All

Table 1. *Cross-resistance among first step mutants resistant to radiomimetic compounds*

Bacterial strain	Radio-resistant designation	Test compound: MC*	NG	CP	CE	NM	NI	AS	DON	PN	u.v.	
		Minimum inhibitory concentration for *Escherichia coli* S†										
		0·038 ± 0·005	0·038 ± 0·006	0·018 ± 0·002	0·021 ± 0·003	13 ± 2	18 ± 3	0·004 ± 0·001	0·04 ± 0·02	2·2 ± 0·2	22 Plated on	
		Resistance factor‡										
											T 7·0	M-9
S/Ni 1a§	—	1	1·2	1	1	1·2	60	1	1	1	1	6
S/As 1a	—	1	0·7	1	1	1	1	16	1	1	1	—
S/Cp 1b	—	1·2	12	9	9·5	0·18	0·22	1·1	1	1	1	—
S/Cp 1a	R_1	2·7	2·0	2·3	1·6	2·9	60	4·0	1	1	15	15
S/Ni 1b	R_2	5·8	19	6·5	6·2	6·2	60	6·8	1	0·34	15	15
S/Nm 1a S/Ni 1c S/As 1b	R_3	9	28	9	9·5	10	60	13	1	1	15	15
S/Ng 1a S/Nm 1b S/As 1c S/Ce 1a S/Cp 1c	R_4	22	45	26	24	20	60	16	1	1	15	15

* The following abbreviations will be used: Mitomycin C, MC; 1-methyl-3-nitro-1-nitrosoguanidine, NG; 1-chlorethyl-3-nitro-1-nitrosoguanidine, CE; 1-chloropropyl-3-nitro-nitrosoguanidine, CP; nitrogen mustard, NM; nitromin, NI; azaserine, AS; 6-diazo-5-oxo-L-norleucine, DON; penicillin, PN; ultraviolet light, u.v.

† μg./ml. estimated from gradient plates (Szybalski & Bryson, 1952) except the u.v. value is expressed in ergs mm.$^{-2}$/hit based on calibration of the u.v. source with T2 phage (Latarjet *et al.* 1953). 'Hit' is defined as $-\ln$ (fraction of survivors). The survivors were plated on tryptone agar pH 7·0 and M-9 minimal agar pH 6·8; the fold-increase, an average of several determinations, represents a comparison with the survival of the parent strain (plated on tryptone agar pH 7·0) set at unity.

‡ The figures in these columns represent the factor of resistance (fold-increase) compared with the parent strain set at unity.

§ Resistance to specific agents is conventionally represented by a bar and the abbreviation of the agent to which the strain was first found resistant. The first number following the bar denotes the selective isolation step to resistance; subsequent letters and numbers identify particular mutants and their families.

the radioresistant mutants exhibited an identical degree of resistance to ultraviolet light, unaffected by post-irradiation growth on defined or complex media. This is in contrast to *E. coli* B (Roberts & Aldous, 1949) and its radioresistant mutant, B/r (Alper & Gillies, 1960; Hill & Simson, 1961) and to *E. coli* S (Woody *et al.* 1961) whose degree of resistance to ultraviolet radiation can be altered by the post-irradiation growth medium. All the radioresistant mutants were equally resistant to nitromin, but differed in their degree of resistance to the other six radiomimetic agents. The R_1 mutant was least resistant to these agents, the R_4 mutants were most resistant, while R_2 and R_3 mutants were intermediate in their resistance. If only ultraviolet radiation and nitromin had been used to characterize these mutants, they would all have appeared identical. However, by using nine test compounds, four different cross-resistance patterns were observed, indicating four different types of radioresistant mutant.

The results of an analysis of 96 first-step and four higher-step mutants show that when Mitomycin C is used as the selecting agent: (1) radioresistant mutants of the classes R_3 and R_4 occurred frequently, while representatives of the classes R_1 and R_2 were not isolated; (2) three radioresistant mutants with hitherto undetected cross-resistance patterns were isolated, including one whose resistance to ultraviolet radiation could be altered by the nature of the post-irradiation growth medium; (3) two types of chemoresistant mutants were isolated—one resistant only to Mitomycin C, the other with a low degree of cross-resistance to nitrosoguanidines and to penicillin and under certain circumstances to ultraviolet radiation; (4) it was possible to select five consecutive steps of increasing resistance, up to 325-fold, to Mitomycin C; (5) the second step conferred increased resistance to Mitomycin C without otherwise effecting the cross-resistance pattern of the first-step radioresistant parent; (6) an R_4-type mutant was derived in a single selective step from a type R_3 parent.

METHODS

Bacterial strains. Escherichia coli strain S, obtained from Dr A. D. Hershey, was the parent strain. The characteristics of the resistant mutants used as reference strains are given in the appropriate tables. Their derivation is described in earlier papers (Mandell *et al.* 1961; Woody *et al.* 1961).

Compounds. The chemicals used were: 1-methyl-3-nitro-1-nitrosoguanidine, purchased from the Aldrich Chemical Co., Milwaukee, Wisc., and recrystallized from ethanol; 1-chloropropyl- and 1-chlorethyl-3-nitro-1-nitrosoguanidine kindly furnished by Dr B. R. Baker, Stanford Research Institute, Menlo Park, California; azaserine, 6-diazo-5-oxo-L-norleucine, Mitomycin C and nitromin, supplied by the Cancer Chemotherapy National Service Center, Bethesda, Md.; and nitrogen mustard, a gift from Merck Sharp and Dohme, Rahway, N.J. All compounds were prepared in sterile distilled water immediately before use.

Media. The media used contained per litre of distilled water:

Tryptone agar. Tryptone, 10 g.; glucose, 1 g.; sodium citrate, 2·0 g.; sodium chloride, 8 g. and agar, 12 g. (BBL, Baltimore Biological Lab., Inc.); adjusted to pH 7·0 with sodium hydroxide or 5·5 with hydrochloric acid.

M-9 agar. Dibasic sodium phosphate, 5·8 g.; monobasic potassium phosphate, 3·0 g.; ammonium chloride, 1·0 g.; sodium chloride, 0·5 g.; glucose, 2·0 g.; mag-

nesium sulphate ($7H_2O$), 250 mg.; calcium chloride, 14 mg.; 1 % gelatin solution, 10 ml.; agar, 8·0 g. (Ionagar, Oxo Ltd., London).

Peptone broth. Peptone, 10 g.; beef extract, 3 g.; glucose, 1 g.; sodium chloride, 5 g.

Diluting fluid. Peptone, 2 g.; sodium chloride, 6 g.; magnesium sulphate ($7H_2O$), 0·5 g.

Phosphate-buffered saline was 1 % sodium chloride in 0·2 M-phosphate buffer, pH 6·8. Tryptone glucose extract agar was a commercial (Difco) preparation, on which cultures were preserved after isolation and identification.

Isolation of resistant mutants. Resistant mutants were isolated from plates of tryptone agar (pH 5·5) containing graded doses over a 100-fold range of Mitomycin C and spread with $3{\cdot}5 \times 10^7$ organisms of the appropriate parent, growing logarithmically in peptone broth.

Measurement of resistance to chemical agents. The methods used to measure the degree of resistance to chemical agents have been given in detail elsewhere (Mandell *et al.* 1961). All isolated clones were grown overnight in peptone broth at 37°, adjusted turbidimetrically to a population density of about $3{\cdot}5 \times 10^8$/ml., and streaked on gradient plates, according to the method of Szybalski & Bryson (1952). Gradient plates were made with tryptone agar (pH 5·5) except that M-9 agar was used in tests involving azaserine, 6-diazo-5-oxo-L-norleucine and penicillin. The minimum inhibitory concentration (MIC) was determined as follows:

$$\frac{\text{length of solid growth}}{\text{total length of streak}} \times \text{maximum concentration of test compound } (\mu g./\text{ml.}).$$

Sensitivity to ultraviolet radiation. The ultraviolet radiation source was a single 15 W. General Electric germicidal lamp with a maximum output at 2537 Å., calibrated with bacteriophage T2 according to the method of Latarjet, Morenne & Berger (1953). Cultures grown overnight in peptone broth were washed twice with phosphate buffered saline, diluted to about 2×10^6 bacteria/ml. in cold buffered saline (pH 6·8) and exposed in 50 mm. Petri dishes containing 1 ml. of bacterial suspension. Exposures were made at a distance of 51·5 cm. from the ultraviolet radiation source. The dish was agitated gently by hand throughout exposure. Appropriate dilutions in cold diluting fluid were plated in duplicate on both tryptone agar (pH 7·0) and M-9 agar, incubated for 24 and 48 hr., respectively, at 37° and counted. All manipulations subsequent to ultraviolet irradiation were carried out in subdued light to minimize photoreactivation. Sensitivity to ultraviolet radiation was calculated as ergs/mm.$^{-2}$/hit, a hit being defined as $-\ln$ (fraction of survivors).

RESULTS

First-step mutants. The results of a survey of 100 potential first-step mutants resistant to Mitomycin C are presented in Table 2. The cross-resistance patterns of the various types of mutants are given in Table 3.

Only four of the 100 isolates were indistinguishable from the parent strain S. The remainder showed some degree of resistance to Mitomycin C. These could be divided into two main groups: those which were cross-resistant with *all* radiomimetic compounds (radioresistant) and those which were not (chemoresistant). The latter group could be subdivided into two subgroups. One, the prototype of

Table 2. *Classification of survivors after treating* Escherichia coli S *with Mitomycin C*

Mytomycin C (μg./ml.)	Surviving colonies (18 hr.)	No. tested	Sensitive	No. of type: Radioresistant R_1	R_2	R_3*	R_4*	R_5*	R_6*	R_7*	Chemoresistant I	II
0·03	Confluent growth											
0·08	300–400	25	4	0	0	8	1	0	1	0	7	4
0·15	200	25	0	0	0	13	11	1	0	0	0	0
0·30	60	30	0	0	0	13	15	2	0	0	0	0
0·50	15	9	0	0	0	0	8	0	0	1	0	0
0·75	5 (8†)	8	0	0	0	0	8	0	0	0	0	0
1·0	1 (3†)	3	0	0	0	0	3	0	0	0	0	0
3·0	0	—	—	—	—	—	—	—	—	—	—	—
Totals		100	4	0	0	34	46	3	1	1	7	4

* Cross-resistant pattern of representative strains of each type S/Mc 1g, S/Mc 1b, S/Mc 1f, S/Mc 1i, S/Mc 1h, S/Mc 1c, and S/Mc 1d, respectively, are shown on Table 1.
† After 42 hr.

which is S/Mc 1c, was resistant (2·5-fold) only to Mitomycin C and is therefore clearly chemoresistant. The other subgroup of which S/Mc 1d is representative, was cross-resistant with nitrosoguanidines and penicillin but not with any of the other chemical agents. The response of S/Mc 1d and S to ultraviolet radiation (Fig. 1) was identical when both were plated on tryptone agar subsequent to irradiation. When, however, both were plated on M-9 agar, the slope of the survival curve of S increased six-fold, but that of S/Mc 1d increased 10- to 12-fold. This is the first instance in which the difference between radio- and chemoresistance is not clearcut. For the present, mutants of this group have been classified as chemoresistant, with the recognition that this is an arbitrary classification of an anomalous strain.

Table 3. *Cross-resistance relationships among first-step Mitomycin C resistant mutants of* Escherichia coli *strain S relative to the sensitive parent strain*

Bacterial strain	Radioresistant designation	Test compound: MC*	NG	CP	CE	NM	NI	AS	DON	PN	u.v.
		Minimum inhibitory concentration for *Escherichia coli* S†									
		0·038 ± 0·005	0·038 ± 0·006	0·018 ± 0·002	0·021 ± 0·003	13 ± 2	18 ± 3	0·004 ± 0·001	0·04 ± 0·02	2·2 ± 0·2	22 Plated on
		Resistance factor‡									T 7·0 / M-9
S/Mc 1d§	—	1·4	1·6	1·4	1·2	1	1	1	1	1·3	1 / 10
S/Mc 1c	—	2·5	1	1	1	1	1	1	1	1	1 / 6
S/Mc 1i	R_6	2·0	3·7	2·7	2·0	2·9	60	4·0	1	1	6 / 15
S/Mc 1a	R_3	9	28	9	9·5	10	60	13	1	1	15 / 15
S/Mc 1e	R_5	14	36	11	13	20	60	16	1	1	15 / 15
S/Mc 1f	—	11	—	—	—	—	—	—	—	—	— / —
S/Mc 1b	R_4	22	45	26	24	20	60	16	1	1	15 / 15
S/Mc 1h	R_7	31	56	36	31	20	60	16	1	1	14 / 14

*, †, ‡, §. See footnotes to Table 1.

Five distinct types of radioresistant mutants were isolated. 54% of the radioresistant mutants, represented by S/Mc 1b, had a cross-resistance pattern identical with that of all previously isolated mutants of type R_4; 40%, represented by S/Mc 1g, were indistinguishable from other members of type R_3. The remaining five radioresistant mutants had cross-resistance patterns not previously observed. S/Mc 1e, S/Mc 1f and S/Mc 1h were identical to R_4 in their degree of resistance to ultraviolet radiation, nitrogen mustard, nitromin and azaserine, and in their lack of resistance to DON and penicillin. However, S/Mc 1e and S/Mc 1f were somewhat less resistant than R_4 to Mitomycin C and the nitrosoguanidines, and S/Mc 1h was

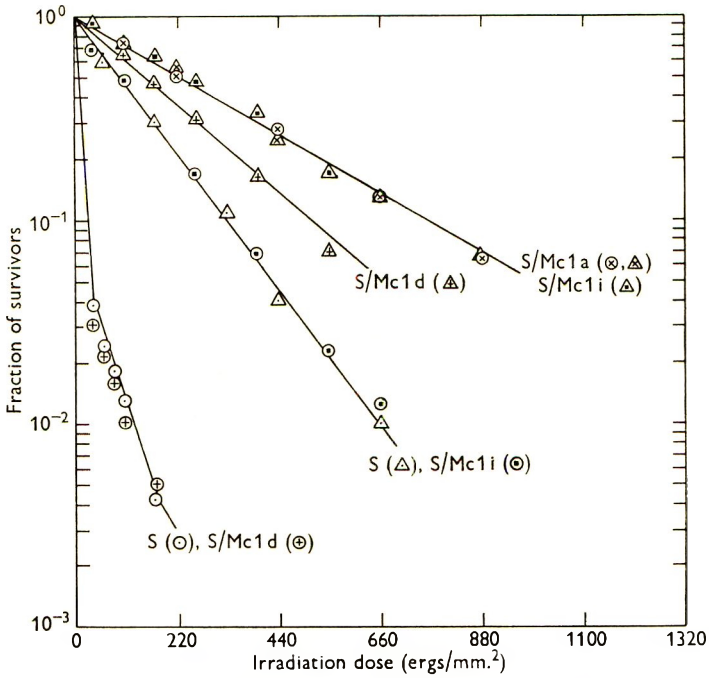

Fig. 1. Survival of *Escherichia coli* S, chemoresistant strain S/Mc 1d, and radioresistant strains S/Mc 1a and S/Mc 1i exposed to ultraviolet radiation. ○, Survivors plated on tryptone agar, pH 7·0. △, survivors plated on M-9 defined medium, pH 6·8.

somewhat more resistant than R_4 to these same compounds. S/Mc 1e and S/Mc 1f differed from each other only in a small, but seemingly consistent, manner on repeated trials, with regard to their resistance to Mitomycin C. They have been arbitrarily classed together as R_5. S/Mc 1h has been designated R_7.

The last, hitherto undescribed, radioresistant mutant, S/Mc 1i, had a cross-resistance pattern very similar to that of R_1. However, unlike R_1, which was 15-fold resistant to ultraviolet radiation irrespective of post-irradiation plating medium, S/Mc 1i was six-fold resistant when plated on complex (tryptone) medium and 15-fold resistant when plated on M-9 agar (Fig. 1). S/Mc 1i is, therefore, considered different from R_1 and has been designated R_6.

The frequency distributions shown in Table 2 should be interpreted with caution. Nevertheless certain facts can be derived from the table. The frequency with which

a type of mutant was selected depended to some extent on the concentration of the drug. The mutants with the lowest degree of resistance to Mitomycin C were selected only from the plate with the lowest concentration of Mitomycin C at which there was not confluent growth. This plate was the only one from which parental, sensitive organisms were picked. As the concentration of Mitomycin C was increased, only the more resistant mutants were isolated. It would appear from the sampling of survivors over a 40-fold range of concentration of Mitomycin C that radioresistant mutants predominated and that most of these were R_3 or R_4. However, half the mutant resistant population sampled from the plate containing Mitomycin C at 0·08 μg./ml. were chemoresistant. It is curious that from this same plate only one R_4-type mutant was isolated. Assuming no sampling prejudice, this would mean that there were 16 R_4 mutants on the plate, whereas at the next higher concentration of Mitomycin C there were 88 and the frequency decreased thereafter as the concentration of Mitomycin C increased. Actually the sampling of the 0·08 μg./ml. plate was probably prejudiced, since there were several colony sizes and an almost equal number of each colony size was picked regardless of the true frequency of the various colony sizes on the plate. Only the mutants of the type S/Mc 1 d and sensitives were found among the smallest colony type.

Table 4. *Cross-resistance relationships among mutants of* Escherichia coli *S representing five consecutive steps in resistance to Mitomycin C*

Bacterial strain	Test compound: MC*	NG	CP	CE	NM	NI	AS	DON	PN	u.v.
	Minimum inhibitory concentrations for *Escherichia coli* S†									
	0·038 ± 0·005	0·038 ± 0·006	0·018 ± 0·002	0·021 ± 0·003	13 ± 2	19 ± 3	0·004 ± 0·002	0·04 ± 0·02	2·2 ± 0·2	22
	Factors of resistance‡									Plated on: T 7·0	M-9
S/Mc 1 a§	9	28	9	9·5	10	60	13	1	1	15	15
S/Mc 2 a	22	28	9	9·5	10	60	13	1	1	15	15
S/Mc 3 a	71	36	26	24	20	60	16	1	1·4	15	15
S/Mc 4 a	235	36	39	38	20	60	16	1	1·4	15	15
S/Mc 5 a	325	36	68	38	20	60	16	1	2·6	15	15

*, †, ‡, §. See footnotes to Table 1.

Granting that the frequency of occurrence of any mutant type depends on the selective pressure of the Mitomycin C, nevertheless mutants of the types R_5, R_6 and R_7 were rare compared to those of types R_3 and R_4.

Sequential steps in resistance to Mitomycin C. Mutants representing five sequential steps in increasing resistance to Mitomycin C were selected as follows: S/Mc 1 a was selected from S, S/Mc 2 a from S/Mc 1 a, S/Mc 3 a from S/Mc 2 a, etc., as seen in Table 4. The second step mutant, S/Mc 2 a, was more resistant than its parent only to Mitomycin C. The third-step mutant, S/Mc 3 a, increased in resistance to all radiomimetic agents. Its cross-resistance pattern was essentially that of an R_4 radioresistant mutant except that S/Mc 3 a was more resistant to Mitomycin C than R_4 mutants. The fourth-step mutant increased in resistance not only to Mitomycin C

but also to 1-chloropropyl- and 1-chloroethyl-3-nitro-1-nitrosoguanidine. The fifth-step mutant increased in resistance to Mitomycin C, to 1-chloropropyl-3-nitro-1-nitrosoguanidine and to penicillin.

The survival curves of the second- to fifth-step mutants in Mitomycin C resistance, as well as those of R_1, R_2, R_4, R_5, and R_7 after exposure to ultraviolet radiation were indistinguishable from that of S/Mc 1a (Fig. 1) and were not altered by post-irradiation growth in complex or synthetic medium.

DISCUSSION

The orginal purpose of the work described here was to determine whether, by cross-resistance studies, relationships could be established among compounds which for various reasons (Mandell *et al.* 1961) were considered to be radiomimetic. It has been shown in this paper and previous papers of this series that there exists a class of compounds such that mutants of *Escherichia coli* S selected for resistance to one member of the class will be resistant to all members of the class and to ultraviolet radiation. Most of the mutants of *E. coli* S selected for resistance to 1-methyl-3-nitro-1-nitrosoguanidine (Mandell *et al.* 1961), nitrogen mustard and nitromin (Woody *et al.* 1961), azaserine and other nitrosoguanidines (Greenberg *et al.* 1961) and, as shown in this paper, Mitomycin C, were also resistant to ultraviolet radiation and to each of the other radiomimetic agents tested. This cross-resistance was not the result of a general increase in resistance to all antibacterial agents, for none of the radioresistant mutants of S was significantly resistant to 6-diazo-5-oxo-L-norleucine or penicillin. The observations regarding mutual cross-resistance among radiomimetic agents and radiation are extensions of earlier observations of Bryson (1948) who found that Witkin's (1947) strains of *E. coli* B, selected for resistance to radiation, were also resistant to nitrogen and sulphur mustard. Conversely, strains selected for resistance to nitrogen mustard were resistant to radiation (Bryson, 1948). Furthermore, the radiation-resistant mutant of *E. coli* B, strain B/r, was similarly found resistant to nitrofuracin, and strains selected for resistance to nitrofuracin were also resistant to radiation (Szybalski & Nelson, 1954).

However, not all mutants selected for resistance to radiomimetic compounds are radioresistant. It was shown earlier that one mutant selected for resistance to nitromin was resistant only to nitromin and not to other radiomimetic agents—not even the chemically similar nitrogen mustard—nor to radiation (Woody *et al.* 1961). It has been shown in this paper that there is at least one first-step mutant resistant to Mitomycin C (S/Mc 1c) which is resistant only to Mitomycin C. There was another mutant which could not so unequivocally be classified as chemoresistant. S/Mc 1d, which appeared as sensitive as the parent S to ultraviolet radiation when plated on complex medium, was reactivated to a much greater extent than S when the organisms were plated on M-9 agar. Furthermore, S/Mc 1d was resistant not only to Mitomycin C but was also resistant to the nitrosoguanidines and to penicillin.

There are other examples where resistance to Mitomycin C and one or more nitrosoguanidines increase or decrease together without any concomitant change in resistance to any of the other agents used in these studies. Mutants classified as R_5 and R_7 (Table 3) have cross-resistance patterns identical with R_4 except for their resistance to Mitomycin C and the nitrosoguanidines, R_5 being somewhat less and

R_7 somewhat more resistant to all these agents than R_4. S/Mc 4a and 5a (Table 4) were characterized by increases in resistance only to Mitomycin C and to one or more nitrosoguanidines; S/Ng 2a, a second-step mutant selected for resistance to 1-methyl-3-nitro-1-nitrosoguanidine displayed an increase in resistance to Mitomycin C (Mandell *et al.* 1961). These data suggest that Mitomycin C and the nitrosoguanidines may have in common a chemically reactive group or a mode of action, not necessarily radiomimetic. The elucidation of the chemical structure of Mitomycin C will tell whether the former possibility is true.

Seven different first-step radioresistant mutants have been isolated to date from the parent strain S. Two of these, R_3 and R_4, have been isolated repeatedly using a variety of different radiomimetic agents for selection. R_3- and R_4-types comprised the overwhelming majority of the radioresistant mutants isolated when Mitomycin C was the selecting agent (Table 2). The other five radioresistant types were rare, R_1, R_2, R_6 and R_7 having been selected once each from among mutants resistant to each of five different radiomimetic agents. Three mutants of the type R_5 were isolated when Mitomycin C was the selecting agent but not when any other radiomimetic agent was used. It should be pointed out, however, that the current survey of 100 potential resistant mutants is the most extensive we have undertaken. It is, therefore, impossible to decide whether R_1 was induced more frequently by 1-chloropropyl-3-nitro-1-nitrosoguanidine, R_2 by nitromin, and R_5, R_6 and R_7 by Mitomycin C, or whether, being rare, they were selected by these particular agents by chance. It is also difficult to decide whether the rare R-types are actually the result of single mutational events, or whether they represent double mutations. R_5 and R_7 might conceivably be R_3 and R_4, respectively, with a second, chemoresistant mutation to Mitomycin C resistance. R_1 and R_2, having such a low degree of resistance to all radiomimetic compounds, would have to be R_3 or R_4, modified by a second mutation to a lower degree of resistance to all radiomimetic compounds. R_6, differing as it does from all other R-types in its plating medium reactivation characteristics, is probably the product of a single mutation. Based on preliminary estimates of the frequency with which the presumed primary mutations to resistance occur ($c\ 3 \times 10^{-6}$ for R_3 and R_4 and the chemoresistant type of S/Mc 1c with Mitomycin C at 0·08–0·15 μg./ml.) it seems unlikely that double mutations would be selected from among the relatively small samples plated (3×10^7 on each of the Mitomycin C plates).

All the radioresistant mutants of *Escherichia coli* S differ in several ways from the radioresistant mutant of *E. coli* B, B/r (Witkin, 1947). The degree of resistance to ultraviolet radiation of all radioresistant types of S, except R_6, was unmodified by the type of medium on which they were plated following irradiation. *E. coli* B/r, on the other hand, appears to be more resistant than strain B to ultraviolet radiation only when both strains are plated on complex medium (Alper & Gillies, 1960). When both B and B/r were plated on defined medium following irradiation, they had indistinguishable survival curves. This is because B is more sensitive to irradiation when plated on complex medium than when plated on defined medium (Roberts & Aldous, 1949), while the opposite is true of B/r. *E. coli* S behaves with respect to plating medium reactivation like *E. coli* B; the radioresistant type R_6 behaves, qualitatively, like *E. coli* B and S, the opposite to B/r, and differently from all the other R-types of S.

B/r also differs from the R-types of *Escherichia coli* S with respect to collateral

resistance to penicillin and the sulphonamides. According to Witkin (1947) and Adler & Haskins (1960), B/r is resistant to penicillin and to sulphathiazole as well as to radiation. None of the R-types of S was resistant to penicillin and none of those tested (R_1 to R_4) was resistant to sulphonamides (unpublished). Moreover, while B/r, R_3 and R_4 do not, as do B and S, respond to the challenge of radiation by forming filaments, filament formation after irradiation is characteristic of R_1 and R_2 (Curry & Greenberg, unpublished).

Assuming all the R-types of strains S are each due to a single mutation, there may be as many as 17 mutations involved in the resistance or sensitivity of *Escherichia coli* to radiation. Witkin (1947) recognized four different groups of radiation-resistant mutants of *E. coli* B. One group, represented by B/r, was resistant to penicillin and sulphathiazole; another was resistant to penicillin but not sulphathiazole; another was resistant to sulphathiazole but not to penicillin; and a fourth group was resistant to neither of these agents. Two of these mutants, the two with concomitant resistance to penicillin, are obviously different from any of the R-types of S. The other two radioresistant mutants of B cannot yet be clearly distinguished from all the R-types of S. Also not yet fully distinguishable from the R-types of S are the radiation sensitive mutants of B, described by Hill & Simson (1961) although B_s and $B_{s\text{-}2}$ appear to be more sensitive than S. With respect to radiation sensitivity together with collateral resistance to chemical agents, *E. coli* B exists in at least seven forms, *E. coli* S in eight. All the forms of B might be counterparts of those in S, differing only in one mutation from B to S, or vice versa. If this were the case, there would be nine mutations controlling radiation sensitivity and collateral resistance to other agents in *E. coli*. If, on the other hand, none of the mutants of B were counterparts of any of those in S, and B and S were different, there might be as many as 16 mutational events controlling radioresistance to *E. coli*. Furthermore, B/r would appear to be about as resistant to radiation as the R-types of S, but *E. coli* H, is, according to our experience, about twice as resistant to ultraviolet radiation as the R-types of S. Assuming, then, that a single mutation controls the difference between S and H, with respect to radiation sensitivity, there may be as many as 17 mutational events controlling radiation sensitivity in *E. coli*. By integrating the published data with our own experience we would estimate that the difference between the most radiation sensitive and resistant forms of *E. coli* (B_s of Hill & Simson, 1961, and H) in resistance to ultraviolet radiation to be about 200-fold.

The isolation of five consecutive steps in resistance to Mitomycin C appears to reflect the interaction between radio- and chemoresistant mutations. S/Mc 2a may be considered to consist of an R_3 plus a chemoresistant mutation similar to or identical with S/Mc 1c. The third-step mutant, S/Mc 3a, probably represents a shift from R_3 to R_4, superimposed on its chemoresistant mutation acquired in the previous step. Previously it was clear that no R-group was a prerequisite for any other R-group; that is, all the R-groups could be selected in one step from the parent sensitive strain. Furthermore, in earlier experiences with nitrosoguanidines, nitromin or nitrogen mustard no R-group was selected from any other R-group. It was as though all R-types were mutually exclusive. Now it is evident from the change in resistance patterns from S/Mc 2a to S/Mc 3a that mutations can occur from one R group to another. Such a shift would appear to require a single genetic change. S/Mc 4a and S/Mc 5a, in turn, may represent either the imposition of additional

chemoresistant steps on a basic R_4 radioresistance or a change from R_4 to R_7. It should be noted that as many as three of the five steps in resistance to Mitomycin C may represent chemoresistant mutations, but only two such mutations were identified as first-step mutants. This might mean that certain chemoresistant mutations can occur only after another one has occurred; or that among the first-step chemoresistant mutants there are two phenotypes for three genotypes; or that one chemoresistant mutation has not yet been discovered; or, as suggested above, one of the steps is a change from R_4 to R_7.

It is evident from this discussion that there are many different radioresistant mutants of *Escherichia coli*, each with a characteristic cross-resistance pattern to ultraviolet radiation, a variety of radiomimetic and some non-radiomimetic agents. Genetic analyses of these mutants are planned, for without such an analysis it is difficult to determine whether the mutants represent different alleles at one locus or mutations at different loci. Information of this nature will be a useful guide for postulating models to account for resistance to the lethal effects of radiation and radiomimetic chemicals.

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The Role of Polygalacturonase in Root-Hair Invasion by Nodule Bacteria

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SUMMARY

The production of polygalacturonase in associations of nodule bacteria and seedlings of leguminous plants was investigated. Plants and bacteria were combined in the following ways: (*a*) different plant species were combined with infective strains (isolated from the same cross inoculation group) as well as non-infective ones (from foreign groups); (*b*) host plant species with different susceptibilities were combined with the same bacterial strain; (*c*) bacterial strains with different infectivities, as measured by the number of infection sites, were combined with the same host species; (*d*) clover strains which had lost their infectivity, as well as transformed and again infective subcultures of these strains, were tested on clover plants. The results indicate that infection of the seedlings was strongly correlated with the production of polygalacturonase. The conclusion is drawn that polygalacturonase plays an important part in the infection process. This function is thought to be a weakening of the cell wall of the root hair which would facilitate the bacterial invasion. The possible role also of indolyl-3-acetic acid in the infection process is discussed.

INTRODUCTION

In a study of the symbiotic relationships between leguminous plants and nodule bacteria one of the first questions which arises is how the bacteria enter the plant. This problem has been comparatively little studied, and no clear picture has so far been obtained of this important step in the development of the symbiosis. In previous work (Fåhraeus & Ljunggren, 1959) some evidence was found that pectic enzymes take part in the infection process. Significant amounts of polygalacturonase were found in associations of seedlings and their specific nodule bacteria, but not in those combinations where no infection occurred. An addition of nitrate, which stops the infection, also inhibited polygalacturonase production. Polygalacturonase was produced by the plant, not by the bacteria, which appears from the fact that an addition of cell-free preparations from the bacteria had the same effect as living bacteria in inducing formation of polygalacturonase (Ljunggren & Fåhraeus, 1959). Since in the above-mentioned work only rather few combinations of plants and bacteria had been examined, the investigation was extended by testing a larger number of plant species and bacteria. Bacterial strains with different degrees of infectivity and plant species with different susceptibilities were also studied. The present paper presents the results obtained, which seem to confirm the importance of the polygalacturonase in the infection process. On the basis of these findings a possible mechanism for rhizobial infection is discussed.

METHODS

Seed material. Commercially available seed of the following plants was used: *Trifolium repens* L. (varieties Morsö and Beta), *T. hybridum* L. (variety Svea), *Medicago sativa* L. (varieties Tuna and Alfa), *Pisum sativum* L. (variety Torsdags II). These were ordered from Svalöv, except *M. sativa*, variety Alfa, which was obtained from Weibullsholm, Sweden. In addition, several wild species were used for the experiments. The seed had been collected by Dr P. S. Nutman, who kindly put it at our disposal. These species were: *Trifolium ambiguum* M. Bieb., *T. glomeratum* L., *T. parviflorum* Ehrh., *T. patens* Schreb., *Melilotus alba* Desv., *Vicia hirsuta* (L.) S. F. Gray, *Lotus angustissima* L., *L. hispida* Desf., *Anthyllis vulneraria* L.

Bacterial strains. Rhizobium trifolii: strains 226, ClF, A 121111, SU 297, all infective and effective nitrogen fixers; strain Coryn, infective but forming ineffective nodules; strains Bart A and A 11, non-infective variants of originally infective strains. It has been possible to transfer infectivity to these strains by adding capsular material produced by the infective strain 226 (Ljunggren, 1961). The transformed strains are called Bart A2 and A11.2, respectively. Other strains: *R. meliloti* AH_2, *R. leguminosarum* 311 and V1, *Rhizobium* sp. (Lotus) L1, *R. japonicum* 507.

The strains 226 and 311 were obtained by the courtesy of Dr G. Bjälfve (Leguminous Plant Laboratory, Uppsala); the others were generously supplied by Dr P. S. Nutman.

Substrates and chemicals. For cultivation of Rhizobium the medium A_5 given by Dorn (1956) was used throughout. For sterility control a standard Difco nutrient agar was used. The pectin used in the determinations of polygalacturonase was low-methoxyl citrus pectin Matheson, Coleman and Bell no. 7366 (Norwood, Cincinnati, Ohio, U.S.A.).

Experimental procedure

The experimental procedure was essentially the same as reported in a previous paper (Fåhraeus & Ljunggren, 1959). Some minor changes in the technique and details which we think are of special importance for the successful performance of the experiments are given in the following paragraphs.

For seed disinfection a $HgCl_2$+formaldehyde mixture (Fåhraeus, 1957) was ordinarily used. Extreme care was exercised to remove traces of mercury and possibly harmful seed exudates (Dadd & Jacobs, 1958; Jacobs & Dadd, 1959). To this end, the seed was allowed to swell overnight in the last wash water and was then rinsed again several times. For hard-coated seeds the procedure with sulphuric acid (Nutman, 1959) was more satisfactory and it was used for *Trifolium pratense*, *T. ambiguum*, *T. subterraneum*, *Lotus hispida*, *Anthyllis vulneraria*, *Melilotus alba* and *Vicia hirsuta*.

Certain batches of seed were very difficult to disinfect by any of these methods. Better results were sometimes obtained when the seed after disinfection was allowed to germinate in 0·01 % (w/v) hydroxylamine (personal recommendation by Dr G. Bjälfve). Nevertheless, in many cases we obtained vigorous growth of a Gram-negative, non-sporulating rod which gave yellow colonies on nutrient agar, it was probably a Flavobacterium.

Following disinfection the seed was transferred to sterile 10 cm. diam. Petri dishes, and the water volume adjusted to 2–3 ml. in each dish. Usually on the

second day the seedlings were inoculated with Rhizobium suspensions made up in distilled water. The suspensions were prepared from agar slopes with 3-day bacterial growth; 0·5 ml. containing about 500 million organisms/ml. was added to each dish, the total volume being adjusted to about 5 ml. with sterile distilled water. The use of distilled water seems to be an important point in this type of work, because even traces of Ca disturb the viscometric analysis. For that reason it is also advisable to rinse all glassware carefully in distilled water.

The dishes after inoculation were kept in an incubator at $20° \mp 1°$, and were illuminated with fluorescent light.

The experiments were terminated 3–4 days after inoculation. The solution was separated from the roots and solid particles by filtration through a layer of cotton wool.

For the determination of polygalacturonase the viscometric technique described previously (Fåhraeus & Ljunggren, 1959) was used throughout. The pectin was dissolved in 0·1 M-acetate buffer (pH 5·0) containing 0·5 % (w/v) NaCl. The solution was filtered on a Buchner funnel through double papers to free it completely from suspended particles. Together with the enzyme solutions to be tested the pectin solution was kept in a constant temperature bath at 30° for temperature equilibration. At zero time the solutions were mixed in Ostwald viscometers (3 ml. test solution + 8 ml. substrate in buffer) and the flow time recorded immediately. Measurements were again made after 1 hr. and 24 hr. The viscometers used had a flow time for water (t_w) between 31 and 35 sec. To get the t_w-value for a 'standard viscometer' with a flow time of 40 sec. we multiplied the values obtained with the different viscometers by the factor $k = 40/t$, where t represents the flow time of water in the individual viscometers.

The decrease in viscosity given in percentage of initial values was calculated from the expression

$$P = \frac{t_0 - t_t}{t_0 - t_w} \times 100,$$

where P = percentage decrease in viscosity; t_0 = initial flow time; t_t = flow time after t min.; t_w = flow time of water.

Sterility control. Since pectin-decomposing micro-organisms are common and the presence of contaminants especially of this kind in our experiments would largely invalidate the results, it was of the utmost importance to keep infection hazards as low as possible. The following precautions were therefore strictly followed.

(*a*) When the seed had been transferred to Petri dishes for germination, about 50 seeds from the disinfection flask were distributed over two plates of nutrient agar. When microbial growth occurred on these plates, the whole experiment was discarded.

(*b*) Immediately before inoculation, nutrient agar plates were streaked with liquid from each dish. All dishes which showed microbial growth were discarded.

(*c*) Before taking samples for analysis, the liquid and a number of roots were examined microscopically. Uninoculated dishes which showed microbial growth and inoculated dishes with growth morphologically distinct from Rhizobium were discarded. Fungal mycelium, when present, usually grew along the roots and was easily observed microscopically.

RESULTS

Cross-infection group specificity

In a series of experiments leguminous seedlings were combined with different species and strains of Rhizobium. As some seeds were available in small quantities only, experiments could not always be performed to determine the most suitable period for incubation. As a rule, however, satisfactory results were obtained by inoculating the dishes when the seedlings were 8–10 mm. long and incubating for another 3–4 days. The experiments are summarized in Table 1, from which it is evident that polygalacturonase was always present, when the seedlings were inoculated with the appropriate bacteria and that there was no, or very little, activity in other combinations. In this connexion it is interesting to notice Nutman's (1959) report that the *Lotus* and *Anthyllis* species showed deformed root hairs and produced nodules but that no infection threads were observed. In our test, however, they did not significantly differ from the other species tested.

Table 1. *Formation of polygalacturonase (PG) in associations of leguminous plants and nodule bacteria*

PG activity expressed as % decrease in viscosity of a 1 % (w/v) solution of low-methoxyl pectin in 24 hr.

Rhizobium strains	Plant: *Trifolium repens*	*Trifolium ambiguum*	*Trifolium hybridum*	*Medicago sativa*	*Melilotus alba*	*Pisum sativum*	*Vicia hirsuta*	*Lotus hispidu*	*Lotus angustissima*	*Anthyllis vulneraria*
	PG activity									
Clover ClF	12·0	—	—	—	2·0	—	—	—	—	—
Clover 226	16·1	—	11·0	−0·8	—	0·0	3·5	3·1	—	1·9
Clover A121111	25·4	10·6	—	—	5·6	—	—	—	0·8	—
Lucerne AH_2	1·0	0·5	—	22·2	20·6	—	—	—	3·7	0·5
Lotus L1	2·8	2·3	—	1·9	—	—	3·5	14·3	46·6	12·5
Vetch V1	2·0	—	—	2·4	—	—	16·6	0·9	—	0·5
Pea 311	1·2	—	—	—	—	16·4	—	—	—	—
Soybean 507	1·1	—	—	—	—	—	—	—	—	—

Host/bacteria effects within the clover group

Host differences. Nutman in a series of papers (reviewed in Nutman, 1956) studied the genetic factors affecting host/bacterial compatibility. An examination of infection density was also performed by Nutman (1959), but in this case without genetical analysis. An examination of his data, however, shows that there was a difference in infection density between different host species inoculated with the same bacterial strain. For instance, the mean number of infected hairs/plant after 11 days was 91·5 in *Trifolium parviflorum* and 7·0 in *T. patens*, when inoculated with Rhizobium strain ClF. The amounts of polygalacturonase produced by the above-mentioned hosts inoculated with one bacterial strain is shown in Table 2. The results indicate that there was a higher polygalacturonase activity in the more susceptible plant species.

Bacterial strain differences. In his work on root hair infection by nodule bacteria Nutman (1959) found great differences in infection density in the same host plant when inoculated with various strains of *Rhizobium trifolii*, and stated that these differences were independent of the host. Table 3 shows the results of two experiments with *Trifolium repens* and *T. glomeratum* inoculated with different strains of clover bacteria. The number of infection threads on the same level on both sides of the root were counted by using the slide technique earlier described (Fåhraeus, 1957) and were compared with polygalacturonase activities found in simultaneous experiments. From Table 3 it is evident that there was a certain correlation between the number of infection threads and the polygalacturonase activity found.

Table 2. *Formation of polygalacturonase (PG) by two species of* Trifolium *inoculated with the same bacterial strain.*

PG activity as % decrease in viscosity of a 1 % (w/v) solution of low-methoxyl pectin in 24 hr.

Host plant	Rhizobium strain	PG activity
Trifolium parviflorum	—	1·4
	ClF	27·6
T. patens	—	0·9
	ClF	14·9

Table 3. *The relation between polygalacturonase (PG) activity and number of root-hair infections in two species of* Trifolium *in association with different strains of* Rhizobium trifolii

PG activity as % decrease in viscosity of a 1 % (w/v) solution of low-methoxyl pectin in 24 hr.

Host plant	Rhizobium strain	PG activity		Mean number of infected root hairs
Trifolium repens	—	−0·7	1·4	0
	ClF	11·2	12·2	3·5
	Coryn	17·6	22·7	18·5
	226	17·0	23·0	21·5
T. glomeratum	—	2·0	—	—
	ClF	16·0	—	48·6*
	S.U. 297	11·6	—	9·8*

* Figures from Nutman (1959).

Experiments with non-infective clover strains. The origin of the Rhizobium strains Bart A, A11 and A121111 was recorded by Nutman (1946). Of these three strains Bart A and A11 have lost their ability to invade the root hairs of their original host plants and hence they are no longer able to induce nodule production. Table 4 shows that the non-infective bacteria were unable to induce polygalacturonase production. In this respect there was no difference between them and quite unrelated bacteria. We have, however, been able to transfer infectiveness to these bacteria from the infective strain 226 by adding capsular material from the latter to seedlings planted on an agar slope and inoculated with Rhizobium strains Bart A and A11. These seedlings produced nodules, and the bacteria isolated from these nodules (Bart A2, A11.2) are infective (Ljunggren, 1961). As shown in Table 4, the transformed

strains are also active polygalacturonase producers. These experiments have in fact given the only unequivocal proof, except serological tests, that Bart A and A11 really are Rhizobium strains.

Table 4. *Formation of polygalacturonase (PG) by* Trifolium repens *in association with infective and non-infective strains of* Rhizobium trifolii

PG activity as % decrease in viscosity of 1 % (w/v) solution of low-methoxyl pectin in 24 hr.

Rhizobium strain	PG activity	
	Expt. 1	Expt. 2
—	−1·0	0·5
Bart A	1·5	2·1
A11	2·5	1·1
A121111	15·0	15·2
226	16·2	15·4
Bart A2	—	10·8
A11·2	—	11·3

DISCUSSION

The correlation found between the infection of leguminous plants by nodule bacteria and the increased production of polygalacturonase in the bacteria + plant associations can be interpreted in two ways: (i) polygalacturonase formation may be the primary effect of bacterial action, (ii) the infection might come first and lead to an increased production of polygalacturonase. We have drawn the conclusion that the formation of polygalacturonase is the primary effect resulting in a partial depolymerization of the cell-wall pectin which, in its turn, facilitates the bacterial invasion. For this succession of events speaks the fact that infection rarely can be observed until after 3 days, whereas an increased production of polygalacturonase may occur as soon as 1 to 2 days following inoculation.

The infection mechanism may be visualized in the following manner. The bacteria secrete water-soluble substances which are highly specific (Humphrey & Vincent, 1959; Ljunggren & Fåhraeus, 1959). Our active preparations are mainly of polysaccharide nature, but transformation experiments carried out by one of us (Ljunggren, 1961) suggest that they might also contain deoxyribonucleic acid. The active principle will pass through the cell wall, reach the protoplasm, and react with some specific cell component. The nucleus, which at this stage is situated in the root hair near the tip, may possibly be involved in the interaction with the bacterial compound. Previous observations (Fåhraeus, 1957; Nutman, 1959) suggest that the nucleus takes an active part in the infection process, but the mechanism is still obscure.

The reaction between the bacterial substance and the specific cell compound results in the formation of an 'organizer' which governs the production of polygalacturonase. This enzyme is always present in the growing root hair in small amounts. The apical hair wall consists mainly of pectic substances, and the function of polygalacturonase may be a continuous softening of this wall, which leads to cell elongation (Ekdahl, 1953). However, when appropriate nodule bacteria are present, they induce, in some hairs at least, a much stronger production of polygalacturonase. This might result in a more pronounced depolymerization of the pectic layer, which

would allow the bacteria to penetrate the cell wall. But it appears also possible that the increased polygalacturonase activity can result in an intensified but very localized growth process giving an infection thread by invagination, in accordance with Nutman's hypothesis (1956). It seems to us that an important role in the infection process can be ascribed to polygalacturonase regardless of whether Nutman's hypothesis be accepted or not.

The mechanism outlined in this way could explain the high specificity which is characteristic for the cross-inoculation groups of leguminous plants, since the

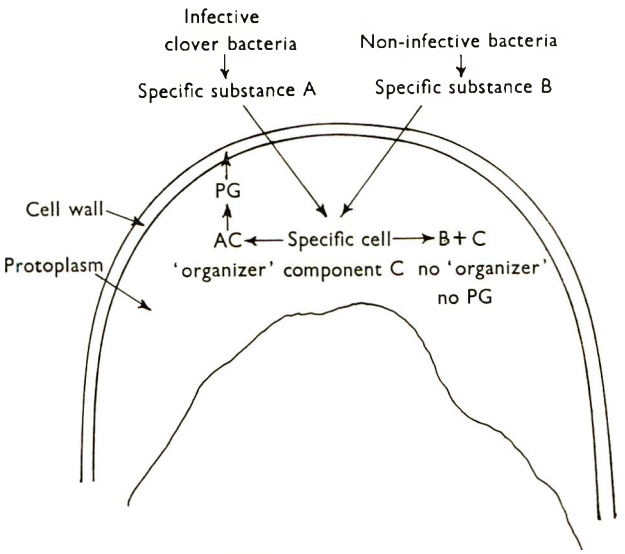

Fig. 1. Induced formation of polygalacturonase (PG) in root hair tip of clover plant.

specificity of polygalacturonase formation, as shown by our experiments, is quite as high. This is further illustrated by the tentative scheme given in Fig. 1. A prerequisite for infection as well as for polygalacturonase production is a certain nitrogen deficiency (Fåhraeus & Ljunggren, 1959). The reason why polygalacturonase formation is stopped at higher nitrate concentrations might perhaps be that nitrogen stimulates synthetic mechanisms in the plant which decrease the formation of depolymerizing enzymes. Even at suboptimal nitrogen concentrations, the increased polygalacturonase activity is probably not a sufficient condition for infection to take place. The well known fact that only a very restricted number among thousands of root hairs are invaded indicates that the infection is a rather complex process. Possibly the cell wall must in some way be 'sensitized' before the polygalacturonase becomes active. It lies near at hand in this connexion to think of indolyl-3-acetic acid (IAA). IAA increases the plasticity of cell walls (Galston & Purves, 1960), and is produced by nodule bacteria (Link, 1937; Kefford, Brockwell & Zwar, 1960). According to Thornton (1936) the deformation of root hairs, which is believed to be caused by bacterial IAA, is a necessary prelude to infection. However, our present knowledge of the action of IAA on cell walls—which might involve an immobilization of pectin methylesterase (Glasziou, 1957; Fåhraeus & Ljunggren, 1959)—are too vague to permit detailed conclusions about the role of

IAA in infection. It is certainly not possible to explain the whole infection process only as an IAA effect (Kefford, Brockwell & Zwar, 1960). Such an explanation does not account for the marked specificity of rhizobial infection, since IAA is produced by almost all types of nodule bacteria, and also by *Agrobacterium radiobacter* which is unable to infect any leguminous plant (Georgi & Beguin, 1939).

A large part of this investigation was made during a visit by one of us (H.L.) at the Rothamsted Experimental Station, Harpenden, Hertfordshire. Thanks are due to the Head of the Soil Microbiology Department, Dr P. S. Nutman, for his great hospitality and interest in the work. Financial support was obtained from M. Bergvall's foundation, Stockholm.

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The Isolation of [^{35}S]Homocystine from Neurospora

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SUMMARY

Evidence is presented inferring accumulation of [^{35}S]homocysteine in methionineless mutants of *Neurospora crassa*. Certain mutants formed five times as much homocystine as did the wild type; [^{35}S]homocysteine was identified as homocystine and homocysteic acid.

INTRODUCTION

Extensive studies have shown the physiological activity of homocysteine in replacing methionine (Butz & Du Vigneaud, 1932; Horowitz, 1947, 1950; Shapiro, 1955; Schlenk & DePalma, 1955; Durell, Anderson & Cantoni, 1957). Thus the role of homocysteine as an intermediate in methionine biosynthesis has become accepted although it has never been isolated from biological material. Because homocysteine usually occurs only as a transitory intermediate in methionine biosynthesis it was felt that methionineless mutants of *Neurospora crassa* might accumulate homocysteine and would be particularly useful in attempts to show directly the occurrence of this compound. The experiments described below show that homocysteine occurs in *N. crassa* and in greater concentration in these mutants than in wild type, as reported in a preliminary note (Leinweber & Liverman, 1959). After this work was completed the authors learned of a communication on the isolation of homocystine from the human adrenal gland (Biserte, 1957); quantitative data, however, were not reported.

METHODS

Wild type strain 5297a and mutants 38706Ra and 80702Ra of *Neurospora crassa* were obtained from Dr M. Fling, Division of Biology, California Institute of Technology, Pasadena, California, U.S.A. Mutants T-27 and T-112 originated in the Genetics Laboratory of the University of Texas and were obtained from Dr Robert A. McRorie, University of Georgia, Athens, Georgia, U.S.A. Mutants 38706Ra, T-27 and T-112 do not grow on any precursor of methionine biosynthesis (Horowitz, 1947, 1950) but require methionine for growth. They are believed to lack an enzyme necessary for the biosynthesis of methionine from homocysteine. With regard to mutants T-27 and 38706Ra, at least two differences are known. Growth of mutant T-27 on methionine is enhanced by augmenting sulphate in the medium; in mutant 38706Ra, sulphate has no effect on growth in the presence of methionine. S-methyl-

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L-cysteine can serve as sole sulphur source for mutant T-27 but not for 38706Ra (Ragland & Liverman, private communication). Mutant 80702Ra does not grow on sulphate or sulphite and was proposed to have a genetic block between sulphite and thiosulphate (Horowitz, 1950).

The various strains of *Neurospora crassa* were grown on 20 ml. of sulphur-free minimal medium contained in 125 ml. Erlenmeyer flasks incubated at 25°. The medium was prepared by replacing the suphates of the Difco Choline Assay Medium (*Difco Manual*, 1953) by the chloride salts in equimolar amounts with respect to the cation. Methionine was supplemented to all strains in a concentration of 5×10^{-4} M which was found optimal for growth and was added aseptically after filter-sterilization. Radiosulphate, purchased from the Oak Ridge National Laboratories, was added either carrier-free or with non-radioactive potassium sulphate. The final concentration of radiosulphate in the medium was 8–15 μc./ml., the specific radioactivity 150–174 μc./μmole unless otherwise stated.

At the end of the growth period, the mycelium was harvested by filtration, freed from culture medium by suction, and repeatedly washed with distilled water. The filtrate was discarded unless otherwise stated. The mycelium was ground in a mortar with sand and 10 ml. of cold 10% (w/v) trichloroacetic acid (TCA), and the homogenate centrifuged. The pellet was twice resuspended and washed with 10 ml. of 5% (w/v) TCA and again centrifuged. The supernatant fluids were pooled and accurately weighed amounts of homocysteine (e.g. 1·5 m-mole; purchased from Nutritional Biochemicals Corporation, 21010 Miles Ave., Cleveland, Ohio, U.S.A.) + homocystine (0·75 m-mole; purchased from California Corporation for Biochemical Research, 3625 Medford St., Los Angeles 63, California, U.S.A.) were added in order to isolate [^{35}S]homocysteine formed in the mycelium. The homocysteine in the TCA extract was then oxidized to the disulphide by the following procedure which was developed in this laboratory. The extract was adjusted to pH 8–9 with sodium hydroxide solution. One drop 0·3 M solution of ferric chloride was added per m-mole of homocysteine-carrier, and oxygen bubbled through the solution until oxidation was complete, as indicated by fading of the violet colour of the iron-sulphydryl complex. The solution was then adjusted to pH 6 to precipitate homocystine. Any contaminating protein was removed by dissolving the precipitated homocystine in 7 ml. of 2N-hydrochloric acid and dialyzing against 50 ml. of 0·5N-hydrochloric acid. The cellophan sack and its remaining contents were discarded. The homocystine in the diffusate was precipitated by adjusting the solution to pH 6. After dissolving the homocystine in a minimum amount of dilute hydrochloric acid it was further purified by subjecting to paper electrophoresis, using a Spinco Continuous Flow Electrophoresis Apparatus, Model CP, at 5°–10°. The homocystine separated as a narrow band when the electrophoresis was carried out at 900 V. in 0·25 M-acetic acid. The homocystine samples thus obtained were recrystallized until the specific radioactivity and the decomposition temperature became constant. Chromatographic assays of the re-isolated homocystine were performed, using four different solvent systems, in one and in two dimensions. The solvent systems used were: (1) methyl ethylketone + *n*-butanol + distilled water + dicyclohexylamine (10 + 10 + 5 + 2); (2) 3-butanol + 90% (w/v) formic acid + distilled water (70 + 15 + 15); (3) phenol + de-ionized water + 0·88 N-ammonia (160 + 40 + 1); (4) *n*-butanol + distilled water + pyridine (1 + 1 + 1).

RESULTS

In a preliminary experiment it was found that *Neurospora crassa* methionineless mutants 38706Ra and T-27 formed [^{35}S]homocystine. The concentrations of [^{35}S]homocystine were rather low. It was, therefore, attempted to enhance the formation of [^{35}S]homocystine by adding non-radioactive sulphate to the medium. In this experiment homocystine was re-isolated from the combined TCA extract and the filtered culture fluid after 9 days of incubation. At a concentration of $8{\cdot}6 \times 10^{-5}$M-carrier-sulphate the [^{35}S]homocystine concentration, expressed as amount [^{35}S]homocystine/g. dry mycelium, approximately doubled as compared to that obtained in the absence of potassium sulphate, as shown in Fig. 1. Further increasing the concentration of carrier-sulphate does not give rise to higher concentrations of [^{35}S]homocystine; since, however, a 3·3-fold increase in carrier-

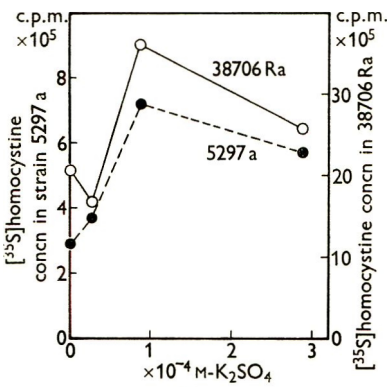

Fig. 1

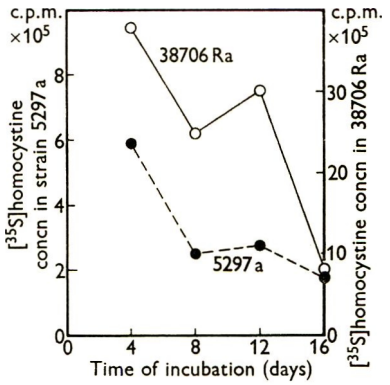

Fig. 2

Fig. 1. [^{35}S]homocystine formation in *Neurospora crassa* as function of sulphate concentration in the culture medium. Left scale refers to wild type strain 5297a, right scale to mutant 38706Ra. The mycelium was incubated for 9 days. Specific radioactivity of sulphate at $8{\cdot}6 \times 10^{-5}$M = 98 μc./μM.

Fig. 2. [^{35}S]homocystine formation in *Neurospora crassa* as function of time of incubation.

sulphate concentration resulted in a decrease in [^{35}S]homocystine of only *c.* 25 %, the optimal sulphate concentration for homocystine formation is apparently higher than $8{\cdot}6 \times 10^{-5}$M.

The data suggest that some optimal concentration of sulphate is required for homocystine formation. As mentioned under Methods and as shown below, sulphate alone does not promote any growth in mutant 38706Ra unless incubated for longer than 2 weeks. Growth, under the conditions employed, therefore, is a function of methionine concentration. Mutant 38706Ra accumulated five times as much [^{35}S]homocystine as did wild-type strain 5297a.

It was expected that increases in incubation time of the mycelia would enhance [^{35}S]homocystine accumulation. Wild-type and mutant 38706Ra were, therefore, incubated for 4, 8, 12 and 16 days in the presence of 5×10^{-4}M-methionine, $8{\cdot}6 \times 10^{-5}$M-non-radioactive sulphate and 15 μc. of [^{35}S]sulphate/ml. medium. As shown in Fig. 2, the maximum accumulation of [^{35}S]homocystine, as measured in the TCA

extract, was noted after four days of growth. Prolonging the incubation period resulted in lower [^{35}S]homocystine concentrations. It is of interest to note that between the 4th and 8th day of incubation the [^{35}S]homocystine concentration in mutant 38706Ra decreased while the mycelial dry weight increased by 58%, indicating that the decrease in [^{35}S]homocystine concentration is not related to depletion of methionine in the culture fluid. Considering the difference in the ordinate scale of a factor of 4 for the plot of mutant and wild type, it can be seen in both figures that the mutant formed 5–11 times as much [^{35}S]homocystine as did the wild type. Measurements of [^{35}S]methionine formation were not made; the results suggest, though, that the block preventing methionine biosynthesis from homocysteine is shown to be incomplete if the incubation period is extended beyond 4 days. In an experiment to test this postulate mutant 38706Ra was grown in parallel on 2×10^{-4}M-methionine or 2×10^{-4}M-potassium sulphate for 4, 12, 17 and 22 days. After 4 days there was no growth on sulphate but after 12, 17 and 22 days the growth was 8, 50 and 86% respectively of that on methionine. This experiment strongly supports the above postulate. In a mutant of *Neurospora crassa* which is blocked between cystathionine and homocysteine and which grows only on homocysteine or methionine it was demonstrated that methionine biosynthesis from [^{35}S]sulphate takes place after prolonged incubation (Strauss & Minagawa, 1959); [^{35}S]cystathionine first accumulated, and later disappeared as [^{35}S]methionine was formed.

Homocystine formation in mycelia of mutant T-112 and 80702Ra and wild-type strain 5297a was compared. It was found that mutant T-112 formed 4·8 times as much [^{35}S]homocystine as did wild type, whereas mutant 80702Ra formed only 0·58 times as much. Such a result might be expected since mutant 80702Ra is blocked before homocysteine in the biosynthetic pathway. The observation that homocystine re-isolated from this mutant was slightly radioactive might indicate that the metabolic block is not complete.

A chromatographic study confirmed that the radioactivity measured in mutants 38706Ra, T-27 and T-112 was due to [^{35}S]homocystine. The ninhydrin-positive material always coincided in size, shape and location with the black spot on the radioautograms. The nitroprusside test for disulphide compounds (Toennies & Kolb, 1951) also supported the identity of the radioactive material as homocystine. Furthermore, when homocystine was oxidized to homocysteic acid with 30% (w/v) H_2O_2 before developing the chromatograms, only one black spot appeared on the film which coincided with the ninhydrin-positive material on the paper. Because the specific radioactivity of homocystine from wild type was too low we were unable to obtain radioautograms in this case. It seems, however, reasonable to attribute the radioactivity measured in homocystine from wild type to the formation of some [^{35}S]homocystine.

The chromatographic techniques used permit differentiation of homocystine from other sulphur amino acids, e.g. methionine, cysteine, cystine, cystathionine, lanthionine, djenkolic acid. Although homocysteine was oxidized during the isolation procedures it is probable that the [^{35}S]homocystine finally identified was due to formation of [^{35}S]homocysteine in the mycelium.

Since varying the growth period or the sulphate concentration never resulted in [^{35}S]homocystine yields larger than 0·3% in terms of ^{35}S initially present in the

medium, no attempts were made to isolate homocysteine without employing non-radioactive homocysteine-carrier.

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The Relationship of the Enterobacterium A12 (Sachs) to *Shigella boydii* 14

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SUMMARY

The relationship of the enterobacterium A12 (Sachs) to *Shigella boydii* 14 is shown by comparison of their biochemical and serological reactions. The aerogenic, mannitol-negative A12 is serologically identical with the typical Shigella serotype, *S. boydii* 14. As mannitol-negative strains of *S. boydii* 14 exist and as the aerogenic A12 organism is otherwise biochemically typical of the *Shigella* group, it is proposed that A12 be regarded as a biochemical variety of *S. boydii* 14 analogous to the accepted aerogenic biochemical varieties of *S. flexneri* 6.

INTRODUCTION

It is well known that there is a wide sharing of antigens throughout the whole family of the Enterobacteriaceae so that the determination of the antigenic structure of a particular enterobacterium is only one part of the jigsaw of identification and subsequent classification. The identification of these organisms must, at least at present, be based on both biochemical and serological criteria. Even so bacteriologists constantly encounter organisms which cannot easily be placed in one or other of the groups of the Enterobacteriaceae, whose main subdivisions, with the increasing use of the more detailed biochemical tests, are now reasonably well defined.

The aerogenic enterobacterium A12 of Sachs (1943) is an example of an intestinal organism whose exact taxonomic position within the Enterobacteriaceae is still not finally determined. Sachs considered this organism a possible member of the non-mannitol-fermenting subgroup of *Shigella*, whereas Ewing & Hucks (1950) considered it an aberrant coliform of the *Escherichia coli* O group 32. Wheeler & Stuart (1946) also discussed A12 and though, in the light of available knowledge at that time, they considered that the organism should not be included in the *Shigella* group, they did suggest that if a serological relationship to other strains of shigella was shown in time and if anaerogenic variants could be produced from it then there would be good reason to include it in the *Shigella* group. These authors drew attention to a similar situation occurring in the biochemical varieties of the Newcastle–Manchester–Boyd 88 dysentery bacilli. This opinion has now been fully justified with further knowledge and it is suggested that the position of A12 can be opportunely reviewed. It is proposed that this organism be regarded as a biochemical variety of the serotype *S. boydii* 14.

SOURCE OF ORGANISMS

There seem to be few records in the literature of the isolation of A12 from either single cases or outbreaks. In the first description of the organism Sachs examined 10 strains isolated in military laboratories on the North West Frontier of India between 1936 and 1941. The single A12 strain examined by Wheeler & Stuart was one of Sachs original strains and Ewing & Hucks strains are described as 'representative cultures', but these authors make no mention of freshly-isolated strains. Since the establishment of the Dysentery Reference Laboratory in 1945 21 strains of A12, freshly isolated in this country, have been identified. One strain was isolated in 1953 from a 14-year-old girl who had just arrived from the Sudan suffering from diarrhoea and from whom *Salmonella uganda* had also been isolated. Nineteen strains had been isolated during an outbreak of dysentery in a mental hospital in 1957; 16 of these strains were isolated from elderly female patients with acute diarrhoea, 1 from a 37-year-old patient who was herself probably infected in India and who undoubtedly introduced the infection to the hospital, and 2 were isolated from symptomless excreters (Dr H. I. Coombs, personal communication). One further strain was isolated from a 21-year-old male laboratory technician suffering from acute dysentery whose infection was acquired in the laboratory during the investigation of the 19 mental hospital strains.

METHODS

Strains. These 21 freshly isolated strains and the stock laboratory culture originally received from Sachs were available for detailed study in comparison with *Shigella boydii* 14. Six strains of *S. boydii* 14 were available and all have been received from Dr W. H. Ewing (Communicable Disease Centre, Atlanta, Georgia, U.S.A.) over the years 1952–57. Three strains of *Escherichia coli* O group 32 were also received from Dr Ewing.

Biochemical tests

Fermentation tests. Carbohydrates (0·5 %, w/v) in 1 % (w/v) peptone water with Andrade indicator, observed for 21 days.

Urease. Christensen (1946).

H_2S *production.* (1) Lead acetate papers over nutrient broth; (2) Triple Sugar Iron Agar (Test 10, *Report*, 1958*a*).

Phenylpyruvic acid test. (1) Henriksen (1950); (2) combined with malonate (Shaw & Clarke, 1955); (3) phenylalanine agar (Test 18, *Report*, 1958*a*).

Methyl red (MR) and Voges-Proskauer (VP) tests. Incubation for 5 days at 30°; VP test (O'Meara, 1931).

Citrate utilization. (1) Koser (1923); (2) Simmons (1926); (3) Christensen (1949) as modified by Edwards & Ewing (1955).

Malonate test. Combined with phenylanine, Shaw & Clarke (1955).

Gluconate test. Shaw & Clarke (1955) modified by using 'Clinitest' Reagent Tablets (Ames Company, Nuffield House, London, W. 1) in place of Benedict qualitative solution.

Gelatine liquefaction. Nutrient gelatine stab culture incubated for 42 days at room temperature (about 22°).

Growth in KCN. Møller (1954) modified by using bijou bottles with the caps very tightly screwed.

Nitrate reduction. (1) 0·1 % (w/v) nitrate broth, tested after 5 days of incubation by Griess–Ilosvay method (*Topley and Wilson's Principles*, 1955); (2) plate test, Cook (1950).

Decarboxylases. Møller (1955).

Indole. 2 % (w/v) peptone water, (1) tested after 24 and 48 hr. with Kovacs reagent (1928); (2) oxalic acid papers (Holman & Gonzales, 1923).

Except as indicated, the temperature of incubation was 35°.

Serological tests

The antigen suspensions used were prepared in mercuric iodide solution (Bridges, 1951) from meat digest agar slopes and as necessary heated in a boiling water bath for 30 min. The several A 12 and *Shigella boydii* 14 antisera used were prepared at the Dysentery Reference Laboratory. The two *Escherichia coli* O group 32 antisera used were kindly supplied by Dr W. H. Ewing and by Dr J. F. Winn (Communicable Disease Center, Atlanta, Georgia, U.S.A.). Tube agglutination tests were performed by a Dreyer technique and incubated in a 50° water bath for 4 hr., read, and incubated further overnight. All tests were done in duplicate with unheated and heated suspensions.

RESULTS

General characters. The A12 organisms are Gram-negative rods, non-sporing and non-acid-fast, indistinguishable in size and appearance from classical shigellas. Capsules have not been demonstrated. They are facultative aerobes and give characteristic shigella-like colonies on the usual 'enteric' media and, like shigellas, they do not grow on bismuth sulphite (Wilson & Blair) agar. Growth in broth and peptone water closely resembles that of accepted shigellas in showing a fairly clear supernatant fluid and a light powdery deposit, quite different from the denser uniform turbidity characteristic of the *Escherichia*, *Hafnia* or *Salmonella* groups.

Biochemical reactions

Table 1 summarizes the biochemical reactions of A12 and *Shigella boydii* 14. The volume of gas produced by the A12 strains from glucose occupied about $\frac{1}{4}$–$\frac{1}{3}$ of the Durham tube; Sachs original strain produced less than the others but it is now an old laboratory culture. In general, only a tiny amount of gas, less than $\frac{1}{4}$ of the Durham tube, was produced from maltose. There was some minor strain variation in the period of incubation required for the fermentation of certain carbohydrates, but the average was fairly consistent. Xylose was generally fermented after incubation for 6 days, dextrin after 5 days, glycerol after 2 days and maltose after 5 days. Sorbitol was rapidly fermented, usually after 1–2 days. Dextrin was the most irregularly fermented carbohydrate. One of the A12 strains and two of the *S. boydii* 14 strains did not ferment it and most of the strains tested produced less than full acidity from it. On testing in Hugh & Leifson (1953) medium all strains showed fermentative metabolism.

Attempts were made by multiple colony selection to find anaerogenic colonies from the aerogenic strains. Though several colonies were found to produce only a tiny bubble of gas no colony failed to produce some gas from glucose.

Apart from the production or otherwise of gas, and the fermentation of mannitol

or otherwise, it is clear that the reactions of the A 12 organisms and *Shigella boydii* 14 are identical. None of the A 12 strains shows any biochemical reaction which would eliminate them from the *Shigella* group as at present defined (*Report*, 1958*a*).

Table 1. *Biochemical reactions*

	A 12		*S. boydii* 14	
	Sachs original strain	D.R.L. 21 strains	4 strains	2 strains
Glucose	AG	AG	A	A
Lactose	–	–	–	–
Mannitol	–	–	A	–
Sucrose	–	–	–	–
Dulcitol	–	–	–	–
Salicin	–	–	–	–
Xylose	(Ag)	(AG)	(A)	(A)
Adonitol	–	–	–	–
Arabinose	A	AG	A	A
Cellobiose	–	–	–	–
Dextrin	(a)	(a)/–	–/(a)	(A)
Glycerol	(A)	(A)	(A)	(A)
Inositol	–	–	–	–
Maltose	(Ag)	(Ag)	(A)	(A)
Raffinose	–	–	–	–
Rhamnose	–	–	–	–
Sorbitol	(AG)	(AG)	A	A
Trehalose	AG	AG	A	A
Urease production	–	–	–	–
H_2S production	–	–	–	–
Motility	–	–	–	–
Indole production	–	–	–	–
Phenylpyruvic acid test	–	–	–	–
MR reaction	+	+	+	+
VP reaction	–	–	–	–
Citrate utilization	–	–	–	–
Malonate	–	–	–	–
Gluconate	–	–	–	–
Gelatine liquefaction	–	–	–	–
Growth in KCN	–	–	–	–
Nitrate reduction	+	+	+	+
Oxidase	–	–	–	–
Catalase	+	+	+	+
Decarboxylases:				
Arginine	–	–	–	–
Lysine	–	–	–	–
Ornithine	–	–	–	–
Litmus milk	No change	No change	No change	No change

A = acid production within 24 hr.; (A) = acid after 48 hr.; (a) = weak acid; G = gas ¼ or more volume of Durham tube; g = gas less than ¼ the volume of Durham tube.

Serological reactions

In slide agglutination tests, all the strains of A 12, *Shigella boydii* 14 and *Escherichia coli* O group 32 examined agglutinated in each of the appropriate antiserums for the three serotypes. None of the A 12 or *S. boydii* 14 strains showed any serological cross-relationship with other members of the *Shigella* group nor with Alka-

lescens-dispar O groups 1–4. The tube agglutination and absorption tests are summarized in Table 2.

The mirror absorption tests showed clearly that A12, *Shigella boydii* 14 and *Escherichia coli* O group 32 are serologically identical. This further confirms the work of Ewing & Hucks (1950) who showed that the somatic antigen of A12 was identical with that of *E. coli* O group 32 and of Ewing (1953) who stated that the then provisional *Shigella* serotype 2770–51 (now *S. boydii* 14) was identical serologically with *E. coli* O group 32.

Table 2. *Serological tests*

Titres are expressed as % of homologous titres for simplicity since more than one serum of each type and several strains were used, each serum giving a slightly different homologous titre.

Antiserum	Absorbing suspension	Antigen suspensions: A12	*S. boydii* 14	*E. coli* O32
A12	—	100	100	100
	A12	0	0	0
	S. boydii 14	0	0	0
	E. coli O32	0	0	0
S. boydii 14	—	100	100	100
	A12	0	0	0
	S. boydii 14	0	0	0
	E. coli O32	0	0	0
E. coli O group 32	—	100	100	100
	A12	0	0	0
	S. boydii 14	0	0	0
	E. coli O32	0	0	0

DISCUSSION

In 1958 the provisional *Shigella* serotype 2770–51 was accepted as a typical member of the *Shigella boydii* subgroup and named *S. boydii* 14 (*Report*, 1958*b*). Ewing & Hucks (1952) in describing 2770-51 stated that 'it appears to us to be coincidental that serotype 2770-51 and type A12 (Sachs) both contain O antigens that are identical with those of *Escherichia coli* O group 32. Culture A12 is considered to be an aberrant intermediate paracolon bacterium because of its biochemical characteristics while serotype 2770-51 is believed to be a typical *Shigella* serotype with all the characteristics of *S. boydii* cultures.' They did not, however, consider the possibility that A12 might be a biochemical variety of *S. boydii* 14. The 2770-51 serotype was further discussed by Ewing, Reavis & Davis (1958) and they noted that six of their cultures (five from one familial outbreak) did not ferment mannitol.

Within the *Shigella* group only one member is at present accepted as having aerogenic varieties; this is the serotype *Shigella flexneri* 6. Scott (Whitehead & Scott, 1934) showed clearly that the aerogenic 'Newcastle' dysentery bacillus of Clayton & Warren (1929), the anaerogenic '88' dysentery bacillus of Boyd (1931) and the aerogenic 'Manchester' bacillus of Downie, Wade & Young (1933) were serologically identical. The biochemical reactions of this serotype are given in Table 3.

The Newcastle variety, which does not ferment mannitol, may or may not produce gas from glucose; this is like the aerogenic non-mannitol fermenting A12 and the

anaerogenic non-mannitol fermenting strains of *Shigella boydii* 14. To date, no strains of *S. boydii* 14 have been found which are both aerogenic and mannitol-fermenting, like the Manchester variety of *S. flexneri* 6. It is generally stated that the characteristic volume of gas produced from glucose by the aerogenic varieties of *S. flexneri* 6 is very small (*Topley and Wilson's Principles*, 1955) or sometimes described as a tiny bubble. This, however, appears from experience in the Dysentery Reference Laboratory to be no longer true. The aerogenic strains of *S. flexneri* 6 now produce a volume of gas quite as large as that produced by salmonellas. Even the original strains of Clayton & Warren, and Downie *et al.*, now produce a similar volume in the peptone water media in current use. There is some evidence that the use of modern peptones may influence the volume of gas produced by these organisms. The amount of gas produced by A12 is very similar to that of the aerogenic strains of *S. flexneri* 6. Table 4 shows the proposed biochemical varieties of *S. boydii* 14, analogous to those of *S. flexneri* 6.

Table 3. *Biochemical reactions of* Shigella flexneri *6*

	Glucose	Mannitol	Dulcitol	Indole
Boyd's 88	A	A	A/–	–
Manchester variety	AG	AG	AG/–	–
Newcastle variety	AG/A	–	AG/A/–	–

Fermentation of dulcitol may be delayed.

Table 4. *Proposed biotypes of* Shigella boydii *14*

	Glucose	Mannitol	Dulcitol	Indole
S. boydii 14 (classical)	A	A	–	–
S. boydii 14 (some strains)	A	–	–	–
A12	Ag	–	–	–

Pathogenicity. Though pathogenicity may be a poor criterion for taxonomy, the *Shigella* group as classified at present contains only organisms accepted as pathogenic and as causing bacillary dysentery. There seems to be no reason to exclude the A12 organism from the bacillary dysentery group. The mental hospital outbreak due to A12 shows that the organism is capable of causing an outbreak of dysentery and, though it might be said that elderly mental hospital patients may be in a special category of susceptibility to such infections, the infection of a young healthy laboratory technician during the handling of the cultures clearly indicates, in the absence of experimental animal infection, that the A12 organism is pathogenic and can produce a clinically typical attack of acute bacillary dysentery exactly similar to that of other shigella infections acquired in the laboratory (K. P. Carpenter, personal observation). Wheeler & Stuart's criteria in 1946 for acceptance of A12 as a member of the *Shigella* group have been fulfilled, and it appears expedient now that Sachs A12 be regarded as a typical aerogenic member of the group and as a biochemical variety of the serotype *Shigella boydii* 14, exactly analogous to the established biochemical varieties of *S. flexneri* 6.

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